ンが受精卵の発生にとって重要であることが知られている．マウスあるいはウシの凍結乾燥精子においても，この充分なカルシウムオシレーション誘導能が維持されていることが報告された[26]．精子によるカルシウムオシレーションの誘導は，精子と卵子との間の膜融合の際に，卵細胞質内に拡散する精子タンパク因子によって惹起されることから，精子の凍結乾燥，復水および ICSI の一連の過程において，精子タンパク因子の活性が維持されていることを示している．また，染色体解析によって，凍結乾燥精子が，染色体レベルでの遺伝的な健全性を比較的良好に維持していることも示されている[27-31]．したがって，凍結乾燥法は，哺乳動物精子の保存に有効な手段のひとつであるように思われる．また，凍結乾燥時における EDTA や EGTA などのキレート剤の添加[21, 27, 28]や中性あるいは酸性溶液(pH7.4-6.0)よりもややアルカリ性(pH8.0)の溶液の方が[29]，染色体構造の維持に有効に働くことが報告されている．新鮮精子と比較して，凍結乾燥精子の正常な染色体像の頻度は低い(100% vs 61-83%)が[27, 29, 30, 32]，放射線照射に対しては，耐性が高いことが報告されている[30]．精巣から採取した未成熟精子は，精巣上体に貯蔵されている成熟精子と比較して，凍結乾燥によって障害を受けやすく，染色体レベルでの遺伝的な健全性を失う．しかし，ジアミドによってチオールを酸化してジスルフィドにすることによって，抵抗性を獲得する[33]．一方，精巣上体精子をジスルフィド還元剤であるジチオトレイトールで処理することによって，凍結乾燥による障害に対する感受性が亢進する[33]．

環境温度下における凍結乾燥精子の長期保存の可能性

遺伝子資源の保存方法のひとつとして，凍結乾燥精子を利用するためには，数年程度の保存性の保証では全く意味を成さず，数十年あるいは数百年にわたる長期保存の可能性の保証が，重要な課題である．Ward らは，4℃で 18 ヶ月間保存した凍結乾燥精子に由来する産仔を得たことを報告したが[32]，より長期間の保存による受精能力あるいは発生支持能を検証することは困難であった．そこで，筆者らは，医薬品の安定性を速度論的に考察するのに利用されている「アレニウスプロット」を用いた加速試験を応用することによって，凍結乾燥精子の長期保存性の予測を実現化した[34]．加速試験とは，ある温度で長期間保存した物質の化学反応速度を短期間で予測する試験系である．ここでは，マウス凍結乾燥精子に対して 30～50℃の温度を 0～7 日間負荷した後に ICSI を実施し，実測した胚の発生率から得られた分解速度定数をもとに，任意の温度の分解速度定数を算出することで，各温度における凍結乾燥精子の保存期間に対する ICSI 後の胚の発生率を予測した(Table 1)．加速試験の結果，現在汎用されている条件で凍結乾燥した精子を 4℃で 1 年間保存した場合，胚盤胞への発生率は 1%，10 年以上保存すると胚盤胞は得られないとの予測値が得られた．一方，-80℃で凍結乾燥精子を保存した場合には，100 年間保存しても胚盤胞までの発生率の低下は，ほとんどないものと予測された．さらに，実際に 4℃あるいは -80℃で 3 ヶ月間から 2 年間保存した凍結乾燥精子を用いた際の胚の発生率は，予測値とほぼ一致しており[31, 34-36]，アレニウスプロットを用いた加速試験を凍結乾燥精子に応用することの妥当性が示されている．また，種々の条件で保存した凍結乾燥精子の DNA の損傷程度について，コメットアッセイを用いて調べたところ，4℃にて 3 ヶ月間および 6 ヶ月間保存した精子には，DNA 損傷を示すコメットテイルが観察されたが，-80℃で保存した凍結乾燥精子と凍結乾燥を施していない新鮮精子においては，コメットテイルを認めなかった(Fig. 1)．4℃にて，長期間保存した凍結乾燥精子から胚盤胞を得ることが困難な原因のひとつは，残存水分による保存期間中の酸化などに起因する精子 DNA の損傷にあると考

Table 1. Estimated rates of development to the blastocyst stage (%) by extrapolation of the Arrhenius plot

Storage temp. (℃)	Storage term						
	0 mo	1 mo	3 mo	6 mo	1 yr	10 yr	100 yr
25	59.00	1.66	0.00	0.00	0.00	0.00	0.00
4	59.00	42.21	21.60	7.91	1.00	0.00	0.00
-20	59.00	58.19	56.00	54.30	49.86	10.96	0.00
-80	59.00	59.00	59.00	59.00	59.00	59.00	58.99

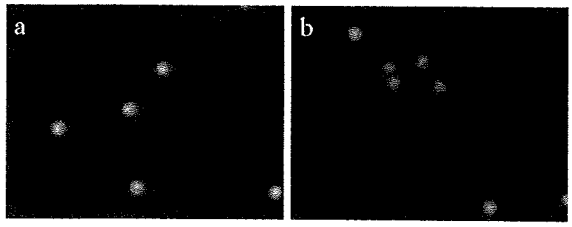

Fig. 1. Comet assay of freeze-dried mouse spermatozoa. Spermatozoa were stored at (a) 4℃, and (b) -80℃ for 6 months each. The presence of comet tails in (a) indicates that fragmented DNA is present in these spermatozoa.

