ABSTRACTS

6th International Symposium on
Canine and Feline Reproduction

&

6th Biennial EVSSAR Congress

European Veterinary Society for Small Animal Reproduction

"Reproductive biology and medicine of domestic and exotic carnivores"

University of Veterinary Sciences
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In his decision making process in the daily veterinary practice the veterinarian should be driven by recent, objective and scientifically proven information. While in human medicine intensive examination of appraising available literature has been conducted in the course of evidence-based medicine (EBM), a systematically work up and appraisal of scientific publication has not been carried out in veterinary medicine yet. The aim of EBM is to base the decisions in the practice of medicine on valid, clinically relevant research data. By summarising information and analysing the results of different clinical trials relating to a specific topic (metaanalysis) concise and advanced conclusion can be formulated. This evidence (certainty that results are true) sets decision making on diagnosis, prognosis, treatment, and risk on the basis of more predictable outcomes.

The objective of this project was to search for published literature on reproduction in dogs and evaluate it in regard to evidence. For this purpose a literature research of online databases PubMed and Vet-CD as well as in the bibliographies of obtained articles was conducted. For appraising the literature 40 criteria in the categories material and methodology, study design, statistics, presentation and information content, practical applicability and conclusions were developed. Subsequently, the criteria were integrated into a questionnaire. This tool can support the practitioner to identify articles with strong evidence and utilise information with high quality. The questionnaire can be found on the website www.tiergyn.de.

The search of veterinary literature of high evidence was difficult. Out of 287 appraised publications only 90 could be classified as clinical trials (31.4 %). The remaining 197 publications (68.6 %) were case reports or contained information based on personal experience. Metaanalyses could not be found in the literature of reproduction in dogs. In half of the cases (49.8 %) generally accepted and science-based conclusions could not be legitimately drawn by the collected data.

For the field of reproduction in dogs this project discovered evidence deficits. The demand for more clinical trials of a higher quality is obvious – even though it is not easy to develop study protocols of high quality in the field of small animal reproduction (e.g. small number of dogs available fitting the inclusion criteria, missing compliance of the owners, individual differences and costs). It has to be assumed that also in other areas of veterinary medicine decisions are often based on sources of doubtful evidence.
BENEFITS OF COMPUTERIZED REPRODUCTIVE RECORDS

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Objective of the presentation is to discuss the value of computerized records to help standardize the minimum data for canine reproductive reports related to the estrus cycle in the bitch, the use of chilled semen, freezing of canine semen, and canine soundness examinations. Furthermore, these standardized records can be shared amongst practitioners and researchers using the program, through an internet site, and therefore, allows for the exchange of reproductive data.

The records related to the bitch consist of ovulation-timing charts that will tract the estrus cycle in the bitch, record times & methods of insemination, type & quality of canine semen used, and information related to the outcome of the breeding. Completed data is compiled into various reports:

### Pregnancy Rates

<table>
<thead>
<tr>
<th>Type of Insemination</th>
<th>Frozen Semen (46)</th>
<th>Fresh Semen (44)</th>
<th>Chilled Semen (32)</th>
<th>Mixed (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural breeder</td>
<td>-</td>
<td>-</td>
<td>90.6%</td>
<td>-</td>
</tr>
<tr>
<td>Vaginal AI</td>
<td>-</td>
<td>30</td>
<td>90%</td>
<td>18</td>
</tr>
<tr>
<td>Transcervical - NF (Norwegian Pipeline)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Transcervical - Endo (Endoscopic)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Surgical</td>
<td>46</td>
<td>90.46%</td>
<td>100%</td>
<td>11</td>
</tr>
<tr>
<td>Mixed</td>
<td>-</td>
<td>-</td>
<td>100%</td>
<td>3</td>
</tr>
</tbody>
</table>

### Motility:

<table>
<thead>
<tr>
<th>Type of Insemination</th>
<th>Frozen Semen (46)</th>
<th>Fresh Semen (44)</th>
<th>Chilled Semen (32)</th>
<th>Mixed (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;35 min</td>
<td>(3)</td>
<td>100%</td>
<td>(1)</td>
<td>100%</td>
</tr>
<tr>
<td>35-55%</td>
<td>11</td>
<td>100%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>55-65%</td>
<td>15</td>
<td>85.71%</td>
<td>(1)</td>
<td>100%</td>
</tr>
<tr>
<td>65-75%</td>
<td>12</td>
<td>100%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>75-85%</td>
<td>11</td>
<td>100%</td>
<td>(1)</td>
<td>100%</td>
</tr>
<tr>
<td>&gt;85%</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>90%</td>
</tr>
</tbody>
</table>

### Quality:

<table>
<thead>
<tr>
<th>Type of Insemination</th>
<th>Frozen Semen (46)</th>
<th>Fresh Semen (44)</th>
<th>Chilled Semen (32)</th>
<th>Mixed (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>-</td>
<td>-</td>
<td>11</td>
<td>62.56%</td>
</tr>
<tr>
<td>F2</td>
<td>(7)</td>
<td>100%</td>
<td>(1)</td>
<td>100%</td>
</tr>
<tr>
<td>F3</td>
<td>10</td>
<td>91.66%</td>
<td>(7)</td>
<td>88.71%</td>
</tr>
<tr>
<td>F4</td>
<td>12</td>
<td>100%</td>
<td>11</td>
<td>61.92%</td>
</tr>
<tr>
<td>F5</td>
<td>-</td>
<td>-</td>
<td>(2)</td>
<td>100%</td>
</tr>
</tbody>
</table>
Insemination & Whelping Information

<table>
<thead>
<tr>
<th></th>
<th>Frozen Semen (RL)</th>
<th>Fresh Semen (FL)</th>
<th>Chilled Semen (CS)</th>
<th>Mixed (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Insemination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average day from start of heat</td>
<td>47</td>
<td>14</td>
<td>47</td>
<td>12 13</td>
</tr>
<tr>
<td>Earliest (day) from start of heat</td>
<td>47</td>
<td>9 47</td>
<td>6</td>
<td>13 9</td>
</tr>
<tr>
<td>Latest (day) from start of heat</td>
<td>47</td>
<td>21</td>
<td>47</td>
<td>20 32 18</td>
</tr>
<tr>
<td>Average P1 value at breeding</td>
<td>(1)</td>
<td>20.05</td>
<td>(1)</td>
<td>12.13</td>
</tr>
<tr>
<td>Chilled P1 value (days after breeding)</td>
<td>(5)</td>
<td>12.58</td>
<td>(5)</td>
<td>12.37</td>
</tr>
<tr>
<td><strong>Second Insemination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average day from start of heat</td>
<td>(2)</td>
<td>19</td>
<td>53</td>
<td>15 14</td>
</tr>
<tr>
<td>Earliest (day) from start of heat</td>
<td>(2)</td>
<td>13</td>
<td>22</td>
<td>4 11</td>
</tr>
<tr>
<td>Latest (day) from start of heat</td>
<td>(2)</td>
<td>19</td>
<td>53</td>
<td>26 16 18</td>
</tr>
<tr>
<td>Average P1 value at breeding</td>
<td>(1)</td>
<td>21.7</td>
<td>(1)</td>
<td>20.21</td>
</tr>
<tr>
<td>Chilled P1 value (days after breeding)</td>
<td>(1)</td>
<td>21.7</td>
<td>(1)</td>
<td>10.99</td>
</tr>
</tbody>
</table>

**Gestation**
- Average gestation: 1st insemination: 64 80 42 40 33 81 - -
- Average gestation: 2nd insemination: (2) 39 20 39 19 06 - -

**Puppy Statistics**
- Average no. of pups conceived: 41 5 36 6 23 7 (4) 7
- Average no. of pups Alive at Whelping: 41 5 36 6 23 6 (4) 6
- Average no. of Puppies Alive at Weaning: 14 5 (5) 6 (6) 6 - -
- Average no. of Puppies Weaned: 41 3 36 3 21 3 (4) 4
- Average no. of Females conceived: 41 3 26 3 23 3 (6) 3
- Average no. of Female Whelped: 40 3 26 3 19 3 (4) 3
- Average no. of Female Weaned: 12 2 26 3 2 (3) 7 - -
- Average no. of Females conceived: 13 2 26 2 (3) 3 - -
- Male:Female ratio - conceived: 41 0.9 26 0.6 23 0.23 (6) 1.20
- Male:Female ratio - weaned: 41 1.43 26 0.84 22 0.85 (4) 0.9
- Male:Female ratio - both: 14 0.7 26 0.6 22 0.85 (4) 2.23 - -

**Type of Deliveries**
- Natural: 13 20 11 - -
- Caesarean: 7 0 2 3 3
- Cesarean: 3 2 1 1
- Cesarean: Medical: 1 1 2 - -

The records related to the male consist of reports related to chilled semen, frozen semen and soundness examinations. Reports detail the volume of ejaculate, concentration per cc, percent motility, and quality of motility based on Pscore. Reports allow for detailed comments on the morphology of the semen. Reports related to chilled semen detail the recipient of the semen and allow the veterinarian collecting the semen to append directions on how the semen is to be handled when received. With respect to the frozen semen report, the computer can calculated the number of breedings based on the data entered for that collection and concentration desired per breeding unit. The computer system will create and print a transfer reports for shipping frozen semen with thawing instructions appended to the report. The computer system allows the input of data related to the post-thaw motility of frozen semen.

The following are examples of a chilled semen report and a freezing semen report:
Chilled Semen Report

Visit Information

Client ID: 60088
Client Name: Elyson, Cyrene
Phones: (717) 702-4500
Visit ID: 3046081
Date: 2-M-2007
Collection Time: 7:20 A
Seamson Owner: Elyson, Cyrene

Collection and Semen Evaluation:

- Libido: Good
- Eros of Coll: Normal
- Color: Light white
- Semen Volume: 8.2 ml
- % Motility: 80%

Total Motile Sperns: 530,726

- Extender Added (ml): 1.8
- Amount of TMActive (ml): 1.8

Bitch Owner: Les Plour
Bitch Registered Name: ""

Send To:

- Name: Dr. Plour
- Hospital: Canine Cryobank, Inc
- Address: 120 N Pacific St, AS
- City: Areal
- Zip: 92089
- Country: USA
- Phone: 760-586-9003

Linked Insemination

Comments:

Freezing Semen Report

Visit Information

Client ID: C18
Client Name: Creamy, Viki
Pet/Stat ID: PM40138
Pet Name: Smooy
Age/Sex: 7 years / M
Breed: Labrador
Visit ID: 2176031
Freezing Date: 9-18-2005
Collection Time: 12:20 P
Seamson Owner: Creamy, Viki

Collection and Semen Evaluation:

- Libido: Dog
- Eros of Coll: Normal
- Color: White
- Semen Volume: 8.2 ml
- % Motility: 80%

Total Motile Sperns: 1,626,626

- Extender Added (ml): 1.8
- Amount of TMActive (ml): 1.8

Soundness
- Concentration/Swirl: 1.0
- Span Poli: A
- Amt. 1 Extender (ml): 1.8
- Amt. 2nd Extender (ml): 1.8

Data:
- Label: A, B, C, D, E
- Storage Location: D4X4, D4X4, D4X4, D4X4
- Dispensation Code: 1

Print Freezing Report
Print Freezing Application
Print Storage Report
Delete
New
Save
Close
Using computer programs as noted above would allow for standardization of canine reproductive records containing a minimum database of information, allow for the compilation and exchange of these records, and would be a source of research information.

**Current reports include:**

* Ovulation-timing charts: tracks progesterone through the bitch’s cycle, vaginal smear data, allows for the projection of LH peak, projects whelping date base on date of insemination and projected LH peak.
* Pregnancy rates: comparing types of semen used, such as fresh, chilled or frozen, quality of semen with respect to concentrations & motility; reports can be viewed for all breeds or an individual breed.
* Whelping & Insemination data: data related to progesterone levels at the time of breeding, numbers and sex of puppies conceived, whelped & alive at weaning.
* Reports related to semen quality and typical morphological changes
* Other types of reports can be added using the database structure of the program and reports can be exported to other database applications for additional analysis.
COMPARISON OF SELECTED ENDOCRINE PARAMETERS DURING LUTEAL PHASE AND PREGNANCY IN GERMAN SHEPHERD DOGS AND BEAGLES

Beste N, Nottorf S, Eschricht F, Hoppen HO, Dieleman S, Einspanier A, Günzel-Apel AR
Unit for Reproductive Medicine of Clinics - Small Animal Clinic, University of Veterinary Medicine, Foundation, Bünteweg 15, 30559 Hannover, Germany

Introduction - An investigation in German Shepherd Dogs (GSD) has revealed disturbed luteal function and pregnancy in short cycling bitches [5]. The nonpregnant short-cycling bitches had lower progesterone concentrations than the normocyclic controls, indicating decreased luteal activity. In the pregnant short-cycling group progesterone supplementation was accompanied by a low prolactin level from day 20 to 60 after ovulation compared with the same period in the pregnant controls. Relaxin concentrations showed a similar and about synchronous pattern in these two groups. The aim of the present study was to further elucidate these findings regarding a) a possible suppression of prolactin secretion by external progesterone or a primarily deficient prolactin secretion during pregnancy, and b) a causal connection between prolactin and relaxin secretion in beagle bitches. Furthermore the effects of progestin treatment on progesterone and prolactin secretion during luteal phase were examined.

Materials and methods - Blood samples were collected in normocyclic beagle bitches (group 1 non pregnant, n=5; group 2 pregnant, n=5) at 5 day intervals from day 5 to day 60 after ovulation in two consecutive luteal phases or pregnancies, respectively. Group 1 received medroxyprogesterone-acetate (MPA, 10 mg per dog) or a placebo orally from day 30 to 40 of the first or second luteal phase. Group 2 was treated intramuscularly with progesterone (2.0 mg kg\(^{-1}\)) or a placebo every second day during the same period of the first or second pregnancy. Concentrations of progesterone, prolactin and in pregnant bitches also relaxin were measured for the periods day 5-15, day 20-30, day 35-45, and day 50-60 after ovulation. Progesterone was additionally analysed daily during the treatment period. For hormone determination assays were used as previously described [5]. MPA concentrations in blood serum were analysed by RIA (Immunometrics, London, UK). The hormone concentrations of the beagle groups were statistically compared with those obtained in our previous study in German Shepherd bitches [5].

Results - Normocyclic nonpregnant bitches: from day 20 to 60 after ovulation mean progesterone concentrations were higher in the beagles compared with the GSD. From day 50 to 60 the difference was significant (beagles 5.2 ±2.2 ng ml\(^{-1}\), GSD 2.6 ±1.4 ng ml\(^{-1}\), p<0.05). Mean prolactin concentrations were markedly higher from luteal phase day 35-60 (day 35-45: beagles 5.5 ±3.2 ng ml\(^{-1}\), GSD 3.7 ±4.4 ng ml\(^{-1}\); day 50-60: beagles 7.0 ±2.8 ng ml\(^{-1}\), GSD 4.5 ±1.0 ng ml\(^{-1}\)). MPA treatment did not affect progesterone and prolactin secretion in the normocyclic non pregnant beagles. MPA serum concentrations were about 10 ng ml\(^{-1}\) during the 10 day treatment period and decreased to 1.1 and 0.1 ng ml\(^{-1}\) within 5 respectively 15 days after the end of treatment. Normocyclic pregnant bitches: in the GSD mean progesterone concentrations were only slightly below those in beagles, but significantly lower in late pregnancy (day 50-60: beagles 3.9 ±4.2 ng ml\(^{-1}\), GSD 1.5 ±0.6 ng ml\(^{-1}\), p<0.05). The prolactin secretion was at a significantly lower level throughout pregnancy (day 20-30 beagles: 6.9 ±2.9 ng ml\(^{-1}\), GSD: 4.5 ±1.0 ng ml\(^{-1}\); day 35-45 beagles: 22.4 ±9.9 ng ml\(^{-1}\), GSD: 8.3 ±2.4 ng ml\(^{-1}\); day 50-60 beagles: 27.4 ±11.0 ng ml\(^{-1}\), GSD: 12.9 ±8.6 ng ml\(^{-1}\), p<0.01 or 0.05). Mean relaxin concentrations were markedly lower in GSD than in the beagles (day 20-30 beagles: 1.2 ±0.8 ng ml\(^{-1}\), GSD: 0.8 ±0.4 ng ml\(^{-1}\); day 35-45 beagles: 2.3 ±1.6 ng ml\(^{-1}\), GSD: 1.3 ±0.6 ng ml\(^{-1}\);
day 50-60 beagles: 3.9 ±4.2 ng ml\(^{-1}\), GSD: 1.5 ±0.6 ng ml\(^{-1}\)). In the pregnant beagles during and after progesterone supplementation from day 30 to 40 after ovulation concentrations of progesterone as well as of prolactin and relaxin were markedly higher than in the placebo group.

**Discussion** - The results confirm the suspicion of deficient luteal function in GSD bitches, which may primarily result from a decreased prolactin secretion. Considering the higher prolactin concentrations found under progesterone supplementation in the beagles, the suspected suppression of prolactin secretion by exogenous progesterone in GSD, as has been previously discussed [5] can be denied. The synchronous and parallel secretion patterns of prolactin and relaxin found in the two breeds, indicate interaction of prolactin and relaxin in terms of a stimulatory effect of relaxin on prolactin secretion as previously discussed [1,3]. As had already been demonstrated by other authors [2,4] treatment with the synthetic progestin MPA does not affect progesterone secretion and even prolactin release does not seem to be influenced. By this oral MPA administration may be useful for monitoring luteal function in pregnant bitches with suspicion of hypoluteidism [4], but duration of application has still to be evaluated to avoid delay of parturition.

**References**


GROWTH FACTORS, CYTOKINES, AND PROSTAGLANDIN SYNTHESIS IN THE UTERUS OF PREGNANT BITCHES – THE FEATURES OF PLACENTAL SITES

S. Schäfer-Somi*, I. Kücükaslan, R. Agaoglu, N. Gülteken, S.S. Ay, D. Kaya, S. Aslan
*Centre for Artificial Insemination and Embryo Transfer, University of Veterinary Science, Vienna, Austria. E-mail: sabine.schaefer@vu-wien.ac.at

Objectives - The aim of the present study was to investigate uterine tissue from pregnant bitches for the expression of growth factors, cytokines, and enzymes participating in prostaglandin synthesis, probably important for the implantation and development of canine embryos.

Materials and methods - Twenty bitches of different breeds and ages were spayed. They were identified as early pregnant by means of embryo flushing with PBS solution after ovariohysterectomy (1; day 10 to 12 after mating, n=10) or at implantation time by means of ultrasonography before the operation (day 20 to 35, n=10). Four non-pregnant bitches (day 10 to 12 after mating) served as controls. Uterine tissue, from the middle of the horn of early pregnant and non-pregnant animals and from placentaion sites of later pregnant bitches, was assessed for the expression of mRNA. Qualitative RT-PCR was performed after extraction of mRNA from frozen tissue (-80°C) with TRI Reagent and species-specific primers, except for IGF-1 and IGF-2, where equine primers were used (for sequence, length of amplicon and references of primers used in the present study see 2).

Results - Factors detected in the preimplantation uterus were cyclooxygenase (COX)-1, the receptor molecule CD-8, interferon (IFN)-γ, the growth factors transforming growth factor (TGF)-β, hepatocyte growth factor (HGF) and insulin like growth factor (IGF)-1, the cytokines interleukin (IL)-2, -4, -10 and leukemia inhibitory factor (LIF). During the implantation and placentaion stage until day 35, the same factors as in the preimplantation period were assessed, however, we additionally detected mRNA for IGF-2 and granulocyte macrophage colony stimulating factor (GM-CSF). The diestrus uterus differed from the pregnant uterus because of the additional expression of tumor necrosis factor (TNF)-α, IL-6 and CD-4, but not CD-8, IL-4 and IFN-γ. At least, we could not detect COX-2, or IL-1β,-8,-12 and leptin in any tissues investigated.

Discussion - Thus, CD-8, IL-4 and IFN-γ were exclusively expressed in the pregnant uterus, probably as a reaction to maternal contact with the preimplantation embryo. Interleukin-4 is a typical T-helper-2 cell cytokine which is important for regulation of B-cell proliferation and immunoglobulin secretion. IFN-γ is believed to regulate endometrial angiogenesis, as has been shown in pigs (3) and mice (4). However, in the present study, the growth factors IGF-2 and GM-CSF were not up-regulated before the implantation stage and seem to have special functions during this stage of pregnancy. In the horse, the expression of IGF-2 in fetal and placental tissue starts at day 14 of gestation, which coincides with the time of embryo adhesion (5). In most species, conceptus growth is promoted by GM-CSF. In addition, GM-CSF together with CD8 suppressor-type cells protected mice embryos against cytotoxic mechanisms (6). We conclude, that these facts point towards a growth supporting effect of IGF-2 and GM-CSF and probably a modulation of maternal immune reactions during the invasion phase.
References


EXPRESSION OF MHC-I AND -II IN UTERINE TISSUES FROM EARLY PREGNANT BITCHES

Sabine Schäfer-Somi*, Hakki B Beceriklisoy, Ingrid Walter, Sonja Sabitzer, Dieter Klein, Halit Kanca, Duygu Kaya, Ali Reha Agaoglu, Hakki İzgür, Selim Aslan
*Centre for Artificial Insemination and Embryo Transfer, University of Veterinary Science, Veterinärplatz 1, A-Vienna. E-mail: sabine.schaefer@vu-wien.ac.at

Introduction - The major histocompatibility complex (MHC) is a cluster of genes, important in the immune response to infections. Furthermore, MHC genes are responsible for acute rejection of allogeneic tissue and tumor grafts. MHC genes products interact with bound peptides and present these antigens to T-cells, which is important for the regulation of the immune response (1). During pregnancy, MHC-I and –II antigens located on invasive cells within the feto-placental unit have to be downregulated or silenced, or act as T-cell recognition molecules and modulate maternal immune reactions (2), to avoid attacks from the maternal immune system. The function and expression of MHC-I and –II antigens during canine pregnancy has not been investigated so far.

Objectives - Aim of the present study was to investigate the expression of MHC-I and –II mRNA in uterine and placental tissues from pregnant and non-pregnant bitches, taken at different time periods after mating.

Materials and methods - The pregnant bitches were ovariohysterectomized during the preimplantation (group 1, n=6, day 5 to 12 days after mating), implantation (group 2, n=7, 15 to 19 days after mating) and placentation stage (group 3, n=10, 20 to 30 days after mating). Non-pregnant animals in diestrus served as controls (group 4, n=7). The expression of MHC–II in salpinx, apex, middle horn, corpus uteri and at implantation sites was determined by immunohistochemistry as well as qualitative and quantitative RT-PCR, however, MHC-I expression was only determined at the mRNA-level due to non-reproducible results in immunohistochemical demonstration of MHC-I. For the quantitative RT-PCR, two housekeeping genes, namely GAPDH and ß-Actin were used.

Results - MHC-I mRNA was detected in all tissues during all pregnancy stages. The relative expression of MHC-I mRNA did not change significantly during the here observed gestation time (p>0.05). At the apex and corpus site, the average number of MHC-II positive cells increased significantly from the preimplantation to the postimplantation stage (apex: 1.54 to 3.82; corpus: 1.62 to 5.04; p<0.05). Moreover, highest numbers of MHC-II positive cells were observed at placentation sites (6.64). In the salpinx, significantly higher numbers of MHC-II positive cells were found in the tissues of pregnant animals than in the control group (p<0.05). However, the MHC-II expression increased markedly from the preimplantation towards the implantation and placentation stage (GAPDH: 6.9 ± 9.59, 8.45 ± 5.8, 10.7 ± 10.95, p>0.05), which corresponds to the results obtained with immunohistochemistry. In addition, in pregnant animals at all gestation stages, the relative expression of MHC-II was markedly higher than that of MHC-I. This is supposed to be induced by pregnancy associated factors like IFN-γ or the contact with fetal antigen. At least, in the present study, we investigated only the expression of MHC-I or -II antigens on canine maternal tissue. However, in a former study, we could not detect MHC-I and -II mRNA in canine preimplantation day 10 embryos (3), which supports the theory that in canines as in other mammals, the absence or down-regulation of MHC-I and -II genes in embryo cells is important to evade attacks from the maternal immune system.
**Conclusions** - the increase in MHC-II towards placentation seems to be pregnancy related, even though the impact on the maintenance of pregnancy has to be further investigated.

**References**


(2) Shiroishi M, Tsumoto K, Amano K et al. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G, Proc Natl Acad Sci USA 2003;100:8856–8861.

EXPERIMENTAL STUDY OF THE POST-PARTUM INVOLUTING GENITAL TRACT OF THE BITCH. PART I: CLINICAL, BACTERIOLOGICAL AND CYTOLOGICAL FEATURES

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Objective of the work. The aim of this study was to describe the clinical, bacteriological and cytological features of the post-partum involution of the genital tract of the bitch, after normal whelping.

Materials and methods. We used nine primiparous Beagle-breed bitches, which had whelped normally, and we monitored them for up to three months later. Detailed clinical examination and sampling was performed on D0 (day of whelping), D1, D2, D4, D7, D10, D14, D21 and weekly thereafter until D84. The dimensions of the vulva were measured, by using a cutimetre: (i) total vertical length × total horizontal width and (ii) distance from anus to upper vulval commissure. The uterus was palpated through the abdominal wall and the diameter of uterine horns was subjectively estimated. Presence of vaginal discharge was evaluated; if there was any, it was described. Samples were collected after inserting a sterile swab into the anterior part of the vagina through a Hannovert-type vaginoscope. Bacteriological examination was carried out by using conventional techniques. Giemsa-stain smears were evaluated by means of semi-quantitative observational method to report number of cells; results were reported on a 1 (few, scattered cells) to 4 (slide full of cells) scale. Finally, ovario-hysterectomy was carried out to one of the experimental animals on each of D7, D21, D28, D35, D42, D56, D70 and to two animals on D84 (days after whelping), for a detailed examination of the ovaries and the uterus (Part II of the present study). Differences in findings throughout the study were detected with the Wilcoxon Signed Rank test. For binary responses, the Sign test was used.

Clinical findings. All animals whelped normally 5 to 9 puppies (median value: 7) and remained clinically healthy throughout the experimental period. Median dimensions (cm; total vertical length × total horizontal width) of the vulva were 3.6×2.6 on D0, to 3.2×2.6 on D28, to 3.2×2.5 on D56, to 3.2×2.4 on D84. Median distance (cm) from anus to upper vulval commissure was 9.0 on D0, 8.1 on D28, 7.7 on D56 and 7.3 on D84. The uterus was palpable through the abdominal wall until D84 with a mildly hard consistency; median diameter (cm) was approx. 3.0 on D0 to <1.0 cm on D28 and subsequently. After whelping the discharge was thick and dark green, progressively becoming mucous to clear and brownish to red to pink to colourless. Discharge was intermittently present up to D56 (3/4 animals) and to D77 (2/2).

Bacteriological findings. Bacteria, in pure or mixed culture, were isolated from the anterior vaginal samples of every bitch at some point during the study, but temporal differences between the bitches were observed. For six bitches, the samples were positive on D0 and for the remaining three they had become positive by D4. Bacteria were isolated from 47/105 samples and median duration of infection was 7 days. Anterior vaginal infection was more frequent and of shorter duration early post-partum. Bacteria were isolated from 29/45 samples obtained from D0 to D7; median duration of infection was 3.5 days. They were isolated from 9/31 samples from D10 to D28 and from 9/29 samples from D35 to D84; median duration of infection for each period was 7 days (p<0.05 for frequency and duration of infection). Of the 48 bacterial isolates, most were identified as Escherichia coli (n=20) or Arcanobacterium
pyogenes (n=13); other isolates were Streptococcus sp., Pasteurella multocida and Staphylococcus aureus. There was no association between presence of discharge and isolation of bacteria ($p<0.1$).

**Cytological findings.** Number of cells observed in swab samples from the anterior part of the vagina, decreased throughout the study. Median cell-score in samples obtained from D0 to D7 was 2, from D10 to D28 was 1 and from D35 to D84 was 1. Approximately half (40-60%) of these cells were epithelial (mainly lysed or intermediate vaginal cells and clusters of endometrial cells). Their proportion remained constant throughout. Leucocytes were also present; up to D28, most were neutrophils (>85%), but subsequently (D35 to D84) their proportion decreased (=60%), whilst that of macrophages and lymphocytes increased. Trophoblast-like cells were frequently seen up to D4. Finally, erythrocytes were also observed occasionally.

**Discussion and conclusions.** In general, detailed descriptions of the normal post-partum involution of the genital tract of the bitch are lacking. Principally, the findings provide baseline results for further studies into post-partum diseases. Clinical evidence (long-standing vaginal discharge) suggests that normal involution of the genital tract in the bitch can last up to three months. Anterior vaginal infection is more frequent immediately after whelping, but still present at later stages. It lasts shorter at early involution, perhaps due to the improved defences (e.g. increased number of leucocytes) of the genital tract. One may suggest that presence of respective discharge should not be considered pathological or indicative of sub-involution, if not coupled with other genital or systemic signs.
PULMONARY MATURATION OF CANINE FETUSES ALONG GESTATION TO PARTURITION

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Introduction - Development of the fetal respiratory system includes pulmonary growth and maturation. The lung maturation step can be divided into 5 phases, according to histological appearance: embryonic, pseudoglandular, canalicular, sacular and alveolar (5). During pseudoglandular phase, vessels have little blood flow and line apart from the epithelium, which makes the respiratory function impracticable (5). At the canalicular period, the respiratory epithelium becomes thin and the epithelial lining of the alveoli differentiates into pneumocytes type I and II (2). During transition to the sacular period, the formation of sacular sacs and the development of a capillary network are observed; consequently the gas exchange airway expands (5). Lung functional maturation is considered to occur during sacular phase, the period in which surfactant begins to be synthesized by the alveolar type II cells (5). At the alveolar period, the site of gas exchange is established and so are the functional and structural characteristics of the fetal lung. Alveolar maturation and growth are extended to neonatal period, when alveolar sacs continue to be formed and mature to alveoli (2). In Human Medicine, it is notified a higher incidence of respiratory distress in the male gender newborn (6). This high prevalence can be explained by differences in structural lung development during the gestational period, according to the fetal gender. High intrauterine androgen concentration is one of the main causes of deficiency in surfactant synthesis (1). The aims of this study were to distinguish fetal lung maturation phases during gestation till parturition, to determine the canine gestational period in which surfactant production begins and to compare pulmonary development of male and female fetuses.

Material and methods - This research was previously approved by the Ethical Committee of Faculty of Veterinary Medicine - University of São Paulo. 34 pregnant bitches were submitted to elective ovariohysterectomy and allocated into 4 groups according to the gestational period: 30 to 40 days of pregnancy (n = 10); 41 to 50 days (n = 10); 51 to 60 days (n = 10) and bitches at the first stage of labor (n = 4). Gestational timing was performed through the fetal crown-rump length (3). After hysterectomy, fetuses were euthanised through lethal intravenous injection of a barbituric agent (5% sodium thiopental) at uterine artery. After euthanasia, the fetuses were removed from the uterine cavity, sexed and a pulmonary lobectomy was performed. Fetal lungs were stored in 10% formol at room temperature. Pulmonary specimens were processed to a paraffin block and sectioned at 5 microns. Sections were stained with hematoxylin-eosin and evaluated by optical microscopy (10 –100x).

Results - Pulmonary development phases were determined according to the main characteristics observed microscopically. Pseudoglandular phase was identified between the 35th and 46th day of gestation; the onset of canalicular and sacular periods was observed respectively from the 48th and 60th day of pregnancy. It was not possible to accurately determine the onset and end of each lung maturation period. During the pseudoglandular phase, tubular structures containing cuboid epithelium origin the primitive alveolar ducts. High colunar epithelium compounds the upper airways, whereas cuboid epithelium lines the down airways. Yet at the canalicular period, epithelium tubes with a muscle cells surface were localized. It is believed that these structures represent the primitive conduct airways. Hence, cuboid epithelium tubes with the absence of muscle cells will origin the functional airways. The histological analyses revealed that respiratory tract development occurs in a centrifugal
way direction, from upper to down airways. In addition, during the transitional phases, the central regions develop faster than the peripheral ones. Therefore, it is possible to diagnose distinct development periods in different portions of the same organ (1). At birth, canine fetuses presented at the sacular phase, thus the neonatal period includes the progress to the alveolar period (4). Species with an active and independent behavior at birth, as ruminants and equines, present lungs at the alveolar phase of development. Histologically, no differences were verified between male and female lungs, as they showed similar pulmonary development along pregnancy. Hence, we assume that androgen influence in pulmonary maturation is of higher importance in monotocus species. In polytocus animals, as the canine species, the placental circulation is widely shared among mother and fetuses. As a consequence, both gender fetuses have unrestricted contact with the circulating androgens.

**Conclusion** - We can conclude that the sacular phase of lung development begins around 57 and 60 days of pregnancy, the period in which surfactant production is believed to occur. In addition, both fetal genders present similar pulmonary development during pregnancy till parturition. Further studies are necessary to determine the exact moment in which pneumocytes type II cells are formed and, ultimately recognize when surfactant production begins. For this objective it is greatly advised the use of a specific dye to histologically detect the alveolar type II cells.

**References**

FETAL LUNG DEVELOPMENT AND SURFACANT PRODUCTION IN THE DOG

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Introduction - Professional dog breeders frequently request elective Cesarean sections in bitches with histories of uterine inertia. Properly timed elective Cesarean sections reduce the risks of intrapartum fetal asphyxia and injury. However, without a known date of the ovulatory LH surge, elective Cesarean sections often result in the delivery of preterm fetuses that rarely survive or require elaborate neonatal support. The goal of this pilot study was to evaluate the fetal lung development and surfactant production between 59 and 62 days after the LH surge.

Materials and Methods - Four pregnant bitches with synchronized LH surges were used. One bitch was anesthetized and prepared for ovariohysterectomy on each of the 59th to 62nd days following their respective LH surges. The reproductive tracts of all bitches were excised in toto. Amniotic fluid was aspirated from each gestational sac and a foam stability test was performed on each amniotic fluid sample separately. Briefly, 1 ml of 95% ethanol and 1 ml of amniotic fluid were added to a glass tube and vigorously shaken for 15 s. If a concentric ring of bubbles was present at the air-fluid interface 15 min later, surfactant concentration was deemed sufficient for extra-uterine life. Complete blood cell counts and serum chemistry panels were performed on blood samples collected from the umbilical cords of all pups used in the study. Fetuses were then euthanized via intracardiac injection of pentobarbital (0.5 ml). Fetal body and lung weights were recorded.

