



In: **Recent Advances in Equine Reproduction**, B.A. Ball (Ed.)

Publisher: International Veterinary Information Service (www.ivis.org), Ithaca, New York, USA.

Hysteroscopic and Low-dose Insemination Techniques in the Horse (20-Dec-2004)

B. A. Ball

Department of Population Health and Reproduction, School of Veterinary Medicine, University of California-Davis, Davis, CA, USA.

Introduction

The number of equine sperm used for artificial insemination (AI) in the mare was largely established by research conducted at Colorado State University in the 1970s [1-3]. The minimum insemination dose of 500 million progressively motile spermatozoa (PMS) established in these studies has been widely used as an industry standard. Clinical observations suggest that this insemination dose provides a conservative and effective insemination dose for the mare. Other authors have suggested that individual stallions may require fewer sperm for maximal fertility (100 to 250 million PMS) and that adjustment for the percentage of morphologically normal sperm may be desirable [4,5].

A more widespread application of cryopreserved equine semen and the application of techniques for sex-sorted sperm in the horse have provided considerable incentive to reduce the number of spermatozoa used per insemination in order to maximize the efficiency of these techniques. Two reports in 1998 provided information that suggested that deposition of relatively low numbers of spermatozoa at the uterotubal papilla via endoscopically guided techniques could result in pregnancy in mares [6,7] (Table 1). Although pregnancy rates were low (22 to 30%) after insemination of 1 to 3.8 million PMS, these studies demonstrated the feasibility of dramatically lowering the number of sperm inseminated by direct deposition of sperm at the uterotubal junction (UTJ) via hysteroscopically guided techniques.

Subsequent to these reports, a number of studies have examined low-dose insemination techniques in the mare based upon both hysteroscopic and deep intrauterine techniques for deposition of fresh, chilled, cryopreserved as well as sex-sorted sperm (Table 1).

Sperm Reservoir Formation

Although relatively large numbers of sperm are deposited into the uterus at the time of natural service (10^9) or AI (10^8), relatively few (10^2 - 10^3) sperm reach the site of fertilization at the ampullar-isthmic junction of the oviduct of mares [8-11]. The progression of sperm through the female reproductive tract is associated with a marked reduction in the number of sperm as well as a marked improvement in the percentage of motile, morphologically normal sperm [8,12,13]. The uterotubal junction as well as the initial portion of the oviductal isthmus is believed to play an important role in the selection of sperm as well as in the formation of an effective sperm reservoir [8,14]. Sequestration of sperm within this site occurs rapidly after mating or insemination such that sperm are effectively isolated from the uterine environment within approximately 4 hours after insemination [15,16].

Formation of an effective sperm reservoir within the initial portion of the isthmic oviduct appears to depend upon a cell-cell adhesion between sperm and oviductal epithelial cells that is capable of maintaining the fertile life of equine sperm for several days during estrus in mares [8]. Sperm that are adherent to the oviductal epithelium are effectively blocked from undergoing the pre-fertilization change of capacitation which acts to maintain a viable, intact population of sperm to participate in ovulation at the time of ovulation. The adhesion of sperm to oviductal epithelium maintains low intracellular calcium in adherent sperm [17]. At the time of ovulation, sperm are released from this reservoir and undergo the prefertilization changes necessary for successful binding of the zona pellucida and fertilization.

Low-Dose Insemination - Results to Date

A relatively large number of reports have examined various aspects of reduced-dose insemination in the mare over the past 5 years (summary - Table 1). In the first large trial that compared the effect of sperm number on subsequent pregnancy rates in

mares after hysteroscopic insemination at the UTJ, Morris et al., [18], demonstrated that as insemination doses of 10, 5, or 1 x 10⁶ motile sperm deposited at the UTJ (Fig 1a, Fig 1b) ipsilateral to ovulation resulted in pregnancy rates of 60 - 75% and that insemination doses as small as 1,000 motile sperm achieved pregnancy (1/10 mares inseminated).