えられるが，その詳細は明らかではない．

凍結乾燥精子の長期保存に向けて

液体窒素を用いた精子の凍結保存の代替として凍結乾燥を利用し，その特性を最大限に利用するという観点からは，比較的高い保存温度で長期間保存可能な凍結乾燥条件を見出すことが必須であり，このことが凍結乾燥精子の実用化のための大きな課題であると思われる．比較的高い温度（例えば室温）で長期間保存可能な凍結乾燥条件を見出すためには，添加物を含む凍結乾燥溶液の組成の改良や凍結乾燥時の真空度の変更などがあげられる．なかでも凍結乾燥時の昇華により大部分の水分を除去する一次乾燥期の真空度は極めて重要な条件の一つであると考えられるが，この条件の検討に関する報告はなされていなかった．これまで，一次乾燥期の真空度は0.03～0.04 mbar (1 bar = 100 kPa)が用いられているが[18, 24, 27-34]，これを約10倍程度緩やかにすることによって，凍結乾燥精子の保存性が向上することが示されている(Tables 2, 3)[35]．筆者らは，一次乾燥期の真空度を0.04 mbar，0.37 mbarおよび1.03 mbarに設定して凍結乾燥した精子を，30℃で3日間あるいは4℃で6ヶ月間保存後，ICSIによって受精させた卵子の胚盤胞への発生率を比較したところ，凍結乾燥直後の精子の場合は0.04 mbarでは59%，0.37 mbarでは71%，および1.03 mbarでは33%であった[35]．また，30℃で3日間保存後の凍結乾燥精子を用いた場合の胚盤胞への発生率は，0.04 mbarでは20%，0.37 mbarでは54%，および1.03 mbarでは19%であった[35]．さらに，4℃で6ヶ月間保存後の発生率については，0.04 mbarでは13%，0.37 mbarでは50%，および1.03 mbarでは36%と(Table 2)，いずれの保存条件においても，一次乾燥期の真空度を0.37 mbarにした場合の発生率が，他の実験区に比べて有意に高い成績であった[35]．発生した胚盤胞の受容雌への移植後の産仔への発生率についても，一次乾燥期の真空度を0.37 mbarとした場合が，統計学的に有意な差異ではないものの，他の実験区と比較して高い傾向が認められた(Table 3)．これらの成績から，4℃で6ヶ月間保存した凍結

Table 2. Effect of vacuum pressure at primary drying on the in vitro development of embryos generated by ICSI of freeze-dried spermatozoa stored at 4℃ for 6 months

Vacuum pressure (mbar)	No. of oocytes injected	No (%) of oocytes survived	No. (%) of oocytes fertilized[a]	No. (%) of embryos developed to 2-cell stage[b]	No. (%) of embryos developed to blastocyst stage[b]
0.04	522	404 (77)[a]	367 (91)[a]	346 (94)[a]	48 (13)[a]
0.37	213	156 (73)[a]	145 (93)[ab]	142 (98)[a]	73 (50)[b]
1.03	267	187 (70)[a]	182 (97)[b]	179 (98)[a]	66 (36)[c]

Different superscript letters within a column indicate significantly different values (P<0.05).
[a]Percentage of oocytes survived. [b]Percentage of oocytes fertilized.

Table 3. Effect of vacuum pressure at primary drying on the in vivo development of embryos generated by ICSI using freeze-dried spermatozoa

Vacuum pressure (mbar)	Storage temperature (℃)	Sperm storage time (months)	No. of blastocysts transferred	No. (%) of implantation sites	No. (%) of live-term fetuses
0.04	RT	Non-stored	194	137 (71)[a]	58 (30)[a]
0.37	RT	Non-stored	132	93 (70)[a]	48 (36)[a]
1.03	RT	Non-stored	99	70 (71)[a]	20 (20)[a]
0.04	4	6	48	28 (58)[a]	4 (8)[a]
0.37	4	6	73	39 (53)[a]	15 (21)[a]
1.03	4	6	66	56 (85)[b]	16 (24)[b]

Values within a column with the same superscript are not significantly different (P>0.05).

Table 4. Fertilization and development of oocytes by ICSI using air-transported (Japan ⇔ Belgium) freeze-dried spermatozoa

Vacuum pressure (mbar)	No. of oocytes injected	No. (%) of oocytes survived	No. (%) of oocytes fertilized[1)]	No. (%) of embryos developed to 2-cell[2)]	No. of 2-cell transferred	No. (%) of implantation sites	No. of live term fetuses
0.04	180	125 (69)[a]	120 (96)[a]	115 (96)[a]	115	7 (6)[a]	1 (1)[a]
0.37	198	145 (73)[a]	141 (97)[a]	134 (95)[a]	134	43 (32)[b]	22 (16)[b]
1.03	180	119 (66)[a]	114 (96)[a]	108 (95)[a]	108	16 (15)[a]	5 (5)[a]

Values within a column with the same superscript are not significantly different (P>0.05)
Air transportation of the freeze-dried spermatozoa consisted of roundtrip jet service between Japan and Belgium as check-in baggage (12,000 miles, 7 days, mean temperature: 18℃, range: 0.5-27℃).
Freeze-dried spermatozoa were stored at -80℃ until use.
1)Percentage of survived oocytes. 2)Percentage of fertilized oocytes.