Results - Results from the foam stability test suggest that female and male fetuses ≥61 and ≥62 days past the LH surge respectively, have sufficient surfactant to support extra-uterine life. From day 59 to 62 the mean fetal hematocrit and plasma protein concentration decreased from 65 to 54.5% and 3.7 to 3.45 g/L, respectively. The lung:body weight ratio remained unchanged at 0.3.

Discussion - A practical test for the presence of fetal surfactant in the amniotic fluid of dogs was established. Using this qualitative assay designed for premature babies, canine fetuses do not possess adequate surfactant levels for extra-uterine life prior to 62 days past the LH surge. Sex differences observed in this study correspond to reports in other species indicating that premature female fetuses are capable of surviving in an extra-uterine environment at an early gestational age than their male counterparts. Further studies are necessary to compare the results of this test with actual neonatal viability and survival of pups delivered by elective Cesarean sections. Further studies are also required to develop a harmless technique of collecting amniotic fluid from unborn puppies before these findings can find practical application for the timing of elective Cesarean sections in dogs. Transabdominal, ultrasound-guided needle aspiration may provide such a technique.

References

ACID-BASE EVALUATION OF CANINE NEONATES BORN UNDER DISTINCT OBSTETRIC CONDITIONS

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Introduction - Studies accomplishing canine neonatal blood gases and acid-base are scarce in specialized literature comparing to Human Neonatology research. The importance of such knowledge consists on establishing physiological values aiming medical intervention and ultimately, help to reduce neonatal mortality. Maternal or fetal dystocia are the main reasons for the high index of neonatal mortality (3). Prolonged parturition or dystocia cause direct alterations mainly represented by fetal distress or injuries. Important consequences can be attributed to dystocia: fetal tachycardia or cardiac arrest; reduction or absence of fetal movements and tonus; hypoxia by placenta blood flow compromise; metabolic acidosis due to reduced blood oxygenation and consequently acid lactic production. Moreover, administration of oxytocin during maternal dystocia may compromise fetal well-being due to maternal hypotension and increased fetal stress. The aims of the present study were to evaluate canine neonatal acid-base balance during eutocia and verify the consequences of dystocia treated medically by oxytocin administration and assisted through manipulative management or cesarean section.

Material and methods - Forty eight neonates of different genders and breeds were allocated into 3 groups according to the obstetrical condition: eutocia (group 1; n=27), manipulative obstetric assistance or cesarean section (group 2; n=10) and ecbolic treatment with oxytocin administration (group 3; n=11). Immediately after birth, at 5 and 60 minutes postnatal, puppies were submitted to a clinical evaluation by Apgar scoring (respiratory effort, muscle tone, heart rate, irritability reflex and mucous color). Venous blood samples were collected from jugular vein immediately after birth and 1 hour later for acid-base evaluation with the use of the i-STAT (Abbott®) device which measures: pH, PO2 (mmHg), PCO2 (mmHg), HCO3 (mmol/L), BE (base excess-mmol/L), TCO2 (mmol/L), anion gap (mmol/L) and SO2 (%). Data were analyzed using a repeated measures analysis of variance (ANOVA) and the post hoc Newman-Keuls comparisons were conducted to establish overall differences among groups at p<0.05.

Results - Apgar score was statistically lower at birth than the other periods of evaluation (5 and 60 minutes), regardless of the obstetrical condition. Comparing the results among groups, puppies born through obstetrical assistance or C-section showed significantly lower Apgar score at birth. However, no difference was registered among groups at 5 and 60 minutes after birth. Although Apgar score is a subjective assessment, it can be a practical parameter to recognize neonates under serious illness and to formulate therapeutic interventions. Analyses of neonatal blood gases can provide more reliable information of critically ill patients. During parturition, uterine contractions and abdominal straining decrease uterine blood flow, thereby causing fetal hypoxia. Low concentration of blood oxygen leads to anaerobic metabolism and hypoxic lactic acidosis (4, 1, 2). Indeed, hypoxemia was observed at birth in all newborns and this low oxygenation statistically worsened after 1 hour. Puppies of Group 3 presented a significantly higher PO2 at birth compared to groups 1 and 2. The reflex hyperventilation triggered by hypoxia helped to eliminate dioxide carbon delivered to the lungs, balancing acid-base rate. All neonates showed metabolic acidosis at birth, however statistically lower BE values were verified in puppies born under therapeutic approach (groups 2 and 3). The reliability of the BE results allows for the statement that the whelping condition adversely
affects neonatal acid-base balance. After 1 hour of birth, a significant improvement was observed for pCO₂, BE and pH. Nevertheless, based on BE values, neonates remained under metabolic acidosis even after 1 hour of birth, regardless of the obstetrical management. On the other hand, after 1 hour, pH and pCO₂ data were considered consistent to neonatal normal range. Antagonistic results among BE and pH/pCO₂ are attributed to venous samples, in which metabolic acidosis can only be differentiated by BE values. To clarify such condition, future studies should be undertaken in order to evaluate the reliability of arterial blood samples. Despite the stated injury related to oxytocin therapy in canine dystocia, the present work showed no additional damage caused by this ecbolic treatment. No acid-base disorders were exclusively attributed to oxytocin that wasn’t verified in the other dystocia group. Hence, oxytocin administration to maternal dystocia can be safely instituted, unless fetal distress has been previously diagnosed.

**Conclusion** - Canine neonates born under abnormal parturition present a severe metabolic acidosis and this acid-base condition remains for an additional hour. Thus, it is recommended that neonates born through manipulative or surgical treatment receive special attention to their vital parameters and eventually be subjected to intensive care. Moreover, oxytocin treatment does not worsen a dystocia complication and can be safely employed.

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**References**

AN EXPERIMENTAL MODEL TO STUDY RESISTANCE INDEX AND SYSTOLIC/DIASTOLIC RATIO OF UTERINE ARTERIES IN ADVERSE CANINE PREGNANCY OUTCOME

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Introduction - Doppler ultrasound has been a satisfactory and valuable method for evaluation of fetal condition during pregnancy in many species [2,6,8]. Uterine blood flow can be assessed by the resistance index (RI) and systolic/diastolic ratio (S/D) of the uterine arteries using Doppler ultrasound. Resistance index, also known as Pourcelot’s ratio, is defined as (S-D)/S where S is the peak systolic velocity and D the end diastolic velocity while S/D ratio is S/D [3]. Both RI and S/D progressively decrease throughout normal gestation [2,3,6,8]. In pregnant women, increasing RI and S/D indicate a high risk of adverse obstetric outcome [3,9]. Similar data is missing in veterinary medicine where no Doppler ultrasound indicators of potential abortion have been yet identified in dogs [4,5]. The aim of this study was to describe the RI and S/D of the uterine arteries in an experimental model of abnormal canine gestation pharmacologically induced.

Materials and methods - For this purpose, 16 cross and pure-bred pregnant (30-35 days from first mating) bitches, were randomly assigned to one of the following treatment groups: medicated group (MG; n = 8) which was administered a cabergoline and cloprostenol protocol to interrupt gestation [1] and a control group which did not receive any drug (CG; n = 8). Color and pulsed-wave Doppler examinations (Toshiba Core Vision Pro, Shimoishigami, Otawara-Shi Tochigi-Ken-Japan, 8-MHz linear-array transducer) of uterine arteries were carried out before the initiation of the study and then every other day up to abortion or parturition. In all the cases pregnancy and pregnancy termination were ultrasonographically diagnosed [4]. Resistance indexes and S/D of the left and right uterine arteries, before and after treatment, were compared between groups using T test. To further characterize results, a correlation analysis was carried out between days to abortion or parturition and both RI and S/D in the MG and CG, respectively (SPSS®, Chicago, USA, 10.01). P < 0.05 was considered significant.

Results and discussion - There were no RI nor S/D differences between left and right uterine arteries neither in the groups (CG vs. MG; P > 0.6 and P > 0.4) nor the periods (before vs. after treatment; P > 0.2 and P > 0.3) of the study, therefore results were calculated including both arteries. Pre treatment RI did not differ between CG and MG (0.57 ± 0.0 vs. 0.53 ± 0.0; P > 0.2). Neither did S/D (2.54 ± 0.3 vs. 2.22 ± 0.1; P > 0.1). All MG (8/8) but none (0/8) of the CG bitches terminated their pregnancy by abortion 6 ± 1.2 days after medication with a range of 2 to 12 days. Post treatment RI of the MG and CG were 0.62 ± 0.1 and 0.53 ± 0.1 (P < 0.01), respectively. Post treatment S/D of the same groups were 2.96 ± 0.9 and 2.23 ± 0.3 (P = 0.01). Correlation between days to abortion or parturition and RI were 0.75 (P < 0.01) and - 0.78 (P < 0.01) for the MG and CG, respectively. The same correlation for the same groups were 0.79 (P < 0.01) and - 0.73 (P < 0.01) for S/D. As expected, this pharmacological protocol was useful to develop an experimental model of impending abortion as all medicated animals terminated their pregnancy. The similarity of RI and S/D between left and right arteries was also expected and probably due the even distribution of fetuses in the uterine horns [4]. In the present study, RI and S/D of uterine arteries progressively decreased to parturition and increased to abortion. Abnormal trophoblastic invasion of the hemochorial...
placenta has been described as the cause of increasing RI and S/D in women [3,9]. In spite of placental differences, increasing RI and S/D may also reflect histological abnormalities of the endotheliochorial carnivore placenta. In women, it has also been suggested that uterine blood flow increases with rising concentrations of serum progesterone [7]. Moreover, administration of oral progesterone was followed by a significant decrease in spiral arteries RI in one study [3]. Therefore, in the present canine model increasing RI and S/D could have been provoked by the pharmacologically induced progesterone deprivation [2]. In line with human obstetrics [3,9], in dogs, increasing RI and S/D seem to be predictors of adverse obstetrics outcome. If this is probed many cases of abnormal gestation could be diagnosed and prevented before other ultrasound signs appear. Further work on spontaneous abnormal gestation cases is still needed to confirm these initial experimental findings.

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References

A MULTI-CENTRE CLINICAL TRIAL EVALUATING THE EFFICACY AND SAFETY OF ALFAXAN® ADMINISTERED TO BITCHES FOR INDUCTION OF ANAESTHESIA PRIOR TO CAESAREAN SECTION

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Introduction - Alfaxan® (Jurox Pty Ltd, Rutherford, NSW) is an injectable formulation of the neuroactive steroid anaesthetic drug alfaxalone (10 mg/mL), which has been formulated for use in small animal anaesthesia. This investigation was a special population study designed to corroborate the clinical efficacy and safety of Alfaxan® used by veterinary clinicians in Caesarean section bitches under practical, clinical conditions. The study was a prospective, multi-centre, positive-controlled, randomised efficacy study involving two parallel groups: 1) Alfaxan® and, 2) Rapinovet X® (propofol 10 mg/mL, Schering-Plough Animal Health, North Ryde, NSW, Australia).

The study was conducted according to Good Clinical Practice guidelines (VICH GL9 (GCP) June 2000) and with Animal Ethics Committee approval (No. JAEC/05/012).

Materials and methods - Bitches satisfying the inclusion criteria were enrolled in the study. A total of 74 bitches were enrolled in the study with 48 and 26 receiving Alfaxan® and Rapinovet X®, respectively, for induction of anaesthesia. Individualized anaesthetic plans were developed by each Investigator based on consideration of the health of the bitch according to the American Society of Anesthesiologist’s Physical Status Classification. No premedicants were administered. Anaesthesia was induced in each bitch by intravenous injection of the selected agent. All bitches were intubated and maintained on isoflurane and oxygen. Physical parameters of each bitch were assessed prior to, during and after anaesthesia. The survival and vigour of pups delivered by Caesarean section were also assessed. Variables were described using parametric descriptive statistics. Comparisons between groups were made using Student’s T-test. Associations were evaluated using linear or logistic regression where appropriate. All statistical procedures were performed in Microsoft Excel or SAS for Windows, version 8.02 (SAS Institute Inc, Cary, NC, USA).

Results - The average induction dose of Alfaxan® was 1.9 mg/kg body weight. Immediate post induction apnoea, defined as a cessation of breathing for 30 seconds or greater, occurred in 25% and 15% of Rapinovet X® and Alfaxan® cases, respectively. Cardiovascular and respiratory parameters were well maintained during the induction, maintenance and recovery periods for both treatment groups.

Anaesthetic induction was subjectively scored for each bitch as: 1 (unacceptable where intubation was not possible even with additional induction agent), 2 (acceptable when induction agent in excess of maximum calculated dose was required, or intubation was difficult, with a large amount of jaw tone) or, 3 (excellent where smooth loss of consciousness occurred and the dog was readily intubated after administration of no more than the calculated induction dose). The average quality of anaesthesia was numerically superior during induction and anaesthesia with Alfaxan® compared to Rapinovet X®. Ninety-eight (98) and 81% of
bitches scored a top score of 3 for induction and anaesthesia with Alfaxan®, respectively, while only 89 and 65% scored a 3 for the same parameters with Rapinovet X®.

Anaesthetic recovery was subjectively scored as: 1 (poor, where a rough recovery with vocalization, opisthotonos and/or clonic-tonic seizures occurred), 2 (fair, where there was paddling or thrashing when moving), 3 (good where smooth recovery with only minor paddling or tremors was experienced and, 4 (excellent, where there was a completely smooth recovery. Average scores for recovery were similar between the two treatment groups with 96% of bitches scoring a good or excellent rating. Bitches tolerated a number of concurrent medications throughout the peri-operative period. No bitch fatalities were observed in this study.

There were no statistically significant differences between treatment groups for the puppy variables; however, puppy vigour scores for Alfaxan® were numerically superior versus Rapinovet X®. For puppy vigour scores the four graded categories (Alfaxan® vs Rapinovet X®) were: 1) limb withdrawal reflex (96 versus 93%), 2) sucking reflex (94 vs 84%), 3) anogenital reflex (83 versus 81%) and flexion reflex (90 versus 83%). The reflexes were scored as present or absent (+/-) in each puppy. Puppies born by Caesarean section to bitches having been administered Alfaxan® or Rapinovet X® had similar survival rates 24 hours after birth (i.e. 97 and 98%, respectively).

This study confirms the safety and efficacy of Alfaxan® for the purpose of anaesthetic induction for Caesarean section in the bitch. In addition, Alfaxan® had a negligible effect on the neonate with > 95% of puppies alive 24 hours after the bitch had recovered from anaesthesia with Alfaxan® induction.
INTEREST OF ELECTIVE CAESAREAN SECTION ON NEONATES’ SURVIVAL RATE

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Introduction - Dystocia is a common complication of parturition in the bitch. Most of the time, dystocia requires an emergency surgical treatment in order to save the dam and the puppies. Nevertheless, according to the literature and despite adequate management, up to 12.7% of mortality is reported among newborns. Emergency C-section is thus an important cause of financial losses for the breeders. Also parturition is an important physiological and stimulatory process for the fetus and the bitch to undergo and routine circumvention of vaginal birth is not recommended, early planning of the C-section for bitches of high risk of dystocia may be beneficial to reduce neonates’ death. The purpose of this study was to evaluate the interest of early planning of parturition and C-section with regard to mortality of pups at birth.

Material and methods - Sixteen bitches were included in the study: 8 bitches of high risk of dystocia that were prepared for elective C-section; and 8 bitches randomly chosen among bitches submitted to emergency surgery during the same period (March to November 2007). The risk for dystocia was considered relevant for single puppy syndrome, brachycephalic breeds and bitches previously submitted to a C-section with high proportion of neonates’ mortality. The ovulation timing was precisely determined (blood progesterone) for all the bitches that were submitted to elective C-section. Ultrasound examinations were performed by day 59-63 to evaluate the vitality of the fetuses and to predict the parturition day. Induction of the parturition was obtained with 15 mg/kg (SC) of aglepristone and the surgery was performed 18 hours later, before uterine contractions take place. After preoxygenation of the dam, the anesthesia was induced with propofol and was maintained with isoflurane. An antibiotic treatment (30 mg/kg cephalexine, IV) was initiated at time of induction. The C-sections were performed routinely as fast as possible. Neonates’ resuscitations were performed immediately. The dam and the puppies were discharged as soon as possible with antibiotics (cephalexine 20 mg/kg BID for 6 days) and metoclopramide (1 mg/kg/d for 3 days). For each dog, neonates’ mortality and complications were reported.

Results - Early planning (day 59-63 post ovulation, mean 61.1±1.3) of the c-section was associated with no sign of fetal distress (ultrasound examination), no fetal death during the surgical procedure and a higher survival rate (34/37 living pups 15 days after the surgery vs. 25/40 for emergency C-section group).

Discussion - According to these preliminary results, planning C-section is safe and can be beneficial to reduce neonates’ death. Nevertheless, planning C-section should only be recommended for bitches with high risk of dystocia because parturition is important to facilitate attachment of the dam to the puppies. Moreover, the stress and the pain may interrupt the milk production. Timing of surgery is the key-point of planned C-section. Indeed, fetus viability has to be access carefully before induction of the parturition. Precise determination of the timing of ovulation is thus beneficial to avoid premature surgery and to reduce pups’ mortality. Aglepristone is a competitor of progesterone and allows inducing parturition and final maturation of the fetus. According to this preliminary study, the use of aglepristone leads to satisfactory results and may help the breeder in obtaining the support of
a competitive and larger team to take care of the dam and the pups during and just after the surgery.

**Conclusion** - Planning elective C-section with aglepristone seems to be a safe procedure which could be developed in the future. Our results may be of great help for the practitioners to avoid dystocia in bitches. However, this procedure should be reserved only to the bitches with high risks of dystocia.
DEFICIENT DIESTROUS PERIOD IN BITCHES WITH LONG-TERM IMPLANTATION OF GNRH AGONIST DESLORELIN

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Objectives - The study aimed to investigate the duration of diestrus from the serum progesterone level in bitches following proestrus and estrus induced by subcutaneously implantation with GnRH agonist, deslorelin in two patterns; short-term and long-term implantation.

Materials and methods - Dogs used in the experiment were 9 healthy and fertile female beagles, 3-7 years old and weight 12-16 kilograms with normal reproductive status. During anestrus, dogs were divided into 3 groups. Gr 1 (n=3), dogs were subcutaneously implanted with 4.7 mg GnRH agonists, deslorelin and removed on day1 at the stage of estrus. Gr 2 (n=3), dogs were subcutaneously implanted with 4.7 mg GnRH agonists, deslorelin and removed at the end of the experiment. Gr 3 (n=3), dogs were let into estrus naturally for the confirmation of normal reproductive cycle of the dog in the experiment. All dogs were investigated for estrus signs, behavior changes, physical appearance, vaginal cytology and serum progesterone everyday following the appearance of proestrous signs. Then, serum progesterone levels were examined once a week during diestrus. The diestrous period was defined when serum progesterone was higher than 1.5 ng/ml.

Results - dogs in group 1 and 2 processed into proestrus and estrus shortly after GnRH agonist implantation. The durations of diestrus from dogs in group1 and 3 were in the range of normal diestrous interval in bitch (58.3±1.5 and 61.3±0.6 days) while dogs in group 2 had shorter diestrous period (32.0±16.5 days).

Conclusions - the implantation of 4.7 mg GnRH agonists, deslorelin can induce estrus in bitches. When the implants were removed at the first day of estrous stage, the bitches processed estrus cycle and diestrous stage as in normal estrous cycle. However, when the implants were remained for a longer time, the diestrous period became significantly shorter. The result revealed that if the bitches were bred and fertilized at the induced estrus in the long-term GnRH agonist implantation, the deficient diestrous interval may result in the pregnancy failure which may benefit the purpose of the GnRH agonist long-term implantation for contraception.
ULTRASOUND AS A MECHANICAL METHOD FOR MALE DOG CONTRACEPTION

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Introduction - Ultrasound (US) is an acoustic vibration, with a frequency greater than the upper limit of human hearing. US waves when applied to a tissue, propagate through it with a progressive loss of energy, inducing a tissue heating (1). It is well known that heat affects spermatogenesis by reducing sperm production (2). As hot water (60°), infrared and microwave, US has been used to suppress spermatogenesis in several animal species thanks to the heat production. Although, compared with other techniques, the latest was found to be more effective at lower temperature because US combine both thermal and mechanic effects (3). With the aim to apply a non-invasive technique for male dog contraception, we investigated the effects of testicular US application either on testicles modification (size, consistency, and volume) as well as on sperm cell concentration and motility.

Materials and methods - Five clinically health mixed breed male dogs, ranged from 2 to 5 y.o. and 23.2±3.7 kg, b.w., were used in this trial. All of them were known to be of proven fertility. Both testis of each subject, were exposed to 1,5 watt/sq cm of US (Vetri-son Portable, Physiomed® Elektromedizin AG, Germany; 2.5 cm² transducer, 1 MHz) for 5 minutes each, in alternate day for one week. To facilitate US penetration into underlying tissues, scrotum hairs were clipped and a water soluble US gel was spread over the skin just before placing the probe on gonads surface. Morphological evaluations were made before and two weeks after the end of treatments. Size and testicular consistency, performed by digital palpation before the first application, resulted to be within a physiological range. Length (L) and testicular width (W), were echographically measured (Sonomed Concept/MCV; 6.5 MHz mCX, USA) to calculate the volume according to the formula for a prolate spheroid: L x W² x 0.52 (4). Semen was collected by digital manipulation in the presence of a teaser bitch and the second sperm-rich fraction of the ejaculate was examined, within 1 hour, by using an integrated visual optical system for semen analysis (IVOS Version 12.2; Hamilton Thorne Biosciences Inc., Beverly, MA, USA). A sperm drop (4 µl from sample at 37°/30min) was mounted on a 4 chamber Leja® slide at 37°C and six different randomly microscopic fields were counted. Each sample was measured for sperm concentration and for percentage of total and progressively motile sperms. Data concerning testicular volume were statistically analyzed with “Wilcoxon matched pairs signed rank sum” test and considered significant for P≤0.05.

Results and Discussion - In all patients no remarkable local or systemic adverse effects and no skin burns occurred, but after US treatment, testicles, at palpation, exhibit marked tenderness and decreased size. The ultrasonographic evaluation showed a statistically significant reduction of the volume of both testis (left 9.2±3.8 vs 5.5±3.8/cm³; right 8.7±2.5 vs 3.6±1.3/cm³; P≤0.05). Before the US treatment, mean volume of ejaculates was 12±4.5 ml, sperm concentration was 301.9±28.8 x10⁶/ml with an average percentage of total and progressively motile sperms of 87.2±5.5 and 58.4±7.7, respectively. Semen analysis, performed two weeks after ultrasonic applications, showed a zero sperm count. Classical neutering in male dogs has been obtained through surgical methods of sterilization (orchiectomy and vasectomy); even though permanent sterilization is desired, surgical methods can be expensive and may cause post-surgical complications. In addition, many owners are reluctant to consider surgery (5) and feral dog populations are still growing (6). Currently, several safe, reliable and reversible alternatives to surgical sterilization are being investigated. Hormonal
treatments, immunocontraception, intratesticular or intraepididymal chemical injections (7, 8), were already tested. In this trial, dogs treated with three applications of US at 1.5 watt/sq/cm² showed a significant decrease of the testicular parenchyma and a suppression of spermatogenesis without any systemic or topical adverse effects, providing a suitable alternative method for a controlled dog contraception. Moreover the findings of this work will form the basis of future investigation on the deep effects and mechanism of Ultrasound on male reproduction.

References

PYOMETRA AND CYSTIC ENDOMETRIAL HYPERPLASIA IN BITCHES

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Pyometra is a polysystemic disease of old bitches in diestrus, which it’s clinical and pathological signs becomes to apparent in other organs in addition to uterus. Eventually, the first factor is defect on estrogen and progesterone metabolism by uterus.

During 5 years ago 28 bitches (5-10 years old) were referred with dehydration, depression, polydipsia and palpable uterus enlargement. All bitches except 9 had vaginal discharges. Some of them had fever and some had temperature lesser than normal. By experiments such as physical examinations, C.B.C, vaginal cytology, radiology and ultrasonography, pyometra was distinguished. Ovariohysterectomy were accomplished for all. In histopathologic examinations on 21 samples cystic endometrial hyperplasia were found.

With correct hygiene management and absence use of old bitches on breeding we can be able to decrease this disease.

References

MANAGEMENT OF ENDOMETRITIS/PYOMETRA IN DOGS FOR PRESERVING BREEDING CAPACITY

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Introduction - Inflammatory uterine diseases – endometrium/pyometra (E/P) in bitches are hormonally mediated, multifactor diseases which may cause the end of breeding ability. The pathogenesis of E/P is not absolutely clear, but predisposing factors - middle age, luteal phase, abnormal uterine response to progesterone (P4) and bacterial infection- considered being responsible for diseases (4.). Ovariohysterectomy is the most often applied treatment leads to interruption of reproduction and genetic line. In the last few years several new medicines and treatment protocols appeared in the veterinary practice (1, 2).

Objectives - Our aim was to develop a case-specific surgical, hormonal or combined therapy of E/P and to establish a breeding protocol for following heating period, to maintain breeding potency in genetically valuable dogs. Our hypothesis was, to get similar pregnancy rate applying more invasive (surgical-hormonal) treatment protocol in serious cases (pyometra) and less invasive (hormonal) in mild endometrial inflammations (endometritis).

Material and methods - During four years (2004-2007) 35 breeding bitch (average: 5,2 years old) with E/P were diagnosed at our clinic. Only 18 bitches were selected to the treatment based on following criteria: good general condition, less than 7 years age, intact renal and hepatic function. Patients were divided two groups according to the general symptoms, ultrasonic examination (1.) and laboratory results: Group 1. (G1): pyometra: echogen uterine picture with enlarged horns (lumen diameter exceeds 1cm). Group 2. (G2): endometritis: echogen uterine picture with less than 1cm uterine lumen vaginal discharge and elevated white blood cell rate. In G1. (n=9) a surgical-hormonal combined therapy was used to prevent more serious general symptoms and local degeneration of endometrium by purulent contents. Under general anaesthesia {premedication: 0,04mg/BW Fentanyl inj. (Richter Gedeon , Hungary) + Midazolan 0,5mg/BW (Dormicum inj. EGIS Hungary) with inhalation narcosis (Isofluran CP, CP-Pharma Germany) median laparotomy was performed. Uterine horns were elevated from the abdomen, sectioned in 0,5cm close to the bifurcation and flushed by 1% Polykrezulen solution (Vagothyl 50, POLFA, Poland) and 1% Povidon-iodine solution (Betadine , EGIS Hungary) via 22G venous catheter inserted in the apex of the uterine horns. Abdomen was closed in a routine way. Post-operative therapy: Aglepristone (Alizine inj VIRBAC France) 10mg/BW on day 1 and day 2 (2.). Amoxicillin+clavulanic acid 1ml/20kg/day (Synulox inj. Pfizer) was used for antibiotic therapy. From 3rd day Prostaglandin F2α 0,15mg/BW/day (Enzaprost 25 inj, CEVA-Phylaxia, Hungary) and Cabergolin 5µg/BW (Galastop, CEVA-Phylaxia) were applied for 7 days to stimulate luteolysis. In G.2 (n=9): only the hormonal therapy (see post-operative therapy) was used for 10 days. Patients were controlled by ultrasonic examination at 10th day and 4 weeks later. At the next oestrus bitches were inseminated artificially with diluted semen (Triladyl canine, MINITÜB, Germany) to minimise the risk of bacterial contamination. Optimal time of AI was determined by vaginal cytology, vaginoscopy and measuring the serum P4 level. Antibiotics were given for 7 days according to the result of bacterial culturing from vagina in prooestrus.

Results - Clear, small amount vaginal discharge but no echogen contents in uterus were found by ultrasound at 10th day after the onset of treatment. From the treated patients 13 bitches (G1: 7, G2: 6 bitches) were inseminated at the following oestrus. Fertility results were in G1:
4 pregnant (57.1%) 1 non-pregnant, and 2 dogs recurrence of pyometra G2: 4 pregnant (66.6%), 1 non-pregnant and 1 bitch had pyometra again. In both group number of newborn pups ranged from 3 to 8 (average: 5.8).

**Discussion** - During four years approximately 50% of the E/P diagnosed breeding dogs were sufficient for hormonal or surgical-hormonal combined therapy. The average pregnancy rate was 61.5% after treatment. There were no significant differences in pregnancy rate between two groups (G1:57.1%, G2: 66.6%). No recurrence of E/P was found in whelped bitches at following cycles. These pregnancy results are comparable to others found in the literature. (3.). These results, although based on limited number of cases, indicate that surgical, hormonal or combined therapy and “low-bacterial contamination” breeding protocol produce 61% pregnancy rate after E/P.

**References**

LACTOFERRIN IN THE DOG: A PYOMETRA STORY

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Introduction - Lactoferrin is an 80 kDa glycoprotein belonging to the family of non-heme iron-binding monomeric glycoproteins (7). The predominant cell types involved in a lactoferrin synthesis comprise a secretory epithelia and myeloid cells including neutrophils (1). Plasma lactoferrin concentrations may or may not correlate with the neutrophil count (7), depending on the extent of degranulation and perhaps the contribution of other organs. Lactoferrin plasma levels change during pregnancy, and vary also with the menstrual cycle and increase during infection or inflammation (3). Lactoferrin has many functions (4;7) including effects on the immune system. Lactoferrin affects both proliferation and differentiation of immune cells through its ability to modulate cellular signaling pathways. However, there are conflicting data regarding to its effects on lymphocyte proliferation. While some (5) report stimulatory effects, the others (8) suggest an inhibitory role. It was generally accepted that dogs produce very limited amount of lactoferrin (if any). In 2007, the lactoferrin was visualized in dog neutrophils using flow cytometry (9), simultaneously it was isolated from the neutrophils and its high resembling properties with human lactoferrin were demonstrated (2). Pyometra is a serious disease syndrome affecting mainly older nulliparous bitches in the luteal stage of the sexual cycle and is associated with neutrophilia and impaired some immune functions including activity of lymphocytes (6). A wide range of factors including lactoferrin has been proposed to explain this inhibition.

Aim - To measure lactoferrin concentration in the dog and analyze its possible role in a suppressed lymphocyte activity in bitches with pyometra.

Materials and methods - Samples were: sera from 18 bitches with pyometra before and 10 of them as well 7th day after ovariohysterectomy and antibiotic treatment, 38 healthy dogs (formed control group altogether) from which were 6 pregnant and 20 non-pregnant bitches, and 12 male dogs to see the influence of the gender itself and pregnancy respectively. Sandwich ELISA was created to measure lactoferrin in dogs’ sera (Maxisorp plate from Nunc, Denmark, anti-human lactoferrin from Sigma conjugated and non-conjugated with horseradish peroxidase, human lactoferrin from Sigma as standard). The neutrophil counts were determined and lymphocyte transformation test performed in all sera samples. The neutrophil counts were correlated to both lymphocyte activity and lactoferrin levels. The ability of sera from bitches with pyometra to decrease lymphocyte activity was proved by adding these to lymphocytes from healthy bitches in this test as well. The influence of lactoferrin on lymphocyte activity was checked by precipitating serum lactoferrin with its antibody (Sigma) and lymphocyte activites were compared.

Results - The average serum lactoferrin concentration in bitches with pyometra was 47.82 ± 11.69 ng/ml which was significantly higher (p<0.001) than in the control group (14.44 ± 2.0 ng/ml). 10 bitches with pyometra that were checked before and after therapy showed significant (p<0.05) decrease from 32.59 ± 9.46 ng/ml to 26.84 ± 8.29 ng/ml in7 days whereas after therapy the concentration was statistically comparable (p>0.05) to the control group. As for differences within the group of healthy adult dogs, although the results seem to be different in each group (male dogs: 16.68 ± 3.90 ng/ml, non-pregnant bitches: 11.26 ± 2.34
ng/ml, pregnant bitches: 22.33 ± 8.09 ng/ml), none of these differences was proved as significant (p>0.05). According to Spearman’s correlation, lactoferrin demonstrated to be in no relationship neither to neutrophil counts (p>0.05; r = 0.2279) nor to the lymphocyte activity (p>0.05; r = -0.1658). Capability of sera from bitches with pyometra of suppressing lymphocyte activity was proved (p<0.001), although the rate of this suppression shows no correlation to lactoferrin levels (p>0.05; r = 0.1991). The suppressive influence of these sera remained the same (p>0.05) even after removing lactoferrin using its antibody.

Conclusion - Although serum lactoferrin levels in bitches with pyometra are increased, in contrast to previous hypotheses, lactoferrin seems to play no role in suppression of lymphocyte activity that is connected with pyometra in the bitch.

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References

GENE EXPRESSION OF PROSTAGLANDINS PRODUCTION ENZYMES IN PYOMETRA AND IN NORMAL DIESTROUS AND ANESTROUS ENDOMETRIA.

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Introduction - Cystic endometrial hyperplasia-complex is the most frequent endometrial disorder in the bitch. Gram negative are the most frequent bacteria isolated in cases of pyometra and are associated with more and severe systemic signs. Endotoxins strongly stimulate prostaglandin release. Synchronized up-regulation of COX-2 and mPGES, together with increased production of PGE2 has been noted in response to proinflammatory stimuli, including LPS. Measurement of the PGF2α metabolite allows for the differentiation between pyometra and CEH cases (1).

Objectives - The aim of this study was to evaluate the gene expression of PG production enzymes in the endometrium of anestrous (n=6) and diestrous uteri with (n=8) or without pyometra (n=7).

Materials and methods - Uteri were collected during routine OVH and uterine contents were cultured. Phase of estrous cycle was determined through the measurement of plasmatic progesterone concentration, observation of ovarian structures and recorded estrous date. Gene expression of COX-1, COX-2, mPGES-1 and PGFS were analysed by relative real time PCR and normalized with the housekeeping gene ß2MG.

