

Comparison of five extenders for canine semen freezing

Comparaç o entre cinco diluidores na congelaç o de s men de c es

Silvia Edelweiss Crusco dos SANTOS¹; Camila Infantsi VANNUCCHI¹; Sandra SATZINGER¹; Mayra Elena Ortiz D'Avila ASSUMPÇ O¹; Jos  Antonio VISINTIN¹

CORRESPONDENCE TO:
Jos  Antonio Visintin
Departamento de Reproduç o
Animal
Faculdade de Medicina Veterin ria
e Zootecnia da USP
Cidade Universit ria Armando de
Salles Oliveira
Av. Orlando Marques de Paiva,
87. 05508-000 - S o Paulo - SP
e-mail: visintin@usp.br

¹-Departamento de Reproduç o
Animal da Faculdade de
Medicina Veterin ria e Zootecnia
da USP-SP

SUMMARY

Semen was collected from six stud dogs to compare five extenders in the semen freezing process. Each ejaculate was divided in five parts and added to tris-fructose-citric acid, glycine, lactose, skim milk and tris-fructose-citrate extenders. The semen was diluted at 37°C in extenders without glycerol, in the ratio 1:1 and cooled for 60 minutes to reach a 5°C temperature. Then, extenders with glycerol in the ratio of 2:1 were added to give the final prefreezing concentration of 4% of glycerol. The diluted semen with the cryoprotectant was maintained for a further 60 minutes in refrigeration to equilibrate the spermatozoa in the glycerol and packaged in 0.5 ml plastic straws. The straws were maintained for 30 minutes in vapor, plunged and stored in liquid nitrogen. Sperm morphology was evaluated before and after freezing, whereas progressive motility (%) and velocity of forward progression (0-5) were appraised in different periods of the freezing process. The extender tris-fructose-citric acid showed the best post thaw progressive motility and velocity of forward progression compared to the others extenders. Semen freezing increased major sperm morphological abnormalities, regardless of the extender.

UNITERMS: Diluents; Freezing; Semen; Dogs.

INTRODUCTION

Small animal reproduction technologies, especially in dogs, have been in evidence in the last years due to the introduction of new techniques as artificial insemination with frozen semen. The exchange of canine frozen semen among many countries is widely applied, therefore an increase in the number of artificial insemination banks has been noticed.

Rowson²⁰ was the first researcher to freeze canine semen, thenceforth studies were developed to find a good extender and cryoprotectant to preserve canine semen. An ideal extender should have nutrients as an energy source, substances that buffer against harmful changes of pH, that provides a physiological osmotic pressure and concentration of electrolytes, that prevents bacterial growth and protects the cells from cold shock during the freezing and thawing processes⁴. One of the first researchers to compare different

extenders in canine semen freezing was Foote⁹. Since then, many works have been done to evaluate the cryoprotectant levels, the freezing methods and the extenders.

The aim of this experiment was to compare five semen extenders analyzing post thaw progressive motility (%), velocity of forward progression (score from 0 to 5) and sperm morphology (%).

MATERIAL AND METHOD

Animals

Six dogs of different breeds (Rottweiler, English Bulldog, 2 German Shepherd, and 2 mixed-breed dogs), with an average of 2.5 years of age, were used. The animals were receiving commercial dog food and water *ad libitum*.

Extenders

1. Tris-fructose-citric acid (3.187 g tris; 1.781 g citric acid; 1.136 g fructose and 80 ml distilled water), according to Andersen²;

2. Lactose (11% lactose) in accordance to Yubi *et al.*²⁶;

3. Skim milk (12% skim milk; 1.2% solution of glucose, sodium citrate and fosfate), in agreement with Takeishi *et al.*²³;

4. Glicine, according to Tekin *et al.*²⁴.

Solution I = 1.2 g glucose; 1.2 g fructose; 60 ml distilled water. This solution was warmed at 95°C for 15 minutes.

Solution II = 2.0g sodium citrate; 0.94 g glicine; distilled water to complete 100 ml.

The glicine extender was constituted by 30 ml of the solution I and 50 ml of the solution II.