乾燥精子を用いた ICSI 後のマウスの生産効率（ICSI によって受精した卵子 100 個から得られる産仔数）を算出したところ，0.04 mbar では 1.1，0.37 mbar では 10.3，および 1.03 mbar では 8.8 と，0.37 mbar の生産効率が最も高い成績であった[35]．これらの成績は，一次乾燥期の真空度が，凍結乾燥精子の保存性を左右する重要な因子のひとつであることを明確に示していると考えられる．

おわりに

マウスにおいては，精子の凍結乾燥の技術開発と並行して，遺伝子資源の簡便な保存法・輸送法としての実用性の検証も行われている．Wakayama and Yanagimachi は，3 週間の旅行に凍結乾燥精子を携帯し（この間の環境温度は，5℃～30℃），旅行 1 週間後に ICSI を行い，得られた受精卵を移植した結果，移植胚の 16%の産仔を得たことを報告している[17]．また，筆者らは，一次乾燥期の真空度を 0.04 mbar，0.37 mbar あるいは 1.03 mbar の条件で凍結乾燥したマウス精子を 4℃あるいは-80℃で 2～2.5 年間保存後，陸路(1,740 マイル，5 日間，平均環境温度：22℃，温度幅：17～24℃)あるいは空路(12,000 マイル，7 日間，平均環境温度：18℃，温度幅：0.5～27℃)で輸送を行い，ICSI 後の発生支持能について検討している(Table 4)[36]．一次乾燥期の真空度が 0.04 mbar の場合，4℃で 2 年間保存されて凍結乾燥精子由来の産仔を得ることはできなかったが，-80℃の保存においては，2.5 年を経過しても移植胚の 28%が産仔へ発生することが示された[36]．さらに，輸送によって環境温度に曝露された凍結乾燥精子においては，一次乾燥期の真空度が 0.37 mbar であった場合に，移植胚の 16%が産仔へと発生した(Table 4)．この移植後の発生率は，十分に実用に耐え得る成績であることから，一次乾燥を 0.37 mbar として凍結乾燥を行ったマウス精子を-80℃に保存しておくことによって，環境温度下における数日間の輸送が現実的となったことを示している。

しかしながら，冷蔵庫温度あるいは室温などの，より高温度における長期間の保存に耐え得る条件は見出されておらず，一次・二次乾燥期の真空度や懸濁液組成を含む凍結乾燥過程のより詳細な条件検討が求められるところである．

文　　献

1) Polge, C., Smith, A. U. and Parkes, A. S.: Revival of spermatozoa after vitrification and dehydration at low temperature, Nature, **164,** 666-667 (1949)
2) Sherman, J. K.: Freezing and freeze-drying of human spermatozoa, Fertil. Steril., **5,** 357-371 (1954)
3) Yushchenko, N. P.: Proof of the possibility of the spermatozoa in dried state, Proc. Lenin. Acad. Agr. Sci., **22,** 37-40 (1957)
4) Leidl, W.: Experiments in freeze-drying of bull semen, In Proc. 3rd Congr. On Animal Production, Vol. 3, p. 39-41, Cambridge (1956)
5) Bialy, G. and Smith, V. R.: Freeze-drying of bull spermatozoa, J. Dairy Sci., **40,** 739-745 (1957)
6) Sherman, J.K.: Freezing and freeze-drying of bull spermatozoa, Am. J. Physiol., **190,** 281-286 (1957)
7) Meryman, H.T. and Kafig, E.: Survival of spermatozoa following drying, Nature, **184,** 470-471 (1959)
8) Meryman, H.T.: Drying of living mammalian cells, Ann. N. Y. Acad. Sci., **85,** 729-739 (1960)

9) Albright, J.L., Erb, R. and Ehlers, M.H.: Freeze-drying bovine spermatozoa, J. Dairy Sci., **41**, 206 (1958)

10) Singh, S.G. and Roy, D.J.: Freeze-drying of bovine semen, Indian J. Vet. Sci., **37**, 1-7 (1967)

11) Larson, E.V. and Graham, E.F.: Freeze-drying of spermatozoa. p.343-348. in International symposium of freezing biological products, Vol 36. Cabasso, V.J. and Regamey R.H. (eds.), Karger, S., Basel, Switzerland. (1977)

12) Saacke, R.G. and Almquist, J.O.: Freeze-drying of bovine spermatozoa, Nature, **192**, 995-996 (1961)

13) Nei, T. and Nagase, H.: Attempt to freeze-dry bull spermatozoa, Low Temp. Sci. Ser. B, **19**, 107-115 (1961)

14) Meryman, H.T. and Kafig, E.: Freeze-drying bovine spermatozoa, J. Reprod. Fertil. **5**, 87-94 (1963)

15) Jeyendran, R.S., Graham, E.F. and Schmehl, M.K.L.: Fertility of dehydrated bull semen, Cryobiology, **18**, 292-300 (1981)

16) Jeyendran, R.S., Hunter, A.G. and Graham, E.F.: Alteration of seminal proteins during freeze-drying of bovine semen, J. Dairy Sci. **66**, 887-891 (1983)

17) Wakayama, T. and Yanagimachi, R.: Development of normal mice from oocytes injected with freeze-dried spermatozoa, Nat. Biotechnol., **16**, 639-641 (1998)

18) Liu, J.L., Kusakabe, H., Chang, C.C., Suzuki, H., Schmidt, D.W., Julian, M., Pfeffer, R., Bormann, C.L., Tian, X.C., Yanagimachi, R. and Yang, X.: Freeze-dried sperm fertilization leads to full-term development in rabbits, Biol. Reprod., **70**, 1776-1781 (2004)

19) Kimura, Y. and Yanagimachi, R.: Intracytoplasmic sperm injection in the mouse, Biol. Reprod., **52**, 709-720 (1995)

20) Hirabayashi, M., Kato, M., Ito, J. and Hochi, S.: Viable rat offspring derived from oocytes intracytoplasmically injected with freeze-dried sperm heads, Zygote, **13**, 79-85 (2005)