Results - All selected cases of pyometra were hyperplasic and E. coli was the only isolated bacteria. Anestrous and diestrous uteri did not present signs of CEH and were negative for bacteriology. COX-1 gene expression did not differ among groups (p>0.05). COX-2 gene expression was 19 and 28 times higher in pyometra cases than in diestrous and anestrous endometria, respectively (p<0.001 and p<0.002). The gene expression of mPGES-1 was 9 times higher in pyometra cases compared to normal uteri (p< 0.02). PGFS gene expression had a 3 and 600 fold increase in pyometra cases compared to normal diestrous and anestrous endometria, respectively (p< 0.02 and p< 0.001). No difference was observed between normal diestrous and anestrous endometria (p>0.05). This study suggests that in the bitch, the up-regulation of COX-2 and mPGES in the endometrium is associated with endotoxin in pyometra caused by E. coli. In addition, the observed increase in PGFS expression might be associated with a higher systemic PGFM concentration as previously reported.

This work was supported by CIISA 74 – Endometrial Hyperplasia.

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REPRODUCTIVE TUMORS IN TWO OVARIOHysterECTOMIZED DOGS

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**Case 1** - A 16-year-old, spayed, poodle bitch weighing 4.1 kg, was presented for investigation of attractiveness to male dogs, allowance to mate and difficulty in defecation. The bitch had been spayed when it was 10 year-old and had not had clinical signs until a year prior to presentation. On clinical examination the bitch was normal other than vulvar swelling. Complete blood count and blood biochemistry demonstrated leukocytosis and uremia. Vaginal cytology revealed a > 80% degree of vaginal epithelial cornification. Abdominal ultrasonography showed an irregular shaped cauda-abdominal mass and two masses with anechoic areas next to each kidney. A midline exploratory laparotomy was performed and a uterine mass with both ovaries were identified and excised. Histopathologic diagnosis of the excised tissues was uterine leiomyoma, cystic left ovary and papillary cystic adenoma of the right ovary. Six months later, the owner reported that the bitch was clinically healthy with no complaints.

**Case 2** - A 10-year-old, spayed Cocker spaniel cross-bred bitch weighing 17 kg, was presented with a six months history of vulvar bleeding, perineal swelling and tenesmus. The bitch had undergone elective ovariohysterectomy about two years before the examination. Accompanying complaints were vomiting, polyuria and polydipsia. Clinical symptoms included bilaterally symmetrical alopecia of the trunk, vulvar bleeding and a firm, non-painful round ball-shaped mass of tissue, as big as a tennis-ball in the dorsal aspect of the vulva. The dog had anemia and a normal serum biochemistry profile. Abdominal ultrasonography demonstrated a mass with anechoic spots next to the caudal pole of the right kidney and a thickened uterine stump. The remnant ovary and the uterine stump were identified and removed via a midline laparatomy. An episiotomy was performed and the vaginal mass was excised. All the excised tissues were submitted for histopathological examination which revealed vaginal transmissible venereal tumor and metritis. No ovarian pathology was determined. The bitch recovered well and was sent home after the surgical procedure.

**Discussion** - Development of reproductive tumors after an elective surgery is extremely rare. In the literature, a few cases of ovarian granulosa cell tumor and uterine adenocarcinoma has been previously reported in ovariohysterectomized dogs and cats (1, 2, 3). As in current cases, remnant tissues may affect the life quality of an animal. Therefore, in ovariohysterectomy, sufficient abdominal wall incision, proper ligation and total removal of the ovaries and the uterus have crucial importance in prevention of development of neoplastic or non-neoplastic conditions.

**References**

FIRST REPORT OF VAGINAL PROLAPSE IN A BITCH TREATED WITH ESTROGEN

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Introduction - Indications for estrus induction in the bitch include missed breeding opportunities or conception failure, the treatment of primary or secondary anestrus, synchronization of ovulation for embryo transfer programs [7] and also the treatment of hypogonadal obesity and hormonal urinary incontinence in bitches [1]. The administration of estrogens, either synthetic or naturally occurring, has been used to induce estrus in animals that are anestrus, especially in the bitch.

In spite of this therapeutic effect of estrogen, some adverse effects such as vaginal discharge, [5] pyometra and infertility [3] have been documented after administration of estrogen. While a search of the available literature failed to reveal any report of type III prolapse caused by estrogen administration to induce estrus in bitch.

Case presentation - An 18 month-old intact bitch, weighing 40 kg was referred for evaluation of a mass protruding from the vulva. The mass had become apparent 1 day after administration of estrogen [Estradiol benzoate, (0.3 mg.kg\(^{-1}\), IM)] to terminate prolonged anestrus. On the basis of the clinical findings, the bitch was diagnosed with type III vaginal prolapse and it was elected to perform an ovariohysterectomy (OHE) followed by resection of the protruding vaginal mass. During celiotomy, no signs of matured or early ruptured follicles were seen in both ovaries. Finally, vulvar sutures were placed to prevent immediate recurrence after surgery. There was no evidence of recurrence of the vaginal prolapse for 30 days after surgery.

Result and Discussion - True vaginal prolapse may occur near parturition, as the concentration of serum progesterone declines and the concentration of serum estrogen increases [2, 6]. In the bitch, true vaginal prolapse is a very rare condition [9]. Memon et al. [8] reported that vaginal prolapse occurs primarily during proestrus or early estrus stages of the cycle. In this case of study, although the absence of large or recently-ruptured follicles in the ovaries at the time of OHE reduces the probability of endogenous estrogen having caused the prolapse, the possibility cannot be ruled out as some smaller follicles may have gone unnoticed during gross inspection of the ovaries [4]. Nevertheless, the fact that the prolapse occurred within one day after the estradiol benzoate treatment, together with the absence of large or recently ruptured follicles supports the conclusion that the estradiol benzoate may have caused the prolapse.

Conclusion - In conclusion, this case report, to the best of our knowledge, represents the first description of type III vaginal prolapse as a side effect of estrus induction in a bitch. This case demonstrates that type III vaginal prolapse should be considered in the list for side effects of estrus induction hormonal therapy in the bitch.

References
INTRALUMINAL VAGINAL CYSTS CAUSING VAGINAL DISCHARGE IN THREE BITCHES
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Introduction - Mullerian ducts, Wolffian ducts (mesonephric ducts) and the urogenital sinus contribute to the development of the vagina [3]. Vaginal cysts arise from cystic enlargement of embryonic remnants of these structures within the floor of the vagina. Also traumatic inclusion of vaginal mucosa have been implicated in cyst pathogenesis [2].

Case history - Two spayed and one intact bitch were presented because of mucous vaginal discharge. Diagnosis was obtained by ultrasonography and vaginal endoscopy revealing in each case fluid filled spherical masses protruding into the lumen of the cranial vagina. For therapy cysts were fenestrated using a biopsy device at vaginal endoscopy and the fluid removed. The intact bitch was spayed. In all three bitches the clinical signs resolved. Ultrasonography and endoscopy identified fluid-filled masses within the lumen of the cranial vagina. The mucus was drained from the vaginal cysts via vaginoscopy. The intact bitch was spayed. In all three bitches the clinical signs permanently resolved.

Discussion - Cysts of vaginal origin are rare in bitches and often asymptomatic [5]. Nevertheless they have to be considered as a reason for different clinical symptoms. Depending on their location and size vaginal cysts may cause fecal tenesmus [1] dysuria [3, 4, 6] and dyschezia. [4]. In the present three cases the only symptom was mucous vaginal discharge probably originating from a leakage of an intraluminal vaginal cyst or from irritation of the vaginal mucosa.

A complete clinical examination including vaginal cytology, vaginal endoscopy and ultrasonography is necessary to differentiate the vaginal cysts from other disease associated with vaginal discharge. To improve diagnostic findings a biopsy of the cystic wall should be examined histologically. In case of recurrence the resection of the cystic wall may be needed.

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DIAGNOSIS AND SURGICAL TREATMENT WITH CO2 LASER OF A VAGINAL SEPTUM IN A BITCH

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Introduction - Congenital abnormalities of the canine vagina, vestibule and vulva are often under diagnosed. That is the reason why, the incidence and the heritability of this syndrome are not well determined [3]. Vaginal septa and circumferential vaginovestibular strictures are the most commonly reported congenital vaginal abnormalities of the dog [1-3]. Normal embryological development involves fusion of the mullerian ducts to form the urogenital sinus. The hymen separates the vagina and vestibule and is formed by the epithelial lining of the paramesonephric ducts and urogenital sinus. The hymen normally disappears by birth in the dog [1-3]. This syndrome is a cause for an apparently normal bitch to refuse to permit breeding and later if an insemination was done a dystocia in the parturition time [2].

History - A 4-years-old, spayed female Bernese mountain dog was presented at the Veterinary College for recurrent and purulent vaginal discharges associated with attraction of the male dog and infection of the lower urinary tract since two years. One year before the beginning of this clinical signs, the bitch was ovariohysterectomized by another veterinarian. During the last two years, two vaginal bacteriologic cultures associated with antimicrobial susceptibility testing were performed. However, antibiotics treatments did provide no result. Moreover, regular vaginal washing did not allow resolving vaginal discharges.

Clinical signs and diagnosis - Physical examination revealed a normal bitch except for vaginal discharges. Vaginal smear revealed the presence of more than 90% of cornified cells numerous white blood cells. The progesterone concentration was <0.3 nmol/L. The ultrasonography revealed an enlarged uterine stub with hyperechoic areas (pus-like appearance) in the lumen; caudally to both kidneys, ovaries were detected. The endoscopic examination of the vagina revealed the presence of a vaginal septum (5 cm length, from the urethral os), which delimited two distinct tubes (1 cm in diameter).

Treatment - First, an exploratory laparotomy was performed and did confirm the presence of one complete ovary and the other, partly remnant, ovary. Withdrawal surgery was completed. The uterine stub was removed, curetted and omentalized. One month later, the symptoms were persistent despite antibiotics and anti-inflammatory treatments. After an episiotomy, the vaginal septum was divided using CO2 laser (Novapulse LX 20 SP, Lumenis France). At the time of withdrawal of the suture, the symptoms were disappeared. Three months later there was no vulvar discharge.

Discussion - Chronic vaginitis or recurring urinary tract infections are potential sequelae to vaginal obstructions, sometimes causing the bitch to lick at the vulvar area excessively because of pain or discomfort. Vaginitis probably results from chronic retention of a small amount of normal vaginal secretion or urinary present in any bitch. If the defect prevents normal clearing of these secretions, they have the potential for serving as a medium for bacterial overgrowth. Therefore, the problem may be the subsequent irritation of the vaginal lining or ascending urinary tract infection [1, 2]. In this clinical case, the remnant ovarian syndrome did potentially worsen the symptoms and induced the uterine stump pyometra. The use of the CO2 laser allowed avoiding the abundant bleedings, decreased the inflammatory...
reaction and accelerated the cicatrization. Thus, it prevented any secondary stenosis within the vagina which would have lead to the failure of the treatment.

References

CHEMOTHERAPY- TREATED HIGHLY INFILTRATIVE PERINEAL TRANSMISSIBLE VENEREAL TUMOUR IN THE BITCH. TWO CLINICAL CASES

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Introduction - Transmissible venereal tumour (TVT) is still endemic in some regions of Greece. Affected, intact, free-roaming dogs serve as reservoirs and contribute to its dissemination. Diagnosis is usually straightforward, based on its characteristic clinical appearance (reddish cauliflower-like tumour in the external genitalia) and cytological examination. Its size, when diagnosed, seldom exceeds that of a walnut (diameter <5 cm). However, in some severe and neglected cases it may become quite enlarged and erosive, like the ones described in this report.

First clinical case - A 9-year-old 20.5-kg mongrel bitch was presented with an ulcerated mass in the perineal region. She was living in northern Greece, semi-stray, with loose, but regular human care. Five years ago, she had been hysterectomised through a program of stray dog spaying. After that, she continued showing "heats" and accepting copulations. However, during her last oestrus, which had occurred two months before admission, she was reluctant to mate, seemingly due to perineal discomfort. On admission, the bitch was febrile (39.7 °C), mildly depressed and showed severe pain because of an ulcerated, suppurated, malodorous and very large (15×20×15 cm) perineal mass. The vulva was distorted and indistinguishable from the affected tissues. A slight bilateral enlargement of the superficial inguinal lymph nodes was detected, while other palpable lymph nodes were normal. Thoracic and abdominal radiography did not reveal any metastatic foci. Apart from a hypochromic, microcytic anaemia, haematological and serum biochemical profiles of the bitch were within normal limits.

Second clinical case - A 7-year-old 7.2-kg mongrel bitch was presented with a perineal ulcerated mass, which had been growing for last 12 months. The bitch had been recently homed in a stray-dog shelter in central Greece, before that living stray, outdoors, with distant attendance of a shelter member. She was intact, regularly showing “heats” and accepting copulations. On admission, the bitch was emaciated and showed severe pain due to an ulcerated, suppurated, malodorous and very large (18×15×15 cm) mass invading the perineal area. The vulva was impossible to distinguish among affected tissues. All palpable lymph nodes were of normal size. A mild normochromic, normocytic anaemia and peripheral blood eosinophilia was noted, while serum biochemical profile was within normal limits.

Cytological findings - In both cases, Giemsa-stained impression smears revealed many large, discrete, round-shaped cells with high N:C ratio, large round nuclei of varying size with coarse chromatin and one or two large round nucleoli, with pale basophilic vacuolated cytoplasm. All these features were highly indicative of malignant, round-cell tumour. In smears from the second case, many mitotic figures were also observed. The background consisted of abundant bacteria, mature - degenerated neutrophils and foreign material indicative of superimposed bacterial infection.

Diagnosis - In both cases diagnosis was based on the history (multiple copulations in TVT endemic regions), the clinical features (genital - perigenital location of an ulcerative dripping tumour) and the characteristic cytological findings (round cell tumour).
Treatment - Both bitches were treated with vincristine sulphate (dose: 0.6 mg m⁻² i.v., once weekly for six weeks. Additionally, during the first week of treatment amoxycillin-clavulanic acid was prescribed to eliminate infections; multiple warm washings of the lesion with mild antiseptic (povidone iodine scrub, diluted 1:20) were suggested to accelerate healing, up to the end of the treatment. Before each vincristine treatment, clinical and routine haematological examinations were performed to evaluate the response and to avoid probable chemotherapy induced side effects.

Treatment progress and response - In both bitches, lesions responded to treatment, with a 5-30% size reduction after each consecutive treatment. The lesions tended to decrease faster when regularly scavenged with antiseptic washes. The first bitch showed fever (40.2°C), slight depression and leucopenia one week after the first vincristine administration; therefore, the second session was postponed for one week, whilst antibacterial drugs were continued for a further week. Remaining treatments continued uninterrupted, although slight leucopenia was detected one week after the 2nd, 3rd, 5th and 6th session, with no other side effects. After the fourth session, most superficial impression smears from healing lesions were negative of TVT cells, although minor biopsy impression smears of deeper tissues revealed such cells. Nevertheless, one week after the last treatment a considerable volume of tumour (TVT-cells as confirmed by cytological examination) and minor skin defects still remained at the perineal area. A second course of vincristine administration had been planned, but ultimately was not deemed necessary, since the mass had completely subsided with no further treatment five months after end of treatment. At that time, only inactive scars and concavities of minor importance were evident at the vulva and vestibulum. Till now, two years after last treatment, this bitch remained healthy, without any signs of TVT recurrence. In the second bitch, the treatment course had no postponements. The enlargement had mostly subsided at seven days after the end of the treatment, bar from an ulcerated deformation of the perivulvar skin, which had to be surgically reconstructed to reposition the vulva. This bitch remained healthy and TVT free for five months after the last treatment, when she died because of unrelated reasons.

Discussion - Untreated neglected TVT cases may extraordinarily extend into and erode neighbouring tissues. Even in such severe cases, evident metastases do not always occur. Sizeable TVT and related lesions may satisfactorily heal with vincristine treatment. Parallel antiseptic washings of the ulcerated lesions may hasten recovery. Although a considerable tumour remnant may still be present at the end of the treatment, further healing may take place subsequently (up to three months or even more). Impression smears of surgically-excised deep tissue or fine needle biopsy may be needed to reveal TVT cells when these become pocketed under superficial healing. Remaining vaginal scars or deformations may need plastic reconstruction.
GERM CELL DEPLETION IN AN AZOOSPERMIC COLLIE

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A 2,5 years old, healthy, smooth coated, male Collie was used to breeding without success. Semen was collected twice, two weeks apart, and no sperm was detected in the ejaculates. Sexual behaviour of the dog was normal. Penis was normal and testes were descended normally into the scrotum. Testes were small (sin: length: 3,4cm, width: 1,6cm, height: 2,3cm; dx: length: 3,2cm, width: 1,7cm, height: 2,4cm) and soft. Consistence of epididymes was normal. Alkaline phosphatase (AP) of semen was measured (70 900 U/l). The dog was chromosomally normal 78 XY, (SRY gene present, ZFY gene present). Signs of inflammation in genitals were never detected. The dog was neutered six months later, and histology of testes and epididymes was performed. Very few spermatogonia were present but no later spermatogenic stages. The germinative cells were almost totally absent in seminiferous tubules. Half of the tubules were Sertoli cell only. No spermatocytes and no spermatids were observed in any of the studied tubuli. There were some focal lymphosytic infiltration areas around seminiferous tubules. The proportion of tubular area in histologic slides was decreased compared to testicular slides of normal control dogs. The reason for azoospermia in this Collie was absence of spermatogenesis possibly due to an unidentified genetic reason.
HORMONAL EVALUATION AND IMMUNOHISTOCHEMICAL ANALYSIS IN BITCH (CANIS FAMILIARIS) AT DIFFERENT STAGES OF THE ESTROUS CYCLE AND PIOMETRA

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Introduction - The cystic endometrial hyperplasia-pyometra complex (CEH) is a chronic or acute pathology, that occur after estrous and attack adult and nulliparous bitches (2). The pyometra pathogeny is not perfectly known, in spite of decades of oriented studies to the disease etiology. The classic theory mention that the cystic endometrial hyperplasia-pyometra is considered a result of chronic hormones stimulus and unleash for endometrial hyperplasia scenery, increasing endometry susceptibility to secondary bacterial infection, causing pyometra (1). Nevertheless, the most recent theory suggest that a subclinic uterine infection may occur primarily, promoting stimulus to endometrium hyperplasia and hypertrophy. The association between hypertrophy and the increase of glandular secretion could aggravate the primary infection causing a secretion increase by endometrial glands and luminal epithelium cells proceeding to pyometra (3).

Objectives - The aim of present study were evaluate estradiol and progesterone serum hormonal levels, as well as estrogen and progesterone receptors expression by immunohistochemical techniques in health bitches (control group) and pyometra (experimental group) at different stages of the estrous cycle.

Materials and methods - Fifty-one bitches were submitted to ovariohysterectomy, which thirty normal dogs and twenty one with pyometra. The animals were classified on different stages of estrus cycle through progesterone serum levels and vaginal cytology. Blood samples were collected directly before OSH for progesterone serum levels determination. Auto Gamma / Cobra II, Packard Bioscience Company meter was used to hormonal the serum levels, using radioimmunoassay technique on solid stage, through COAT-A-COUNT®, DPC – Med Lab. progesterone commercial kits. The Clone PPG5/1-Dako/M7292 was used for estrogen receptor, using immunohistochemical techniques.

Results - The means and standard error for the 51 dogs ages were 68,88 ± 4,93 months. The minimum and the maximum age were 6,00 and 144,00 months, respectively. The variation of 2,30 kg and 52,00 kg were observed for animals weight, with means and standard error of 18,83±2,00 kg, respectively. The progesterone levels for all animals varied between 0.11 e 38.00 ng/ml, with mean and standard error of 7.46±1.52 ng/ml, respectively. The analysis of variance exhibit results statistically significant (P<0,01) of progesterone levels to estrous cycle stages within comparative groups. Control group animals had significantly higher serum hormonal progesterone levels than experimental groups (P<0,01), especially at diestrus stage. However, results statistically significant (P<0,01) were noted in animals with pyometra in diestrus and anestrus stages. To diestrus stages were observed results statistically significant (P<0,01) to comparative groups, been higher progesterone levels associated to control groups when compared with experimental groups. The samples cytologically examined were classified according estrus cycle stages. Herewith, the animals were classified according
estrus cycle stages, where in control groups eight, five, five and twelve animals were in pro-estrus, estrus, diestrus and anestrus stages, respectively. To the experimental groups were observed seventeen and four animals with diestrus and anestrus, respectively. The uterus samples were histologically examined, and the animals were classified as CEH absent or present. To healthy animals were observed only 6.6% with cystic endometrial hyperplasia (CEH) and 93.4% without CEH. To experimental group, 100% showed CEH. The analysis of variance exhibit results statistically significant (P<0,01) to the effect of tissue within each stage and within each comparative groups when considered the effects of comparative groups, estral cycle stages within each comparative groups, as well as, tissue within each stage and within each comparative groups. Evaluating control groups within each stage and tissue on diestrus stage, the tissues with bigger immunopositive cells percentages were glandular epithelium and myometrium, which did not exhibit results statistically significant (P>0,05) between each other. Immunopositive cells percentages to estrogen receptor were statistically significant lower (P<0,01) in stromal tissue compared with other tissues for animals with pyometra on diestrus stage. Evaluating comparative groups within the same stage and tissue, as much anestrus as diestrus stages, was noticed that experimental group was statistically significant different (P<0,01) compared with those of control group for all evaluated tissues. Significant results by Qui-square test (P<0,01) considering all animals and comparative groups were showed for scores obtained by optical microscopy. In the control groups were observed that 20,41% and 36,73% presented marcation score 1 and 2, respectively. During analysis, were not observed results statistically significant (P>0,05) for score 3 in control group. However, scores 1 (4,08%), 2 (20,41%) and 3 (18,37%) were verified to animals with pyometra.

Conclusions - The exact CEH aetiology is not perfectly known in biitches and steroid hormones and their receptors presented an important connection with visible histologicals changes on epithelium, during estrus cycle. Auspiciously, progesterone and estrogen receptors modifications had been described in biitches uterus with CEH, but they are probably not the only involved factors. A detailed studies need to be done in normal and with pyometra dogs seeking best knowledge of pyometra aetiology, as well as, the association between those and others factors and estrogen and progesterone receptors.

References


UNILATERAL MUCOMETRA IN A PREVIOUSLY SPAYED CAT. AN UNUSUAL CASE OF “UTERINE HORN REMNANT SYNDROME”

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Introduction - Feline ovarian remnant syndrome is usually suspected when signs of overt oestrus occur in previously ovario- or ovariohysterectomised cats (6, 8). This syndrome commonly results from surgical technique errors i.e. failure to remove both ovaries or all ovarian tissue (7), while it is seldom attributed to the presence of ectopic - supernumerary ovaries or sex-steroid producing adrenal glands, adenomas etc. Uterine and/or ovarian anatomic congenital abnormalities, like uterine segmental aplasia may lead to incomplete ovarian removal like in the following case.

History - A 13-months old, grey-striped-tabby, domestic shorthaired queen was referred two months after partial ovariohysterectomy because she continued presenting regular oestrus activity (oestrus of 6 days, interoestrus intervals of 10 days). According to the referring practitioner, whose surgical experience was certified, during that surgery the cat’s inner genital tract appeared to be unicorn, as only the left uterine horn was normally joining the uterine body. At its cranial part the left uterine horn was normally joining the oviduct, the infundibulum and the left ovary, which were all removed. At that time, no uterine continuity to or ovary at the right sublumbar side could be identified, so the cat was presumed as being devoid of right uterine horn and ovary.

Clinical and ultrasonographic findings - On admission, the cat was clinically healthy and showing overt signs of oestrus. Vaginal cytology revealed plenty of anucleated and nucleated superficial epithelial cells also indicative of oestrus. Abdominal palpation did not reveal any abnormalities. However, abdominal ultrasonography (B-mode at 10 MHZ, Logic Book XP, General Electric) revealed a long cystic-tubular structure of maximum diameter 1.7 cm, with homogeneously anechoic content and thin hyperechoic wall. At its cranial pole a small isoechoic oval structure (approx. 0.78 x 0.63 cm) was distinguished, close to the caudal pole of the right kidney. Routine haematological and serum biochemical profiles of the cat were within normal limits.

Diagnosis and treatment - A tentative diagnosis of distended uterine horn along with respective remaining ovary was set, based on the history, the clinical and the ultrasonographic findings. Then the cat was subjected to exploratory laparotomy under general anaesthesia.

Exploratory laparotomy and gross pathological findings - A single, distended, thin-walled uterine horn was found at the right side of the abdominal cavity. At its cranial ending, the oviduct, the infundibulum and respective right ovary were found normally developed. The caudal part of the right uterine horn was blind-ended. Corresponding right broad and round ligaments, ovarian and uterine arteries and veins were also normally present. The other intra-abdominal organs seemed normal. The utero-ovarian remnants were excised and the abdomen routinely closed. The horn was incised to reveal plenty of intra-luminal homogenous, seromucous discharge. The uterine wall was very thin. The endometrium was reddish and slightly coarse. The ovary had 3 discrete follicles (diameter: 3 mm). In cytological examination the discharge showed to be sub-cellular, with few scattered endometrial cells and no inflammatory cells. The discharge was aerobically cultured to show no bacterial growth. Histopathological examination of the excised tissues was not performed.
Discussion - Aplasia of one uterine horn and its respective ovary has been occasionally encountered in the cat (5). This was also the initial but fault diagnosis of the referring practitioner, who first and partially ovariohysterectomised the present cat. Despite surgical experience, any surgeon might become embarrassed when unexpectedly handling morphologically abnormal tissues. So, in the absence of uterine continuity, the remnant horn may be easily missed especially when abdominal cavity is not fully scrutinized. However, as indicated by the abdominal exploratory findings (second surgery) the final diagnosis for this case was uterine segmental aplasia. Until now, only four such cases have been reported in cats (1, 2, 3 & 4). Under regular ovarian activity, the production and accumulation of sterile uterine secretion (mucometra) in the blind-ended uterine horn remnant should be awaited, given its outflow impotency. The authors believe that segmental aplasia of the cat’s reproductive system should not be underestimated and might be included in the differential diagnosis in cases of suspected ovarian remnant syndrome or localized uterine distensions.

References

GENITAL SIDE EFFECTS AFTER THE USE OF MEGESTROL ACETATE FOR PREVENTION OF OESTRUS IN CATS DURING THREE YEARS

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Objectives - Megestrol acetate is an oral progestogen, indicated for the management of oestrus cycle in cats. Because some clients don’t want to have their female cats permanently infertile, however, do not like the oestrus cycles behavioral disorders, we decided to use megestrol acetate for the prevention of oestrus.

Materials and methods - First of all the probable side effects were described to clients. Then 5mg Megestrol acetate per each cat was used every 15 days. Within 7 years, 215 cats were referred to the clinic and their clients tended to use Megestrol acetate for the prevention of their cats oestrus cycles. Some of them have already used this drug for 4 years. Ultrasonography of the abdomen with focus on genital system and mammary glands were accomplished every 6 months to exclude any masses or abnormalities.

Results - To date, eight cats died because of different causes; all necropsies and histopathologic examinations of ovaries, uteri and mammary glands were negative for tumor cells or other abnormalities. So Megestrol acetate can be recommended for a few years for the prevention of oestrus cycle in cats, since this apparently doesn’t have important side effects.

References
OVARIOHISTERECTOMY IN CATS: EFFECTS OF ELECTROACUPUNCTURE AND MORPHINE ON CARDIORESPIRATORY PARAMETERS AND ANHESTESIC CONSUMPTION

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Introduction - Pain perception is associated with behavioral and emotional reactions. In cats, continuous pain can lead to hiporexia and inadequate food intake (Dohoo & Dohoo, 1998). Thus, any surgical procedure requires adequate anesthetic protocol to achieve analgesia during the transoperative period. However, as some anesthetics can cause respiratory depression and hypotension, electroacupuncture (EA) became an alternative for transoperative analgesia, mainly in elevated risk patients. The acupoints mainly associated with surgical analgesia are Zusanli (E36) associated to Sanjinqiao (BP6), or Hegu (IG4), or Quchi (IG11), or Yanglingquan (VB34) (Luna, 2002), and analgesia occurs due to the release of endogenous opioids, serotonin, glycine and acetilcoline.

Objectives - The aim of the present study was to compare the effects of electrostimulation of acupoints Zusanli (E36) and Yanglingquan (VB34) or morphine on cardiorespiratory parameters and isoflurane consumption in cats submitted to elective ovariohysterectomy.

Materials and methods - Eighteen health, cross-bred, adult cats, were randomly divided into three groups: GC (control group), GE (EA group) and Gm (morphine group). After 8 hours of food and water fasting, the animals were premedicated with apecromazine 0.2% (0.2 mg·kg⁻¹ IM) and after 20 minutes induced with propofol IV in a dose to allow an easy oral intubation. The anesthetic maintenance was performed with isoflurane, in an anesthetic circuit without gas reinhalation, at 200ml·kg⁻¹·min in 100% O₂. The acupoints needles were inserted on acupoints Zusanli (E36) and Yanglingquan (VB34) (Drachmpael e Zohmann, 1997) bilaterally. The needles were connected to an acupuncture pulse stimulator (Multiple Electronic Acupuntoscopy WQ-10D1®), a frequency of 2 and 100 Hz was maintained, in an alternating square-wave current. The Gm animals received morphine (0.3 mg·kg⁻¹ IM). In both GC and Gm animals acupoints needles were inserted in false acupoints (sham), 10 to 20 mm laterally to the truth acupoints. Variables were recorded immediately before apecromazine administration (Mbasal); 10 minutes after apecromazine (Macep); after induction of anestesia and anestesic stabilization (M0); 30 minutes after electrostimulation and/or morphine (M10); and every 10 minutes after M10, for 60 minutes (M20 to M70). The recorded variable were rectal temperature (Tr); respiratory frequency (f); heart rate (HR); oxygen saturation (SaO₂); invasive mean arterial pressure (MAP); propofol consumption (CProp); and total isoflurane inhalated volume (VIsof). A multivariate statistical analysis was performed.

Results - The media CProp was 7.21±1.07mg·kg⁻¹, which is recommended to cats. In the GE and Gm animals, the VIsof was significatively smaller than in the GC animals. The acupoints electrostimulation produced a decrease of 58.33% in VIsof, while morphine decreased 22.02%. This smaller isoflurane consumption in the GE animals may be associated with the release of various endogenous substances by electrostimulation, which alterate the conduction, perception and modulation, besides blockage of the nociceptive stimulus conduction.
At M20, during the ovarian pedicle manipulation moment, MAP was smaller in the GE animals. Comparing the three groups, the GE was the unique group which did not present differences in MAP during all the moments evaluated. This was probably due to the analgesia produced by electrostimulation, as it is able to induce the release of endogenous opioids, serotonin, glicine, glutamate, and hypothalamic colecistocinine. Moreover, electrostimulation produces blockage of the nervous impulse in myelinic and amyelinic C fibers. A significative increase of MAP was observed during the transoperative period in GC animals and occurred due to few or no analgesia produced by the anhestetics used. An increase of MAP was also verified in GM animals and this may have related to alteration of transmission, modulation and pain perception produced by morphine, but not in its conduction. In all the three groups the media MAP remained between 80 and 120 mmHg.

There were no statistical differences in mean HR in all groups during the transoperative period. However, there was an increase in HR in Macep compared to Mbasal, and this taquicardia was probably due to hypotension caused by acepromazine.

There were statistical differences for $f$ in all three groups in Macep (decrease) compared to Mbasal. Except in this moment (Macep), there were no differences in $f$ neither in moments nor between groups. Although the main adverse morphine effect is respiratory depression, in this study $f$ was similar between groups during the anhestesia.

A gradual decrease in Tr was observed in all groups since acepromazine administration until the end of surgery. However, there were no differences between groups. In the present study, various factors may have contributed to a continuous decrease in Tr until the end of surgery, as the nervous depression produced by anhestetics, peripheral vasodilatation due to acepromazine and propofol, and also the abdominal opening for ovariohysterectomy. The inhibition of thermoregulation tonus induced by anhestetics produces a temperature decrease more accentuated at the beginning of anhestesia, which is stabilized after 3 or 4 hours (Sessler, 1993). In this study, a decrease in Tr was also detected at the beginning of the anhestesia, mainly in the GE and Gm animals. No statistical differences for SaO2 were observed, with a media SaO2 between 96 and 98%. This is suggestive that the O2 therapy used in the experimental protocol was efficaceous. It is concluded that EA during ovariohysterectomy in cats produces cardiorespiratory stability and decrease in the isofluorane consumption, and is suggestive to produce a potent analgesia.

References


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WİDESPREAD METASTASIŞ İN A CAT WITH MAMMARY ADENOCARCİNOMA

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Case History - An 8 year-old sexually intact, mixed breed queen was presented with a four months history of multiple mammary masses. The cat developed abdominal enlargement, lethargy and anorexia four days before presentation. On physical examination, abdominal distention as well as a large, ulcerated and hemorrhagic mass measuring approximately 7-8 cm were identified in the left third and fourth mammary glands. Another open wounded 3-4 cm mass was found in the right third mammary gland. Evaluation of regional or distant metastasis by radiography or ultrasonography was not performed because of the cost. Mastectomy was initiated but the owner elected to euthanize the cat during the surgery because of the unfavorable prognosis. At necropsy, the abdomen was distended and contained clear fluid. Numerous nodules measuring 0.2-0.3 cm were detected on the diaphragm, abdominal wall, omentum and mesentery. A large area of the liver was in yellowish-green color and had a swollen appearance. A solitary nodule, 3 cm in diameter was present in the left lateral lobe of liver. Right quadrate and right lateral lobes had necrosis. Multifocal nodules were determined in the peripheral part of pancreas and capsules of the kidneys. Another solitary, 0.7 cm cream-colored nodule was found in the spleen. No gross lesions were identified in the lungs. For histopathologic examination, specimens were initially fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 4-5 µm and then stained with haematoxylin and eosin (HE). Microscopic examination of the mammary glands consisted of numerous atypical epithelial cells which were arranged in adenoid structures with central necrosis. Tumor cells had high mitotic index. Examination of the samples from liver, spleen, mesentery, diaphragm and various lymph nodes revealed a similar morphology to the cells observed within the mammary gland. According to the classification of World Health Organization (5), a final diagnosis of a grade II, cribriform carcinoma with metastasis to lymph nodes, liver, spleen, peripheral part of pancreas and capsules of kidneys was made.