5. Tris-fructose-citrate (2.526 g tris; 1.3868 g sodium citrate; 1.053 g fructose; distillate water 80 ml) in accordance to Foote; Leonard¹⁰.

Semen collection and evaluation

Semen was collected by digital manipulation¹⁴, with a 48 hour interval between the collections. The semen was collected in a glass funnel connected to a glass tube immersed in water at 37°C in a nursing bottle¹⁴. The sperm-rich fraction and some drops of the third fraction of the ejaculates⁷ were collected. The volume and color were immediately appraised. Progressive motility and velocity of forward progression were evaluated in a 100x microscopic examination utilizing a drop of semen between a warmed glass slide and coverslip. Sperm count was performed in a 400x examination objective using a hematimeter chamber with a 1:200 dilution rate of the semen in saline formaline. Sperm morphology was evaluated utilizing William's method and wet preparation with saline formaline in a differential interference contrast microscope.

Table 1

Means and standard deviations of the sperm parameters in 30 ejaculates of the 6 dogs. São Paulo, 1994.

Seminal	Volume	Motility	VFP ¹	Sperm Count	Defects		
Character	MI	%	(0-5)	Millions/ml	major %	minor %	total %
Mean (x)	4.68	90.67	4.73	288.50	3.83	2.5	6.08
(±SD)	(±2.84)	(±4.08)	(±0.27)	(±82.68)	(±2.5)	(±1.6)	(±3.78)

¹VFP = Velocity of Forward Progression.

Table 2

Means and standard deviations of spermatozoa progressive motility (%) of 30 ejaculates from 6 dogs during the different steps of the freezing process*. São Paulo, 1994 (p<0.05).

Step Extender	Fresh	A0	A30	A60	B0	B30	B60	D
Tris	90.67	87.18	83.50	76.47	70.70	70.83	70.33	36.75
	(±4.08)	(±4.54)	(±7.50)	(±15.62)	(±11.61)	(±9.15)	(±8.77)	(±20.49)
Glicine	90.67	84.33	83.17	80.67	75.33	71.83	71.33	32.30
	(±4.08)	(±6.12)	(±5.34)	(±5.31)	(±3.82)	(±7.08)	(±6.62)	(±8.12)
Lactose	90.67	71.67	58.83	56.50	44.17	41.33	37.17	10.27
	(±4.08)	(±10.63)	(±16.27)	(±11.27)	(±18.09)	(±16.18)	(±16.66)	(±6.19)
Milk	90.67	43.17	34.00	24.50	10.20	8.23	5.93	3.98
	(±4.08)	(±13.02)	(±11.98)	(±8.30)	(±6.56)	(±3.69)	(±4.26)	(±4.52)
Citrate	90.67	74.67	57.67	36.63	18.73	23.30	11.13	1.67
	(±4.08)	(±4.08)	(±11.65)	(±20.07)	(±17.98)	(±34.27)	(±12.97)	(±1.65)

Fesh = immediately after the collection; A0 = immediately after the A extender addition; A30 = after 30 minutes of refrigeration; A60 = after 60 minutes of refrigeration; B0 = immediately after the B extender addition; B30 = 30 minutes after the B extender addition; B60 = 60 minutes after the B extender addition; D = after thawing.

Freezing and thawing procedures

The five ejaculates obtained from each dog were divided into 5 parts and diluted in the 5 extenders. The initial dilution without glycerol was performed at 37°C (A fraction). This mixture was maintained at 37°C in a becker with water and cooled during 60 minutes to reach a 5°C temperature. After this period, the B fraction (with glycerol) was added to reach the final pre-freezing concentration of 4% glycerol. Final dilution rate of all samples was 1:3.

Semen was packaged in 0.5 ml straws, each one containing 50x10⁶ spermatozoa¹. Straws were maintained at 5 cm of the nitrogen liquid column (nitrogen vapor) for 20 minutes¹, plunged and stored in liquid nitrogen. Straws were thawed in water bath 37°C for 30 seconds.

Semen was evaluated for progressive motility and velocity of forward progression immediately after the

collection, immediately after the A extender addition, 30 and 60 minutes after the initial cooling period; immediately, 30 and 60 minutes after de B extender addition and after thawing.