Figure 1a. Hysteroscopic views of the uterotubal junction (UTJ) of mares during estrus. Arrows indicate the location of the UTJ. - To view this image in full size go to the IVIS website at www.ivis.org . -



Figure 1b. Hysteroscopic views of the uterotubal junction (UTJ) of mares during estrus. Arrows indicate the location of the UTJ. - To view this image in full size go to the IVIS website at www.ivis.org . -

Sperm Preparation Techniques: A variety of extenders and media have been used for sperm preparation prior to endoscopic insemination. Preparation of sperm prior to insemination may influence subsequent pregnancy rates because of factors such as the removal of seminal plasma as well as the potential influence of some extender components on the ability of sperm to adhere to the oviductal epithelium. We have previously shown that seminal plasma in low dilutions (1:100) may block the ability of equine sperm to bind oviductal epithelium in *in vitro* studies (Dobrinski and Ball, unpublished data). Normally transit through the female reproductive tract results in removal of much of the seminal plasma; however, with deposition of sperm near the UTJ, there may be less opportunity for seminal plasma proteins to be removed prior to sperm reservoir formation. Likewise, inclusion of extenders in sperm suspensions used for GIFT procedures in the horse appears to reduce resultant fertilization rates [19]. However, there are no controlled studies available to address the potential roles of either extender components or seminal plasma on fertility achieved after low-dose insemination.

Centrifugation across density gradients, such as Percoll, has been used to remove seminal plasma, and extender components as well as to enrich the population of motile sperm used for low-dose insemination via hysteroscopy [18]. In other reports, more practical methods for sperm preparation that do not result in complete removal of extender components or seminal plasma have achieved acceptable pregnancy rates. In studies with frozen-thawed equine sperm, direct deposition of cryopreserved sperm after thawing without removal of freezing extenders has also resulted in acceptable pregnancy rates.

Media used for sperm suspension prior to insemination include a variety of semen extenders, including skim-milk or skim-milk/egg yolk, cryopreservation media as well as a number of sperm media such as Tyrode's medium (TALP). Again, there are no reports of critical comparison among these media and acceptable pregnancy rates have been achieved using both routine semen extenders, cryopreservation extenders (frozen semen) as well as TALP. The use of media such as TALP requires careful sperm handling conditions as sperm suspended in these media appear to be more susceptible to cold shock.

Insemination volume: The volume of inseminate deposited at the UTJ by hysteroscopy typically varies between 20 to 200 ul. When frozen-thawed sperm are inseminated directly, a volume of 250 to 500 ul (corresponding to a single 0.25- or 0.5-ml straw) is typically inseminated.

Hysteroscopy vs. Deep Intrauterine Insemination: Although the initial reports dealing with low-dose insemination techniques in the mare were based upon endoscopically-guided procedures to deposit the sperm on the UTJ, other workers have used a transrectally guided technique to direct a flexible insemination catheter to the tip of the uterine horn ipsilateral to the preovulatory follicle. In a report by Brinsko et al., [18,20], pregnancy rates after insemination with 5 x 10⁶ motile sperm at the tip of the uterine horn by either pipette or endoscopic procedures were similar (Table 1). In other reports in which similar numbers of sperm were deposited at the horn tip by pipette, pregnancy rates varied between 35 - 50% (Table 1). These studies suggest that insemination doses of approximately 5x10⁶ motile sperm with deep-intrauterine techniques can achieve acceptable pregnancy rates without the need for endoscopic procedures.

Site of Insemination: Although most of the effort with low-dose insemination has been directed toward deposition of sperm on or near the UTJ, at least one study indicates that low dose insemination into the uterine body may also result in acceptable fertility. Morris et al., [21], compared fertility after insemination of 14x10⁶ motile, frozen-thawed sperm into the uterine body

by pipette or at the UTJ via endoscopy. Pregnancy rates achieved with the two techniques were similar (Table 1). Pregnancy rates were significantly lower when 3×10^6 motile, frozen-thawed sperm were deposited via endoscopy into the uterine body compared to deposition at the UTJ. There are likely important stallion effects related to the minimal number of sperm needed to achieve acceptable fertility with these techniques; nonetheless, the ability to achieve reasonable fertility rates after deposition of 14 million motile sperm into the uterine body offers intriguing possibilities to improve the efficiency of frozen-thawed equine semen.

Results with Subfertile Stallions: Anecdotal reports indicate that low-dose, hysteroscopic insemination procedures have been successful in improving fertility of stallions with low sperm numbers in the ejaculate. Experience at the University of California, Davis (UC-Davis) has not yielded success in managing stallions with oligospermia which included poor sperm morphology.