21) Nakai, M., Kashiwazaki, N., Takizawa, A., Maedomari, N., Ozawa, M., Noguchi, J., Kaneko, H., Shino, M. and Kikuchi, K.: Effect of chelating agents during freeze-drying of boar spermatozoa on DNA fragmentation and on developmental ability in vitro and in vivo after intracytoplasmic sperm head injection, Zygote, **15**, 15-24 (2007)

22) Keskintepe, L., Hassan, A., Khan, I. Stice, S.L.: Bovine embryo development after lyophilized sperm injection, Theriogenology, **55**, 505 (2001)

23) Keskintepe, L., Pacholczyk, G., Machnicka, A., Norris, K., Curuk, M.A., Khan, I. and Brackette, B.G.: Bovine blastocyst development from oocytes injected with freeze-dried spermatozoa, Biol. Reprod., **67**, 409-415 (2002)

24) Kwon, I. K., Park, K.E. and Niwa, K.: Activation, pronuclear formation, and development *in vitro* of pig oocytes following intracytoplasmic injection of freeze-dried spermatozoa, Biol. Reprod., **71**, 1430-1436 (2004)

25) Hoshi, K., Yanagida, K., Katayose, H. and Yazawa, H.: Pronuclear formation and cleavage of mammalian eggs after microsurgical injection of freeze-dried sperm nuclei, Zygote, **2**, 237-242 (1994)

26) Liu, Q.C., Chen, T.E., Huang, X,Y. and Sun, F.Z.: Mammalian freeze-dried sperm can maintain their calcium oscillation-inducing ability when microinjected into mouse eggs, Biochem. Biophys. Res. Commun., **328**, 824-830 (2005)

27) Kusakabe, H., Szczygiel, M.A., Whittingham, D.G. and Yanagimachi, R.: Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa, Proc. Natl. Acad. Sci. USA, **98**, 13501-13506 (2001)

28) Kaneko, T. and Nakagata, N.: Improvement in the long-term stability of freeze-dried mouse spermatozoa by adding of a chelating agent, Cryobiology, **53**, 279-282 (2006)

29) Kaneko, T., Whittingham, D.G. and Yanagimachi, R.: Effect of pH value of freeze-drying solution on the chromosome integrity and developmental ability of mouse spermatozoa, Biol. Reprod., **68**, 136-139 (2003)

30) Kusakabe, H. and Kamiguchi, Y.: Chromosomal integrity of freeze-dried mouse

spermatozoa after ^{137}Cs γ -ray irradiation, Mutat. Res., **556**, 163-168 (2004)

31) Kaneko, T. and Nakagata, N.: Relation between storage temperature and fertilizing ability of freeze-dried mouse spermatozoa, Comparative Med., **55**, 140-144 (2005)

32) Ward, M.A., Kaneko, T., Kusakabe, H., Biggers, J.D., Whittingham, D.G. and Yanagimachi, R.: Long-term preservation of mouse spermatozoa after freeze-drying and freezing without cryoprotection, Biol. Reprod., **69**, 2100-2108 (2003)

33) Kaneko, T., Whittingham, D., Overstreet, J.W. and Yanagimachi, R.: Tolerance of the mouse sperm nuclei to freeze-drying depends on their disulfide status, Biol. Reprod., **69**, 1859-1862 (2003)

34) Kawase, Y., Araya, H., Kamada, N., Jishage, K. and Suzuki, H.: Possibility of long-term preservation of freeze-dried mouse spermatozoa, Biol. Reprod., **72**, 568-573 (2005)

35) Kawase, Y., Hani, T., Kamada, N., Jishage, K. and Suzuki, H.: Effect of pressure at primary drying of freeze-drying mouse sperm reproduction ability and preservation potential, Reproduction, **133**, 841-846 (2007)

36) Kawase, Y., Tachibe, T., Jishage, K. and Suzuki, H.: Transportation of freeze-dried mouse spermatozoa under different preservation conditions, J. Reprod. Dev. **53**, 1169-1174 (2007)

Journal of Reproduction and Development, Vol. 54, No. 2, 2008

—Research Note—

Preimplantation Development of Embryos in Labrador Retrievers

Yasuyuki ABE[1], Yoshinori SUWA[2], Yoshiko YANAGIMOTO-UETA[1] and Hiroshi SUZUKI[1,3]

[1]Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, [2]Hokkaido Guide Dog Association, Sapporo 005-0030 and [3]Department of Developmental and Medical Technology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

Abstract. Preimplantation development of canine embryos is not well understood. To understand the timing of preattachment embryogenesis relative to the luteinizing hormone (LH) surge, early embryonic development was examined in Labrador Retrievers after artificial insemination. The embryos migrated from the oviduct to the uterus beginning on day 11 after the LH surge. This transport must be completed within 24 h. By day 13 after the LH surge, all of the embryos had moved and were localized in the uterus. The embryos developed to the morula stage within 11–13 days and to the blastocyst stage within 14 days after the LH surge, respectively. These findings add to the current understanding concerning the physiology of preimplantation development and should help further develop assisted reproductive techniques in canine species, such as cryopreservation and subsequent embryo transfer.
Key words: Dog, Embryo, Preimplantation development

(J. Reprod. Dev. 54: 135–137, 2008)

The dog is a monoestral polyovulatory nonseasonal species. Canine reproductive physiology is considerably different from other mammalian species. The plasma progesterone concentration of the dog begins to increase a few days before ovulation. Preovulatory luteinization is typical in dogs. The oocytes of dogs are ovulated at the germinal vesicle stage (an immature diploid stage), and they complete meiotic maturation in the oviduct. Thus, canine oocytes and embryos spend a long time prior to implantation in the reproductive tract. Due to these singular reproductive features, the actual situation and mechanisms of early development, such as oocyte maturation, fertilization and subsequent embryogenesis, have not been fully elucidated for canine species compared with many other domestic mammalian species [1].