Discussion - Feline mammary tumors (FMTs) rank third after lymphoma and skin tumors accounting for 10.3% to 12% of all diagnosed feline tumors (1, 3). Most of the FMTs are considered to be malignant with 75% to 86% being adenocarcinoma (2, 3). Metastasis in FMTs varies from 22.7% to 70.6% depending on the study (4, 6). The most common sites for metastasis are the lungs and the regional lymph nodes although involvement of other organs like liver, adrenals, kidneys, spleen and heart has been previously reported (2, 4, 6). The present case is one of few cases of FMTs with an extensive metastasis to a wide range of organs which suggests the crucial importance of early detection and immediate intervention following the diagnosis.

References

EXPRESSION OF PROLACTIN IN FELINE MAMMARY ADENOCARCINOMAS

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Introduction - Mammary tumors are the third most common tumor in cats after cutaneous neoplasia and lymphosarcoma. More than 80% of feline mammary tumors are adenocarcinomas, which are similar to human mammary adenocarcinomas in their biologic behavior and histologic characteristics. Despite treatment with radical mastectomy, feline mammary adenocarcinoma is associated with a high incidence of metastasis to regional lymph nodes, spleen, liver and lungs. Prolactin (PRL), a protein hormone synthesized by the anterior pituitary gland, stimulates mammary epithelial cell proliferation and differentiation. In many mammalian species, PRL is also produced within the mammary gland. In humans and rodents, PRL is an important mitogen for mammary neoplasia. However, the role of PRL has not been investigated in feline mammary adenomas and adenocarcinomas. The purpose of this study was to determine if feline mammary tumors expressed PRL.

Materials and methods - Formalin-fixed paraffin-embedded sections of archived mammary tissues (n=6) previously submitted to the Oregon State University Veterinary Diagnostic Laboratory were cut to 6 μm and studied by a modified indirect immunohistochemistry protocol with a polyclonal PRL antibody. Anterior pituitary tissue was used to verify the validity of the procedure as PRL expression by lactotrophs in the tissue had positive and specific staining.

Results - Prolactin expression was positive and specific in the mammary gland of cats with malignant adenocarcinoma (n=2). Prolactin was not expressed in sections from benign mammary adenomas (n=2) or fibroblastic hyperplasia (n=2).

Discussion - These results suggest a role for PRL in the development of feline malignant mammary neoplasms. Further exploration into the relationship between PRL and feline mammary adenocarcinomas is needed.

References

NECESSITY OF THE OVARY FOR THE MAINTENANCE OF PREGNANCY IN CATS

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Introduction - Plasma Progesterone (P4) levels are maintained in pregnant cats until delivery, but become low in pseudopregnant cats 40-45 days after copulation while infertile [3]. This difference in P4 levels is considered to be due to P4 secretion by the placenta of pregnant cats [4]. In support of this, Malassine and Ferre detected 3-HSD, an enzyme metabolizing pregnenolone to P4, in the cat placenta [1]. Thus, it is considered that ovariectomy (OVX) in cats on day 45-50 of pregnancy or later does not result in abortion [2]. However, neither the necessity of the ovary for pregnancy maintenance nor the dependency of P4 secretion on the placenta in the latter half of pregnancy has been clearly shown. Therefore, to clarify these points, we performed OVX in various stages of pregnancy, examined the pregnancy status, measured LH and P4 levels in peripheral, ovarian, and uterine venous blood, and conducted histological examination of the corpus luteum.

Materials and methods - Twenty-five 2- to 8-year-old cats, which were allowed to mate several times on the third day of estrus and became pregnant, were used in this study. Six 4- to 11-year-old male cats with normal semen qualities, mating ability, and fertility were used for mating. OVX was performed in each group of 5 cats on day 35 (I), 40 (II), and 45 (III), or 50 (IV). At the time of OVX, peripheral and bilateral ovarian and uterine venous blood was collected, and the number of corpora lutea and fetuses was counted. Blood was collected at 3-day intervals from 5 pregnant cats, as controls, as in the experimental groups, for hormone measurements. These cats also underwent surgery on day 45 of pregnancy to count the number of corpora lutea and fetuses. The postoperative cats were bled for 3 consecutive days, and then at 5-day intervals until the day of abortion or delivery. Plasma LH and P4 levels were determined by RIA and EIA, respectively. In addition, the cats were examined for abortion or delivery every morning, daytime, and evening. The experimental cats were examined for fetal survival using an ultrasound imaging device.

Results - We found that the number of ova ovulated and fetuses was 3-7 (mean, 4.5 ± 0.2) and 1-5 (mean, 3.9 ± 0.3), respectively. After OVX, abortion occurred in 100% (5/5), 80% (4/5), 40% (2/5), and 60% (3/5) of group I, II, III, and IV cats, respectively. In the remaining cats, normal delivery took place on days 63-69 (mean, 66.1 ± 1.1) of pregnancy. The 5 control cats normally delivered 2-6 kittens (mean, 4.4 ± 0.8) on day 62-69 (mean, 66.0 ± 1.5). The time to abortion after OVX was 4-8 (mean, 5.6 ± 0.8), 3-17 (mean, 8.0 ± 3.6), 10 and 11, and 2-4 (mean, 3.0 ± 0.7) days in experiments I, II, III, and IV, respectively, showing that abortion occurred early after OVX on day 50 of pregnancy. The levels of LH were not correlated with the stage of pregnancy, and were similar in ovarian, uterine, and peripheral venous blood, but were elevated in peripheral blood after OVX. The plasma P4 levels were 1-2 ng/ml in all groups on the day after OVX, decreasing to less than 1 ng/ml from the second day onward. The levels of P4 in ovarian venous blood at the time of OVX decreased with the stage of pregnancy, and were consistent with the histological findings of the corpus luteum, but clearly higher than those in peripheral blood. However, the P4 levels in bilateral uterine venous blood were similar to those in peripheral blood, regardless of the number of fetuses and stage of pregnancy. After OVX, pregnancy was maintained without abortion in 20%
(1/5), 60% (3/5), and 40% (2/5) of group II, III, and IV cats, respectively. OVX on day 50 of pregnancy was associated with a higher rate of abortion, compared with that on day 45 of pregnancy, probably not because of the influence of the OVX-induced decrease in blood P4 levels, but because of a greater influence of surgery due to uterine enlargement. In the control group, abortion did not occur despite surgery on day 45 of pregnancy.

Conclusions - These results suggest that P4 in pregnant cats is secreted by the ovarian corpus luteum, not by the placenta, but indicate that P4 is not essential for the maintenance of pregnancy in cats from day 40 of pregnancy onward.

References

PROLONGED DURATION OF FERTILITY OF DOG OVA

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Objectives - The fertile period for natural mating in dogs extends from approximately 7 days before ovulation until day 5 after ovulation. This relates to intra-uterine survival of canine sperm for up to 7 days or more, the delay in maturation of oocytes until 2 to 3 days after ovulation in the lower oviduct, and viability of secondary oocytes for an additional 48-60 h or more [1,2]. We have also observed in dogs with uterine fistulas that spermatozoa did not enter the uterus after vaginal insemination in the last stage of estrus, even if mating occurred [3]. Verstegen et al. [4] confirmed late-estrus “cervical closure” occurring on average 5 days after ovulation, but also observed that, even 72 h after the cervical, one of five intrauterine inseminations resulted in conception, indicating that canine ova can be fertile perhaps up to 8 days after ovulation and 6 days after oocyte maturation. Therefore, in the present study conducted to further clarify the duration of fertility of canine ova, we examined pregnancies after intrauterine artificial insemination (IUAI) between 6 and 9 days after ovulation, with the time of ovulation initially estimated from plasma progesterone and later confirmed by LH levels.

Materials and methods - Beagles used were 1.9 to 6.7 years old, including 27 females at 4.3 ± 0.3 (mean ± sem) yr and 5 males at 6.9 ± 0.8 yr. Plasma was collected and assayed for progesterone (EIA) daily from day 8 after onset of vulval bleeding, and IUAI was performed at known days post-ovulation, based on the day of ovulation being estimated as the first day that progesterone exceeded 2 ng/ml [5]. At a later date, plasma LH levels measured by RIA allowed a re-estimation of ovulation time as having occurred 2 days after the preovulatory LH peak. The IUAI was performed surgically in 5, 7, 8, and 7 dogs on days 6, 7, 8, and 9 post-ovulation, respectively. Each IUAI deposited 2 x 10⁷ sperm in 100µl into the upper portion of the uterine horn ipsilateral to the ovary with the greatest number of ovulations. The vulva was examined for the appearance of sperm at 0.5, 1, and 2 h post-IUAI by microscopic exam of smears obtained by saline-moistened swabs from the vulva and vestibule. Pregnancy was diagnosed by ultrasound (7.5-MHz probe) exam and fetal development in pregnant dogs was examined at 5-day intervals beginning at 20 days post ovulation. The number of pups was counted on the day of birth.

Results - The day of ovulation estimated from plasma progesterone was corrected using LH levels in 3 (11.1%) of the 27 dogs and thus changed to a date 1 day earlier or 1 day later in 1 and 2 dogs, respectively. The rates of conception following IUAI at 6, 7, 8, and 9 days after ovulation were 100% (5/5), 71.4% (5/7), 37.5% (3/8), and 0% (0/7), respectively. Of the 13 conceptions, complete resorption of the litter occurred in 20% (1/5), 40% (2/5), and 33.3% (1/3) of bitches inseminated at 6, 7, and 8 days after ovulation, respectively, yielding an average litter resorption rate 30.8% (4/13). In the remaining 9 pregnancies, partial-litter fetal resorption occurred in 50% (2/4) and 33.3% (1/3) of dogs artificially inseminated at 6 and 7 days after ovulation dogs. The mean litter size and time from ovulation to delivery were 4.3±1.6 pups and 64.0 ± 0.0 days (n=4), 4.0 ± 1.4 pups and 66.3 ± 0.4 days (n=3), 1.4 pups and 67,69 days (n=2) for IUAI at 6, 7, and 8 days after ovulation, respectively. The litter size was larger than the estimated number of ova ovulated on the inseminated side in only 2 (2/5, 40%) and 1 dog (1/5, 20%) after IUAI at 6 and 7 days after ovulation, respectively. Spermatozoa were observed in the vestibule within 2 h in all dogs except one day-8 (complete...
litter resorption) dog and one day-9 (non-conception) dog. The timing of the appearance of sperm in the vestibule varied among dogs and was unrelated to time of IUAI after ovulation.

**Discussion** - The high pregnancy rates with IUAI at 6 and 7 days after ovulation, confirms that many canine oocytes are fertile at 4-5 days after maturation. The high rate of post-implantation partial or full litter resorption was presumably due to the aging of ova and/or asynchrony between embryonic development and the intrauterine environment. That asynchrony becomes an increasingly important factor as day of insemination post-ovulation increases agrees with the prolonged (67-69 d) gestation lengths relative to the day of ovulation seen in most of the pregnancies resulting from insemination 7 or more days after ovulation. The 2 pregnancies resulting from IUAI at 8 days post ovulation confirm that, in the extreme, some oocytes remain viable until that time and thus 6 d after oocyte maturation. The low percentage of fertilization of ova from the contralateral ovary in this study was presumably due to the decreased motility and fertility of spermatozoa in the intrauterine environment at 6 days after ovulation or later. The appearance of spermatozoa in the vaginal vestibule at by 2h post IUAI suggests that failure of spermatozoa to enter the uterus from the vagina (or remain viable after doing so) at 6 days or more after ovulation is not due to the physical closure of the cervical canal, but because of other and possibly biochemical changes in the cervical or intrauterine environment.

**References**

PLASMA HORMONE LEVELS AND SEMEN QUALITIES IN MALE CATS DURING THE BREEDING AND NON-BREEDING SEASONS

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Objectives - Male and female cats are known to be annual and seasonal breeders, respectively. However, despite there being only a few studies, opinion is divided as to the influence of the season on the semen qualities and plasma hormone levels in male cats [1-5]. Importantly, none of these studies examined the influence of the season on the plasma hormone levels and spermatogenic ability in the same male cat. Therefore, in the same male cat, we compared the plasma levels of LH and testosterone (T) and the qualities of semen collected using an artificial vagina in the breeding season (BS) and non-breeding season (NBS). During both seasons, cats were subjected to natural hours of daylight at a constant room temperature.

Materials and methods - We used 5 male cats born in our colony showing normal mating ability, spermatogenic ability, and fertility: 1 (3.25 year old), 3 (3.5-year-old male siblings), and 1 (3.75 years old). They were kept separately in an animal house with transparent glass windows on the east, west, and south sides and without artificial light sources. Since the BS of female cats kept under these conditions was from January to July, we performed experiments in the BS and NBS in April and November, respectively. Although semen can be collected with stable qualities at 2-day intervals from male cats in the BS, we performed semen quality examination ten times at 3-day intervals, considering experiments in the NBS. Using a self-made artificial vagina and a female cat in estrus, two consecutive ejaculates were collected and evaluated. At the time of semen collection, male cats were also observed for libido. The main experiment was started after four preliminary experiments at 3-day intervals. Semen was examined for volume, sperm motility, the sperm viability, sperm count, sperm abnormality, and immature sperm. Coincident with semen collection, blood (3 ml) was collected at 4 p.m. for the determination of plasma LH and T by RIA and EIA, respectively.

Results - Although no problems with libido and ejaculating ability were encountered in collecting semen from experimental cats in the BS, one 3.5-year-old cat was able to ejaculate in the NBS only in three out of the ten occasions of semen collection. There were no problems collecting semen from the remaining 4 cats in the NBS, as in the BS. Semen in the BS was clearly superior in semen volume in 2, sperm motility in 2, the sperm viability in 3, and immature sperm in 1 cat (P<0.01 or P<0.05). None of the cats showed a significant difference in the sperm abnormality. However, even the cats showing no significant differences in semen qualities were superior in all parameters of semen analysis in the BS. Moreover, all 5 cats clearly excelled in all parameters in the BS (P<0.01 or P<0.05). The plasma levels of LH and T in the BS were significantly higher in 2 and 3 cats, respectively, and the plasma levels of both hormones were significantly higher in all cats in the BS (P<0.01).

Conclusions - Thus, although the spermatogenic ability and sex hormone-secretory ability of male cats were maintained during the NBS of female cats, these abilities were clearly higher during the BS, indicating that the reproductive function of male cats is also influenced by the season (hours of daylight).

References
RELATIONSHIP BETWEEN PLASMA LH SURGE AND OVULATION INDUCTION AND CONCEPTION RATES IN CATS MATED ONCE OR THREE TIMES ON DAY 1 OR 5 OF ESTRUS

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Objectives - Although cats ovulate after mating, the number of times of mating and its timing for ovulation induction and conception have not been extensively studied. The rate of ovulation induction in cats mated once during estrus is low, but is considered to increase in those mated on day 3 of estrus or later [1,2,3]. Glover et al. [2] noted that the rate of conception was low in cats mated once, even if ovulation occurred. To clarify these points, in the present study, we examined the presence or absence of LH surge, and ovulation induction and conception rates in cats mated once or three times on day 1 or 5 of estrus.

Materials and methods - We examined 26 female cats ranging in age from 1 to 5 years, with a mean of 2.35 ± 0.25 (SE). Six 2- to 5-year-old male cats (with a mean age of 4.50 ± 0.55 years) with the mating and fertility ability were used as mating partners. They were allowed to mate once or three times on day 1 or 5 of estrus. When male and female cats had mated three times, the same male was used at 10- to 15-min intervals. The plasma LH surge level was measured before mating and at 1, 2, 3, 4, and 24 h after mating, and thereafter every 3 days until 9 days after mating. At each time point, 3 ml of blood was collected, and the plasma was immediately separated by refrigerated centrifugae and stored at -40°C. The occurrence of ovulation induction was confirmed by plasma progesterone (P4) levels of at least 5 ng/ml 9 days after mating. Plasma LH and P4 levels were determined by RIA and EIA, respectively. Using an artificial vagina, semen was collected from the 6 male cats five times at 3-day intervals. The mean number of sperm per ejaculate was calculated from the total number of those in the five ejaculates from each cat. Pregnancy was diagnosed using an ultrasound imaging device 15-20 days after mating.

Results - As a result, the ovulation induction and conception rates after a single mating on day 1 of estrus were 60% (6/10) and 33% (2/6), respectively, and those on day 5 of estrus were 83.3% (10/12) and 40% (4/10), respectively. The ovulation induction and conception rates after three matings on day 1 of estrus were 66.7% (6/9) and 83.3% (5/6), respectively, and those on day 5 of estrus were 100% (10/10) and 100% (10/10), respectively. In cats that had ovulation after a single mating, the LH level peaked to 3.77-20.02 ng/ml (mean, 11.77 ng/ml) at 2-4 h after mating on day 1 of estrus, and to 3.47-15.83 ng/ml (mean, 8.05 ng/ml) at 1-2 h after mating on day 5 of estrus. The level of LH did not increase in non-ovulating cats, but increased to 6.32 ng/ml at 1 h after mating in only 1 cat that had been mated on day 5 of estrus.

Cats that mated three times showed a clear increase in LH levels: in cats subjected to mating three times on day 5 of estrus, the LH level reached a peak of 12.0-40.39 ng/ml (mean, 21.03 ng/ml) at 1-2 h after mating; and in 2 cats that were subjected to mating three times and had no ovulation, their LH levels slightly increased to peaks of 4.73 ng/ml and 5.66 ng/ml, respectively, at 2 h after mating.

Litter size was not related to the number of times of mating. The number of sperm per ejaculate ranged from 57.9 x 10⁶ to 103.7 x 10⁶, with a mean of 82.0 ± 8.0 x 10⁶.
Conclusions - Thus, an increase in the number of times of mating on day 1 of estrus did not result in an increase in the ovulation induction rate, suggesting that plasma E₂ levels were not sufficiently elevated to induce a high pituitary response to mating stimulation. The ovulation rate was 83.3% in rats mating once on day 5 of estrus, indicating that the stimulus of mating more than once is required to elevate LH levels despite the increase in E₂ levels. In this study, a few cats had ovulation, although LH levels after mating were low at about 4 ng/ml, suggesting that the plasma level of LH required for ovulation induction varies greatly with the individual cat. The conception rate after a single mating was low, suggesting that the number of sperm per mating is not sufficient. These results suggest that mating more than once in the middle of estrus is required to improve the ovulation induction and conception rates in cats.

References

DETECTION OF LIPID PEROXIDATION REACTION IN FROZEN-THAWED EPIDIDYMAL CAT SPERMATOZOA USING BODIPY581/591 C11

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Introduction - Lipid peroxidation, a reaction between reactive oxygen species and polyunsaturated fatty acids, occurs on mammal sperm cell membranes, particularly during the cryopreservation process and post-thaw incubation of spermatozoa. This reaction induces sperm membrane damage leading to losses of sperm membrane fluidity and integrity (1) and can be stimulated by the presence of ferrous ion (2). Lipid peroxidation reaction is considered as one of the most important causes of impaired fertility in many species, i.e. men and stallion (1, 3). Recently, a lipophilic dye probe fluorescence assay (BODIPY581/591 C11) has been used as an infertility indicator for the lipid peroxidation reaction in the sperm membrane of several animal species (2) but information for the felidae is scarce.

Objectives - The present study aimed to evaluate the lipid peroxidation reaction test on frozen-thawed epididymal cat spermatozoa using the BODIPY581/591 C11. A lipid peroxidation reaction promoter was used as a model.

Materials and Methods - Spermatozoa were collected from eight adult cats after orchidectomy by slicing the cauda epididymides into warmed Tris buffer. The sperm sample from each cat was extended with a Tris egg yolk extender containing Equex STM paste, loaded into one-quarter each of three mini-straws and cryopreserved according to Axnér et al. (4). The sperm samples were thawed at 37 °C for 15 sec in a water bath and emptied into a small Eppendorf tube containing 65 μL of warmed Tris buffer (1:1 v:v). One straw was used for treatment and the other two were used as controls, with and without the presence of freezing extender after thawing (EYT and TM groups). The sperm samples from the treatment groups were equally divided into two aliquots and evaluated with and without the presence of the freezing extender (EYT and TM grous), treated with lipid peroxidation reaction promoter (100 mM ferrous sulphate; FeSO₄), incubated for 6 h, and processed for evaluation of the lipid peroxidation reaction. Post-thaw sperm samples from the control groups were incubated for 6 h, and thereafter processed for evaluation of lipid peroxidation reaction. The lipid peroxidation reaction was evaluated by loading the sperm samples with a final concentration of 10 μM lipophilic dye probe BODIPY581/591 C11 and incubating them for 30 min at 37 °C. Unbound dye probe was removed by centrifugation. The sperm sample was resuspended with warmed Tris buffer. Total of 50,000 sperm specific-events from each sample were evaluated as the percentage of lipid peroxidation reaction using a BD LSR flow cytometry according to Aitken et al. (2). Briefly, the probe shifts from red to green upon reactive oxygen species or oxidation reaction. An argon-ion laser (488 nm) was used for excitation. Green fluorescence (FL1) was detected with a band pass filter 530/28 nm and red fluorescence (FL3) was measured using a long pass filter (>670 nm). The statistical analyses were performed using an ANOVA and paired-test.

Results - With the BODIPY581/591 C11, the frozen-thawed epididymal cat spermatozoa showed green and red fluorescence. The percentage of lipid peroxidation reaction in the
treatment groups were significantly higher than the control groups (Table 1) (Group I and Group II ($P= 0.01$); Group III and Group IV ($P= 0.001$)).

Table 1. The percentage of lipid peroxidation reaction in frozen-thawed epididymal cat spermatozoa resuspended with or without freezing extender (EYT or TM group) and with or without lipid peroxidation reaction promoter (100 mM ferrous sulphate; FeSO$_4$)

<table>
<thead>
<tr>
<th>EYT Group</th>
<th>TM Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Group II</td>
</tr>
<tr>
<td>without FeSO$_4$</td>
<td>with FeSO$_4$</td>
</tr>
<tr>
<td>(control)</td>
<td>(treatment)</td>
</tr>
<tr>
<td>2.3 ± 2.3a</td>
<td>12.9 ± 8.3b</td>
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</table>

<table>
<thead>
<tr>
<th>Group III</th>
<th>Group IV</th>
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<tbody>
<tr>
<td>without FeSO$_4$</td>
<td>with FeSO$_4$</td>
</tr>
<tr>
<td>(control)</td>
<td>(treatment)</td>
</tr>
<tr>
<td>4.9 ± 4.3a</td>
<td>51.3 ± 23.9b</td>
</tr>
</tbody>
</table>

Mean ±SD. N=8.
Means within row with different letters differ significantly ($P<0.05$)

**Conclusion** - This study demonstrated that ferrous sulphate induces the lipid peroxidation reaction in feline sperm cell membranes and that this reaction can be detected by the lipophilic dye probe fluorescence assay (BODIPY$_{581/591}$ C11). This was confirmed by the higher percentage of the lipid peroxidation reaction in the treatment groups compared to the control groups. The concentration of BODIPY$_{581/591}$ C11 (10 μM) used in this study seems appropriate for the detection of the lipid peroxidation reaction that occurs on the cat sperm membranes.

**References**

DETERMINATION OF PROGESTERONE (P4) IN CANINE BLOOD SERUM FOR DETECTION OF OVULATION USING AN ENZYME-LINKED FLUORESCENCE ASSAY (ELFA) IN COMPARISON TO A VALIDATED RADIOIMMUNO ASSAY (RIA)

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Introduction - The detection of ovulation is very important for the breeding management of bitches. In addition to the clinical examination (vaginal cytology, vaginoscopy, external genitalia and behaviour), the level of P4 in peripheral blood is an important parameter. In the bitch, P4 levels are rising from the time of the LH-peak and ovulation is assumed to occur when a concentration of 5 ng/ml is reached (Guenzel-Apel et. al 1990a). A number of laboratories offer the determination of P4 with quantitative methods (RIA, EIA) but in these cases blood samples have to be transported and this may lead to a significant delay. In a clinical setting, the information about the P4 level should be available immediately because the combination of clinical and endocrinological data is necessary to predict ovulation with a good reliability. A semi-quantitative P4 assay is available for practitioners to allow for a rapid in-house testing of blood samples (Guenzel-Apel et al 1990b). Due to the nature of these tests, a certain subjective component cannot be excluded and preference should be given to a test yielding reliable quantitative data and being similarly rapid and easy to handle. For use in humans a whole array of test kits meeting these demands is available. However, and according to own experiences, application of such test kits for the determination of P4 in canine blood in general does not provide reliable information, most likely due to matrix effects (high concentrations of corticosteroid binding globulin).

Objectives - This communication compares P4 - data obtained with a commercial ELFA validated for use in human and an in-house RIA validated for use in dogs. The enzyme-linked fluorescence assay is provided by Biomerieux, France, and is to be performed on the MiniVidas automated analyser. With this assay results are available within 45 min.

Materials and methods / Results - According to the information provided, P4 and a P4-derivative (P4D), coupled to alkaline phosphatase (AP), compete for binding sites of an anti P4-mouse monoclonal antibody coated to a solid phase (FPR) which also serves as the MiniVidas pipetting device. Different to other test systems, a multi well strip rather than individual tubes is provided for the various pipetting steps. Pipetting of P4 and P4D is sequential and all steps are performed automatically following initiation of the procedure. In a final step, AP hydrolyses the substrate, 4-Methyl-umbelliferyl phosphate, into a fluorescent product (4-Methyl-umbelliferone) and the fluorescence is measured at 450 nm. The intensity of the fluorescence signal is inversely proportional to the concentration of P4 in the sample. With each batch of reagents a calibrator and batch specific reference data are provided. After an initial calibration of the instrument, recalibration in 14 day intervals is necessary. Following computation by the instrument the results are expressed in ng/ml, the range covered is 0.25 – 80 ng/ml. Each kit allows for 60 assays.

Blood samples from a total of 27 female dogs of different breeds were used for this study. Blood serum was collected by centrifugation (10 min, 2000 U/min) and stored at -20 Celsius until assayed. The progesterone kits and the blood samples were allowed to equilibrate at
ambient temperatures 30 minutes before the assay was performed with 200 μl serum for each sample.

To check the intra-assay reproducibility blood samples from five bitches were tested 10 – 12 times in the same run. The mean values obtained were 61.8, 6.8, 51.4, 43.7, and 1.1 ng/ml and the respective coefficients of variation (CV) were 3.4, 6.7, 2.6, 3.1, and 25.4%.

Four serum samples from different bitches were tested singly in 10 separate series to test the inter-assay variability. The mean value obtained were 47.2, 15.1, 49.4, 4.0 ng/ml and the corresponding CV were 2.1, 2.1, 3.1 and 4.3% respectively.

Samples from three dogs were used to test the linearity of the assay. Samples were diluted (½, ¼, 1/8, 1/16) with charcoal-stripped human serum (Biomerieux, France) and tested in 3 runs. The expected values were met in a range of 62.8 – 100%.

To test the assay for its correctness when applied to dog blood, the MiniVidas assay and a RIA validated for the dog (Hoffmann et al. 1992) were applied to 19 serum samples from female dogs of different breeds. Test application was as described above (MiniVidas Assay) or published (Hoffmann et al. 1992). The P4 concentrations determined by RIA covered a range of 0.41 to 46.2 ng/ml. With one exception the absolute values obtained with the MiniVidas showed a high agreement (mean deviation 19%), deviations were in both directions and the correlation coefficient was 0.989.

The results of the intra- and inter-assay variability demonstrate that the assay provides a high reproducibility. The CV was generally not higher than 6.7% except in one sample with a mean value of 1.1 ng/ml (CV 25.4%). However, samples with such low values simply indicate the lack of functional corpora lutea. The comparison with the results of a RIA validated for the dog demonstrates that the MiniVidas Assay, different to many other commercial kits provided, truly measures P4 in the dog.

As a conclusion the use of the MiniVidas system for determination of P4 in peripheral blood of the bitch can be recommended.

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DETECTION OF SERUM RELAXIN AS A DIAGNOSTIC TOOL FOR EARLY PREGNANCY DIAGNOSIS IN BITCHES

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Objectives - With the object of finding a suitable and reliable method of early pregnancy diagnosis in bitches, the study was undertaken to investigate the efficacy of trans abdominal palpation, ultrasound scanning and serum relaxin detection was conducted in 45 apparently healthy bitches.

Material and methods - Out of this, ten animals were selected at random for pregnancy diagnosis at 16 to 20 days, 21 to 24 days and 25 to 30 days post breeding. Body weight was recorded and blood samples were collected for the estimation of haemoglobin, packed cell volume and erythrocyte sedimentation rate at the day of breeding and also at above gestation periods.

Results - In the present study, it was found that abdominal palpation was difficult in diagnosing pregnancy between 16 to 20 days of gestation while it gave an accuracy of 50% and 70% at 21 to 24 and 25 to 30 days post breeding. The percentage accuracy of ultrasound scanning at 16 to 20 days was 50%, which improved to 80% and 100% at 21-24 and 25-30 days post breeding where foetal heartbeat could be observed in all the positive cases. The earliest positive result obtained for serum relaxin detection was obtained at 20th day post breeding and the percentage accuracy was 50% at this period, as against 100% at 21-30 days of gestation. It was found that serum relaxin test was not influenced by pseudo-pregnancy and pyometra. There was significant variation in haemogram (P<0.01) at the day of breeding and at different gestational age where haemoglobin level and packed cell volume values were found to be decreasing with an increase in erythrocyte sedimentation rate. The body weight of all the ten animals varied significantly (P<0.01) where it had shown a steady and progressive increase as the pregnancy advanced. The study revealed that abdominal palpation was not very useful in diagnosing early pregnancy. By ultrasound scanning, uterus as well as foetus could be visualized after 23 days of gestation. Serum relaxin detection could be used as an early tool for pregnancy diagnosis in bitches from 20 days post breeding

Conclusions - Results of the present study suggest that detection of serum relaxin is a quick, simple and accurate tool for diagnosing early pregnancy under field conditions.
REPEATABILITY TEST FOR QUANTIFICATION OF FECAL PROGESTINES IN BOXER BITCHES

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Introduction - Wild and/or zoo animals’ endocrine monitoring strategy needs to be developed in order to perform an effective reproductive monitoring of those species. An efficient endocrinological investigation with reproductive aims requests a large amount of repeated samples for hormonal analysis, what is very difficult or not possible to be done in non domestical species. In those cases, non invasive collection methods such as urine and feces samples are good options. As urine samples are difficult to be collected from free live animals so feces samples are the best option to perform the endocrine analysis. (SCHWARZENBERGER et al., 1991). Bitches mated at an inappropriate time constitute the commonest cause of apparent infertility in breeding programs. This is caused by 2 characteristics of canine reproduction: short fertile period and spermatozoid longevity in the female genital tract. Non invasive monitoring techniques, in a conservationist perspective, will be helpful to improve reproductive management programs and development/understanding of physiologic mechanisms that involve reproduction, social behavior and ecologic pressures of studied species (MONFORT et al., 1998). Several species have been monitored through non invasive techniques of fecal steroids quantification. In Brazil, some species like “onça pintada” (Panthera onca) (VIAU, 2003), “gato mourisco” (Herpailurus yaguaroundi) (BARNABE, 2004) and “bugio” (Alouatta seniculus) (KUGELMEIER, 2005), among others, have had satisfactory results including information that helps to elucidate important questions of the reproductive cycle.

Objective - The aim of this study is to verify the efficiency of the methodology used for progesterone metabolites extraction from feces (following technique described for Brown et al. (1994), with some modifications), to determine progestine concentration in feces of boxers bitches.

Materials and methods - There were used 17 boxer bitches, keepped in separate areas. Feces samples were collected daily, from the floor and stored at –20°C, for posterior quantification of fecal metabolites of steroid hormone (progesterone). The analysis of fecal progestines were performed in the “Laboratório de Dosagens Hormonais” (LDH) of Department of Animal Reproduction of Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (VRA, FMVZ-USP), using comercial diagnostic kits (COUT-A-COUNT Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA-DPC). Small portions of 0.3 g of feces were placed in 10 ml glass tubes, previously identified, with ethanol as solvent. They were homogenized using vortex device (PHOENIX, MOD AT 56). The samples were boiled in 5 ml of ethanol 90% (90% ethanol: distilled water) for 20 minutes at 90°C under laminar flow. The initial volume of ethanol at 90% were kepted in order to avoid sample drying. Then, they were centrifugated for 15 minutes at 500g. the upper part was placed in clean tubes. There were added more 5 ml of ethanol 90%, homogenized using the vortex device and centrifugated again for more 15 minutes. The upper part of second centrifugation were added to the first one and were drieded in the flow (Láctea) under compressed air (MSI 5,2 ML/100). After the complete drying of the extracts, they were diluted in 1 ml of ethanol (methylc alcohol, Synth) and mixed using Multi Vortex device (VWR Scientific Products, VX-2500) for 10 minutes. The extracts were kept in 1,5 ml “ependorf” tubes and stored at -20°C until the hormonal quantification. Such quantification was performed 10 times, from the...
same sample in each stage (proestrous, estrous and diestrous), in order to verify its normal distribution in the curve (repeatability test of the used technique for hormonal extraction). Komolgorov-Smirnov test was used to verify the association to the normal distribution.

**Results**

Table 1 – Quality control of the extraction technique - Komolgorov-Smirnov test

<table>
<thead>
<tr>
<th>Repeatability</th>
<th>Diestrous</th>
<th>Proestrous</th>
<th>Estrous</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
<td>206.23</td>
<td>34.17</td>
<td>50.24</td>
</tr>
<tr>
<td>Median</td>
<td>204.33</td>
<td>34.06</td>
<td>52.30</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>36.44</td>
<td>6.48</td>
<td>11.93</td>
</tr>
<tr>
<td>Minimum</td>
<td>150.17</td>
<td>23.68</td>
<td>26.67</td>
</tr>
<tr>
<td>Maximum</td>
<td>278.48</td>
<td>48.33</td>
<td>62.58</td>
</tr>
<tr>
<td>size</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Lower Limit</td>
<td>183.64</td>
<td>30.16</td>
<td>42.85</td>
</tr>
<tr>
<td>Upper Limit</td>
<td>228.82</td>
<td>38.18</td>
<td>57.64</td>
</tr>
<tr>
<td>( p )</td>
<td>0.955</td>
<td>0.805</td>
<td>0.963</td>
</tr>
</tbody>
</table>

The variability of each cycle measure is considered low, it demonstrate that measurements are consistent. Considering \( p \) value, the three distributions are very close to normal distribution.