The UTJ represents the ostium of the equine oviduct as it projects into the uterine lumen at the tip of the uterine horn. The morphology of the UTJ varies somewhat from mare to mare but typically is observed as a small papillae that projects approximately 3 to 5 mm into the uterine lumen. The lumen of the oviduct at this point is extremely small (size ~ 2 French [7]) and provides an extremely effective barrier between the uterus and oviduct. Initially at UC-Davis, low-dose insemination was attempted by cannulation of the initial portion of the oviduct prior to deposition of the inseminate. This was quickly abandoned due to the difficulty in cannulation of the oviduct and the insemination was conducted by depositing the inseminate onto the uterine surface of the UTJ.

Other investigators have attempted to improve sperm transit into the oviduct by local administration of prostaglandin E2 (PGE2). Prostaglandin E2 induces relaxation of the circular smooth muscle of the oviduct and acts to facilitate embryo transport into the uterus. Topical pretreatment with PGE2 (250 ug) increased pregnancy rates in mares bred with 25×10^6 sperm (13/18 vs 7/16) into either the uterine horn or uterine body [22]. In a subsequent study in which insemination was conducted via endoscopy, there was no positive effect of pretreatment with PGE2 compared to controls [20].

Technique - Hysteroscopic Insemination

Clearly, appropriate selection of candidate mares for low-dose insemination as well as stallion fertility will influence the success of this procedure. Young, fertile mares without prior history of post-breeding endometritis are optimal candidates. Older mares with poor uterine clearance will be less desirable candidates and are more likely to have intrauterine fluid accumulation after hysteroscopic insemination. In reported studies the incidence of intrauterine fluid accumulation varies. In early reports, as many as 60% (6/10) of mares had intrauterine fluid accumulation after the procedure [6]; however, later reports indicate an incidence of 1% intrauterine fluid accumulation after endoscopic insemination [23].

Equipment: The use of a 1.6 M videoendoscope with an operating channel facilitates visualization of the UTJ in most mares. Shorter endoscopes may be used; however, endoscope length may limit easy access to the operating channel and endoscope controls in larger mares. Care of the operating channel of the endoscope is important to assure that no debris remains in the channel after the procedure and to assure that all of the chemical disinfectant used in processing the endoscope is removed. The small volumes of inseminate used in this procedure are extremely susceptible to contaminants remaining in the biopsy channel of the endoscope.

Typically a double-lumen catheter is used for the insemination procedure. The double-lumen helps prevent contamination of the inseminate during passage through the endoscope biopsy channel [6]. The catheter length should be a minimum of 20 cm longer than the biopsy channel in order to provide adequate working length for insemination¹. A minimum of three personnel are typically needed to complete the procedure.

Mare Preparation: Typically, low-dose insemination is conducted following ovulation induction with either hCG or deslorelin acetate (Ovuplant®) administration. Intervals of 24 to 30 hours between administration of hCG and insemination are most often used (Table 1) with slightly longer intervals being more useful after administration of Ovuplant®.

Optimally, for endoscopic insemination mares should be restrained in stocks. Depending upon the mare's temperament, sedation (detomidine / butorphanol) may be required. The mare's rectum should be evacuated, and the location of the preovulatory follicle should be confirmed. Careful preparation of the vulva and perineum for the vaginal procedure should be performed.

Two methods of introduction of the endoscope into the uterus may be used. In the standard hysteroscopic approach, the endoscope is introduced through the cervix, the uterus is insufflated and the endoscope is directed visually up the uterine horn ipsilateral to the preovulatory follicle. Alternatively, the endoscope can be introduced through the cervix and guided by manipulation per rectum up the uterine horn prior to insufflation. Using the first method, it is important to confirm location of the endoscope ipsilateral to the preovulatory follicle because under visual guidance, it is easy to become disoriented as to left versus right uterine horns. Once the UTJ is identified, the insemination catheter should be passed through the biopsy channel and the inseminate deposited directly onto the UTJ (Fig 2 & Fig 3). No attempt is made to cannulate the oviduct during this process. Some authors advocate the introduction of air "bubbles" during deposition of the inseminate in order to maintain a greater contact of the inseminate in the region of the UTJ by surface tension. After insemination is complete, the air that was introduced during the procedure should be removed by suction as the endoscope is removed from the uterus.