Statistical analysis

Square means for F test were stipulated according to the rules showed by Stell & Torrie²². Analysis of variance were shown separately.

RESULTS

The sperm parameters of the fresh ejaculates from 6 dogs are listed in Tab. 1.

The analysis results of the post-thaw progressive motility of each extender and steps of the freezing process are presented in Tab. 2.

Table 3

Means and standard deviations of spermatozoa velocity of forward progression (0-5) from 30 ejaculates of the 6 dogs during different steps of freezing process*. São Paulo, 1994. (p<0.05).

Step Extender	Fresh	A0	A30	A60	B0	B30	B60	D
Tris	4.73 (±0.27)	4.43 (±0.29)	4.13 (±0.27)	3.83 (±0.40)	3.50 (±0.41)	3.33 (±0.51)	3.40 (±0.50)	2.85 (±0.88)
Glicine	4.73 (±0.27)	4.67 (±0.27)	4.30 (±0.41)	4.16 (±0.34)	3.63 (±0.32)	3.70 (±0.50)	3.67 (±0.65)	2.93 (±0.56)
Lactose	4.73 (±0.27)	3.60 (±0.50)	3.36 (±0.59)	3.03 (±0.29)	2.63 (±0.58)	2.60 (±0.43)	2.40 (±0.77)	1.42 (±0.55)
Milk	4.73 (±0.27)	3.03 (±0.38)	2.76 (±0.66)	2.16 (±0.58)	1.30 (±0.48)	0.76 (±0.39)	0.93 (±0.50)	0.48 (±0.28)
Citrate	4.73 (±0.27)	3.83 (±0.19)	3.03 (±0.28)	2.80 (±0.28)	1.53 (±0.75)	1.10 (±0.54)	1.00 (±0.59)	0.50 (±0.45)

* Fresh = immediately after the collection; A0 = immediately after the A extender addition; A30 = 30 minutes after refrigeration; A60 = 60 minutes after refrigeration; B0 = immediately after the B extender addition; B30 = 30 minutes after the B extender addition; B60 = 60 minutes after the B extender addition; D = after thawing.

Table 4

Means and standard deviations of major, minor and total sperm morphological abnormalities from 30 ejaculates of the 6 dogs, before and after freezing. São Paulo, 1994. (p<0.05).

Defect	Fresh	Extender				
		Tris	Glicine	Lactose	Milk	Citrate
Major	3.83 (±2.35)	8.45 (±4.11)	10.32 (±2.94)	7.8 (±3.89)	8.85 (±3.85)	7.93 (±3.83)
Minor	2.25 (±1.68)	2.33 (±1.23)	3.03 (±1.69)	2.10 (±1.16)	1.67 (±1.46)	2.83 (±1.10)
Total	6.08 (±3.78)	10.68 (±4.99)	13.35 (±4.53)	9.90 (±4.90)	10.52 (±5.03)	10.76 (±4.69)

There was a significant effect of extender (p<0.05) on major and total defects.

There was a significant effect of extender and steps ($p<0.05$) on progressive motility. The most dramatic reduction in motility occurred during step B60 and D.

Analysis results of the spermatozoa velocity of forward progression in each of the extenders during freezing process are presented in Tab. 3.

There was a significant effect of the extender and the steps of the freezing process ($p<0.05$) on the velocity of forward progression. The most dramatic reduction in the velocity of forward progression occurred during step B60 and D.

Analysis results of the post-thawing sperm morphological abnormalities of each extender are presented in Tab. 4.

DISCUSSION

The present data indicated that tris-fructose-citric acid and glicine extenders provide best recovery after thawing of dog semen. The damage to sperm progressive motility and velocity of forward progression occurred progressively.