**Conclusion** - The used methodology to extraction of fecal progesterone metabolites, performed following technique described for Brown et al. (1994), with some modifications, is efficient for determination of fecal progestin concentrations in Boxer bitches, using Solid Phase Radioimmunoassay (RIA) kits (DPC Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA). The extraction method showed low variability, the non correlated results aren’t related to the methodology, which obtained results very close to normal distribution.

**References**


THE EVALUATION OF CLASSIC METHODS FOR BRUCELLA CANIS ISOLATION AND IDENTIFICATION

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Introduction - Brucellosis are diseases which are made by gram negative bacteria from Brucella genus, which presently are 6 species: Br. abortus, Br. melitensis, Br. suis, Br. canis, Br. neotomae și Br. ovis. Recently it was isolated and characterized another two species which affected different aquatic mammals: Brucella cetaceae and Brucella pinnipediae, who produce more types of lesions to common dolphins, marsuins, seals (especially otteria) and walrus. Because of economical damages and human health danger, brucellosis represented a priority problem for comparative pathology. This group of diseases is present to all continents, but in last decades was ascertain a modifying of spread area and an apparition incidence of this disease for different species and population of animals. The extension of canine brucellosis is still a little know and study although is certain its spread in all countries from European continent. In natural conditions the canines can infected with different species of Brucella (Br. abortus, Br. melitensis, Br. suis) but normally the principal etiologic factor is Brucella canis. Although practical the dogs aren’t an important host, with directly implication in epidemiological link of brucellosis, the role of this don’t be ignore. Usually, more practical importance has done for canine brucellosis by Brucella canis. Brucella canis was describe and identified for the first time to Carmichael and Bruner in 1968. Since that time till now, although it was effectuate studies in many countries and that subject it was tackle of different researches, hadn’t completely analyzed and resolved yet all the elements referring of this pathological entity.

Materials and methods - It was harvested a total number of samples to come from 285 clinical cases (dogs), presumptive diagnosed with brucellosis. From total cases it was investigated a number of 257 cases it was represented of males which are suspicion was determined by sign of orchids or orchit-epididimits and a number of 28 cases it was represented by female with spontaneous abort (regular in 15 – 20 days of gestation). For each presumptive diagnosed case with brucellosis it was draw out a complete observation file which was contained all signs. Also, was established a comparatively diagnosed scheme for obtaining a rigorous scientific data.

Results - On the 285 samples prevailed by dogs with presumptive brucellosis it was isolated and identified belong Brucella canis species a total number of 10 stems. The best result of used isolating medium was obtained in case of Columbia agar associated with nalidixic acid or vancomycin, which don’t permit to develop other bacterial species. The Brain-Hearth Broth with 5 % glucose and with 10 % horse serum, or Brain-Hearth Agar with 5 % sheep blood, it was the optimal medium for developing of Brucella canis stems. The Brucella canis culture was preserved through freezing at -20 degrees; in this condition it was kept the vitality properties for a long time. The all 10 stems of identified Brucella canis stems had the same biochemical behavior.

References

VALIDATION OF PREPUTIAL CYTOLOGY IN THE DOG

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Introduction - The preputial epithelium is described as a target tissue of oestrogens¹. Characteristic changes in exfoliated preputial epithelial cells may occur as a result of modifications of the oestrogens plasma level. Elevated concentrations of estrogen in serum, as sometimes found with testicular tumours in male dogs, could cause differentiation and cornification of preputial epithelial cells. For this reason preputial cytology is often already used in practice when an oestrogen impregnation is suspected in male dogs. However, no investigator has validated this complementary test. The aim of this study is to analyse if preputial cytology is a reliable method of detecting modifications of preputial cells.

Materials and methods - Ten preputial smears were realised in 10 different dogs (5 have normal testis, 3 suffering from Sertoli cell tumors, 1 suffering from orchitis, and the last one showing a retained inguinal testis). Exfoliated cells were obtained by passing a humidified cotton-tipped swab into the prepuce. The swab was gently rolled over a clean microscope slide. The air-dried smears were stained with a Harris-Shorr staining technique and observed under the microscope (40 x). A classification system based on cell morphology was applied. Three different cell types were defined as in vaginal smears: parabasal cells (round to oval cell with a normal-appearing nucleus), intermediate cells (cells of variable size with a prominent nuclei that appear normal), and superficial cells (large angular cells with a pyknotic, faint, or non distinguishable nucleus). For each slide, proportion of the different cell types was defined by counting 100 cells / slide. Three different readers were included in the study. Each reader observed 10 slides three times. The test was completely blind due to a codification of the slides.

Results - For each reader, the proportion of different cell types did not vary significantly in the 3 consecutive readings. For each slide, no statistical differences were observed between each reader. However, there were statistical differences between the 10 slides. The correlation between the three different readers was significant, for the three different cell types. A good correlation between readers was obtained for superficial cells ($r^2$ is 0.52 between reader 1 and 2, 0.56 between reader 2 and 3, and 0.81 between reader 1 and 3).

Discussion - In the different slides the superficial cells represented 0 to 100 % of the cells depending of the slide. Whatever the reader, a good discrimination between the different cell types was obtained. The discrimination was easier in case of the superficial cells. The difference between parabasal cells and small intermediate cells was not evident.

Conclusion - Preputial cytology may be accurately used to determine the changes within exfoliated preputial epithelial cells.

References

PREPUTIAL EPITHELIAL CYTOLOGY AS A DIAGNOSTIC TEST FOR ADRENOCORTICAL DISEASE IN CASTRATED MALE FERRETS

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Introduction - The current pet ferret population in the U.S. is estimated to be 8 - 10 million. Adrenocortical disease is one of the most common diseases in domestic ferrets, with prevalence reported to be as high as 25% in the United States. The purpose of this study was to determine if preputial epithelial cytologic findings would correlate with endocrine and clinical findings associated with adrenocortical disease in castrated male domestic ferrets.

Materials and methods - Twenty-one castrated male ferrets were used in this study, eight of which exhibited one or more clinical signs of adrenocortical disease and thirteen of which served as healthy controls. Blood and preputial lavage samples were collected. Serum samples were submitted for endocrine assay at the University of Tennessee Veterinary Diagnostic Laboratory. Differential cell counts were performed on preputial lavage preparations to determine the percentage of cornified epithelial cells. Cytology results were compared to serum hormone concentrations as well as to clinical signs of adrenocortical disease.

Results - Preputial epithelial cornification was not significantly correlated with serum estradiol 17-β or androstenedione concentrations. However, there was a significant correlation between preputial epithelial cornification and serum 17-hydroxyprogesterone (17-HPG) concentration (Figure 1). The percentage of cornified preputial epithelial cells was higher in castrated ferrets with clinical signs of adrenocortical disease (70.6±15.9%) compared to non-clinical control animals (55.2±16.4%) (p=0.041).

Discussion - Further investigation is needed to elucidate the mechanism of preputial epithelial cornification in castrated male ferrets.
VERIFICATION OF THE PLASMA RELAXIN LEVELS AS A POTENTIAL PROGNOSTIC MARKER IN BITCHES WITH MAMMARY GLAND TUMOURS

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Introduction - One of the most consistent effects of relaxin (RLX) is its ability to stimulate the breakdown of collagen to allow tissue remodelling, which is required for physiological, such as implantation, and pathological situations, such as breast cancer. Human breast tissue is able to produce RLX locally. This RLX production is particularly potent in neoplastic breast cancer cells and can be measured in the periperal blood (2). Human breast cancer patients with an infaust prognosis show elevated serum RLX concentrations (1). The aim of the present study was to investigate the plasma RLX levels as a prognostic marker in bitches with mammary gland tumours.

Material and methods - In this prospective study 93 bitches were included, presented for surgical therapy of a mammary gland tumour. Preoperatively and up to 24 months after surgery the concentration of RLX in blood plasma from the patients and a healthy, tumour free control group (n = 28) was determined. Six months after surgery the thorax was radiographically checked for pulmonal metastases. The stage of the disease was characterised by clinical parameters according to the TNM-Score of the WHO. Bitches initially suffering from stage four of the mammary tumour disease were excluded from the study.

Results - The bitches in this study were of different races with an average age of 9.7 years. 81.7% were intact and in 92.5% histopathological investigations revealed malignant mammary tumours. No significant differences between the plasma RLX of patients and the control group or the plasma RLX of intact and spayed patients were found. Furthermore, no significant correlation could be obtained between the plasma RLX level and tumour dignity, tumour size, stage of the disease according the TNM-Score, presence of metastases in the regional lymph node, ulceration of the mammary tumour, presence of lung metastases 6 months after surgery or incidence of local recurrence. We could confirm, that these tumour properties were negative prognostic factors for the bitch to survive the first year after surgery.

Summary - The parameters examined so far do not allow to consider plasma RLX as a reliable prognostic marker for mammary cancer in the bitch. However, further investigations are required concerning the growth characteristics of the mammary tumour related to local remodelling factors.

This study was founded by the Kynologische Gesellschaft

References

Breast cancer is a disease that occurs in bitches and women. cDNA arrays is a powerful tool for studying gene expression in many different organisms. Access to cancerous and normal tissue is critical in order to know its regulation. This study used cDNA array in cross-species hybridization with canine breast cancer RNA. Fragments of normal and cancerous tissues were obtained from canine mammary glands after routine mastectomy surgery. The tissues were dissected and small pieces were quickly frozen in liquid nitrogen and stored frozen at -80°C. Total RNA was isolated by phenol-chloroform extraction (TRIzol®, GibcoBRL). The cDNA synthesis was done by using reverse transcriptase reaction. Nylon membranes were hybridized to the labeled probe and after 24 hours of exposure images were digitalized. Scanned images were visually and analyzed. Results showed a set of 222 up-regulated genes (p < 0.01). Among all samples some genes were expressed in all cancerous tissue when compared with normal tissue. These genes were: ESR2, PGR, RL23, CCL3, MYL3, NME1, RPS9, RPS11, RPS23, SLC25A5, HDAC2, HSPE1, CDC45, ESRRB, ATP5J2, NUTF2, FOSB, TSK, FHL5MG, C4767, IGF1 and RHSPA5. Many thousands of canine breast cancer components and candidate molecular markers can now be studied and compared with human breast cancer using microarray technologies. Acknowledgements: This work was supported by Fundação de Amparo À Pesquisa do Estado de São Paulo (FAPESP) – Brasil and Waltham Foundation.
ANALYSIS OF QUANTITY AND QUALITY OF BITCH OOCYTES RECOVERED BY USING VARIOUS SYSTEMS FROM ANIMALS OF DIFFERENT ESTRUS CYCLE STAGE

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Introduction - The effect of estrus cycle stage on in vitro meiotic and maturation competence of bitch oocytes has been well described in several studies [2,3]. Using an in vitro maturation system, De Avila Rodrigues B et al. indicated that in vitro maturation (IVM) of bitch oocytes is not influenced by the in vivo reproductive status of the females [2,3]. Moreover, they showed that the quality of gametes assessed by morphological evaluation is a more important indicator of its maturation and its developmental and meiotic competence than the hormonal environment of the individual female. Although the full characteristics of bitch oocyte morphology have been well defined, little is known about the effects that different gamete recovery systems have on the quantity and quality of oocytes. The quality and number of oocytes recovered from bitches of different age has been described by Rocha et al. (2006) [1]. However, determining the quality of gametes isolated from bitches in different reproductive stages requires future investigation.

Aims - The aim of this study was to evaluate the quality of oocytes collected from ovaries of bitches by incising ovarian tissue or by multi-slicing the ovarian surface with many razor blades.

Materials and methods - Collection was followed by the morphological evaluation of recovered COCs from bitches at various reproductive stages. The bitches were divided into groups based on reproductive cycle stage; estrus (n=10), metestrus (n=15), and anestrus (n=15). The oocytes were retrieved from anesthetized bitches by laparotomy. Classification of oocytes was based on their morphological evaluation and assessed as DI, DII, or degenerated.

Results - Follicles with a diameter of 500 µm to 1000 µm were observed in the ovaries of all 40 of the investigated animals. In all investigated groups of animals, we found a statistically significant increase in the number of COCs isolated by multi-slicing of the ovarian surface as compared to the procedure based on incising the ovary (P<0.05). The number of DI and DII COCs was higher in metestrus bitches as compared to estrus and anestrus females (P=0.032, P=0.042, and P=0.31, P=0.42, respectively). The differences in the number of DI and DII oocytes between estrus and anestrus bitches were not statistically significant (P=0.19, P=0.36, respectively). Moreover, we observed a significantly increased number of degenerated oocytes in anestrus bitches as compared to estrus (P<0.001), and in metestrus as compared to estrus (P=0.043). We postulated that increased numbers of high quality oocytes isolated from metestrus bitches may be associated with an increased concentration of progesterone at this stage. An increased number of degenerative oocytes in metestrus and anestrus bitches may result from a decreased concentration of estrogen, which has a protective role in the development of oocytes.
References

IGF GROWTH FACTOR EFFECTS ON MATURATION IN VITRO OF CANINE OOCYTES (CANIS FAMILIARIS): EVALUATION OF BOTH NUCLEAR AND CYTOPLASMIC MATURATION

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Objectives - This research aims at evaluating the effects of insulin like growth factor (IGF-I) on both nuclear and cytoplasmic in vitro maturation (IVM) of canine ovarian oocytes.

Materials and methods - The cumulus oocyte complexes (COCs) were matured in vitro in synthetic oviductal fluid. Ovaries of 37 bitches submitted to elective and therapeutic hysterosalpingo-oophorectomy (respectively as common spay procedure and pyometra correction) were used. The donators were classified according to race, age, reproductive condition and phase of estrus cycle. The COCs (n=1474) were freed from the follicles by the ovary slicing technique and matured for 72 hours, except for the 0M (zero moment) group. The base means of cultivation was synthetic oviductal fluid (SOF), supplemented or not with IGF. Only COCs classified as degree I were selected for MIV. COCs were distributed into three groups: 0M (processed on the collection day in the same way as the others), C (SOF) and E (SOF + IGF-I). After the incubation period or at the day of collection (0M), the oocytes were stained with bizbenzamide (Hoechst 33342) and for the cytoplasmic maturation with Lens culinaris.

Results - The percentages of COCs/donators of all bitches was 35.21% for cross-bred; 39.69% for adults (>10 months); 39.83% for multiparous, and 39.20% for in diestrus bitches (P<0.05). The percentage of degenerated oocytes was very high, both in the total amount (74.00%) and the experimental groups (0M=59.43; C=75.44 and E=80.5), the differences being statistically significant (P<0.05). In the nuclear maturation, only 3.02% of all oocytes submitted to this evaluation reached metaphase stage I (0M=2.83, C=4.19 and E=2.10%). Just like the meiotic retake, the cytoplasmic maturation rates were very low: 15.83% for the total amount, and 12.90, 25.58 and 8.69% for 0M, C and E groups, respectively. These outcomes indicate that supplementation or not of the SOF means for nuclear and/or cytoplasmic maturation did not improve IVM rates in canine oocytes. Cross-bred bitches, adults, multiparous and in diestrus provided more degree I COCs. Further study is needed in order to determine the lacking or deleterious components to canine COCs. This information can contribute to the optimization of MIV protocols, fundamental stage for in vitro embryo fecundation and production, which is important for the application of biotechnologies of the canine species to wild canines.

Key words - Oocyte, IVM, IGF-I, SOF, bitch, nuclear maturation, cytoplasmic maturation.
THE ASSOCIATION BETWEEN THE NUMBER AND QUALITY OF BITCH COCS AND SELECTED DONOR FACTORS

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Introduction - The number and quality of canine cumulus-oocyte complexes (COC) suitable for in vitro maturation and consequent embryo culture depends on several factors. Moreover, the method of COC collection is considered one of the most important in the process. An average number of COCs collected from a bitch may range from 10 up to several dozen or even over 100 [2,4,5]. On the other hand, regardless of the oocyte collection method, the number and morphology of collected COCs is influenced by some bitch-related factor, such as: season, breed, age, size and weight of ovaries, presence or lack of functional structures on the ovarian surface, stage of oestrus cycle, and the physiological or pathological state of uterus [1,3,5]. Previously published data on the importance of the above mentioned factors on oocyte number and quality is quite often contradictory.

Aims - The aim of this study was to investigate whether selected factors that are attributed to oocyte donors can affect the number and quality of canine cumulus-oocyte-complexes (COCs).

Materials and methods - The following parameters were considered: female age and body mass, ovary weight, presence of functional ovarian structures (eg. corpus luteum, visible follicles), and ovarian and uterine pathology. Altogether 10 077 COCs were collected, on average 125.6 per bitch.

Results - The number and quality of collected COCs were significantly affected by female age and presence of functional ovarian structures. We found an increased total number of COCs in up to 8 month-old bitches compared with up to 3-year-old and up to 7-year-old females (P<0.001). Moreover, older females produced more COCs displaying higher quality than the younger ones. However, the differences between those groups were not statistically significant (P=0.469, P=0.346). A higher number of COCs was collected from ovaries with a smooth surface (202.8) or with visible follicles (121.1), in comparison to ovaries with corpus luteum (97.6), pyometra and pathological status (82.8). No influence was observed of the bitch’s body mass and ovary weight on the number of COCs, although usually a higher number of oocytes was collected from the right ovary. The quantity and morphological quality of bitch COCs varied significantly among individual females, which occurred in an age-dependent manner. An increased number of COCs isolated from younger bitches may be associated with higher reproductive potential and hormonal activity of these females. We suggest that age affects the total number of collected COCs but has no influence on the quality of bitch oocytes.
References

COLD STORAGE OF CANINE OVARIES DID NOT AFFECT APOPTOSIS IN CUMULUS CELLS OR OOCYTES

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Objective of the work - The domestic dog is a model for developing in vitro maturation/fertilization protocols for wild canid species, where prolonged post-excision storage of ovaries frequently occurs due to long-distance transport. Apoptosis may be triggered by maintaining cells under suboptimal conditions. So far, the effect of canine ovary storage on cumulus cell and oocyte apoptosis has not been assessed. The aim of the present work was to compare the apoptotic rates of cumulus cells and oocytes recovered in Grade-1 cumulus-oocyte complexes (G1-COCs) collected from fresh (FO) and stored (SO) canine ovaries.

Materials & Methods - Ovaries were obtained from 12 post-pubertal animals undergoing ovario-hysterectomy (OVH). In 6 animals (FO) oocytes were collected immediately after OVH (<6 h at 22-23ºC in 0.9% NaCl with antibiotics). Ovaries of the other 6 animals (SO) were used to collect oocytes after overnight storage at 4ºC in 0.9% NaCl 0.9% with antibiotics. Both FO and SO groups were constituted by the same number of animals in the following reproductive stage and age group: anestrous animals aged 1-3 (n=2), 4-6 (n=2) and 7-10 (n=1) years old, and one pregnant female (1-3 years old). The ovarian cortex was sliced and washed in PBS supplemented with 1 mg PVP/ml at 37ºC. At the stereomicroscope, COCs with multilayered compact cumulus-oophorus and a dark, evenly granulated cytoplasm, with a diameter over 100 µm (Grade 1/G1), were selected. Between 20-30% of the total G1-COCs obtained were immediately fixed overnight at 4ºC in 4% paraformaldehyde/PBS. A similar percentage of oocytes were denuded of all cumulus cells before fixation, in order to assess oocyte nuclear maturity status and apoptosis. Apoptosis was detected with a TUNEL assay kit (Roche, Mannheim, Germany). One-way ANOVA was used to compare the number of collected G1-COCs between groups. The effect of overnight storage on apoptosis of cumulus cells and oocytes was assessed by chi-square test. A significance level was attributed to P<0.05.

Results - There was no difference in the number of G1-COCs recovered (p=0.80) and in the proportion of apoptotic cumulus cells (p=0.98) between the two groups. From a total of 194 G1-COCs observed for the presence of TUNEL-positive (apoptotic) cumulus cells, 63% (FO) and 65% (SO) presented apoptosis in less than 15% of the cumulus cells. There were also no significant differences between the two groups regarding oocyte nuclear maturity status and apoptotic rates. From 170 oocytes, over 72% were at the Germinal Vesicle (GV) stage and were non-apoptotic. Only 8% (FO) and 15% (SO) of the oocytes were apoptotic. In both groups all degenerated oocytes, were either apoptotic or no DNA was identified (9% in FO-group and 11% in SO-group).

Conclusions - Storage of canine ovaries overnight at 4ºC does not induce apoptosis in cumulus cells and in oocytes from G1-COCs.

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THE INFLUENCE OF OXYGEN TENSION ON CUMULUS CELLS VIABILITY OF CANINE COCs MATURED IN HIGH-GLUCOSE MEDIUM

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Introduction - The low rate of meiotic maturation of canine oocytes cultured in vitro is a main obstacle to the in vitro production of canine embryos. It has been suggested that the addition of serum to maturation medium can increase the rate of degenerated oocytes [2] [3], as well as to increase the incidence of apoptosis [1]. Cumulus cells surround and intercommunicate with oocytes during follicular development and after ovulation, suggesting that the incidence of apoptosis in cumulus cells could influence the developmental capacity of the oocyte. It is known that high oxygen tensions in vitro are associated with high levels of reactive oxygen species (ROS), which are implicated in the occurrence of apoptosis [5].

Objectives - The objective of this study was to determine the influence of oxygen tension on cumulus cells viability from canine oocytes matured in high-glucose medium.

Materials and Methods - Canine ovaries were obtained after ovariohysterectomy (OVH) surgery from 9 bitches at unknown reproductive stages, and with ages ranging from 1 to 12 years. The ovaries were transported to the laboratory at room temperature in phosphate buffered saline (PBS) and were processed within 2h of collection. The ovarian cortex was sliced and washed in PBS supplemented with 1% of foetal calf serum (FCS) to release cumulus-oocyte complexes (COCs). COCs were placed in TCM199 with 25 mM HEPES (M-2520; Sigma), and COCs with a multilayered compact cumulus-oophorus and dark cytoplasm (Grade 1) were selected to in vitro maturation (IVM). The oocytes were then distributed into three groups and two oxygen tensions (5% or 20%). The control group (CG) was matured in TCM199 with 2.2 mg/ml sodium bicarbonate (11150; Gibco) and supplemented with 10% (v/v) of foetal calf serum (FCS) (Nutricell), 50 µg/ml gentamicin, 22 µg/ml pyruvic acid, 20 µg/ml oestradiol (E-8875; Sigma), 0.5 µg/ml follicle-stimulating hormone (FSH) (Folltropin-V; Vetepharm Inc), 0.03 IU/ml human gonadotropin (hCG) (Chorulon®; Intervet), and 1.0 µg/ml human somatotropin (hST) (Humatrope, Lilly). The two others groups, which were designed as T5 and T20, were matured in a high-glucose medium, constituted by TCM199 with 2.2 mg/ml sodium bicarbonate (11150; Gibco), and supplemented with 0.1% Polyvinyl Alcohol (PVA) (P-8136; Sigma), 0.991 mg/ml glucose (108337; Merck), 50 µg/ml gentamicin, 22 µg/ml pyruvic acid, 20 µg/ml oestradiol (E-8875; Sigma), 0.5 µg/ml follicle-stimulating hormone (FSH) (Folltropin-V; Vetepharm Inc), 0.03 IU/ml human gonadotropin (hCG) (Chorulon®; Intervet), under 5% or 20% oxygen tension, respectively. After 48h of IVM the cumulus-cells were removed by mechanical cell displacement using a small-diameter glass micropipette and stained with propidium iodide (1 mg/ml) in PBS. Viability in cumulus cells was evaluated by assessing cytoplasmic features and integrity in nuclear morphology, as previously reported [4]. Incidence of apoptosis (marginated chromatin, pyknotic appearance, multiple nuclear fragments, and apoptotic bodies) in cumulus cells was assessed as percentages. Counts were performed at various randomly selected fields of the cumulus mass pipetted onto a microscope slide and mounted with coverslip sealed with incolor nail polish.

Statistical Analysis: Statistical analysis was performed using the data analysis software SPSS, version 13.0. Chi-square test with adjusted residual was used to compare differences among apoptotic groups.
Results - Cumulus cells were removed from a total of 405 COCs: i) CG (n = 136); ii) T5 (n = 135); T20 (n = 134). A total of 2700 cumulus cells were counted, being 900 for each group. Rates of apoptosis in cumulus cells were statistical different between groups, with 57.9% (521/900), 38.9% (350/900), and 54.4% (490/900) for CG, T5 and T20, respectively ($P<0.001$). Predominant features in the 1361 counted apoptotic cells were those containing multiple nuclear fragments, and observed with 94.0% (1280/1361) incidence.

Conclusions - Cumulus cells of canine COCs cultured in high-glucose medium presented significant less apoptosis than those cultured in medium with FCS. The FCS might be responsible for the high level of apoptosis observed in the control group. Low level of oxygen tension was efficient to reduce the occurrence of apoptosis in canine cumulus cells.

References

CUMULUS CELLS VIABILITY AND THE RELATIONSHIP WITH NUCLEAR MORPHOLOGY IN OOCYTES FROM PRE-PUBERTAL AND ADULT BITCHES AT 0, 24, 48 AND 72 HOURS AFTER IN VITRO MATURATION

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Introduction - In vitro maturation of oocytes in canine species is a complex process. Progression of meiosis is essential for the achievement of fertilization in vitro. At the present time both processes have been observed with low success in the bitch. Initial morphology of cumulus oocyte complexes (COCs), before in vitro culture is one important parameter inside these processes, and has been viewed as a predictor of oocyte competence.

Objective - The objective of this experiment was to observe the relationship of viability between the components of COCS, which was established through cumulus cells and oocyte nuclear morphology analysis. Individual COCs evaluations were done at different moments of in vitro culture (0, 24, 48, and 72 h). Furthermore, comparisons of above mentioned parameters were established between COCs from pre-pubertal and adult bitches.

Materials and methods - Ovaries were collected from pre-pubertal bitches (6-8 months) (n=03), and from adult bitches (2-10 years) (n=03) at unknown reproductive status, undergoing elective ovariohysterectomy. Oocytes from pre-pubertal bitches (n = 74) and adult bitches (n = 90) were obtained by slicing of ovarian cortex in PBS supplemented with foetal bovine serum (FBS) (Nutricell, São Paulo, Brazil) at 37°C. Oocytes of high quality (grade 1) with more than two compact layers of cumulus cells, dark pigmented and uniform ooplasm, and observed as having the largest diameter among their counterpartners (subjectively assessed) were selected and individually matured in vitro. Evaluation of viability of each oocyte and its respective cumulus cells was performed after 0, 24, 48 and 72 h of culture, by denudation with strained glass pipettes. Fluorescence microscopy was used to observe the progression of nuclear maturation by using the fluorescent dye Hoechst 33342 as described previously [1]. Viability of cumulus cells (200 cells per COC) was performed by using a differential staining with propidium iodide (PI) (1µg/ml) and Hoechst 33342 (5µg/ml). The total of cumulus cells counted for each COC, were distributed into groups of viability as following: <50%, 50-70%, and >70%.

Statistical analysis: Statistical analyses were performed using the data analysis software SPSS, version 13.0. Data were analyzed using Chi-square analysis with adjusted residual. Fisher’s exact test was used for comparison of nuclear maturation in oocytes. The values were considered statistically significant when P<0.05.

Results - The results showed an intimacy of the viability between the cumulus cells and chromatin configuration in COCs, both in adult and in pre-pubertal bitches. In adult bitches this statement was statically confirmed by the rates of oocyte nuclear degeneration (20/23; 87%), when incidence of viability in cumulus cells was <50%, and by observation of meiosis progression to the MI stage (4/27; 15%) when incidence of viability in cumulus cells was >70%, while in pre-pubertal females rates of 100% (12/12) of nuclear degeneration in oocytes were observed at cumulus cells viability < 50%, and 9% (3/32) meiosis progression to the MI stage, when incidence of viability in cumulus cells was >70% (P < 0.001). Rates of degeneration, as measured by numbers of PI positive cells, were enhanced in pre-pubertal (20/27; 74%) and different from the rates in adult females (2/40; 5%), when comparison was established between groups with 50-70% of viability in cumulus cells (P<0.001).
Furthermore, in the group with cumulus cells viability <50%, rates of 12.5% (3/24) of degeneration in COCs from adult bitches at 0h interval time, increased to rates of 22.7% (5/22) at 48h, and 59% (13/22) at 72h of *in vitro* culture (*P*<0.001).

**Conclusions** - Viability of cumulus cells has an intimate relationship with nuclear chromatin in immature oocytes. Also, the results of this experiment showed that with the progression of interval time of *in vitro* culture, oocyte nuclear morphology was associated with the viability features presented by cumulus cells.

**Reference**

EFFECTS OF NALOXONE ON IN VITRO MATURATION OF OOCYTES RECOVERED FROM ANESTROUS BITCHES

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Introduction - Supplementation of in vitro maturation (IVM) medium with opioid agents, agonist or antagonists, was shown to affect oocyte maturation in several species such as rat (1), bovine (2) and equine (3). The pharmacological effects of these compounds are mediated by G protein-coupled receptors (4). The mu opioid receptor (MOR) is expressed in oocytes of bovine (2), humans (5), equine (3) and canine (6) and, in seasonal breeders, is expressed with higher intensity in anestrous specimens (3). This study reports the effects of supplementing IVM medium with naloxone (Nx), an opioid antagonist, on nuclear and cytoplasmic maturation rate of oocytes recovered from anestrous bitches. Cytoplasmic maturation was examined in terms of mitochondrial (mt) distribution. In order to confirm the receptor-mediated action of Nx, the expression of MOR in oocytes of anestrous bitches was analyzed.

Methods - Cumulus-oocyte complexes were recovered from the ovaries of 4 bitches in anestrous. The IVM culture was performed as described by Otoi et al. (7). Naloxone was used at the concentrations of 1x10^-6, 1x10^-8 1x10^-10 M. Control oocytes were cultured in absence of Nx. After IVM, nuclear maturation of oocytes was classified as metaphase I (MI), metaphase II (MII) and MI+MII. The distribution of active mitochondria was revealed by using MitoTracker Orange CMTM Ros and confocal laser scanning microscopy. Heterogeneous mt distribution patterns were considered as indicative of cytoplasmic maturity as reported in other species (8). Western blot analysis was performed on oocytes examined after IVM culture in control conditions, as previously reported (9).

Results and Discussion - Nuclear maturation rates to MI, MII and MI+MII stages were 0%, 6% and 6% in control group (n=33 oocytes); 6%, 22% and 28% in oocytes cultured with 1x10^-6 M Nx (n=32); 7%, 3% and 10% in the group cultured with of 1x10^-8 M Nx (n=30); 7%, 0% and 7% in oocytes cultured with 1x10^-10 M Nx (n=27). Overall maturation rate of oocytes cultured in presence of 1x10^-6 M Nx (28%) was significantly higher than those of control group (6%; P<0.05). The treatment with Nx did not affect cytoplasmic maturation. All MII stage oocytes, control or Nx-treated, showed homogeneous small granular mt distribution pattern. In denuded oocytes and in corresponding cumulus cells, a doublet of 65 and 50 kDa was observed. We conclude that, in oocytes of anestrous bitches, MOR is expressed and the opioid antagonist Nx significantly improves nuclear maturation rate. Further studies could be performed to elucidate the expression of other opioid receptors, such as delta and kappa, and possible interactive effects of their antagonists on canine oocyte maturation.

References

ULTRASTRUCTURAL STUDY OF THE CANINE ZONA PELLUCIDA SURFACE DURING IN VITRO MATURATION

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Introduction - Zona Pellucida (ZP) is an extracellular matrix that plays important functions during gamete interaction and fertilization (De los Reyes y Barros, 2000). During maturation, the oocyte undergoes nuclear and cytoplasmatic changes, including oocytes investment. In the course of acquiring these competencies variations in the architecture of the ZP have been described in some species (Vanroose et al., 2000; Michetmann et al., 2007). The objective of this study was to evaluate by scanning electron microscopy (SEM) morphological changes of the ZP surface of immature and in vitro matured bitch oocytes cultured during two different periods (48 and 72 h.)

Materials and Methods - Cumulus oocytes complex (COC’s) were obtained from normal bitch ovaries following ovary hysterectomy and selected according homogeneous dark cytoplasm with more than three compact cumulus cells layers. The selected COC’s were randomly allocated into three groups: a) one group was incubated for in vitro maturation in TCM 199;Earle’s salt, buffered with 25mM Hepes, supplemented with 10% fetal calf serum, 2.5 µL/mL pyruvic solution (11.2 mg/mL pyruvic acid), 10 IU/ mL of human chorionic gonadotrophin and 5µL/mL antibiotic solution (12.2 mg/mL penicillin and 20 mg/mL streptomycin) for 72h; b) the second group was cultured in the same medium for 96h, and c) the third group was processed at immature state in PBS medium. Immature and in vitro matured oocytes for 72 and 96h were processed for SEM (Barros et al., 1984). After removal of the cumulus cells, oocytes were fixed for 1 hour in 2.5 % (v/v) gluteraldehyde in a 0.1 M sodium cacodylate buffer, pH 7.4, and post fixed in 2% osmium tetroxide. After that, the oocytes were dehydrated in increasing concentration of acetones, critical point dried, mounted and sputtered with palladium-gold target. The samples were evaluated with a “LEO” 1420 VP SEM. The ZP network surface (holes diameters) were measured by “AutoCad 2006” software on a total of 93 canine oocytes, 30 oocytes were evaluated at immature state, 31 after 72 h and 32 after 96 h of culture. The results were analyzed by ANOVA using Tukey’s test to determine the differences p<0.05.

Results - Significant differences (p<0.05) between the ZP surface of immature and in vitro matured oocytes were found. Before IVM, the ZP surface showed numerous tight holes (mean 0.69 µm) and after culturing, the ZP surface was characterized by large holes. A significant difference was also observed between each time period (p<0.05; 72 and 96 h). Oocytes cultured for 72 h showed wider holes than oocytes cultured for 96 h (1.56-1.42 µm respectively).