On the other hand, large numbers of canines are produced and used as working dogs as well as companion animals in many parts of the world. It seems likely that application of assisted reproductive techniques, such as *in vitro* fertilization, embryo transfer, artificial insemination and cryopreservation of gametes and zygotes, would be useful for improved breeding of working dogs, such as guide dogs for the blind, as has proven to be the case in other domestic animals. However, development of assisted reproductive techniques for canines has been poor, with the exception of artificial insemination [2]. In order to develop methods of transfer and cryopreservation of embryos, it is essential to understand early embryonic development *in vivo* in all mammals. However, very little information has been reported regarding the preimplantation development of embryos after mating in dogs. It is believed that fertilized eggs develop to the 2-cell stage 6–10 days after the luteinizing hormone (LH) surge and that they migrate to the uterus 11–12 days after the LH surge in domestic dogs [3, 4]. However, the timing of preattachment embryogenesis relative to ovulation has yet to be determined [5]. Thus, to obtain information regarding early embryonic development in the Labrador Retriever, one of the most utilized working dog breeds in such roles as guide dogs for the blind, embryos were recovered from excised reproductive tracts after artificial insemination, and then the stages and localizations of the embryos were determined.

Accepted for publication: November 28, 2007
Published online: January 17, 2008
Correspondence: H. Suzuki (e-mail: hisuzuki@obihiro.ac.jp)

Materials and Methods

Both male and female Labrador Retrievers from a breeding colony for guide dogs for the blind were used in the present study. The animals were basically maintained by volunteers in their homes and were moved to the Hokkaido Guide Dog Association when the bitches exhibited signs of estrus. The embryo donors were 13 nulliparous animals that were 9–18 months of age and one multiparous animal that was 8 years of age (Table 1). The plasma progesterone concentrations of the bitches were measured daily by enzyme-linked fluorescent assay (SV-5010; Spotchem Vidas, Arkray, Kyoto, Japan) after the appearance of blood-tinged vaginal discharge. The day the plasma progesterone concentration exceeded 2 ng/ml was considered the day of the LH surge (day 0) [2]. A total of 14 bitches were inseminated by injecting freshly ejaculated semen into the vagina on day 4–6 after the estimated LH surge. The semen was collected from the dogs by digital manipulation. On day 9–15 after the LH surge (day 4–10 after insemination), the reproductive tracts of the bitches were excised after ovariohysterectomy at private clinics. The embryos were flushed from the oviduct, isthmus and uterus with phosphate buffered solution. The developmental stages of the collected embryos were determined under an inverted microscope. The number of ovulated oocytes was estimated by counting the number of corpora lutea present in both ovaries.

The animals used in this study were treated and cared for under the Guiding Principles for the Care and Use of Research Animals

Table 1. Developmental stages of embryos in Labrador Retrievers after artificial insemination

Bitch	Age (M)	Multipara or nullipara	Days post LH surge	Days at AI post LH surge	Days after AI	No. (%) embryos collected/ no. CL	Developmental stage							
							UF	4C	8C	16C	M	ExB	LEB	F
WIN	16	N	9	5	4	5/8 (63)	0	0	5	0	0	0	0	0
ROS	18	N	10	6	4	7/7 (100)	1	0	6	0	0	0	0	0
KOK	16	N	10	4	6	5/8 (63)	1	0	0	4	0	0	0	0
KOH	17	N	11	5	6	7/7 (100)	0	3	1	0	1	0	0	2
HIN	14	N	11	4	7	4/5 (80)	0	0	0	4	0	0	0	0
HAR	10	N	11	4	7	7/8 (88)	0	0	0	6	1	0	0	0
ELU	10	N	12	6	6	9/9 (100)	0	0	0	8	1	0	0	0
EMI	9	N	12	5	7	4/7 (57)	0	0	0	4	0	0	0	0
PET	13	N	13	4	9	4/5 (80)	0	0	0	0	4	0	0	0
PEZ	11	N	14	6	8	8/8 (100)	0	0	0	3	1	0	0	4
DYI	10	N	14	5	9	1/7 (14)	0	0	0	0	0	0	0	1
BET	107	M	14	4	10	5/5 (100)	0	0	0	0	0	5	0	0
JIN	15	N	14	4	10	7/7 (100)	0	0	0	0	0	0	7	0
BIA	9	N	15	5	10	7/10 (70)	0	0	0	0	0	0	7	0
Total			–	–	–	80/101 (79)	2	3	12	29	8	5	14	7

Embryos were collected surgically after non-surgical flushing of the reproductive tract. UF: Unfertilized egg. 4C: 4-cell stage. 8C: 8-cell stage. 16C: 16-cell stage. M: Morula. ExB: Expanded blastocyst. LEB: Large expanded blastocyst. F: Fragmented.

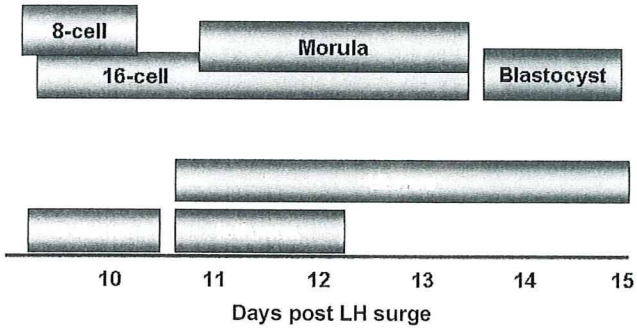

Fig. 1. Preimplantation development and localization of embryos in the Labrador Retrievers. The day the plasma concentration of progesterone exceeded 2 ng/ml was considered to be the day of the LH surge (day 0). The embryos developed to the morula and blastocyst stages by 11–13 and 14 days after the LH surge, respectively. The embryos began to migrate from the oviduct to the uterus on day 11 after the LH surge. By day 13 after the LH surge, all of the embryos had moved and were localized in the uterus.