Seminal parameters as volume, progressive motility, velocity of forward progression, sperm count and sperm morphology used in this experiment were also applied in other studies^{4,5}. Only dogs that had progressive motility higher than 70% and less than 50% of total sperm morphological abnormalities were used (Tab. 1), in agreement with Linde-Forsberg¹⁴, and Dobrinsky *et al.*⁵. Semen was collected according to Seager²¹ and Linde-Forsberg¹⁴ using the sperm rich fraction and some drops of the third fraction to avoid harmful effects of the prostatic fluid during the freezing process^{6,7}. In a general way, these dogs showed a good adaptation to the semen collection by manual massage of the penis. Nevertheless, the collection of some animals was not possible due to excessive work and stress. The presence of a female with signs of estrous proptiated in some dogs a better libido, total volume of the ejaculate, progressive motility, velocity of forward progression and sperm count. Progressive motility, velocity of forward progression and sperm count were applied as semen evaluation parameters through the freezing process of the five extenders (Tris-fructose-citric acid; glicine; lactose; skim milk; tris-fructose-sodium citrate).

The best extender for freezing canine semen in this experiment was tris-fructose-citric acid, which showed the best progressive motility after thawing (36.75%) compared to the other extenders (Tab. 2). These results are in accordance with Martin¹⁵, Joshua¹² and Olar¹⁷. The effectiveness of the tris-fructose-citric acid extender was showed by Olar *et al.*¹⁸, Ivanova *et al.*¹¹ and Belluzi *et al.*³. The tris-fructose-citric acid extender contains in its composition several types of buffer substances as tris, citric acid and the egg yolk. The increased concentration of buffer solutions in the extender reduces the deleterial effects of the great amount of hydrogenic ions

produced as a consequence of the metabolic activity of the spermatozoa. It is known that a pH decrease of the extender solution promotes an integrity damage of the spermatozoal membrane, consequently interfering with the fertilizing capacity of the sperm.

Spermatozoa progressive motility after thawing in glicine extender was 32.30% (Tab. 2). On the other hand, Foote⁹ and Foote; Leonard¹⁰ reported different results, while Rowson²⁰ suggested the possibility of using the glicine extender in canine semen freezing procedures. Tekin *et al.*²⁴ used the glicine extender in stallion chilled semen and observed a greater success compared to the skim milk extender. At present, the glicine extender is not an outspread diluent for cryopreservation of canine semen, since it is believed that the composition of the extender offers an unsatisfactory electrolyte composition to the viability of the frozen-thawed semen.

Post-thaw spermatozoa progressive motility of the Lactose extender was 10.27% (Tab. 2), however in other works the values ranged from 24.17%²⁶ to 50%¹³. Yubi *et al.*²⁶ demonstrated that the lactose extender showed poor results when compared with the Tris-fructose-citric acid extender. The lactose extender has the greater density compared to the other diluents utilized in this experiment, which is deleterious to the post-thaw progressive motility assessment of the semen. For this reason, not only the evaluation of the seminal parameter could have been altered, but the movement of the spermatozoa itself was affected as a consequence of the extender viscosity.

The post-thaw spermatozoa progressive motility of the skim milk extender was 3.98% (Tab. 2), although values of 35% to 70% were observed by Takeishi *et al.*²³. Although the skim milk extender contains favorable characteristics for the cryopreservation of semen as: viscosity, buffer capacity, energy source, and initial antibacterial activity; there are no manners to avoid milk acidification and consequently damage to the integrity of the spermatozoa. Moreover, it is necessary to heat the milk (92-95°C during 10 minutes) in an attempt to destroy the milk spermicidal substances⁸.

The tris-fructose-sodium citrate extender showed a sperm progressive motility after thawing of 1.67% (Tab. 2), which was in accordance with other studies^{2,15,25}. Extenders which contain sodium citrate are less favorable to dog sperm survival¹⁵, since it is noticed that the density of the extender is harmful to the progressive motility of the spermatozoa⁹.

A decrease in progressive motility after thawing and during the different steps through the freezing process was noticed (59.47% for tris fructose-citric acid and 98.16% for Tris fructose citrate) (Tab. 2). The velocity of forward progression was described as an evaluation parameter for canine spermatozoa by Dobrinsky *et al.*⁵ and England⁶, however no specific study was done to establish the velocity of forward progression rates for the post thaw semen. Tris

fructose-citric acid and glycine extenders showed the best velocity of forward progression means, followed by lactose, skim milk and tris-fructose-citrate (Tab. 3). There was a significant increase of the total sperm defects after the freezing process (Tab. 4), as also described by Oettl¹⁶, Rodriguez-Martinez *et al.*¹⁹ and Belluzzi *et al.*³. Thus, the freezing method is responsible for the increase in the total sperm defects and not the extenders.