Figure 2. Hysteroscopic view of the UTF and insemination catheter prior to insemination. - To view this image in full size go to the IVIS website at www.ivis.org . -



Figure 3. Hysteroscopic view of the UTJ and insemination catheter after insemination. - To view this image in full size go to the IVIS website at www.ivis.org . -

The low number of sperm as well as the small volume of the inseminate require more careful handling and loading sperm for this procedure. This is particularly true if cryopreserved sperm are used as these cells are much more susceptible to cold shock and the small volumes used make heat transfer and thermal shock more likely. Prior to the insemination, catheters and lab-ware used for semen handling should be prewarmed to body temperature. Once the endoscope is in place and the UTJ is identified, the inseminate should be drawn into the distal end of the insemination catheter by aspirating it carefully much as an embryo would be aspirated into a transfer pipette. Insemination should be accomplished rapidly after the catheter is loaded.

Deep Intrauterine Insemination Techniques

Mare preparation would be similar to that used for endoscopic insemination although the need for sedation would be less common in this procedure. Again, palpation per rectum should confirm the location of the ipsilateral follicle and evacuate feces from the rectum. After preparation for the vaginal procedure, insemination is conducted by passing the insemination catheter through the cervix. After the catheter is introduced into the cervix, the catheter is guided per rectum to the tip of the uterine horn ipsilateral to the preovulatory follicle. Entry into the uterine horn can be facilitated by grasping the base of the contralateral uterine horn and applying caudal traction. This maneuver tends to straighten the opposite uterine horn and facilitate passage of the catheter to the tip of the horn.

Our program prefers the insemination catheter from Minitube². This catheter allows the introduction of a 0.5-ml straw for insemination and also allows the use of multiple 0.5-ml straws if needed without withdrawing the catheter (requires two operators to accomplish this). This catheter in the longer length (65 cm) has adequate flexibility for deep intrauterine insemination.

Table 1. Summary of current literature regarding low-dose and hysteroscopic insemination in the mare.

Study	Semen Type	Sperm Number	AI Location/Method	AI Timing	AI No.	Pregnancy Rate	
[6]	Fresh	3.8x10 ⁶ PM	UTJ endoscope	24 h post hCG	1	3/10	
[7]	Fresh	10x10 ⁶ PMMN	UTJ endoscope	hCG at time of insemination	1	0/11	
		10x10 ⁶ PMMN				2/9	
[24]	Fresh	500x10 ⁶ PM	UT body pipette	34 or 40 h post Ovuplant® (deslorelin acetate)	1	18/20	
		25x10 ⁶ PM	Horn tip pipette			12/21	
		5x10 ⁶ PM				7/20	
	Sexed, Fresh	25x10 ⁶ live	Horn tip pipette	34 h post Ovuplant® (deslorelin acetate)	3/10 (SMG ext)		
		25x10 ⁶ live			5/10 (EY ext)		
	[18]	Fresh	10x10 ⁶ motile	UTJ endoscope	32 h post hCG	1	6/10
5x10 ⁶			6/8				
1x10 ⁶			16/25				
0.5x10 ⁶			4/14				
0.1x10 ⁶			3/11				
0.001x10 ⁶			1/10				
[22]	Fresh	25x10 ⁶	UT body pipette	24 h post hCG	1	14/30	
			UT horn pipette			14/32	
[26]	Frozen	50x10 ⁶	Horn tip pipette	Pre-OV	1	0/10	
			UT body pipette	2/10			
		150x10 ⁶	Horn tip pipette	Pre & Post-OV	2	4/10	
			UT body pipette			5/10	
		400x10 ⁶	UT body pipette	5/10			
		800x10 ⁶	UT body pipette	Post-OV		3/10	
[27]	Frozen	10x10 ⁶ motile	UTJ endoscope	Post-OV	1	4/12	
		4400x10 ⁶ motile	UT body pipette			4/12 (Percoll)	
						0/12	
[28]	Frozen	800x10 ⁶	UT body pipette	Post-OV	1	12/20	
		400x10 ⁶		24 & 40 hr post hCG		2	11/20
		200x10 ⁶		24 & 40 hr post hCG (Ovuplant?)			10/20
		200x10 ⁶	Horn tip Pipette		4/20		

Table 1. Summary of current literature regarding low-dose and hysteroscopic insemination in the mare.