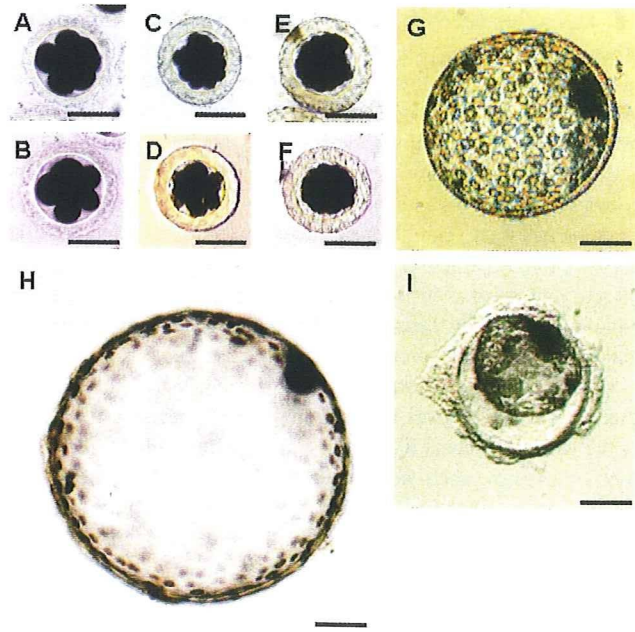

Fig. 2. Preimplantation stages of embryos recovered from the female reproductive tract after artificial insemination in the Labrador Retriever. A, B: 8-cell stage. C, D: 16-cell stage. E, F: Morula. G: Expanded blastocyst. H: Large expanded blastocyst. I: Shrunken blastocyst. Bars indicate 100 μm.

established by Obihiro University of Agriculture and Veterinary Medicine.

Results and Discussion

To be able to utilize assisted reproductive technologies in research and clinical practice, it is essential to be able to determine the timing of ovulation. Although the onset of vulvar bleeding, vaginal smears and mating behavior have been used to assess the timing of ovulation, their relative accuracy has proven to be highly variable. However, ovulation is much more closely related to the LH surge and progesterone concentration. There is little variation in the 2-day interval between the preovulatory LH surge and ovulation (36–50 h) [1]. The blood progesterone concentration at ovulation is also relatively stable and is thus extensively used to estimate ovulation time [1]. Since measurement of the blood LH concentration is difficult to routinely carry out (i.e., it is time-consuming), we utilized measurement of the progesterone

Table 2. Localization of embryos in the reproductive tract of Labrador Retrievers after artificial insemination

Bitch	Days post LH surge	Days after AI	No. embryos collected	Localization of embryo		
				Oviduct	Isthmus	Uterus
WIN	9	4	5	5	0	0
ROS	10	4	7	7	0	0
KOK	10	6	5	5	0	0
KOH	11	6	7	0	7	0
HIN	11	7	4	0	3	1
HAR	11	7	7	2	1	4
ELU	12	6	9	0	0	9
EMI	12	7	4	0	0	4
PET	13	9	4	0	0	4
PEZ	14	8	8	0	0	8
DYI	14	9	1	0	0	1
BET	14	10	5	0	0	5
JIN	14	10	7	0	0	7
BIA	15	10	7	0	0	7
Total	–	–	80	19	11	50

Embryos were collected surgically after non-surgical flushing of the reproductive tract.

concentration to predict the LH surge in the present study.

Table 1 shows the developmental stages of the embryos from the Labrador Retrievers after artificial insemination with freshly collected semen. A total of 80 embryos were collected from 14 bitches that possessed a total of 101 corpus lutea (recovery rate: 79.2%). The recovery rate was 100% in 6 of the 14 animals (44%). The mean ovulation rate of the Labrador Retrievers used in the present study was 7.2 (n=14). The blastocyst stage of the embryos did not appear within 13 days after the LH surge. Beginning 14 days after the LH surge, blastosysts were recovered from the uterus (Fig. 1). In a previous study using Beagles, blastocyst stage embryos were recovered on days 9 and 10 after the LH surge [6]. These results indicate that there is a considerable breed-related difference in the preimplantation development of embryos in canine species. In the present study, a majority of the expanded blastosysts were found to be shrunken at the time of collection or during washing of the embryos (Fig. 2-I). However, shrinkage of the blastocyst stage embryos was not observed during recovery.

Table 2 shows the localization of the embryos in the reproductive tract of the Labrador Retrievers after artificial insemination with freshly collected semen. Embryos that developed into morula migrated from the oviduct to the uterus beginning on day 11 after the LH surge. By day 13 after the LH surge, all of the embryos had moved to and were localized in the uterus. Although the embryonic stages of the collected embryos were synchronized in a majority of the bitches, the fact that the collected embryos from one particular bitch (KOH) exhibited a wide range of developmental stages, from the 4-cell stage to the morula stage, might indicate that ovulation can extend over a long period of time in some animals (Table 1). It is generally believed that ovulation from both ovaries is completed in a maximum of 24 to 36 h [1]. When freshly ejaculated semen was inseminated 4–6 days after the estimated LH surge, morula- and blastocyst-stage embryos (Fig. 2), which appear to be suitable for cryopreseravation and embryo transfer, might be collectable from the uterine horns of Labrador Retrievers on days 13 and 14, respectively (Fig. 1).