Conclusions - These results show that structural changes in the ZP surface occur during in vitro maturation of canine oocytes; this mesh-like arrangements of the ZP after maturation, could influence sperm binding and penetration during the gamete interaction.
References


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FOLLICULOGENESIS AND MORPHOMETRY OF OOCYTE AND FOLLICLE GROWTH IN THE FELINE OVARY

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This study was designed to describe, both quantitatively (morphometry) and qualitatively (histological differentiation), follicle and oocyte growth in the feline ovary.

Materials and methods - The ovaries of 43 cats were collected and processed for histology. The diameters of 832 follicle / oocyte pairs were measured, with and without zona pellucida, and a special emphasis was placed on the study of early folliculogenesis.

Results - Primordial, primary, secondary, preantral and early antral follicles were measured at 44.3 µm, 86.2 µm, 126.0 µm, 155.6 µm and 223.8 µm in diameter respectively. A biphasic pattern of follicle and oocyte growth was observed. Before antrum formation, follicle (x) and oocyte (y) size were positively and linearly correlated (y = 0.500 x + 20.01, r² = 0.89). Antrum formation occurred when the follicle reached 160 – 200 µm in diameter (when oocyte was at 102 µm). After antrum formation, a decoupling was observed, a minimal increase in oocyte size contrasting with a significant follicle development (y = 0.001 x + 114.39, r² = 0.01). The preovulatory follicle diameter was ~ 3500 µm and the maximal oocyte diameter was 115 µm. The zona pellucida, absent in primordial and primary follicles, appeared at the secondary stage and reached almost 6 µm at the preovulatory stage.

Conclusions - These results suggest that (1) in feline ovary, follicle and oocyte growth pattern is similar to that observed in other mammals, (2) the antrum forms in 160 – 200 µm follicles, which represents 5% of the preovulatory diameter, (3) the oocyte had achieved more than 90% of its maximal growth at the antrum formation.
THE EFFECTS OF VARIOUS PROTEIN SUPPLEMENTATION ON IN VITRO MATURATION OF FELINE OOCYTES

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Objectives - This study has been conducted to evaluate the effects of various protein sources [Foetal Calf Serum (FCS), Bovine Serum Albumin (BSA) and Oestrus Cat Serum (OCS)] on in vitro maturation of feline oocytes.

Materials and methods - The study was conducted on 1013 oocytes obtained from cats that underwent ovario-hysterectomy surgeries for the purposes of spaying. Ovaria were brought to the laboratory in PBS solution at 25-30°C. The recovered oocytes were left to mature for 48 h in Ham’s F-10 with FSH 1µl/mg, LH 1µl/mg and different protein supplements: Group I: 5% FCS, Group II: 3 mg/ml BSA, Group III: 5% OCS, Group IV: without protein. Incubator conditions were 38.5°C temperature, high humidity and a gaseous mixture (5% O2, 5% CO2, 90% N2). At the end of the incubation period, the oocytes were fixated in 1:3 acetic acid:ethanol for at least 24 h and painted with 2% aceto-orcein. The maturation state was determined under a phase-contrast microscope at 400x magnification.

Results - Maturation rates (Metaphase II) were 6.2% (14/225), 16.5% (43/260), 13.1% (36/273) and 13.3% (30/225) in groups I, II, III and IV, respectively. The maturation rate in the group in which 5% FCS was used as protein additive was significantly lower (p<0.001) than other groups. The ratio of oocytes reaching Metaphase I + II stage was 28.6% (73/225), 40.3% (105/260), 31.1% (85/273) and 30.2% (68/225), respectively to the groups. The difference in the BSA group was found to be significant (p<0.05).

Conclusions - it was determined that using BSA as the protein additive for the medium will be beneficial for in vitro maturation of feline oocytes.
FUNCTIONAL ROLE OF FELINE ZONA PELLUCIDA PROTEIN B2 TREFOIL DOMAIN: A SPERM RECEPTOR OR STRUCTURAL COMPONENT OF THE DOMESTIC CAT ZONA PELLUCIDA?

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Introduction - In general, the trefoil domain is a protein structure characterized by 6 cysteines, which form a typical 3 loop structure by 3 disulphide bridges. It is assumed that two of these loops generate a hydrophobic groove, which can be a binding pocket for carbohydrate residues or proteins [3]. Zona pellucida B proteins contain such a trefoil domain (TFD). The feline ZPB2 (= ZP4) trefoil domain (fTFD) also represents a B-cell-epitope [2]. The suggested carbohydrate/protein binding property of trefoil domains allows us to assume a potential sperm receptor function. Additionally gastrointestinal trefoil peptides are stable against proteases [3]; therefore a structural role of TFD within the ZP might also be possible.

Aims - The aim of the present study was to characterize the function of feline ZPB2 trefoil domain (fTFD) by (i) determining the protease stability of native and mutated fTFD, (ii) identifying the binding of fTFD to cat spermatozoa and (iii) testing the influence of TFD on sperm binding to native zona pellucida of cat oocytes.

Materials and methods - Two modifications of the feline TFD peptide (aa 175-218 of fZPB2) were synthesized – the native peptide (fTFD) and a mutated form (fTFD-CS), where all cysteine residues were changed to serine. Within the mutated peptide, the trefoil structure cannot be formed due to the avoidance of disulphide bridges. Additionally, rabbit polyclonal antibodies were produced against solubilized feline zona pellucida [1] and a peptide representing partly loop 1 of feline TFD (aa 180-190 of fZPB2) to detect zona pellucida and fTFD epitops on cat oocytes and to test sperm binding in vitro. Co-immunoprecipitations applying the fTFD and anti-fTFD antibody were aimed to isolate sperm proteins, which specifically bind the fTFD.

Results - We could show that the native fTFD expresses the typical protease resistance that was lost under reducing conditions and after substitution of cysteine residues within the peptide (= mutated version). Specific binding of anti-fZP and anti-fTFD antibodies to cat zona pellucida was successfully proven by immunofluorescence (intact oocytes) and immuno electron microscopy. Interestingly, the antibody titre of the two antibodies differed markedly if different ZP-sources were used. Using a feline ZP lysat (lithium -3,5-diiodsalicylate, [1]) in an ELISA, both antibodies expressed quite high and almost comparable titres of 1:10^5 and 1: 10^6 (for anti-fTFD and for anti-fZP-antibody, respectively). Binding to native ZP, however, was only detectable using undiluted anti-fTFD, whereas the anti-fZP-antibody was still active at a 1:25,000 dilution. We suggest that a structural masking of the fTFD domain within the intact ZP might be responsible for this poor interaction. Accordingly, the inhibition of sperm binding to in-vitro matured cat oocytes was also only shown for the anti-fZP-antibody. Pre-incubation of cat sperm cells with fTFD did not result in any detectable binding of the fTFD to sperm cell surface (by antibody detection via immunofluorescence). This might be caused by either low-affinity interaction of fTFD with sperm cells or masking of fTFD by bound sperm molecules to the antibody epitope, but co-immunoprecipitations and immuno electron microscopy also failed to identify a sperm surface binding of fTFD. The final prove for any
sperm cell interaction with fTFD would be the induction of acrosome reaction of cat sperm cells.
To summarize, there is increasing evidence that the trefoil domain of feline ZPB2 has rather a structural than a sperm binding function. Both the stability against trypsin and the result of the antibody-binding experiments support the idea that TFD contributes to the stability of feline zona pellucida which is known to be more resistant against chemicals and enzymes then ZPs of other species.

References

TESTIS AND EPIDIDYMIS OF ASIATIC BLACK BEAR (URSUS THIBETANUS) EXPRESS MU-OPIOID RECEPTOR

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Introduction - Reproduction of endangered species is of great interest in management of modern zoos. Asiatic Black Bear (Ursus thibetanus) is included among endangered species by IUCN (International Union for Conservation of nature and Natural Resources). The onset of puberty in Male Japanese Asiatic Black Bears (Ursus thibetanus japonicus) is between 2 and 4 years of age (1). Reproduction is seasonal and occurs from late spring to summer (2), in fact testosterone serum concentration is high in the breeding season, lower in March-April and in November –January (3). Mammals reproduction is negatively affected by endogenous opioid peptides (EOPs), such as β-endorphin, able to bind mu-opioid receptors (MORs) (4) and to modulate gonads activity. It was demonstrated that chronic opioids administration is capable to reduce gonadotropin plasma levels and to inhibit testicular enzymes (5). MORs are expressed in gonads and gametes of Vertebrates (6,7), including carnivores (8). The aim of the present study was to detect MORs presence in testis and epididymis of an Asiatic Black Bear.

Materials and Methods - Testes and epididymes were collected from an adult male Asiatic Black Bear died in August 2007 at the Zoo Safari of Fasano (Br, Italy) and frozen at -80°C until μ-opioid receptor assessment.

Western blot analysis
Western blot analysis was performed on proteins extracted from testicular and epididymal tissues homogenized with a motor-driven homogenizer in lysis buffer containing protease inhibitors (PBS/0.1% Triton X-100, 0.1mg/ml PMSF, 1 g/ml leupeptin, 1 g/ml aprotinin, 0.1mg/ml benzamidine, 1 g/ml pepstatin A, 8 g/ml Calpain I and II pH 7.2). The homogenate was centrifuged at 10000 xg for 30 min at 4°C to remove nuclei and mitochondria. Protein concentration of the supernatant was measured using a BCA assay (Pierce, Milan, Italy). An aliquot of 30 g protein was loaded on a precasted 12% SDS/PAGE (Bio-Rad, Milano, Italy). After electrophoresis, proteins were electrotransferred (semi-dry apparatus, BioRad, Milano, Italy) to Immobilon-P membranes (Millipore, Bedford, MA, USA). Filters were blocked in 20mM Tris-HCl, pH 7.5/0.15M NaCl/1% Triton X-100/5% non fat milk (blocking buffer) for 1h and blotted overnight at 4°C against the primary antibody (anti MORs third extracellular loop polyclonal antibody, Chemicon Int. Inc. Temecula, CA) diluted 1:7500 in blocking buffer. Membranes were washed in blocking buffer and incubated with a 1:10000 dilution of peroxidase-conjugated goat anti-rabbit secondary antibody for 2 h at room temperature. After washing, reactive bands were visualized by Supersignal West Pico Chemiluminescent substrate (Pierce, Milano, Italy). As positive control, protein extract from rat brain were used.

Results - Our research evidenced MORs expression both in testis and epididymis by a positive immunoreactive doublet of approximately 50 and 65 KDa plus an additional band of approximately 45 KDa molecular mass. Anyway, MORs expression, was very low. Both the cortical and medullar part of the testis were positive, but the band of 45 KDa, that is very evident in the former, is not present in the latter. In epididymis signals were very faint and only in the body the additional 45KDa band is present.
Discussion - Some EOPs, such as β-endorphin, are known to impair reproductive performance. It has been demonstrated that in the Indian major carp (Cirrhinus mrigala) its hypothalamic level is low during the reproductive period, increases during the non-reproductive phase, exerting an inhibitory control on the reproductive axis (9). The weak immunoreactive signal observed, both in testis and epididymis, is probably correlated to the fact that organ collection was carried out in the full breeding season of this species. We hypothesize, as already shown in other species (10), that MORs expression is different according to the reproductive phase, increasing out of the breeding season thus causing a stronger biological response to some EOPs such as β-endorphin. The different molecular sizes of MORs immunoreactive bands detected may reflect a specific pattern of post-translational modifications as observed before in other species (7) Further studies are required to investigate on the functional meaning of the different immunoreactive bands to find out, if exists, a correlation between functionality and post translational modifications of MORs.

References

PRECISION AND ACCURACY OF THE ACCUREAD® SPECTROPHOTOMETER TO DETERMINE SPERM CONCENTRATION IN THE DOG

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Introduction - Freezing dog semen requires a precise and accurate way for sperm counting, as sperm concentration in straws or pellets plays a great role in freezing rate success. Actually, hemocytometers (counting chambers) remains the gold standard to assess sperm concentration. However, a relatively high number of spermatozoa (300 to 400) should be counted to achieve a high level of precision. That is why great variations between operators can occur frequently, as difficulty to count spermatozoa increases with sperm concentration. The Accuread® (IMV, L’Aigle, France) spectrophotometer, first developed for large animals semen evaluation, could be a reliable and time efficient alternative to counting chambers. However, dilution rate which should be used for sperm counting are species specific. The aim of our study was to control if the same dilution rate as for counting chambers may be used and then to compare the two methods.

Material and methods - 63 dog semen samples were collected by masturbation in the Alfort Veterinary College and divided in the three different fractions of the dog ejaculate (pre-spermatic, spermatic and post-spermatic fractions). The spermatic fraction was conserved and used for sperm counting with the Thoma® chamber hemocytometer and the Accuread® spectrophotometer. With the Thoma counting chamber, a 1/100 dilution rate was realized (10µL of semen spermatic fraction added to 990µL of sterile saline fluid, NaCl 3%) then diluted samples were loaded by moistening the pillows of the hemocytometer prior to applying the cover slip. The sperm suspension was then loaded to fill the counting chamber exactly and a total of 5 large squares of 1/250mm³ each were counted under light microscopy. This procedure was realized twice by two different operators. Sperm counting with the Accuread® spectrophotometer was done according to the manufacturer’s instructions. The same dilution rate was used, and 20µL of semen were added to 1980µL of sterile saline fluid (NaCl 0,9%). The zero was determined by using 2000µL of NaCl 0.9%, and then sperm concentration was evaluated. Statistical analysis was carried out with Microsoft Excel® Analysis Toolkit. Repetability of each method was assessed by calculating the CV for the 5 repeats of each ejaculate. Comparison between methods was initially carried out using a 1-way analysis of variance (ANOVA) and direct comparisons between treatments were compared with reference to the least significant difference. Since there was no significant difference between methods, further analysis between samples was carried out by correlation.

Results - Each method ensured a good repeatability. CV was 8.4% for the Thoma® counting chamber and 0.8% for the Accuread® spectrophotometer. No significant difference was found between the two operators (p<0.0001). Sperm concentration varied greatly among samples, ranging from 18.75 to 4520 million spermatozoa/mL with the Thoma chamber and 14.1 to 4360 million/mL with Accuread®. Mean dog semen output with the counting chamber was 771.2 million spermatozoa/ejaculate (SD=688.4 million) and 700.2 million spermatozoa/ejaculate (SD=582.8 million) with the spectrophotometer. A one way ANOVA demonstrated no significant difference between the two methods (p<0.0001). These data were supported by high correlation coefficient (r²=0.951).
**Discussion** - Counting chambers are laborious for routine use and subject to human error, whereas spectrophotometers appear to be an easier, time efficient and more effective alternative. We demonstrated that Accuread® dog sperm counting with the dilution rate of 1/100 was as efficient as Thoma counting chamber. There was an excellent correlation between the two methods for a wide range of sperm concentrations, from 14 to 4360 million spermatozoa/mL. This great variation was depending on the volume of prostatic fluid collected with the spermatic fraction. It is difficult to determine whether this spectrophotometer is more precise and accurate as their counts are based on calibrations using hemocytometers. However, we think it is a valuable and affordable tool to optimize our work in canine semen bank and for practitioners who deal with canine reproduction.

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Morphology analysis of dog sperm

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Introduction - The testicles of breeding males are marked by their high sperm cell production; however these cells include a deficiency in high quality cells naturally. A series of factors in the internal and external environment influence the production of this cell population. Morphologic abnormalities of sperm in different species of animals have been evaluated in connection with decreased fertility by a number of authors. According to Oettlé (3) the fertility of fresh dog semen is markedly reduced when the percentage of morphologically abnormal cells in the semen is higher than 40%. The development of ‘so-called’ strict morphology analysis (2) allows expanded and more accurate morphological diagnosis. This method was noted while developing the computer programme SASMO (Strict Analysis of Sperm Morphology; 5). Cryopreservation intervention reduces dog ejaculate quality and thus also the fertilisation success rate. Nevertheless, the quality of sperm from some dogs declines more after cryopreservation than sperm from other dogs. This may be caused by variations in the quality of cell membranes (1). Due to the fact that resistance of dog spermatozoa to the process of cryopreservation is low, it is important to monitor the integrity of the sperm plasma membrane when performing resistance tests in fresh semen.

Objectives - The aim of the first part of the study was to perform detailed morphology assessment of dog sperm. The aim of the second part was to evaluate sperm membrane quality during short-term survival test to predict post-thawing semen quality.

Materials and methods - Experiment 1: Forty ejaculates of forty dogs (mean age 4.8 years; range 1.5-11) with a history of good fertility and without being exposed to any factor that could seriously impair their spermiogenesis were analyzed after collection. Motility, viability and detailed morphological evaluation by SASMO program (5) were evaluated (viability by eosin-nigrosin staining; sperm morphology by optic microscopy examination of slides stained acc. to Karras). The morphological examination was done according to Kruger strict morphology criteria both after collection and after the 2h sperm survival test (as you see below; 4). The entire spermatozoon was taken into account and emphasis was placed on the multiparametric examination of spermatozoa, i.e. all changes present in the spermatozoon are evaluated and the teratozoospermic index was determined (total number of defects/number of spermatozoa with defects; 6). Any deviations from normal shape and structure are considered abnormal. Following sperm morphological defects of sperm head, neck, mid-piece and tail were studied: detached heads, macrocephalic head, microcephalic head, tapered head, amorphous head, pyriform head, ridge head, pinhead head, double head, knobbled acrosome, swollen acrosome, detached acrosome, vacuoles in the head area, flat implantation site, abnormal head-mid-piece junction, disintegration of the head-mid-piece junction, proximal droplet, thickened mid-piece, thinned mid-piece, partial absence of mitochondria, kinked mid-piece (corkscrew defect), double mid-piece, bent tail, coiled tail, tightly coiled tail (Dag defect), broken tail, stumped tail, and double tail. Experiment 2: Forty ejaculates from 20 dogs included with experiment 1 were analyzed after collection and split into two portions: one was incubated in buffered saline (pH 7.2) at 22°C for 2h (2h sperm survival test); the second part was cryopreserved in TRIS-fructose extender and maintained in LN2. Motility and percentage of sperm with intact membranes in the acrosomal area (as indicator of sperm membrane integrity) were evaluated after collection, after 2h survival test, and after thawing. Sperm with intact membranes in the acrosomal area were detected by morphological evaluation after staining acc. to Karras.
Results - Experiment 1: Mean total sperm count was 783.9±917.73 x 10^6 spermatozoa (range 28.8-4774.4 x 10^6 spermatozoa), sperm motility was 74.8±11.82% (50-90%), sperm viability was 82.1±10.12% (57.6-97%), percentage of sperm with normal strict morphology was 66.8±13.76% (32.5-92%), and percentage of sperm with primary morphological defects 14.1±8.91% (2-37%). We found 17.1±9.95% (1.5-37.5%) of sperm with head abnormalities; 5.8±7.48% (0-38%) with neck abnormalities; 1.1±1.42% (0-6%) with mid-piece abnormalities; 13.5±10.08% (2.5-52.5%) with tail abnormalities, and 0.8±1.97% (0-9%) of sperm with detached heads. Mean teratozoospermic index was 1.18±0.11 (1-1.44). Detailed morphological evaluation showed that bent tail, swollen acrosome, proximal droplet, detached acrosome, tightly coiled tail (Dag defect) and coiled tail were found to be the most frequented sperm defects in fresh dog semen. Secondary acrosomal defects (swollen and detached acrosome) showed significant increase after 2h survival test (p<0.001). These defects indicate disturbed sperm membrane integrity in the head area. Experiment 2: Based on post-thaw motility, ejaculates were assessed as poor (group 1) and good freezers (group 2) (the threshold was 40%). Membrane integrity was more impaired in group 1 vs. group 2 during the survival test and cryopreservation (p<0.01). The percentages of sperm with intact membranes in the acrosomal area were decreased by 11.4±6.56% and 4.9±3.83%, resp. The decrease of sperm with intact membranes during the survival test correlated with their decrease during cryopreservation (r=0.707; p<0.001) and sperm motility after thawing (r=-0.521; p<0.01). Conclusion: Bent tail, swollen acrosome, proximal droplet, detached acrosome, tightly coiled tail (Dag defect) and coiled tail were found to be the most frequent sperm defects in fresh dog semen. High decrease in sperm membrane resistance during 2h survival test leads to low survival rate after thawing of the cryopreserved cells. The decrease in sperm membrane resistance during survival test should not exceed 10% of initial value in order to get good sperm survival rate in cryopreservation process.

References


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THE SEASON EFFECTS ON SEMINAL CHARACTERISTICS IN DOGS IN A TROPICAL ZONE

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Introduction - In equatorial regions, photoperiod and temperature are important factors that may influence the reproductive cycle of several species [1, 4]. The effect of day length on breeding seasonality seems to disappear below 30º of latitude [2]. Usually at lower latitudes there is little seasonal variation in environmental temperature and marked variation in rainfall [2].

Objectives - The objective of this study was to investigate the seasonal influence in seminal characteristics in different breeds of dogs living in identical conditions in a tropical zone, at 23º 35’ S latitude and 48º 59’ W longitude. In this region, the variation in day length between winter and summer solstice is approximately two hours and thirty minutes.

Materials and methods - Eight adult dogs of 3 breeds (2 Blood Hound, 2 Golden Retriever, 4 Springer Spaniel), aged between 1.2 and 6 years were used in the study. The dogs were active breeding animals that were housed in the same kennel throughout the 14-month period of the study (July 2002 to August 2003). Semen samples were collected by digital manipulation each 15 days. Immediately after the collection, spermatic progressive motility, progressive velocity, pH and abnormalities of the acrosomes, midpieces, sperm tails, and head defects were evaluated. The year was divided into 4 seasons and each season was related to the mean environmental temperature and pluvial mean rainfall during that season: winter 2002 (I - July to September 2002), spring 2002 (II - October to December 2002), summer 2003 (III - January to March 2003), autumn 2003 (IV - April to June 2003) and winter 2003 (V - July and August 2003). Environmental temperature and pluvial index data were obtained from the Information Center for Agronomy and Meteorology (www.iac.org.sp). Differences between seminal characteristics (means ±SD) and environmental data were determined by ANOVA followed by a two-paired t-test. Values were considered significant at p≤0.05. Correlation coefficients (r – values) were calculated between seminal characteristics and environmental data using Pearson’s correlations.

Results - Throughout the research period, temperature ranged from 10.2ºC to 32.8ºC and the pluvial index from 33 mm to 476 mm. The highest temperatures and greatest rainfall were observed during the summer periods. Median values of semen characteristics in 8 stud dogs, during seasons were: pH (6.4, 6.2, 6.3, 6.1, 6.2), sperm motility (84.0, 84.2, 89.6, 88.0, 88.8%), sperm velocity (4.2, 4.1, 4.4, 4.5, 4.6), total concentration (346.5, 369.5, 403.9, 461.2, 466.0x10⁶ spermatozoa/mL), normal sperm (61.5, 61.9, 65.5, 73.8, 73.5%), major defect (24.9, 27.7, 22.4, 17.2, 16.4%) and minor defects (12.0, 10.5, 12.7, 9.0, 10.3%), respectively to I, II, III, IV and V. Semen characteristics were within normal values for the specie, except for the percentage of total morphological defects (20%), it is believed that fertility has not been compromised, because as fertility is impaired when the percentage of morphologically normal spermatozoa is below 60% [8] and the average percentage of normal cells was greater than 61%. Significant increase was observed in progressive spermatic motility during Summer 2003 versus Spring 2002 (84.2% versus 89.6%, p=0.004). Variations averages of seminal parameters were similar to the study done in the north hemisphere [7.10] and higher than results obtained in Germany [9]. In this study, similar the literature [10], was observed an increase of semen quality in summer, in contrast to other...
authors, which has been observed a decrease [7]. Although high temperature and high pluvial index result in high humidity, which may be the major stressor in summer, where was observed that serum canine testosterone concentration was a significantly decrease (xx), but was not enough to decrease the semen parameters. The dogs studied remained fertile throughout the year, as observed in horses [5] and sheep [6]; unlike wild dogs, which have a minor testicular performance during female seasonal anestrus [3].

In conclusion, in the domestic canine in tropical zones, the seasonality has only a minimal influence on testicular function.

References


OSTEOPONTIN IN SEMINAL PLASMA AND SPERM MEMBRANE OF DOGS

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\textbf{Introduction} - Seminal plasma and membrane sperm proteins have been associated with fertility in various species. Killian et al. \cite{4} studied seminal plasma of bulls using electrophoresis and suggested 4 proteins associated with fertility. Two of these proteins were associated with high-fertility and one was identified as an osteopontin. In stallions, Brandon et al. \cite{1} identified the SP-1, one protein from seminal plasma positively correlated to fertility; this protein showed similarity with bovine osteopontin.

\textbf{Objectives} - Thus, the aim of this study was verified the presence the bovine osteopontin in the canine seminal plasma and membrane sperm extracts, using western blot method.

\textbf{Materials and methods} - The semen was collected from 30 dogs, of different breeds, with recent paternity history. One aliquot of each ejaculate was separated to analyze membrane proteins. In this sample was added an aliquot of buffer (1:1) contained protease inhibitor. Thus, the samples were centrifuged for 3 times, 700xg, during 5’, at 4°C. The supernatant was eliminated and the pellet was diluted in the same buffer containing protease inhibitor. Futhermore, it was added Nonidet P-40 0.1% and the samples were sonicated. After sonication, the samples were vortexed for 3 minutes and centrifuged 15,000xg, during 30’, at 4°C. The supernatant, constituted of protein sperm membrane extract, was separated and frozen. The seminal plasma was separated by centrifuged 5,000xg, during 30’, at 4°C and frozen. Sample pool seminal plasma and sperm membrane extract was used to one-dimensional electrophoresis (12% - SDS-PAGE). Bovine seminal plasma sample was used as positive control. After electrophoresis, the bands were transferred to nitrocellulose membrane and western blot was performed using the bovine anti-osteopontin antibody (kindly provided by Dr. Gary Killian, PenState University, USA). Diaminobenzidine was used as a substrate.

\textbf{Results} - Two and 12 bands were marked in the seminal plasma (77.2 kDa and 15.6 kDa) and sperm membrane extract (70.6 kDa to 26.6 kDa), respectively (Figure 1); from 12 marked bands in sperm membrane extract, only 3 (46.4 kDa, 37.7 kDa and 36.5 kDa) were strongly marked. The band 15.6 kDa marked by anti-osteopontin antibody is found in high concentration in seminal plasma and it is a heparin-binding protein described by Souza et al. \cite{6}; these last authors suggested that band is an arginine esterase subunit, protein with enzymatic action, named canine prostate specific esterase (CPSE). The CPSE is 30% of total proteins from seminal plasma \cite{3}. The meaning of its \textit{in vivo} proteolitic activity is obscure, but it can be related to cervical mucus hydrolysis and, uterus and uterine tubes motility regulation during the fertilizing process \cite{2,3}. Martins \cite{5} showed the band \textasciitilde{}15.6 kDa positively correlated with pre- and post-thawed sperm motility and integrity membrane. Therefore, this author found one other seminal plasma protein (band \textasciitilde{}15.3 kDa) in lower concentration in dog with higher results from frozen semen evaluation. Additionally, suggested that 2 bands (\textasciitilde{}15.6 and \textasciitilde{}15.3 kDa) could be arginine esterase subunits. The binding to anti-osteopontin antibody is not confirmation that these proteins will be related to...
fertility (positively or negatively), but it could conduct other studies to identify important molecules to fertilization process.

Figure 1: Nitrocellulose membrane resulted of western blot. MP: sperm membrane extract (1: 46.4 kDa; 2: 37.7 kDa and 3: 36.5 kDa) and SP: seminal plasma.

References

EFFECTS OF THE CHLOROFORMIC EXTRACTS FROM WASHED AND NON-WASHED PAPAYA SEEDS (CARICA PAPAYA) ON THE SPERM CONCENTRATION OF DOGS.

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Introduction - Control campaigns against dog overpopulation are based on systematic euthanasia and surgical gonadectomy (orchiectomy and ovariohysterectomy). The use of non-surgical methods for orchiectomy may be more humanitarian, economical and easier to perform on large scale sterilization campaigns. Several studies have shown the effect of papaya seeds (Carica papaya) on the sperm parameters of rabbits, humans and monkeys (Lohiya et al., 1999; Lohiya et al., 2000; Lohiya et al., 2002), but not studies have been reported in dogs. The objective of this study was to determine the effects of chloroformic extracts of papaya seeds on the sperm concentration of domestic dogs.

Material and methods - Nine healthy mongrel dogs with normal sperm characteristics were used. Group 1 consisted of three dogs treated orally with 50 mg/kg/day of a chloroformic extract of washed dried papaya seed for 120 days. Group 2 (n=3) was similarly treated but using a chloroformic extract of non-washed dried papaya seed. Group 3 (n=3) was control. Dogs were ejaculated by digital manipulation before treatment (day 0) and every two weeks until day 120. First and second fractions of the ejaculated were collected and semen evaluated (Ortega-Pacheco et al., 2006). A monthly ultrasonographical evaluation of the testicles was made. Results from semen evaluation were expressed as means ± SD. Difference values of sperm concentration between treatments and control group were tested using a paired T-test. The level of significance was set at p< 0.05.

Results and discussion - Non significant decrease on sperm concentration was observed in dogs from Group 1(non-washed seeds) compared to control group, but a large variation was observed especially 90 days after the beginning of the treatment (199.7 ± 133.7 sperm cells x10⁶). Dogs from group 2 showed a progressive decline on sperm concentration to reach significant low levels on day 120 post-treatment. No detectable ultrasonographic changes were visible in the testes of either group. Azoospermia was not produced in treated dogs, contrary to what was observed in langur monkeys fed with chloroformic extracts of papaya seeds (Lohiya et al., 2002). This may indicate different species susceptibility to the compound. However, reduction in sperm concentration found in this study may reflect some damage on the Sertoli cells and as consequence reduced spermatogenesis on the treated dogs. Several compounds present in the non-washed papaya seed extracts may interfere with the elements involved in the processes affecting the spermatogenesis.

Conclusion and further studies - Results from the present study indicate an important effect of the chloroformic extract from washed seed of C. papaya on the spermatogenesis in dogs at a dose of 50mg/kg/day. It is necessary to evaluate higher dosages of the extracts, to identify the compound (s) involved and determine the possible ultra-structural changes in testicular tissue for a better understanding of the mechanism of action.
References

SYMPTOMATIC BENIGN PROSTATIC HYPERPLASIA IN DOGS: A RETROSPECTIVE STUDY (2001 – 2007)

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Introduction - Benign prostatic hyperplasia (BPH) is the major neoplastic disease in both the aging dog and man and therefore the dog has been increasingly used as an experimental animal model for the study of this important human disease (3). The normal prostate in the intact male dog increases in weight, due to normal growth and glandular hyperplasia, for the first 1 to 5 years, with a peak at 4 years of age. As many as 16% of dogs have been reported to have histologic evidence of benign hyperplasia and hypertrophy of the prostate by 2 years of age (5). The incidence of BPH increases over 80% with advanced age (4). Senile involution of the prostate occurs in animals aged 11 years or more (5). BPH is not always correlated to clinical signs (2), but when it is symptomatic is usually associated to non specific symptoms as lower urinary tract and/or defecation disorders (1,6,7). It has been reported that 11/177 dogs were affected by BPH; the 27% and the 9.1% of these animals showed respectively lower urinary tract and gastrointestinal symptoms, while the other dogs were asymptomatic (6). Other Authors reported that the 60% of dogs with prostatic disorders showed urinary symptoms, systemic signs were observed in the 48% of the cases and defecation problems in the 36% of the animals (8). In literature, data about correlations between BPH and symptoms are confused and obtained by analysis of animals affected not only by this pathology. The aim of this paper is to provide data about BPH symptomatology acquired by a retrospective study performed on a large number of clinical cases.

Materials and methods - The database of clinical cases records at the Clinical Veterinary Department of Alma Mater Studiorum – University of Bologna was searched for sexually intact male dogs at least 1 year old, observed between January 2001 and January 2007. From these, animals presenting the most common symptoms referable to BPH have been selected. The BPH clinical signs have been divided into lower urinary tract or gastrointestinal symptoms. The lower urinary tract symptoms detected were intermittent or persistent hematuria and sanguineous urethral discharge unassociated with micturition, dysuria, urinary incontinence or retention. The gastrointestinal symptomatology referable to BPH included fecal tenesmus and dyschezia. The diagnosis of BPH has been made on the basis of signalament, history, clinical evaluation, ultrasonographic examination, prostatic cytology and bacteriology. The clinical examination, particularly referred to the uro-genital system, included evaluation of penis (inspection and palpation), testicles (scrotal position, scrotal size), and prostate (shape, size, consistence, mobility).

Results - On a total of 5065 intact male dogs observed, 173/5065 (3.60%) showed the lower urinary tract symptomatology mentioned above and 125 of them (72%) were diagnosed as affected by BPH. In 165/5065 (3.12%) dogs, gastrointestinal clinical signs above described were identified and 38 (23%) of these animals resulted affected by BPH. In conclusion, BPH unassociated to other pathologies was diagnosed in a total of 170/5065 (3.36%) dogs. The remainder of the animals (n = 168) resulted affected by different diseases that caused the symptoms reported.

Discussion - The high number of animals observed permits to obtain important information about BPH. A comparison with other studies in literature is difficult because of the low number of clinical cases reported or the different methods of study applied. It is important to
clarify that in this study only symptomatic animals were evaluated and, when BPH was diagnosed, the presence of other pathologies correlated with the symptoms reported has been excluded. This aspect has never been well defined in literature. Krawiec et al (1992) (6) reported the presence of prostatic pathologies in 2.5% of dogs and BPH was diagnosed in 6.2% of these animals. These data permit to calculate that in 0.15% of total of cases observed, BPH was detected. It has also been reported that BPH is the most commonly diagnosed prostatic disease (58%) in dogs (9). In the same paper, in 19 of 20 cases, in which blood urethral loss was the only clinical sign, a diagnosis of BPH was made. Other Authors (7) identified BPH as the most prevalent subclinical prostatic disease (44.8%). In our study, on a total of 5065 visits performed, the 3.36% (n = 170) of symptomatic dogs resulted affected by BPH. This paper, besides the occurrence of symptomatic BPH, analyzes the relationship between BPH referable symptoms and the real presence of this pathology. This provides useful data about the possibility to detect BPH in sexually intact male dogs showing the lower urinary tract or gastrointestinal symptoms mentioned above.