Study	Semen Type	Sperm Number	AI Location/Method	AI Timing	AI No.	Pregnancy Rate
[29]	Fresh	5x10 ⁶ motile	UTJ endoscope	6 h post hCG	1	4/10
	Fresh, sorted					6/16
	Frozen			30 h post hCG		6/16
	Frozen, sorted					2/15
[30]	Fresh	5x10 ⁶	UTJ endoscope	At time of hCG	1	5/10
			UT body pipette			0/10
	Fresh, sorted		UTJ endoscope			5/20
[31]	Fresh, cooled	500x10 ⁶ PM	UT body pipette	12 & 24 h post hCG	2	4/11
	Frozen	50x10 ⁶ PM	Horn tip pipette			7/11
[32]	Fresh 15°C sorted	20x10 ⁶	UTJ endoscope	30 h post hCG	1	18/25
	Fresh 5°C Sorted		UTJ endoscope			12/22
	Fresh 5°C Sorted		Horn tip pipette			9/24
[21]	Frozen	14x10 ⁶ PM	UT body pipette	30 - 32 h post hCG	1	8/12
		14x10 ⁶ PM	UTJ endoscope			9/14
		3x10 ⁶ PM	UTJ endoscope			16/34
			UT body endoscope			2/14
			Contralateral UTJ - endoscope			1/12
[27]	Frozen	50 - 75 x10 ⁶	UTJ endoscope	6 hr Post-OV	1	95/166

UTJ = uterotubal junction; PM = progressively motile; PMMN = progressively motile, morphologically normal; sorted = sex-sorted sperm; Percoll = Percoll separated; Post-OV = post-ovulation; pre-OV = pre-ovulation

Footnotes

- 1- V-EFIS-2-200 200 cm long coaxial catheter set - 3 French tapering to 2 French double lumen Global Veterinary Products, Inc.
- Large animal insemination set (catalog #ins200) - 200 cm long, 2 French double lumen. Mila International, Inc.
- 2 - Universal Pipette and AI Gun - 65 cm. Minitube

References

1. Demick DS, Voss JL, Pickett BW. Effect of cooling, storage, glycerolization and spermatozoal numbers on equine fertility. *J Anim Sci* 1976; 43:633-637.
2. Householder DD, Pickett BW, Voss JL, et al. Effect of extender, number of spermatozoa and hcg on equine fertility. *J Equine Vet Sci* 1981; 1:9-13.
3. Pickett BW, Voss JL. The effect of semen extenders and sperm number on mare fertility. *J Reprod Fertil Suppl* 1975; 23:95-98.
4. Cooper WL. Artificial Breeding of Horses. *Vet Clin North Am Large Anim Pract* 1980; 2:267-275.
5. Brinsko SP, Varner DD. Artificial insemination and preservation of semen. *Vet Clin North Am Equine Pract* 1992; 8:205-218.
6. Vazquez JJ, Medina VM, Liu IKM, et al. Nonsurgical uterotubal insemination in the mare. In *Proc Annu Conv Am Assoc Equine Pract* 1998; 44:68-69.
7. Manning ST, Bowman PA, Fraser LM, et al. Development of hysteroscopic insemination of the uterine tube in the mare. In *Proc Annu Conv Am Assoc Equine Pract* 1998; 44:70-71.
8. Scott MA. A glimpse at sperm function in vivo: sperm transport and epithelial interaction in the female reproductive tract. *Anim Reprod Sci* 2000; 60-61:337-348.
9. Parker WG, Sullivan JJ, First NL. Sperm transport and distribution in the mare. *J Reprod Fertil Suppl* 1975; 23:63-66.
10. Boyle MS, Cran DG, Allen WR, et al. Distribution of spermatozoa in the mare's oviduct. *J Reprod Fertil Suppl* 1987; 35:79-86.
11. Bader H. An investigation of sperm migration in the oviducts of the mare. *J Reprod Fertil Suppl* 1982; 32:59-64.
12. Thomas PGA, Ball BA, Miller PG, et al. A subpopulation of morphologically normal, motile spermatozoa attach to equine oviductal epithelial cell monolayers. *Biol Reprod* 1994; 51:303-309.
13. Scott MA, Liu IKM, Overstreet JW, et al. The structural morphology and epithelial association of spermatozoa at the uterotubal junction: A descriptive study of equine spermatozoa in situ using scanning electron microscopy. *J Reprod Fertil Suppl* 2003; 56:415-421.
14. Troedsson MHT, Liu IKM, Crabo BG. Sperm transport and survival in the mare. *Theriogenology* 1998; 49:905-915.
15. Brinsko SP, Varner DD, Blanchard TL, et al. The effect of postbreeding uterine lavage on pregnancy rate in mares. *Theriogenology* 1990; 33:465-475.
16. Brinsko SP, Varner DD, Blanchard TL. The effect of uterine lavage performed four hours post insemination on pregnancy rate in mares. *Theriogenology* 1991; 35:1111-1119.
17. Dobrinski I, Smith TT, Suarez SS, et al. Membrane contact with oviductal epithelium modulates the intracellular calcium concentration of equine spermatozoa in vitro. *Biol Reprod* 1997; 56:861-869.
18. Morris LH, Hunter RH, Allen WR. Hysteroscopic insemination of small numbers of spermatozoa at the uterotubal junction of preovulatory mares. *J Reprod Fertil* 2000; 118:95-100.
19. Coutinho da Silva MA, Carnevale EM, Maclellan LJ, et al. Oocyte transfer in mares with intrauterine or intraoviductal insemination using fresh, cooled, and frozen stallion semen. *Theriogenology* 2004; 61(4):705-713.
20. Brinsko SP, Rigby SL, Lindsey AC, et al. Pregnancy rates in mares following hysteroscopic or transrectally-guided insemination with low sperm numbers at the utero-tubal papilla. *Theriogenology*. 2003; 59:1001-1009.
21. Morris LHA, Tiplady C, Allen WR. Pregnancy rates in mares after a single fixed time hysteroscopic insemination of low numbers of frozen-thawed spermatozoa onto the uterotubal junction. *Equine Vet J* 2003; 35:197-201.