CONCLUSIONS

The results of this study allowed the conclusion that the tris-fructose-citric acid extender is effective in the freezing process of canine semen, in comparison to glycine, lactose, milk and tris-fructose-citrate ($p < 0.05$). The skim milk, lactose

and tris-fructose citrate extenders showed the lowest rates of progressive motility (%) and velocity of forward progression (0-5). Recent procedures for freezing canine semen remarkably diminish sperm progressive motility and velocity of forward progression and increase major sperm defects (acrossome, head, midpiece and tail). The critic moment for the reduction of progressive motility (%) and velocity of forward progression (0-5) was between the package and the thawing process ($p < 0.05$).

ACKNOWLEDGMENTS

The authors acknowledge the 3rd CIA from 3rd B.P. Chq.- Polícia Militar do Estado de São Paulo for supplying the dogs.

RESUMO

Foram utilizados ejaculados de 6 cães para comparar cinco diluidores no processo de congelação de sêmen. Cada ejaculado foi dividido em 5 partes e adicionadas aos diluidores tris-fructose-ácido cítrico, glicina, lactose, leite desnatado e tris-fructose-ácido cítrico. O sêmen foi diluído a 37°C sem adição de glicerol na proporção 1:1 (fração A) e refrigerado durante 60 minutos até atingir a temperatura de 5°C, quando foi adicionada a fração dos diluidores contendo glicerol (fração B) na proporção 2:1, atingindo a concentração final de 4% de glicerol. O sêmen diluído permaneceu por 60 minutos em refrigeração para equilíbrio no glicerol, sendo envasado em palhetas de 0,5 ml, mantido por 30 minutos no vapor de nitrogênio e imerso e armazenado em nitrogênio líquido. Foram avaliados a motilidade progressiva retilínea, o vigor e os defeitos espermáticos antes da congelação e após a descongelação do sêmen em água a 37°C. Os resultados mostraram que os diluidores tris-fructose-ácido cítrico e glicina apresentaram as melhores médias de motilidade progressiva retilínea e de vigor espermático após a descongelação. A congelação aumentou a frequência dos defeitos espermáticos maiores independente do diluidor.

UNITERMOS: Diluentes; Congelamento; Sêmen; Cães.

REFERENCES

- 1- ALLEN, W.E. Inseminación artificial. In: ALLEN, W.E. **Fertilidad y obstetricia canina**, Zaragoza, Acribia, 1993, cap.21, p.171-6.
- 2- ANDERSEN, K. Insemination with frozen semen based on a new insemination technique. **Zuchthygiene**, v.10, n.1, p.1-4, 1975.
- 3- BELLUZZI, S.; ZAMBELLI, D.; BERGONZONI, M.L. L'inseminazione artificiale nel cane con seme congelato. **Praxis veterinaria**, v.14, n.2, p.12-5, 1993.
- 4- CONCANNON, P.W.; BATTISTA, M. Canine semen freezing and artificial insemination. In: KIRK, R.W. **Current veterinary therapy - small animal practice**. X, Philadelphia: W.B. Saunders, 1989, p.1247-59.
- 5- DOBRINSKI, I.; LULAI, C.; BARTH, A.D.; POST, K. Effects of four different extenders and three different freezing rates on post-thaw viability of dog semen. **Journal of Reproduction and Fertility**, p.291-6, 1993, supplement. 47.
- 6- ENGLAND, G.C.W. The cryopreservation of dog semen: a review. **Journal of Reproduction and Fertility**, p.243-55, 1993, supplement 47.
- 7- ENGLAND, G.C.W.; ALLEN, W.E. Factors affecting the viability of canine spermatozoa. II. Effects of seminal plasma and blood. **Theriogenology**, v.37, n.2, p.373-81, 1992.
- 8- FLIPSE, R.J.; PATTON, S.; ALMQUIST, J.O. Diluters for bovine semen. III /effect of lactenin and of lactoperoxidase upon spermatozoa livability. **Journal of Dairy Science**, v.37, n.10, p.1205-11, 1954.
- 9- FOOTE, R.H. Extenders for freezing dog semen. **American Journal of Veterinary Research**, v.25, n.104, p.37-40, 1964.
- 10- FOOTE, R.H.; LEONARD, E.P. The influence of pH, osmotic pressure, glycine, and glycerol on the survival of dog sperm in buffered-yolk extenders. **Cornell Veterinarian**, v.54, n.1, p.78-89, 1964.