In conclusion, as shown in Fig. 1, the present study demonstrates that embryos migrate from the oviduct to the uterus beginning on day 11 after the LH surge in the Labrador Retrievers. This transport might be completed within 24 h. The embryos developed to the morula and to blastocyst stages by 11–13 and 14 days after the LH surge, respectively. Fundamental research is still required to understand the mechanisms of fertilization and early embryonic development in order to better control and mimic them *in vitro* in canines.

Acknowledgement

This study was supported by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan and funds from the Ministry of Health, Labour and Welfare of Japan.

References

1. **Reynaud K, Fontbonne A, Marseloo N, de Lesegno CV, Saint-Dizier M, Chastant-Maillard S.** *In vivo* canine oocyte maturation, fertilization and early embryogenesis: A review. *Theriogenology* 2006; 66: 1685–1693.
2. **Johnston SV, Root Kustritz MV, Olson PNS.** Breeding management and artificial insemination of the bitch. *In*: Johnston SV, Root Kustritz MV, Olson PNS (eds.), Canine and Feline Theriogenology. Philadelphia: WB Saunders Company; 2001: 41–65.
3. **Concannon PW, Lein DH.** Hormonal and clinical correlates of ovarian cycles, ovulation, pseudopregnancy and pregnancy in dogs. *In*: Kirk R (ed.) Current Veterinary Therapy (Small Animal Practice), vol. 10. Philadelphia: WB Saunders Company; 1989: 1269–1282.
4. **Johnston SV, Root Kustritz MV, Olson PNS.** Canine pregnancy. *In*: Johnston SV, Root Kustritz MV, Olson PNS (eds.), Canine and Feline Theriogenology. Philadelphia: WB Saunders Company; 2001: 66–104.
5. **Senger PL.** Early Embryogenesis and Maternal Recognition of Pregnancy. *In*: Senger PL (ed.) Pathways to Pregnancy and Parturition, 2 ed. Pullman: Current Conceptions Inc; 2003: 284–303.
6. **Tsutsui T, Hori T, Okazaki H, Tanaka A, Shiono M, Yokosuka M, Kawakami E.** Transfer of canine embryos at various developmental stages recovered by hysterectomy or surgical uterine flushing. *J Vet Med Sci* 2001; 63: 401–405.

J. Mamm. Ova Res. Vol. 25, 32–36, 2008

Vitrification of Canine Oocytes

Yasuyuki Abe[1], Dong-Soo Lee[1],
Sang-Keun Kim[2] and Hiroshi Suzuki[1, 3]*

[1]Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[2]College of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon 305-764, Korea
[3]Department of Developmental and Medical Technology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

Abstract: *The objective of the present study was to compare the vitrification method for cryopreservation of canine oocytes. Canine cumulus-oocyte complexes (COCs) were collected from ovaries, and were vitrified by ethylene glycol based (E30S) or DMSO based (DAP213) methods. In the E30S method, COCs were exposed to the vitrification solution, composed of 30% ethylene glycol and 0.5 M sucrose, step-wise transferred onto a cryotop holder, then plunged directly into liquid nitrogen. In the DAP213 method, COCs were exposed to 1 M DMSO and DAP213 solution in a cryotube, and thereafter plunged directly into liquid nitrogen. Although vitrified-warmed COCs in the E30S method showed fewer morphological abnormalities, and higher viability than those in the DAP213 method, there was no significant difference in between. These results indicate that either method of vitrification is available and statistically comparable for cryopreservation of canine oocytes.*

Key words: *Dog, Oocyte, Vitrification, Cryopreservation*

Introduction

Assisted reproductive techniques (ART) of canine species such as *in vitro* maturation (IVM), culture and cryopreservation of the genetic resource materials have limited application *per se*, when compared to those for other experimental and domestic animals. However, they can be useful for improved breeding of companion and working dogs, including guide dogs for the blind. Although guide dogs remarkably contribute to the improvement of the quality of life of blind people in the world, many countries suffer from an acute shortage of guide dogs. Even among Labrador Retrievers, which are particularly suited to the role, only 30–40% of the dogs that are trained become guide dogs in Japan. Current figures indicate that approximately 950 dogs are actively engaged in guiding blind people, however, this number is low in light of the estimated demand, which ranges between 4,800–7,800, including latent needs in Japan. ART would help make it possible to overcome one of the problems. Although there are some reports on IVM of oocytes and culture of embryos in [1, 2], no attempt has been made to cryopreserve canine oocytes and embryos, and then perform embryo transfer (ET). Vitrification has been widely developed to apply to cryopreservation of mammalian embryos. In the mouse, embryo cryopreservation by a vitrification method utilizing a sampling tube with DAP213 solution (2 M dimethyl sulfoxide, 1 M acetamide, and 3 M propylene glycol) as a vitrification solution (DAP213 method) has been proven successful [3]. Moreover, it is possible to vitrify canine ovarian tissues by the DAP213 method [4]. Porcine oocytes were vitrified successfully using a cryotop sheet following exposure to vitrification solution by the step-wise method (E30S method) [5]. However, the suitability of both vitrification methods for canine embryos has not been investigated.

The objective of the present study was to compare the DAP213 with E30S methods for vitrification of canine germinal vesicle (GV) stage oocytes, to improve the breeding management programs for guide dogs for the blind.