22. Woods J, Rigby S, Brinsko S, et al. Effect of Intrauterine Treatment with Prostaglandin E2 Prior to Insemination of Mares in the Uterine Horn or Body. *Theriogenology* 2000; 53:1827-1836.
23. Morris LH, Allen WR. An overview of low dose insemination in the mare. *Reprod Domest Anim* 2002; 37:206-210.
24. Buchanan BR, Seidel GE, McCue PM, et al. Insemination of mares with low numbers of either unsexed or sexed spermatozoa. *Theriogenology* 2000; 53:1333-1344.
25. Rigby SL, Lindsey AC, Brinsko SP, et al. Pregnancy rates in mares following hysteroscopic or rectally-guided utero-tubal insemination with low sperm numbers. In: *Proc 3rd Int Symp Stallion Reprod* 2001; 49.
26. Dell'Aqua JA, Papa FO, Alvarenga FC, et al. Effects of warming rate on sperm parameters and of insemination and dose on the fertility of equine frozen semen. *Anim Reprod Sci* 2001; 68:344-346.
27. Alvarenga M, Trinquete CC, Lima MM, et al. Utilisation of hysteroscopy for the application of stallion frozen semen in commercial programmes. In: *Havemeyer Foundation Workshop on Transporting Gametes and Embryos* 2003; 27.
28. Squires EL, Reger HP, Maclellan LJ, et al. Effect of time of insemination and site of insemination on pregnancy rates with frozen semen. *Theriogenology* 2002; 58:655-658.
29. Lindsey AC, Schenk JL, Graham JR, et al. Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa. *Equine Vet J* 2002; 34:121-127.
30. Lindsey AC, Bruemmer JE, Squires EL. Low dose insemination of mares using non-sorted and sex-sorted sperm. *Anim Reprod Sci* 2001; 68:279-289.
31. Petersen MM, Wessel MT, Scott MA, et al. Embryo recovery rates in mares after deep intrauterine insemination with low numbers of cryopreserved equine spermatozoa. *Theriogenology* 2002; 58:663-665.
32. Lindsey AC, Varner DD, Seidel GE Jr, et al. Hysteroscopic or rectally guided, deep-uterine insemination of mares with spermatozoa stored 18 h at either 5 or 15 °C prior to flow-cytometric sorting. *Theriogenology* 2002; 58:659-662.

All rights reserved. This document is available on-line at www.ivis.org. Document No. A0216.1204.