- 11- IVANOVA, M.G.; NIKOLOV, I.I.; DACHEVA, D.P.; IVANOV, I.H.; IVANOVA, N.B. Testing of different media for cryopreservation of dog semen. *In: INTERNATIONAL CONGRESS ON ANIMAL REPRODUCTION*, 12., The Hague, 1992. *Proceedings*, p.1791-2.
- 12- JOSHUA, J.O. **Reproductive clinical problems in the dog**, Bristol, John Wrighth, 1984, p.49-60.
- 13- LEES, G.E.; CASTLEBERRY, M.W. The use of frozen semen for artificial insemination of German Shepherd dogs. *Journal of American Animal Hospital Association*, v.13, n.3, p.382-6, 1977.
- 14- LINDE-FORSBERG, C. Achieving canine pregnancy by using frozen or chilled extend semen. *Veterinary Clinics of North America*, v.21, n.3, p.467-85, 1991.
- 15- MARTIN, I.C.A. The deep freezing of dog spermatozoa in diluents containing skim milk. *Research Veterinary Science*, v.4, p.315, 1963.
- 16- OETTLÈ, E.E. Changes in acrosome morphology during cooling and freezing of dog semen. *Animal Reproduction Science*, v.12, n.2, p.145-50, 1986.
- 17- OLAR, T.T. Using frozen canine semen: a guide for practitioners. *Veterinary Medicine*, v.80, n.3, p.22-30, 1985.
- 18- OLAR, T.T.; BOWEN, R.A.; PICKETT, B.W. Influence of extender, cryopreservative and seminal processing procedures on post-thaw motility of canine spermatozoa frozen in straws. *Theriogenology*, v.31, n.2, p.451-61, 1989.
- 19- RODRIGUEZ-MARTINEZ, H.; EKWALL, H.; LINDE-FORSBERG, C. Fine structure and elemental composition of fresh and frozen dog spermatozoa. *Journal of Reproduction and Fertility*, p.279-85, 1992, supplement 47.
- 20- ROWSON, L.E.A. Infertility in cow, sow and bitch. *Irish Veterinary Journal*, v.8, n.10, p.216-27, 1954.
- 21- SEAGER, S.W.J. Artificial Insemination in dogs. *In: BURKE, T.J. Small animal reproduction and infertility*, Philadelphia : Lea & Febiger, 1986, p.207-17.
- 22- STEEL, R.G.D.; TORRIE, J.H. **Principles and procedures of statistics: a biometrics approach**, New York : McGraw-Hill Book, 1980. P.633.
- 23- TAKEISHI, M.; MIKAMI, T.; ODAMA, Y.; TSUNEKANE, T.; IWAKI, T. Studies on reproduction in the dog; VIII. Artificial insemination using frozen semen. *Japanese Journal of Animal Reproduction*, v.22, n.1, p.28, 1976.
- 24- TEKIN, N.; WÖCKENER, V.E.; KLUG, E. Konservierungsfähigkeit von pferdsamen bei einatz zweier verdunner und konservierungstemperaturen. *Deutsch Tierärztliche Wochenschrift*, v.95, p.2-15, 1988.
- 25- WALES, R.G.; WHITE, I.G. Viability of diluted dog spermatozoa in vitro. *Journal of Reproduction and Fertility*, v.5, n.1, p.67-76, 1963.
- 26- YUBI, A.C.; FERGUSON, J.M.; RENTON, J.P., HARKER, S.; HARVEY, M.J.A.; BAGYENJI, B.; DOUGLAS, T.A. Some observation on the dilution, cooling and freezing of canine semen. *Journal of Small Animal Practice*, v.28, n.8, p.753-761, 1987.

Recebido para publicação: 20/05/1997
Aprovado para publicação: 23/04/1999