References

THE EFFECT OF BENIGN PROSTATE HYPERPLASIA ON SPERM QUALITY PARAMETERS IN DOGS

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Introduction - Benign prostatic hyperplasia (BPH) may occur in male dogs older than 2 years. The enlargement of the prostate is a physiological process with age, as well, however, the borderline between normal and abnormal is very vague. As the prostatic fluid contributes a great amount to the ejaculate, concerns may arise that biochemical and physical properties of spermatozoa can change in a possibly altered environment in case of BPH. Therefore, our aim was to compare sperm quality parameters in dogs of different age, prostate size and prostate homogeneity.

Materials and methods - In this study, 18 healthy, intact male dogs between 1-9.5 years old age were examined during clinical work. The prostate length was measured by ultrasound, and its homogeneity was recorded. Sperm was collected manually and subjected to macroscopical and microscopical examination. The sperm motility was evaluated subjectively as soon as it was collected (time 0), and again in 20 min (time 20 min), meanwhile keeping the sperm at 33-36 ºC in the collecting container. Slides with a drop of sperm were stained for further morphological evaluation at both times by modified Kovacs-Foote staining.

Results - We found similarly to the literature that the prostatic length increases with age. The length and the homogeneity of the prostate did not result in any significant difference in any of the recorded macroscopical parameters. The sperm motility was not different in dogs with homogenous and inhomogeneous prostates at time 0 (70.6% vs. 70.8%), but at time 20 min the motility was significantly lower in the dogs with inhomogeneous prostate (66.4 vs. 37.5, p<0.034). The various morphological abnormalities of sperm separately were found not to differ substantially between time 0 and 20 min, on contrary, the percentage of normal sperm cells decreased significantly due to the combined effect of prostate length and time. The sperm quality of dogs with possible signs of BPH was not significantly inferior to the ones without the disorder, however, if these dogs are to be used for fresh semen artificial insemination, extra care should be taken of speediness.
LONG TIME FOLLOW-UP OF ULTRASOUND-GUIDED PER-OPERATIVE DRAINAGE OF PROSTATIC CAVITIES: ABOUT 15 CASES

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Introduction - The aim of this prospective study was to evaluate the feasibility and the long-term outcome of an ultrasound-guided drainage and longitudinal omentalization of the prostate. We hypothesized that performing drainage of prostatic abscesses and cysts associated to longitudinal omentalization under ultrasound control would allow treatment of each cavity without injuring the urethra and would decrease the rate of recurrence and the postoperative hospitalization duration in comparison with other published procedures.

Material and methods - For this prospective study, 22 mature dogs diagnosed with prostatic cavities during the period October 2006 to October 2007 were included in our study. A medical treatment (finasteride, 5 mg/kg per day; meloxicam, 0.1 mg/kg per day) was carried out and an ultrasound follow-up was performed when animal’s health allowed it. After at least a three-week treatment period, when the cysts resumption was insufficient and/or the dog presented some abdominal discomfort, we opted for a surgical drainage (n=9). In case of emergency (n=1) or perineal hernia (n=5), the surgery was performed with no delay. A complete blood count and a serum biochemical profile were performed. The prepuce was flushed to allow urethral catheterization. When the dog was entire, a castration was performed and a caudal laparotomy was done. A 7.5-MHz sectorial transducer (8C-RS, used with LOGICe central unit, GE Medical Systems) was placed in an arthroscopic sheet and a sterile glove filled with ultrasound gel. The urethral catheterization allowed a good ultrasonographic visualization of the urethra. All the cysts were located and aspirated with a needle and a syringe under ultrasonographic control. To allow the omentalization, a forceps was introduced through the prostatic cyst under ultrasound control. A leaf of omentum was passed cranio-caudally through the created passage. An abdominal lavage was done and the abdomen was closed using a classical procedure. Two biopsies were performed, one for histological examination and the other for bacterial culture. An ultrasound follow-up was performed at 1, 2, 6, 9 months postoperatively. The size of the prostate, the prostate parenchyma appearance, the absence of prostatic cavity and the absence of clinical signs were the four parameters which were evaluated on each ultrasonographic control. The evolution at one year thereafter was monitored by telephone contact with owners.

Results - No major complication, such as urethral injury or excessive bleeding, was encountered during the surgeries. Within 6 hours, hematuria resolved in 14 out of 15 dogs. The dogs were discharged on average 48 hours later. First, an antibiotic therapy with quinolone was initiated by waiting for results of bacterial culture and pattern of sensitivity to antibiotics. The treatment was continued at least 3 weeks after surgery. In 6 cases, bacteriological culture was positive and yielded a growth of staphylococcus sp in 5 cases and Escherichia coli in one case. The microscopic examination ruled neoplastic process out. An ultrasound follow-up was performed 1, 2, 6 and 9 months after the surgery. The prostatic parenchyma appeared progressively more homogeneous and the prostatic outline more regular. By 1 month after the surgery, the size was reduced on average to 2/3 and (1/2 after 2 months). The omentum leaf was still clearly located at 9 months after the surgery. Only in one dog was identified a prostatic cavity after 9 months but no clinical sign was observed.
**Conclusion** - By performing ultrasound-guided drainage of prostatic cavities during surgery, surgeons are able to treat each cavity even if located dorsally without increasing surgical duration. This technique, combined with a longitudinal omentalization, permits an effective treatment of prostatic cysts and abscesses without injuring the urethra. The complication rate and hospitalization duration are lower as compared to other studies. This is probably related to a less invasive procedure and a more accurate treatment of cavities. After a follow-up of 9 months, only one dog was presented with one little prostatic cavity but did not present any clinical signs of prostatic disease.
MANAGEMENT OF PRIAPISM IN A MALE DOG

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Introduction - Priapism refers to a persistent penile erection in the absence of sexual
stimulation; the penis cannot be manually reduced into the prepuce [1]. The pathogenesis of
priapism is complex, but the condition is generally associated with penile vascular damage or
obstruction, excessive release of erectile neurotransmitters, or prolonged smooth muscle
relaxation, all of which can contribute to an increase in arterial blood flow or a decrease in
venous outflow, causing erection, usually in the absence of sexual stimulation [4]. Priapism is
an uncommon disorder and its incidence in domestics’ animals has not been determined [4].
Serious consequences like penile desiccation and ischemic necrosis may develop as a result of
chronic external exposure of the penis and stagnation of blood in the penis [2, 3].

History - A 5-year-old, entire male Golden Labrador, was presented at the Veterinary College
for lethargy, anorexia and a painful tumescence of penis since 3 days. Two days earlier, a
 treatment for priapism was initiated with hydrotherapy, meloxicam, morphine and
delmadinone acetate.

Examination - When it was presented, three days after the beginning of treatment, the dog
was recumbent, dehydrated and depressed and it had an enlarged and turgid bladder. A
urinary catheter was inserted and 1.6 L of urine was removed; a urinalysis with bacterial
culture was performed. No reduction of the tumescence of the erect penis was observed and a
superficial ischemic necrosis was diagnosed. A penile Doppler ultrasonography was
performed. No flow was observed from bulbus glandis to the cranial region of corpus
cavernosum, whereas it was confirmed unmodified elsewhere. Radiography confirmed the
absence of any fracture of os penis. A complete blood count, a serum biochemical profile and
coaulation test were performed: all results were in normal range.

Treatment - First, administration of atropine (0.01 mg/kg), morphine (0.2 mg/kg),
cephalosporine (15 mg/kg IV, TID), flushing of the prepuce with 0.9% NaCl and glucose,
application of lubricants to prevent desiccation and use of Elizabethan collar to prevent self-
trauma from licking, were setup. Later, a tranquilization was realized with medetomidine
(0.02 mg/kg) and butorphanol (0.2 mg/kg) to release the stricture of the prepuce on the penis.
No positive result was observed. Priapism was also still persistent after anticholinergic action.
Also, surgical aspiration with a needle over the bulbus glandis did provide no result. The
following day, a bilateral incision was performed over the bulbus glandis and pars longa
glandis through the tunica albuginea to expel free blood and thrombi from the corpus
cavernosum penis. But the tumescence persisted. Finally, a penile amputation with castration
and scrotal urethrostomy was performed.

Discussion - In the dog, the priapism may be caused by trauma while mating, urinary tract
infection, constipation, spinal cord lesions, chronic distemper encephalomyelitis, penile
thromboembolism and idiopathic [1-4]. In this dog, no history of mating, nor distemper
disease was reported. The urinary culture was negative. The complete neurologic examination
and the coagulation parameters were normal. After the surgery, the penis was longitudinally
and transversally incised and macroscopically observed. There was no macroscopic anomaly.
In this case, the cause of priapism remained idiopathic.
Conclusion - The priapism is a difficult condition to manage and any opportunity for a treatment should be initiated as soon as possible. Successful pharmacologic treatment of priapism has not been reported in the dog [2]. Penile amputation and perineal urethrostomy may be necessary if the underlying cause cannot be corrected and the penis becomes irreparably damaged [2, 3]. Castration is usually not effective [2, 3].

References

THE ASSOCIATION OF GLUTAMINE WITH LOW DENSITY LIPOPROTEIN (LDL) IN COOLING CANINE SPERM: PRELIMINARY RESULTS

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Introduction - Compared to cryopreservation, refrigeration of canine sperm does not require specific material so is easier to use for practitioners. For a long time advantages of egg yolk have been known in freezing semen. LDL (Low Density Lipoprotein) extracted from egg yolk play an important role in spermatozoa protection during sperm freezing (1,3). Studies have demonstrated cryoprotective action of some amino acids and especially glutamine in conservation of stallion sperm at a concentration of 30mmol (2) and Poitou Jackass sperm at a concentration of 80mmol (4). This study aims to explore the interest of the association of LDL and glutamine in cooling canine semen.

Materials and methods - 20 ejaculates were collected from 7 dogs (Beagle, Golden retriever, Doberman) aged from 3 to 6 years. Samples were diluted in 4 different media: TRIS medium (BM)+20% egg yolk (e.y.), BM+6%LDL (LDL), BM+6%LDL+20mmol Glutamine (LDL+Glut) and INRA96. Spermatic and prostatic fractions were mixed and diluted in each medium in order to obtain a final concentration of 100.10^6 spermatozoa/ml. Samples were kept at 4°C. Each day 50μl were removed, warmed up at 37°C during 10 minutes before being assessed with a HAMILTON THORN CERROS 12 image analyser. Analyses were realized during all days.4 parameters: mobility, Velocity average pathway (VAP), Velocity straight line (VSL) and Curvilinear velocity (VCL) were analysed.

Results:

<table>
<thead>
<tr>
<th>Day</th>
<th>e.y.</th>
<th>LDL</th>
<th>LDL+Glut</th>
<th>INRA</th>
<th>N dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>85.37</td>
<td>89.94</td>
<td>89.45</td>
<td>82.41</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>71.95</td>
<td>74.29</td>
<td>73.26</td>
<td>56.08</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>61.81</td>
<td>64.22</td>
<td>64.41</td>
<td>42.74</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>53.51</td>
<td>58.53</td>
<td>59.16</td>
<td>32.90</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>44.20</td>
<td>52.20</td>
<td>53.05</td>
<td>24.47</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>37.55</td>
<td>46.48</td>
<td>51.03</td>
<td>18.58</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>31.43</td>
<td>37.07</td>
<td>37.36</td>
<td>14.02</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>26.35</td>
<td>33.55</td>
<td>37.75</td>
<td>14.83</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>19.69</td>
<td>24.28</td>
<td>26.19</td>
<td>15.59</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>18.83</td>
<td>16.08</td>
<td>20.83</td>
<td>21.83</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>16.17</td>
<td>10.00</td>
<td>15.58</td>
<td>17.92</td>
<td>3</td>
</tr>
</tbody>
</table>

Table1: Mobility of sperm cells during cooling phenomenon.

Percentage of mobile spermatozoa is higher in LDL and LDL+Glut media than in INRA 96 (p<5%), however with e.y. there is a significant difference at days 0, 4 and 5 (p<5%). INRA’s velocity parameters are significantly inferior to other media. E.y. has a VSL superior to LDL and LDL+Glut but difference is not significant. VAP and VCL are superior in LDL and LDL+Glut than in e.y. (p<5% for VCL). LDL+Glut is superior to LDL but not significantly.

Figure1: Mobility of sperm cells during cooling phenomenon.
Table 2: Average of VAP in µm/s.

<table>
<thead>
<tr>
<th>Day</th>
<th>e.y</th>
<th>LDL</th>
<th>LDL+Glut</th>
<th>INRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>111.1</td>
<td>130.64</td>
<td>127.29</td>
<td>108.62</td>
</tr>
<tr>
<td>1</td>
<td>105.96</td>
<td>106.92</td>
<td>107.45</td>
<td>86.35</td>
</tr>
<tr>
<td>2</td>
<td>97.63</td>
<td>98.12</td>
<td>98.65</td>
<td>74.96</td>
</tr>
<tr>
<td>3</td>
<td>88.94</td>
<td>88.81</td>
<td>90.25</td>
<td>71.98</td>
</tr>
<tr>
<td>4</td>
<td>80.05</td>
<td>81.65</td>
<td>81.43</td>
<td>54.01</td>
</tr>
<tr>
<td>5</td>
<td>66.06</td>
<td>67.80</td>
<td>70.04</td>
<td>38.83</td>
</tr>
<tr>
<td>6</td>
<td>63.17</td>
<td>56.24</td>
<td>60.08</td>
<td>44.73</td>
</tr>
<tr>
<td>7</td>
<td>54.15</td>
<td>58.94</td>
<td>58.90</td>
<td>37.18</td>
</tr>
<tr>
<td>8</td>
<td>58.43</td>
<td>54.44</td>
<td>51.26</td>
<td>44.13</td>
</tr>
<tr>
<td>9</td>
<td>62.77</td>
<td>37.40</td>
<td>34.61</td>
<td>65.61</td>
</tr>
<tr>
<td>10</td>
<td>47.58</td>
<td>34.85</td>
<td>33.45</td>
<td>46.20</td>
</tr>
</tbody>
</table>

Table 3: Average of VSL in µm/s.

<table>
<thead>
<tr>
<th>Day</th>
<th>e.y</th>
<th>LDL</th>
<th>LDL+Glut</th>
<th>INRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>137.31</td>
<td>179.32</td>
<td>170.92</td>
<td>137.54</td>
</tr>
<tr>
<td>1</td>
<td>175.39</td>
<td>190.58</td>
<td>193.19</td>
<td>132.25</td>
</tr>
<tr>
<td>2</td>
<td>162.93</td>
<td>179.49</td>
<td>182.81</td>
<td>114.55</td>
</tr>
<tr>
<td>3</td>
<td>155.11</td>
<td>165.43</td>
<td>172.35</td>
<td>104.96</td>
</tr>
<tr>
<td>4</td>
<td>141.43</td>
<td>156.22</td>
<td>159.42</td>
<td>74.51</td>
</tr>
<tr>
<td>5</td>
<td>120.83</td>
<td>132.53</td>
<td>139.66</td>
<td>55.79</td>
</tr>
<tr>
<td>6</td>
<td>114.73</td>
<td>110.89</td>
<td>118.44</td>
<td>63.67</td>
</tr>
<tr>
<td>7</td>
<td>100.96</td>
<td>117.84</td>
<td>120.08</td>
<td>60.24</td>
</tr>
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<td>8</td>
<td>109.58</td>
<td>102.14</td>
<td>102.88</td>
<td>70.25</td>
</tr>
<tr>
<td>9</td>
<td>109.33</td>
<td>72.43</td>
<td>72.70</td>
<td>91.47</td>
</tr>
<tr>
<td>10</td>
<td>87.16</td>
<td>58.25</td>
<td>66.44</td>
<td>71.08</td>
</tr>
</tbody>
</table>

Table 4: Average of VCL in µm/s.

<table>
<thead>
<tr>
<th>Day</th>
<th>e.y</th>
<th>LDL</th>
<th>LDL+Glut</th>
<th>INRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>101.06</td>
<td>115.93</td>
<td>113.95</td>
<td>100.14</td>
</tr>
<tr>
<td>1</td>
<td>84.59</td>
<td>82.84</td>
<td>82.76</td>
<td>75.37</td>
</tr>
<tr>
<td>2</td>
<td>78.39</td>
<td>75.09</td>
<td>75.82</td>
<td>65.20</td>
</tr>
<tr>
<td>3</td>
<td>69.52</td>
<td>66.68</td>
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<td>62.50</td>
<td>60.80</td>
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<td>48.90</td>
<td>40.95</td>
<td>43.35</td>
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<td>40.60</td>
<td>43.04</td>
<td>41.77</td>
<td>31.71</td>
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<tr>
<td>8</td>
<td>44.02</td>
<td>42.17</td>
<td>36.47</td>
<td>39.52</td>
</tr>
<tr>
<td>9</td>
<td>49.93</td>
<td>29.19</td>
<td>24.75</td>
<td>58.68</td>
</tr>
<tr>
<td>10</td>
<td>35.72</td>
<td>28.88</td>
<td>24.88</td>
<td>40.68</td>
</tr>
</tbody>
</table>

Discussion - Studied parameters show interests of LDL associated to glutamine in cooling canine sperm which could be preserved at least 7 days after taking, while preserving the 30% mobile spz required for artificial insemination. This allows to avoid time of transport problems, time that often exceeds 2 or 3 days. Although in majority there is no significant difference between LDL+Glut and e.y., mobility percentages are always clearly superior for the LDL+Glut association.

Conclusion - This study demonstrates that 6%LDL+20mmol glutamine medium permits to obtain 37.75% mobile spz 7 days after taking, percentage considered as being good enough for an artificial insemination on bitch.

References

EFFECTS OF THE INCLUSION OF EQUEX STM INTO TRIS-BASED EXTENDER ON THE MOTILITY OF DOG SPERMATOZOA INCUBATED AT 5°C

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E-mail: wojtek.nizanski@interia.pl

Introduction - The adding of Equex STM and Orvus ES Paste (active agent SDS-sodium dodecyle sulphate) to extenders that are used for freezing of semen of many species results in enhanced motility, acrosome integrity and high fertilization rates in vivo and in vitro (1,2,3,4). There are no data concerning changes of spermatozoal properties in chilled dog semen extended with diluents containing SDS. The aim of this study was to estimate the effects of Equex STM on sperm motion characteristics in chilled dog semen extended in Tris-based diluent.

Materials and methods - The study was carried out on 36 ejaculates collected from 12 stud dogs. A sperm-rich fraction of each ejaculate was divided into two samples and centrifuged. One sample was resuspended in Tris-citric acid-glucose-egg yolk extender with 1% (v/v) Equex STM addition (sample A) and the second in the same extender without such addition (sample B). The semen was resuspended to give the final concentration of 100 x 10⁶ per mL and incubated at 5°C. The parameters of motility were evaluated for 240 hrs at 24-hrs intervals using Hamilton-Thorne Sperm Analyser IVOS 12.2l. Fluorescent staining was used in order to identify sperm cells and to discriminate between spermatozoa and other cells, debris and egg yolk particles. The fluorescent stain IDENT was dissolved in medium for embryos preservation ZA 454. The UV stroboscope excitation was used to induce the intensive fluorescence of fluorochrome in sperm cells containing highly condensed DNA. The motility parameters obtained by the IVOS analyser were: VAP (average path velocity, µm/s), VSL (straight line velocity, µm/s), VCL (curvilinear line velocity, µm/s), ALH (amplitude of lateral head displacement, µm), BCF (beat cross frequency, Hz), STR (straightness, %), LIN (linearity, %), MOT (total motility, %), PMOT (progressive motility, %), subpopulation of RAPID (%), MEDIUM (%), SLOW (%) and STATIC cells.

Results - PMOT was significantly higher (p<0.05) in samples A during the initial 24 hrs of incubation. RAPID, ALH and VCL were higher (p<0.05) in samples A until 48th hr of incubation. The high ALH resulted in the lower (p<0.05) values of STR and LIN in samples A during the initial 72 hrs after collection and in the higher VCL values in samples A until 48th hr of incubation. There were not any significant differences of MOT, PROG and RAPID between samples A and B at the 72th hr of incubation. After the 72th hr of incubation a dramatic decrease of MOT, PROG and RAPID was observed in group A. Beginning from the 96th hr after semen collection MOT, PROG, RAPID, VAP, VSL were significantly lower in samples A than in samples B. ALH of spermatozoa in samples A was lower (p<0.05) than in samples B at 96, 120 and 192 hrs of incubation. There were no significant differences of ALH between groups at 144 and 168 hrs of incubation. Beginning from the 96th hr significant differences of LIN and STR (except 168 hr) were not observed. Semen samples A and B showed no significant differences in BCF during the whole of the time of incubation.
Table 1 Some of spermatozoal motility parameters of fresh and extended dog semen in Tris-based diluent with and without Equex STM addition during 240 hrs of incubation (mean ± S.D.)

<table>
<thead>
<tr>
<th>Hours of incubation</th>
<th>Sample</th>
<th>VAP (µm/s)</th>
<th>VSL (µm/s)</th>
<th>VCL (µm/s)</th>
<th>ALH (µm)</th>
<th>BCF (Hz)</th>
<th>LIN (%)</th>
<th>MOT (%)</th>
<th>PMOT (%)</th>
<th>RAPID (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>Fresh semen</td>
<td>140.7 ±12.9</td>
<td>129.8 ±11.2</td>
<td>208.3 ±10.5</td>
<td>7.5 ±0.4</td>
<td>32 ±1.6</td>
<td>63.8 ±2.2</td>
<td>76.0 ±5.8</td>
<td>44.5 ±7.3</td>
<td>62.0 ±6.1</td>
</tr>
<tr>
<td>0 hr</td>
<td>A 164.5 ±13.7</td>
<td>153.1 ±13.6</td>
<td>219.7 ±21.7a</td>
<td>7.2 ±1.0a</td>
<td>26.7 ±5.1</td>
<td>70.8 ±6.9</td>
<td>87.9 ±6.9</td>
<td>69.6 ±8.9</td>
<td>80.2 ±7.9a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 163.7 ±16.4</td>
<td>155.1 ±16.6</td>
<td>203.9 ±21.6b</td>
<td>6.5 ±1.0b</td>
<td>24.5 ±5.3</td>
<td>76.4 ±5.7</td>
<td>84.6 ±8.3</td>
<td>63.2 ±10.6b</td>
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<tr>
<td>24 hrs</td>
<td>A 153.1 ±8.3</td>
<td>134.6 ±9.7</td>
<td>226.4 ±28.7a</td>
<td>8.6 ±1.7a</td>
<td>23.1 ±4.2</td>
<td>61.9 ±8.7a</td>
<td>87.4 ±5.9a</td>
<td>63.5 ±11.9a</td>
<td>78.9 ±9.3a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 147.5 ±20.2</td>
<td>134.4 ±19.7</td>
<td>192.4 ±23.8b</td>
<td>6.9 ±1.1b</td>
<td>21.9 ±3.7</td>
<td>70.1 ±5.4b</td>
<td>83.7 ±13.9b</td>
<td>57.0 ±16.6b</td>
<td>70.4 ±17.4b</td>
<td></td>
</tr>
<tr>
<td>48 hrs</td>
<td>A 148.6 ±8.9a</td>
<td>121.0 ±16.7</td>
<td>259.2 ±47.4a</td>
<td>10.5 ±2.2a</td>
<td>24.6 ±2.3</td>
<td>49.8 ±10.9a</td>
<td>82.5 ±14.4</td>
<td>51.4 ±16.9</td>
<td>73.9 ±16.6a</td>
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<tr>
<td></td>
<td>B 132.7 ±26.3b</td>
<td>117.5 ±24.9</td>
<td>192.2 ±33.4a</td>
<td>7.8 ±1.4a</td>
<td>24.1 ±3.9</td>
<td>61.8 ±7.2a</td>
<td>77.0 ±18.9</td>
<td>45.7 ±24.4</td>
<td>59.6 ±23.9b</td>
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<tr>
<td>72 hrs</td>
<td>A 118.6 ±35.2</td>
<td>91.2 ±33.6a</td>
<td>225.5 ±60.8</td>
<td>10.1 ±1.6a</td>
<td>26.4 ±3.6</td>
<td>42.5 ±13.3a</td>
<td>73.4 ±25.6</td>
<td>31.1 ±23.7</td>
<td>57.5 ±28.4</td>
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</tr>
<tr>
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<td>B 129.4 ±21.7</td>
<td>113.2 ±19.7b</td>
<td>202.6 ±40.8</td>
<td>8.6 ±1.8b</td>
<td>25.8 ±5.5</td>
<td>58.3 ±6.5b</td>
<td>72.7 ±15.4</td>
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<tr>
<td>96 hrs</td>
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<td>160.4 ±88.8</td>
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<td>22.8 ±4.7</td>
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<td></td>
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<td>97.4 ±21.1b</td>
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<td>52.5 ±7.7</td>
<td>65.7 ±18.9</td>
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<td>120 hrs</td>
<td>A 91.7 ±31.7a</td>
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<td>170.7 ±87.3</td>
<td>6.7 ±5.3a</td>
<td>30.7 ±7.3a</td>
<td>51 ±19.8</td>
<td>31.1 ±32.2</td>
<td>11.7 ±12.9a</td>
<td>22.0 ±24.4a</td>
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<td>94.6 ±17.3b</td>
<td>205.3 ±41.3b</td>
<td>9.2 ±1.4b</td>
<td>24.9 ±4.8b</td>
<td>47.9 ±7.9</td>
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<td>115.4 ±95.2a</td>
<td>8.1 ±4.5</td>
<td>24.3 ±6.2</td>
<td>56.5 ±19.0</td>
<td>19.8 ±27.7a</td>
<td>5.6 ±10.1a</td>
<td>11.5 ±19.8a</td>
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<tr>
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<td>B 118.0 ±28.4b</td>
<td>95.1 ±28.1b</td>
<td>208.6 ±38.3b</td>
<td>8.8 ±1.3</td>
<td>25.0 ±5.2</td>
<td>46.7 ±10.4</td>
<td>56.5 ±34.9b</td>
<td>29.3 ±28.4b</td>
<td>43.0 ±34.0b</td>
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<tr>
<td>168 hrs</td>
<td>A 62 ±19.2a</td>
<td>50.1 ±13.4a</td>
<td>104.4 ±40.9a</td>
<td>5.8 ±3.9</td>
<td>22.4 ±9.1</td>
<td>52.5 ±17.3</td>
<td>9.0 ±21.2a</td>
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<tr>
<td></td>
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<td>105.2 ±35.2b</td>
<td>191.3 ±55.4b</td>
<td>8.1 ±1.8</td>
<td>23.2 ±6.3</td>
<td>56.1 ±7.8</td>
<td>50.6 ±36.2b</td>
<td>30.1 ±24.5b</td>
<td>40.4 ±31.5b</td>
<td></td>
</tr>
<tr>
<td>192 hrs</td>
<td>A 45.4 ±20.3a</td>
<td>37.2 ±9.3a</td>
<td>74.0 ±55.3a</td>
<td>7±10.0a</td>
<td>15.0 ±2.5a</td>
<td>45.0 ±4.0</td>
<td>5.4 ±13.1a</td>
<td>0.1 ±0.4a</td>
<td>0.6 ±1.5a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 112.4 ±34.2b</td>
<td>90.2 ±32.1b</td>
<td>197.4 ±41.2b</td>
<td>9.4 ±0.7b</td>
<td>23.6 ±5.2b</td>
<td>46.3 ±8.5</td>
<td>40.7 ±35.3b</td>
<td>17.2 ±22.9b</td>
<td>26.9 ±30.0b</td>
<td></td>
</tr>
<tr>
<td>216 hrs</td>
<td>A No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 77.9 ±28.1</td>
<td>57.8 ±16.2</td>
<td>138.9 ±50.1</td>
<td>6.8 ±4.6</td>
<td>21.3 ±9.5</td>
<td>46 ±7.1</td>
<td>21.8 ±20.9</td>
<td>2.5 ±4.3</td>
<td>9.3 ±12.1</td>
<td></td>
</tr>
<tr>
<td>240 hrs</td>
<td>A No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td>No motility</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 42.6 ±17.2</td>
<td>37.2 ±12.3</td>
<td>76.9 ±35.1</td>
<td>7.1 ±0.1</td>
<td>31.2 ±1.6</td>
<td>57.7 ±14.0</td>
<td>14.3 ±4.0</td>
<td>0.7 ±1.2</td>
<td>1.3 ±2.3</td>
<td></td>
</tr>
</tbody>
</table>

Different letters within each pair of the compared rows indicate significant differences (p<0.05)

sample A - Equex STM addition, sample B – no Equex STM added
Conclusions

1. Equex STM changes the sperm motion characteristics in dog semen incubated at 5°C.
2. Equex STM enhances the percentage of motile, progressively motile spermatozoa and subpopulation of rapid sperm cells for the initial 48 hrs of incubation but induces subsequent rapid decrease of these values.
3. The initial changes in the motility pattern in semen samples with Equex STM addition are characterized by increased ALH of spermatozoa, not by any changes in BCF. The initial high values of ALH are similar to those observed in capacitated spermatozoa and may cause a depletion of spermatozoal energy resources resulting in a subsequent decrease in motility.

References

2. Peña AI, Linde-Forsberg C. Effect of Equex, one or two step dilution and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology 2000;54:859-875.
EFFECT OF TWO EGG YOLK-FREE EXTENDERS ON MOTILITY AND MEMBRANE INTEGRITY OF CRYOPRESERVED DOG SEMEN

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Introduction - The egg yolk (EY) is widely used as cryoprotective agent in semen freezing extenders for sperm protection against cold shock and freezing damages, although the mechanisms of sperm protection by EY are unclear. Most diluents for canine semen contain 10-20% egg yolk. Because of microbiological contamination risk presented by ingredients of animal origin, a new generation of semen extenders, free of animal products, were introduced into practice, especially in bovine semen cryopreservation. The aim of this study is to investigate the effects of two egg yolk free extenders on quality of cryopreserved dog semen.

Materials and methods - Sperm-rich fractions of 12 ejaculates from four male dogs, aged between 2-6 years, were divided into three aliquots. The extenders used were: AndroMed (Minitüb Abfull-und Labortechnik GmbH & Co.KG, Tiefenbach, Germany) egg yolk free, Bioxcell (IMV Technologies, L’Aigle, France) egg yolk free and Triladyl Canine (Minitüb Abfull-und Labortechnik GmbH & Co.KG, Tiefenbach, Germany) with egg yolk. The semen was extended to a concentration of $100 \times 10^6$ spermatozoa x ml$^{-1}$ by the one-step method, according to producers’ recommendation. The extended semen was packaged in 0,5 ml straws, then cooled to 4°C over one hour and equilibrated at 4°C for three hours. The freezing was performed by placing the straws horizontally, 4cm above LN$_2$ in a styrofarm box for 10 min and then immersed in LN$_2$. Semen evaluation was performed after collection, equilibration and freezing. The concentration and motility were determined using CASA (IVOS HTB, version 12) and membrane integrity was assessed by hypo-osmotic test. Semen was thawed at 70°C for 10 seconds than at 37°C for 1 min.

Results - The results showed that AndroMed egg yolk-free extender is suitable for dog semen cryopreservation; in this variant, motility and membrane integrity were superior to other two diluents.

Table 1  Semen parameters in fresh dog semen (mean ± SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of sperm rich fraction (ml)</td>
<td>2.24 ± 0,61</td>
</tr>
<tr>
<td>Concentration (x 10^6/ml)</td>
<td>410,33 ± 183,11</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>84,42 ± 7,24</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>67,75 ± 5,42</td>
</tr>
<tr>
<td>Membrane integrity (intact %)</td>
<td>90,83 ± 4,83</td>
</tr>
<tr>
<td>Morphology ( normal spermatozoa %)</td>
<td>89,42 ± 2,63</td>
</tr>
</tbody>
</table>

Table 2  Semen parameters (mean ± SD) after cooling and freezing of dog semen

<table>
<thead>
<tr>
<th>Extender</th>
<th>Motility (%)</th>
<th>Progressive motility (%)</th>
<th>Membrane integrity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>chilled</td>
<td>frozen</td>
<td>chilled</td>
</tr>
<tr>
<td>AndroMed</td>
<td>77±6,9</td>
<td>55,17±6,71</td>
<td>53,25±5,97</td>
</tr>
<tr>
<td>Biladyl</td>
<td>53,92±16,25</td>
<td>21,92±5,47</td>
<td>31,50±14,91</td>
</tr>
<tr>
<td>Triladil</td>
<td>68,58±15,83</td>
<td>42,00±4,47</td>
<td>46,17±14,11</td>
</tr>
</tbody>
</table>
FERTILIZING CAPACITY OF FROZEN EPIDIDYMAL SPERM COLLECTED FROM DOG

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Introduction - The recovery of spermatozoa from epididymis cauda may be an important tool in canine reproduction. This technique recovery viable cells after the death of valuable dogs and endanger species. During sperm maturation inside the epididymis, the plasmatic membrane is biologically modified, which will provide motility and capacity to fertilize the oocytes [2]. The process of sperm freezing can cause irreversible damage in the spermatic membrane and this can interfere with cell viability. The development of techniques to enable the cryopreservation of sperm obtained from epididymis. Thus, the morphological and functional evaluation of these cells after thawing is essential in order to classify them as competent or not for sperm-oocyte interaction.

Objectives - The aim of this study was to evaluate epididymal sperm viability after the freezing process, through morphological and functional parameters and sperm-oocyte interaction test.

Materials and methods - Epididymides were obtained from four adult dogs by elective orchiectomy. After removal and dissection of the testicles, the distal portion of the epididymis and part of the deferens ducts were squeezed, with an anatomic clamp into a Petri dish containing in either 0.9% saline solution (Group 1) or in Ringer without lactate (Group 2). Immediately after the collection, spermatic motility and sperm concentration were evaluated. Samples were centrifuged at 800xg for 10 minutes, the supernatant was removed and the pellet was diluted in one step with a Tris/citric acid/OEP (ovus es Paste)/7% glycerol extender. Samples were packed in 0.5mL French straws with 40 x10⁶ sperm/straw, and cooled to 5ºC, during 60 minutes. After were placed 6 cm above liquid nitrogen for 20 minutes and then emerged into it. Thus they were stored in criogenic container. Semen was thawed in water bath (70ºC/8sec). Later on, submitted to computadorized analysis (Hamilton Thor®). The following parameters were measured for each sample: the percentage of total motile sperm (M); the percentage of progressive motile sperm (PM); velocity average pathway (VAP); the velocity straight line (VSL); the straightness (STR); and the linearity (LIN); stain fluorescent assessed sperm membrane integrit (CUNHA et al., 1996), and proportion of intact cells was determined. Bitch ovaries were sliced in Petri dishes. Grade 1 oocytes were selected and washed in PBS containing BSA and thus, in TALP-FIV medium. The oocytes were transferred to TALP-FIV drops (10 oocytes/drop) and covered with silicone oil. Sperm cells (concentration of 2x10⁶ viable cells/100 µL) were added to the drops and incubated for 18 hours at 38ºC/CO₂. After the incubation period, oocytes and sperm cells were recovered and stained with Hoeschst dye. The interaction sperm-oocyte (%) was evaluated in fluorescence microscope. Results were expressed as means (± SD), a simple linear regression model was used to identify association between sperm-oocyte interaction and the parameters evaluated the sperm thawed. Statistical analyses were performed in the SAEG software. The level of significance was set at P <0.05.

Results - Mean (± SD) of analyzed parameters from Group 1 and Group 2 were: M(%) 44.3 ± 10 and 44.8 ± 15.5; PM(%) 34 ± 10.9 and 26.5 ± 6.4; VAP 70.2 ± 8.6 and 70.6 ± 5.5; VSL 63.7 ± 10.4 and 60.9 ± 7; STR 90.3 ± 5.7 and 85 ± 5.5; LIN 68.5 ± 9.3 and 58 ± 9.9; MI 33 ±
26.4 and 33.8 ± 22.6; Oocyte interacted 34.5 ± 11.4 and 54.3 ± 21.9, respectively. Our results showed that morphological and functional characteristics were similar in both groups. However, the percentage of sperm cells bound to oocytes in Group 2 was significantly higher than in Group 1. The results of interactions sperm/oocytes found in this work are similar 34%(1)and below 72%(3) to those found by other authors for the ejaculate thawed. These results indicate that the canine sperm, following the procedures of freezing and thawing, retains its fertilizing ability. The results showed differences between the isotonic solutions, which probably is due to mineral composition of the ringer that promoted greater protection of sperm to heat stress and obviously the greater interaction between the sperm cells and oocytes. This result suggests that the Ringer without lactate was a better than 0.9% saline to recovery sperm cells from epididymis.

References

AMIDE ON CANINE SEMEN CRYOPRESERVATION

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Introduction - In recent years, the glycerol has been used as the main cryoprotectant to canine spermatozoa \cite{1,2,4,8} and other cryoprotective agents have been tested sporadically, particularly DMSO \cite{5}, ethylene glycol \cite{7}. Studies of extenders containing amides have been limited \cite{3,6,10}.

Objectives - The aim of this study was to assess the effects of extenders containing amides on post-thaw semen quality from dogs. For this purpose, five ejaculates from privately owned dogs from different breeds were collected by digital manipulation. Ejaculate volume and color, sperm motility by CASA (Computer Assisted System Analyzer), percentage of morphologically normal spermatozoa, and membrane integrity (fluorescent staining) were evaluated. Each ejaculate was analyzed and divided in 6 aliquots. The aliquots were centrifuged (800xg, 10 min) and diluted in the extenders. The standard extender used was TRIS, citric acid, glucose, 20% yolk egg and 1% Orvus WA Paste added either glycerol (GLY - 5% or 7%), methyl formamide (MF - 5% or 7%) or dimethyl formamide (DMF - 5% or 7%). Diluted semen was packaged in 0.5 mL plastic straws, equilibrated at 4°C, during 1 h, and frozen in nitrogen vapor for 20 min; thus stored at –196°C. The straws were thawed at 70°C for 8 sec. Thawed semen samples were evaluated for motility by CASA and fluorescent staining.

Results - Obviously, all seminal parameters evaluated on fresh semen were better than post-thawed. The total motility was 95.4% for fresh semen and 60% for GLY 5%, 63% for GLY 7%, 71.6% for MF 5%, 62.2% for MF 7%, 66.4% for DMF 5% and 57.6% for DMF 7%. The extender containing MF 5% showed better in different parameters evaluated. However there was no significant difference between extenders, except to DMF 7%. The membrane integrity of sperm cells was worse in MF 5% (mean 35.2%). The GLY 5% (mean 69.4%) or 7% (mean 59.6%) extenders kept the better membrane integrity. DMF 7% presented the worst result for membrane integrity, followed by MF 7% and GLY 5%. Unlike from our studies Zimmermann et al. \cite{10} found better results for DMF at 7% (motility 46.7%) than in lower or higher concentrations (3.5% and 14%), but these results were lower than the GLY at 6% (61.7%). Anyway, the motility from them results was lower than those found in our work. Oliveira et al. \cite{6} found also better results for DMF 5% (TRIS lactose) than 5% TRIS ethylene glycol or 5% TRIS lactose ethylene glycol. Our study used other sugar in extender (glucose) which could be provided different results. Futino et al. \cite{3} verified a similar motility in semen preserved with GLY 3% and MF 3% (69% \textit{versus} 59%), and lower results to DMF (44%). On the other hand, these authors found lower results (35.8%) to MF in swelling test (HOS). Different from our results, in equine, both MF and DMF protected spermatozoa from cryodamage as effectively as glycerol, but the membrane integrity was not evaluated in this species \cite{9}. In conclusion, the extenders added amides are option to freeze canine semen, but further study were necessary to determined ideal their concentration only or in association with glycerol and their effect \textit{in vivo} fertility.
References


SURVIVAL OF FROZEN-THAWED CAT SPERMATOZOA PRE-COOLED IN THE EPIDIDYMIDES

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Objective of the work - Epididymis is an important source of male genetic materials from endangered animals that died unexpectedly. Sperm recovery from epididymides and preservation require particular media and equipments that are not always available under field conditions. An alternative is to deliver the whole excised testes and epididymides to a laboratory for sperm harvesting and processing. Cat spermatozoa retrieved from the epididymides remain their quality throughout 3 weeks of cold storage in the extender [2] whereas reduction of sperm quality is observed after 72 hours cold stored in the epididymides [3]. The autolytic change occurs in the epididymal tissue has been suggested as the cause of sperm quality reduction [3]. However, a thorough study to compare effects of cat spermatozoa cold stored in extender and in epididymides has not been conducted. Because freezing is the end process for long-term preservation, this study compared quality of frozen-thawed epididymal cat spermatozoa between pre-cooled in an extender and in epididymides.

Materials and methods - Testes with attached epididymides from 23 cats subjected to routine orchidectomy were allocated into two groups; two days (Group A) (n=10) and four days cold storage (Group B) (n=13). Spermatozoa from one of each of the epididymides were recovered, evaluated, extended in a Tris egg yolk extender containing Equex STM paste, cooled to 5 °C and stored for either two or four days prior to freezing. The remaining testis attached with epididymis was stored at 5 °C in a Tris buffer solution either two or four days before spermatozoa were harvested. Sperm recovery was performed by cutting the distal part of the epididymis into four small pieces and placed into a Tris buffer solution. Spermatozoa were frozen using the methods according to Axnér et al [1]. Spermatozoa were evaluated for percentage of motility under a phase-contrast microscope, viability using aniline blue staining and intact acrosome using FITC-PNA/PI. Sperm evaluations were performed immediately after harvested, prior to freezing and after frozen-thawed. The effects of treatment (cold storage in the extender and in the epididymis) were tested using GLM-ANOVA. Pairwise t-test was made to compare sperm quality variables between treatments. The level of significance was set at $P < 0.05$.

Results - There were no differences of epididymal cat sperm quality after cold stored in the extender and in the epididymis for both two and four days of storage ($P > 0.05$) (Table 1 and 2). The quality of frozen-thawed spermatozoa pre-cooled in the epididymis did not differ from that pre-cooled in the extender ($P > 0.05$). However, cold storage of spermatozoa for four days resulted in reduced motility compared to that cold stored for two days ($P < 0.05$). Freezing of spermatozoa pre-cooled for four days resulted in the lower motility, viability and intact acrosome than that pre-cooled for two days than two days ($P < 0.05$).
Table 1  Epididymal cat sperm quality after cold stored for two days (in Tris egg yolk extender or in epididymis) and after frozen-thawed (mean ± SEM) (n=10)

<table>
<thead>
<tr>
<th>Sperm quality (%)</th>
<th>Prior to freezing</th>
<th>Frozen-thawed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extender</td>
<td>Epididymis</td>
</tr>
<tr>
<td>Motility</td>
<td>81.4 ± 5.3</td>
<td>77.0 ± 14.8</td>
</tr>
<tr>
<td>Alive</td>
<td>72.2 ± 1.9</td>
<td>73.1 ± 3.3</td>
</tr>
<tr>
<td>Intact acrosome</td>
<td>49.3 ± 6.0</td>
<td>49.6 ± 6.1</td>
</tr>
</tbody>
</table>

Table 2  Epididymal cat sperm quality after cold stored for four days (in Tris egg yolk extender or in epididymis) and after frozen-thawed (mean ± SEM) (n=13)

<table>
<thead>
<tr>
<th>Sperm quality (%)</th>
<th>Prior to freezing</th>
<th>Frozen-thawed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extender</td>
<td>Epididymis</td>
</tr>
<tr>
<td>Motility</td>
<td>70.0 ± 6.5</td>
<td>64.6 ± 4.0</td>
</tr>
<tr>
<td>Alive</td>
<td>70.2 ± 1.9</td>
<td>72.0 ± 1.8</td>
</tr>
<tr>
<td>Intact acrosome</td>
<td>43.0 ± 4.3</td>
<td>42.8 ± 5.9</td>
</tr>
</tbody>
</table>

**Conclusions** - Cat spermatozoa cold stored in the epididymides provide comparable results as that cold stored in the Tris egg yolk extender regarding the quality before and after frozen-thawed. This is an alternative when spermatozoa need to be transported for long distances to a laboratory.

**Acknowledgement** - The research was funded by Grants for Development of New Faculty Staff and the Faculty of Veterinary Science, Chulalongkorn University.

**References**


NEGATIVE EFFECTS OF SEMINAL PLASMA ON FROZEN-THAWED EPIDIDYMAL CAT SPERMATOZOA

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E-mail: paweena.thuwanut@kv.slu.se

Introduction - In domestic cats, epididymal spermatozoa perform lower initial motility and viability than ejaculated spermatozoa [1]. This may be because of the effects of seminal plasma that mixed with spermatozoa after ejaculation. The components such as inorganic compounds or amino acids in the seminal plasma are beneficial for maintenance of sperm membrane integrity and fertility in mammal [2]. However, the effects of the seminal plasma in relation to fertility in various studies are conflicting [2]. The quality of frozen-thawed epididymal Iberian red deer spermatozoa is improved after spermatozoa are incubated with seminal plasma prior to cryopreservation process [3] whereas chilled spermatozoa quality in dogs was not improved when supplemented with seminal plasma [4]. The study of the seminal plasma on frozen-thawed sperm functions has not been investigated in domestic cats.

Objectives - The aim of this study was to investigate whether seminal plasma could improve epididymal cat spermatozoa quality after freezing and thawing process.

Materials and methods - Seminal plasma was obtained from two proven fertility male cats by centrifugation of the semen, pooled, and kept at -20 °C until used. Epididymal spermatozoa were harvested from eleven cats by transversely cutting of the caudal part of the epididymides and placed into warmed Tris buffer. Sperm sample from each cat was extended with 125 μL of a Tris egg yolk extender containing Equex STM paste, loaded into two mini-straws and cryopreserved according to Axnér et al [5]. Thawing was performed in a water bath at 70 °C for 6 sec. The sperm sample was then emptied into a small Eppendorf tube containing 125 μL of either warmed Tris buffer (control group) or seminal plasma (treatment group) (v:v, 1:1). Motility, membrane integrity and acrosome integrity of the sperm sample of each cat were evaluated at 0, 2, 4 and 6 h after thawing. Motility was subjectively assessed under a phase contrast microscope at 100X magnification. Sperm plasma membrane and acrosome was stained with SYBR-14/EthD-1 and FITC-PNA/PI, respectively, and evaluated under an epifluorescent microscope. The statistical analyses were performed by ANOVA and a paired t-test. The level of significance was set at P < 0.05.

Results - The percentages of motility and membrane integrity were significantly lower in the treatment group compared to the control group whereas the acrosome integrity did not differ between groups (Table 1). Moreover, the frozen-thawed sperm quality in each group was significantly decreased by time.

Table 1. Quality of frozen-thawed epididymal cat spermatozoa after incubated either with Tris buffer (control) or seminal plasma (treatment)

<table>
<thead>
<tr>
<th>Post-thaw incubation time (h)</th>
<th>Motility</th>
<th>Membrane Integrity</th>
<th>Acrosome Integrity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td>0 h</td>
<td>46.4 ± 15.4a</td>
<td>40.0 ± 9.4 b</td>
<td>46.3 ± 9.7 a</td>
</tr>
<tr>
<td>2 h</td>
<td>35.5 ± 12.4a</td>
<td>33.3 ± 8.2 a</td>
<td>35.1 ± 9.3 a</td>
</tr>
</tbody>
</table>
Mean ±SD. N=11.
Means within row with different letters differ significantly (P<0.05)

**Conclusion** - The seminal plasma seems to have negative effects on frozen-thawed epididymal cat spermatozoa. Exposure of frozen-thawed epididymal cat spermatozoa to the seminal plasma could not improve sperm quality.

**References**

1. Axnér E, Hermansson U. Epididymal and ejaculated cat spermatozoa are resistant to cold shock but egg yolk promotes sperm longevity during cold storage at 4 °C. Theriogenology. 2007;67:1239-48.
IMPACT OF 24 HOURS COOLING PRIOR TO CRYOPRESERVATION ON THE SURVIVAL OF DOMESTIC CAT (*FELIS CATUS*) EPIDIDYMAL SEMEN


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Introduction – The possibility of harvesting cat epididymal spermatozoa from excised testis represents an important tool for preserving valuable genetic material from males that die unexpectedly, need to be euthanized or castrated. Unfortunately, sometimes collected material can not be processed immediately, especially when working with free-living animals. Thus, transport of cooled testes, epididymides or retrieved epididymal spermatozoa to an equipped laboratory is necessary. In domestic cat, epididymal sperm viability has been reported after long cold storage at 4 to 5°C [1-6]. Although epididymal sperm cooled for seven days was capable of fertilizing in vitro [4], in many occasions this material needs to be storage for longer periods, aiming to maintain a germ plasm bank for future use. This way, freezing the epididymal sperm after a cooling period would be essential to guarantee the preservation of this valuable genetic material. Previous study has shown that cooling overnight domestic cat testes prior to freeze epididymal sperm did not impair attaching to the homologous zonae pellucidae [1]. The objective of this study was to investigate the impact of a 24 hours cooling period prior to freezing on domestic cat epididymal sperm viability.

Material and Methods - Fifteen adult tom cats from mixed breed were submitted to routine orchiectomy under appropriate anesthetic protocol. Both epididymides were dissected and squeezed using an anatomic forceps to collect epididymal fluid in a Tris-glucose-20% egg yolk extender (TGE). Samples were split into two 1.5 mL plastic tubes, transferred to an aluminum recipient with 600 mL of water at room temperature and placed in a programmed refrigerator at 5°C, resulting in a cooling rate of 0.5°C/ minutes. After cooling for 60 minutes, control group was submitted to the freezing procedure that consisted of glycerolization by dripping TGE extender supplemented with 8% of glycerol at 5°C in a 1:1 proportion, aiming a final glycerol concentration of 4%. After equilibration for 10 minutes at 5°C, samples were loaded into 0.25 mL straws and placed at 6 cm above liquid nitrogen (N2L) during 20 minutes and plunged into N2L. The treatment group was submitted to the same freezing protocol after 24 hours of cooling. Thawing was performed in a water bath at 37°C for 30 seconds. Sperm samples were evaluated at: Moment 1 (M1), immediately after collection; Moment 2 (M2), after refrigeration at 5°C for 24 hours; Moment 3 (M3), after thawing of control group samples; and Moment 4 (M4), after thawing of treatment group samples. Evaluations consisted of sperm motility (1-100 %) and vigor (0–5; 0, no movement; 5, progressive rapid and linear movement), sperm morphology using Rose Bengal and Fast Green FCF and plasma membrane integrity (PMI) using two fluorescent probes (Propidium Iodide and Carboxyfluorescein Diacetate). Statistical analysis was performed using the non-parametric Wilcoxon test, establishing a significance level at p < 0.05.

Results and Discussion – After cooling for 24 hours (M2), a decrease in sperm motility (87.3 ± 5.6% to 77.7 ± 15.7%), sperm vigor (4.6 ± 0.5 to 4.1 ± 0.8) and PMI (81.1 ± 7.1% to 74.7 ± 8.8%) was observed when compared to the group before cooling (M1). Additionally, there was no significant difference for sperm morphology between moments M1 (73.3 ± 7.0%) and M2 (68.6 ± 10.8%). Comparing the results obtained after thawing for the fresh (M3) and cooled (M4) groups, no significant difference was found regarding sperm motility (42.0 ±
24.5% versus 40.0 ± 21.1%, respectively), sperm vigor (2.9 ± 0.8 versus 2.8 ± 0.7, respectively), PMI (42.1 ± 14.4% versus 42.1 ± 14.4%, respectively) and sperm morphology (66.2 ± 8.7% versus 61.8 ± 8.8%, respectively). Conversely, in an earlier report, frozen-thawed samples of cat epididymal spermatozoa overnight cooled in the testes showed lower values for sperm motility and morphology compared to the frozen-thawed samples of fresh epididymal sperm [1]. These discrepant findings maybe due to differences between the freezing protocols used in both studies. For example, we used a glycerol concentration of 4% instead of 3%. Such differences may have offered better conditions during the freezing-thawing procedures, avoiding cell damage especially in the group cooled prior to freezing, since they seem to be more susceptible to cold damage. Unlike previous studies [1,4,6], even though we used a slow cooling rate in this work (0.5°C/ minutes), a loss of motility, sperm vigor and PMI was found after the cooling period. However, despite the fact that the cooling process induced sperm injury, no difference was found after thawing between the groups with or without the cooling period prior to freezing. For this reason, it seems like that the sperm damage observed after cryopreservation of cat epididymal spermatozoa is not attributed to the cooling process but rather to the sperm freezing and possible thawing procedures. The results from the present study revealed that an eventual transportation of domestic cat epididymal spermatozoa at 5°C during 24 hours does not lead to important damages to the sperm cell which could impair the frozen-thaw process.

References


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EFFECT OF CATALASE AND SUPEROXIDE DISMUTASE ON MOTILITY, VIABILITY AND ACROSOMAL INTEGRITY OF FROZEN-THAWED CAT SPERMATOZOA

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Introduction - Sperm cryopreservation is a helpful method for breeding management and ex situ wildlife conservation. However, in many species, the oxidative damage due to the generation of reactive oxygen species (ROS), specifically DNA damage and lipid peroxidation during preservation techniques can cause the declination of sperm motility and fertility capacity.

Objectives - In this study, the effect of antioxidants catalase (CAT) and superoxide dismutase (SOD) on motility, viability and acrosomal integrity of frozen-thawed cat spermatozoa were investigated.

Materials and methods - Semen was collected by artificial vagina from five domestic cats (two ejaculates/cat). Spermatozoa were diluted in egg yolk tris-fructose citrate solution (EYT-FC) without glycerol and cooled at 4°C for 1 hr, then diluted further with EYT-FC with glycerol (7% final concentration) and 400 U/ml of CAT (treatment 1) or SOD (treatment 2) or without antioxidants (control). Diluted spermatozoa were equilibrated for 1 hr before frozen in straws over liquid nitrogen vapour. After thawing, spermatozoa were assessed for motility, viability and acrosomal integrity.

Results - Cryopreservation had a negligible effect (P<0.05) on sperm motility, viability and acrosomal integrity. Moreover, motility, viability and acrosomal integrity of frozen-thawed cat sperm in the EYT-FC with CAT, SOD and without the antioxidants were not significant different. The percentage of mean compared between control, treatment 1 and treatment 2 for frozen-thawed sperm motility were 43.5%, 42% and 38%, viability were 44.8%, 50.6% and 47.1% and acrosomal integrity were 45%, 44.9% and 44.4%, respectively. In conclusion, adding CAT and SOD in EYT-FC did not improve the maintenance of motility, viability and acrosomal integrity in cat sperm cryopreservation.

Keywords: cat, spermatozoa, cryopreservation, catalase, superoxide dismutase
A 7-year-old male German shepherd, unilateral cryptorchid, is presented to investigate its reduced appetite, prostration and testicular hypertrophy. The dog is subject to epileptic convulsions and has been receiving primidone once a day for several years. The clinical examination doesn't show any abnormality, except hyperthermia (39.9°C), dysuria and hematuria. The scrotum is hypertrophic and oedematous. It is hot and swollen, but its palpation doesn't trigger pain. Inside, the testicle is small and a hypertrophic tubular structure is palpable. The penis is normal. Furthermore, the dog urinates like a female.

Ultrasonography shows tubular structures, whose walls contain a lot of small cavities, close to both scrotal and abdominal testicles. A large cavity (6 cm in diameter) is identified near the caudal pole of the kidney. Abdominal testicle parenchyma is not visible. The diagnostic hypothesis is a large atypical bilateral epididymitis, associated with cystitis. A urine sample is collected by urethral catheterization: the urine is dark brown and a Combur test shows proteins 3(+), leucocytes 3(+), blood 4(+). The urinary pH is 6.5. Urine bacteriology isolates Pasteurella multocida and Gemella morbillorum. Serological tests are done to determine if the dog has been affected by brucellosis (B. canis and B. abortus), Q fever (Coxiella burnetti) or chlamydiosis (Chlamydophila abortus) to try to explain potential epididymitis. All the results are negative. During these tests, tetracyclins, sulfamids and non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed for 3 weeks to treat cystitis and potential epididymitis. At the end of the treatment, the dog is seen again: according to the owners, he has been better for the last two weeks, with increased appetite and a decrease in scrotal volume. But, since antibiotics had been stopped, five days earlier, its condition seems to have deteriorated and it is hyperthermic (39.7°C). The scrotal testicle is small and palpation still reveals the presence of tubular structures in the scrotum. Dysuria is described, with occasional hematuria. A second ultrasonography still shows the tubular structures in the abdominal cavity and the scrotum. Antibiotics and NSAIDs are continued, without any improvement: 10 days later, its rectal temperature is 39.7°C, and the dog, looking skinny, is exhausted.

A laparotomy is performed to remove the abdominal testicle, associated with exeresis of the scrotal testicle. The tubular structures in the abdominal cavity appear to be two uterine horns: one connected to a fluid-filled spherical structure, and one to the scrotal testicle through the inguinal ring. The two horns come together up at the uterine cervix, followed by a very dilated blind vaginal sack. No ovaries are recognisable. The scrotal testicle is atrophic, with complete testicular cord (vessels and seminal duct) and normal epididymia at one end, and a connection to a uterine horn at the other. Identically, the abdominal spherical structure has a testicular cord and epididymia on one side, and a uterine horn on the other. The prostate is not visible. A complete exeresis of the internal genitalia is performed. After ablation, a section of the different tissues shows:
- in the abdominal testicle, a cyst full of clear fluid with brown debris, demarcated by unidentifiable pluri-stratified cell population, and a mixed tumor (seminoma and a Sertoli cell tumor);
- in the scrotal testicle, atrophy of the seminiferous tubules;
- in the uterine horns, cystic endometrial hyperplasia;
- in the vaginal sack (8 cm in diameter), a mucosa which looks like that of a female vagina, and presence of clear fluid with brown debris.

Quantitative hormonal assays, done the same day as surgery, show hyperprogesteronemia (11 nmole/L). Estradiolemia and prolactinemia have physiological values for a male. Basal testosteronemia is lower than 1 nmole/L. PCR identifies sexual chromosomes as XXY.

Klinefelter syndrome (KS), affecting 1 in 660 men, is the most common sex chromosome disorder in humans. Affected males carry one or a few additional X chromosomes, which result in male hypogonadism, androgen deficiency and impaired spermatogenesis [3]. An alteration in the hypothalamic-pituitary-gonadal axis has been suspected. KS has been reported in many species including dogs. When found in humans, genital anomalies are generally not recognized as associated features of the syndrome. In dogs, wide variations in phenotypical presentation of the 79, XXY genotype are reported.

Development of male internal and external genitalia occurs during embryonic development and depends on testosterone, produced by embryonic Leydig cells, and on anti-müllerian hormones (AMH) secreted by Sertoli cells in the embryonic testicle. In the male, AMH induces regression of the müllerian duct system, which becomes a female vagina, uterus and uterine horns. Persistence of müllerian duct syndrome (PMDS), a very rare type of male pseudohermaphroditism, is described in dogs, in phenotypically male miniature Schnauzers with normal 78, XY karyotype and testes. Persistent müllerian ducts were associated with unilateral or bilateral cryptorchidism, Sertoli cell tumors in the retained testicle and cystic endometrial hyperplasia or pyometra of the uterus masculinus. In humans, this abnormality is also rare. It is transmitted according to a recessive autosomic pattern and is due, in 84% of cases, to mutations of AMH and AMH receptor type II genes, inducing a lack of regression in the müllerian duct system [1].

In our case report, the dog carries an additional X chromosome characteristic of Klinefelter syndrome, and shows genital anomalies (unilateral cryptorchidism, cystic endometrial hyperplasia of an uterus masculinus) which could be associated with persistent müllerian duct syndrome. Scrotal hypertrophy was due to the presence of the uterus masculinus in the scrotum. Its urinating position (female) may be due to the dog's hormonal status. Progesterone may have been synthesised by the scrotal testicle or abdominal gonad. In dogs as in humans, persistent müllerian duct syndrome is most often discovered unintentionally, as a consequence of cryptorchidism. In our report, the dog lived 7 years before developing symptoms, mainly due to female genitalia disease. The presence of a phenotypical male means that testosterone had been secreted at least during organogenesis. The lack of regression in the müllerian duct system may be due to a defect of AMH or AMH receptors.

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SRY-NEGATIVE XX SEX REVERSAL IN A PUG

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A 4-month-old Pug that had been bought as a male in a pet shop was presented at the Veterinary Teaching Hospital, University of Turin, because of a reddish mass protruding from the prepuce. This appeared more like a vulva, though placed at a longer than normal distance from the anus, and the protruding mass had the aspect of an enlarged clitoris, with a caudoventral direction and a dorsal urethral ostium. Radiography examination confirmed the presence of an internal os. No scrotum was present and a gonad was palpable in the left inguinal region. A blood sample was collected for karyotyping and molecular analysis. Karyological analysis revealed a normal female karyotype (78,XX). Two months later, an ultrasound abdominal examination (7.5 MHz transducer) revealed the presence of a uterine structure together with an abdominal gonad with a testis-like structure, analogous to the appearance of the inguinal gonad. The absence of Sry, the master gene regulating testis differentiation, was confirmed by PCR analysis. The dog was gonadohysterectomised: the right gonad was in the typical ovarian position, and cranial to the right uterine horn; the left gonad had passed through the inguinal canal, followed by the apex of the left uterine horn. A prostate was absent. The histology of both gonads was similar, showing an exclusively masculine character, with seminiferous tubules lined only by Sertoli cells; germ cells were absent, while a moderate number of interstitial cells was present. The epididymi were absent. Histological examinations of the uterus showed a normal structure.

This is an interesting case of an XX sex reversed male. The XX sex reversal syndrome has been sporadically reported in several dog breeds (3): XX sex reversed individuals show different amounts of testicular tissue in one or both gonads, and are classified as true hermaphrodites, with testicular and ovarian tissue, that are fertile and can reproduce like normal females (3), or as XX sex reversed males, exhibiting bilateral testes and alterations in the external genital tract. This disorder is transmitted as an autosomic recessive character in the American Cocker Spaniel (4). The pathogenesis of the XX sex reversal syndrome is not completely understood; the Sry gene, that initiates the development of testes in normal male embryos, has never been found in sex reversed dogs, therefore other etiologic genes should be involved. The XX sex reversal syndrome was also reported in the Pug (8), but it was not possible to look for Sry. To the knowledge of the Authors, this is the first case of an XX sex reversed male showing an inguinal testis, implying a partial activity of the mechanisms leading to abdominal testis translocation along a gubernaculum and transinguinal migration (1). Further analyses will be performed to evaluate the involvement in this case of other genes yet described as responsible for XX male syndrome in others species: Sox9 (2, 9, 7), Rspo1 (6) and PIS locus (5).

References

In this trial, five leash of bitches have laboured 3-4 months before (in early anestrus) were treated by Dopamin agonist (Cabergoline). Such bitches were controlled for the nutrition and estrus signs in their new home (place). All of the bitches under the treatment were given Cabergoline daily 40 days of treatment per oral. Starting 1 month before the beginning of treatment and also during the period of treatment and 1 month after the end of treatment, we measured blood Progesteron concentration in such bitches and obtained vaginal smears to recognize the changes in vaginal cytology. Also we have detected blood Prolactin concentration during the period of treatment every 3rd day.

Side effects of drug such as vomiting, wait loss, changes in hair color and alopecia were registered during the period of treatment after using Cabergoline. The results show that only in 1 leash of bitches proestrus was seen 31 days after the beginning of the treatment (the signs of vaginal bleeding), this bitch was bread after 12 days and in all of the bitches the Progesteron concentrations were low before and during the period of the treatment. In the bitch which has showed the signs of estrus, the progesteron concentration increased above 10 ng/ml by the beginning of estrus and was above 25 ng/ml during pregnancy.

Key words: Dopamin agonists, Cabergoline, Estrus induction in bitches
FINISHING THE PREGNANCY BY USING DOPAMIN AGONIST (CABERGOLINE)

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In this trial,3 leash of bitches,which 22-23 days has gone away(passed)from their matting,were chosen to find out the efficiency and side effects of Dopamin agonist (Cabergoline) on finishing the pregnancy at the first half of pregnancy of such bitches were accepted according to their previous in maintenance of matting and blood progesterone concentration detection (which was 17.53±3.14 a week before the beginning of treatment and was 22.6±3.43 at the beginning of treatment)(P<0.05).all of the treated bitches were taken 10 µg/kg of cabergoline daily,during 10 days of treatment period,per oral.

4 leash of bitches with similar matting previous (without any treatments) were chosen as control group (witness team).blood progesterone concentration was 0.015±0.04 at the begiing of treatment and was reached to 0.06±0.013,72 hours after beginning of treatment (P>0.07).
The plasma progesterone concentration in treated bitches reached to 11.67±3.5 ng/ml,24 hours after the beginning of treatment and 14±0.22 ng/ml,72 hours after beginning of treatment.At the end of 96 hours after beginning of treatment it reached to 1ng/ml (0.5±0.013) (P<0.05).al;l of the treated bitches didn”t have any special side effects during the period of treatment,and during the month after treatment period.They also didn”t show any abortion andvaginal secretion signs.It sounds like that finishing the pregnancy in treated bitches is happened as fetal resorption.All of the dogs in control group have normal (natural) parturition after passing the normal period of natural pregnancy.It seems like that using the high dosage of cabergoline results in finishing the pregnancy as fetal resorption at the first half of pregnancy in treated bitches,without any side effects.
WESTERN BLOT ANALYSIS OF PROACROSIN/ACROSIN IN FROZEN DOG SPERM DURING IN VITRO CAPACITATION

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Introduction - Acrosin, a proteinase found in the spermatozoa of mammalian species, is stored as the zymogen proacrosin in the acrosomal compartment. Proacrosin can self-catalyze its own conversion into the active form acrosin during acrosome reaction (Nuzzo et al., 1990). Freezing and thawing process affect sperm membranes, especially over acrosome region (Cortes et al., 2006); this fact can alter functional mechanisms involved in acrosome reaction inducing an effect on proacrosin activation into acrosin. Thus, the aim of this work was to analyze proacrosin/acrosin in frozen/thawed dog sperm during different times of in vitro capacitation by western blotting.

Materials and Methods - A total of 12 ejaculates were obtained from 3 fertile dogs by manual stimulation. Sperm-rich fraction of each ejaculate was liquated into 2 fractions and centrifuged in tris buffer. The pellet of one fraction was diluted in fert-talp medium and cultured for capacitation 0, 30, 60 and 90 min at 20°C (control). The pellet of the other fraction was diluted in TRIS-citrate fructose-freezing extender, and frozen in liquid nitrogen at -196°C (De los Reyes et al., 2006). After thawing, sperm were cultured in fert-talp for capacitation during the same periods. At each time of culture, fresh and frozen /thawed sperm extracts were made by homogenizing a sperm in buffer containing 1% Triton X-100, NaCl 1M, EDTA 1mM, de PMSF 10 µg/ml, Tris-HCl 20mM pH 7.0 and then centrifuged. Extract proteins were separated on 15% non-reducing SDS-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose paper by western blotting (Lones and Williams, 1990). Blots were blocked with 2% BSA for 1 h and probed with monoclonal antibody C5F10 against acrosin. Optic density (pixels) was evaluated using Photoshop 7.0 Program.

Results - A band of 40 KDa corresponding to proacrosin and two low molecular weight bands corresponding to other molecular forms of active acrosin (alpha-acrosin of 32 kDa and beta-acrosin of 27 kDa) were detected in fresh and frozen/thawed dog spermatozoa in each time of culture. Proacrosin pattern did not change much throughout capacitation time in both fresh and frozen samples. However, beta-acrosin band in frozen sperm was strongest at the beginning of culture and this reactivity decreased over the time, whereas beta-acrosin reactivity in fresh spermatozoa was lowest at 0 h and increased with time. Our results suggest that proacrosin is activated to beta-acrosin earlier in frozen/thawed than in fresh dog spermatozoa.

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