Received: October 4, 2007
Accepted: December 21, 2007
*To whom correspondence should be addressed.
e-mail: hisuzuki@obihiro.ac.jp

Materials and Methods

Collection of cumulus oocyte complexes (COCs)

Ovaries within the ovarian bursa from bitches of mixed breed at random stages of the estrous cycle were collected at slaughterhouses and transported to the laboratory in a thermos flask containing sterile saline at approximately 37°C. Each ovary was cleaned of fat and blood vessels and placed in a Petri dish containing TCM199 medium (Gibco-Invitrogen Life Technologies, NY, USA) supplemented with 10% fetal calf serum, 100 units/ml penicillin G potassium (Meiji, Tokyo, Japan) and 100 μg/ml streptomycin sulfate (Meiji, Tokyo, Japan), for further dissection. Ovarian tissue was sliced by a surgical blade (Feather, Osaka, Japan) repeatedly to collect COCs. Only COCs with more than two layers of cumulus cells and a homogeneous dark cytoplasm ≥100 μm in diameter were used in this study. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO USA) except for those specifically described.

The tissues and cells derived from animals used in this study were treated under the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine.

Vitrification and Thawing

1) DAP213 method

The COCs were pretreated with PB1 medium [6] containing 1 M dimethyl sulfoxide (DMSO) at room temperature (23 ± 2°C). The COCs were transferred into a 1 ml cryotube (Nalge Nunc International, Tokyo, Japan) containing 5 μl of 1 M DMSO, which was then placed in ice water for 5 min to allow DMSO to thoroughly bathe the COCs. Subsequently, 95 μl of DAP 213 solution (2 M DMSO, 1 M acetamide, and 3 M propylene glycol) [3], maintained at 0°C, were added to each cryotube. After the cryotubes had been placed in ice water for 5 min, they were plunged directly into liquid nitrogen and stored until use. For thawing, the samples were taken from the liquid nitrogen and allowed to stand at room temperature (23 ± 2°C) for 60 sec, and then diluted with 900 μl of PB1 medium (37°C) containing 0.25 M sucrose. The recovered COCs were transferred to PB1 medium and washed 5 times.

2) E30S method

The COCs were exposed to PB1 containing 5, 10 and 20% ethylene glycol (EG), and 30% EG containing 0.5 M sucrose for 5, 2, 2 and 1 min, respectively, at room temperature (23 ± 2°C) [5]. They were then placed on a cryotop sheet (Kitazato Supplies, Japan) [7], and the cryotop was immediately plunged into liquid nitrogen. The COCs were warmed at 37°C, and the cryoprotectants were removed in a step-wise manner at 37°C: the cryotop holder was transferred from LN_2 into PB1 with a sequential series of 0.5, 0.25, and 0.125 M sucrose, 1 min in each solution at 37°C, and finally transferred into PB1 for 5 min at 37°C.

Examination of vitrified-warmed GV oocytes

After thawing, the oocytes were denuded of cumulus cells in PB1 using a fine-bore pipette by repeated aspiration and expulsion. The cumulus-free oocytes were stained with 20 μg/ml propidium iodide (PI) in phosphate buffered saline containing 0.1% polyvinyl alcohol and incubated for 15 min in darkness. The oocytes were examined under ultraviolet light using an epifluorescence microscope (Nikon, Japan) and plasma membrane integrity of oocytes was assessed. The oocytes with disrupted plasma membrane were dyed red with PI.

Statistical analysis

Data on oocyte survival were compared using the chi-square test as presented by StatView software (Abacus Concepts, Inc., Berkeley, CA, USA). Differences were considered significant at a level of $P<0.05$.

Results

Morphology of fresh COCs and vitrified-warmed COCs by the DAP213 and E30S methods are shown in Fig. 1. In both the vitrification methods, some vitrified-warmed COCs had partly dispersed cumulus cells and disrupted cytoplasm, however, the majority of vitrified-warmed COCs were morphologically intact. The recovery rates of COCs in the DAP213 and E30S groups were 97.5 and 92.7%, respectively (Table 1). In both the vitrification methods, about 60% of the vitrified-warmed oocytes showed normal morphology. However, there was a tendency for the viability of oocytes, as assessed by PI stain, in the step-wise group vitrified with E30S to be higher than those in the DAP213 group (17.6% and 5.1%, respectively, $P>0.05$) (Table 1 and Fig. 2).

Discussion

Although there were no significant differences in the recovery rates and morphological normality of the denuded canine oocytes between the E30S and DAP213 methods (Fig. 1 and Table 1), the percentage

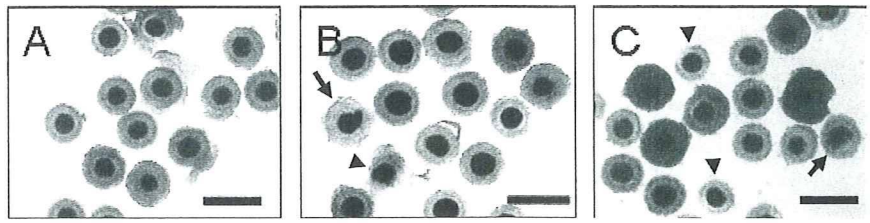

Fig. 1. Morphological figures of the cumulus oocyte complexes (COCs). (A) Fresh; (B) vitrified-warmed by the DAP213 method; (C) vitrified-warmed by the E30S method. Some vitrified-warmed COCs had partly dispersed cumulus cells (arrow head) and disrupted cytoplasm (arrow). Bar = 200 μm.

Table 1. Viability of canine GV oocytes after vitrification

Method	No. of vitrified oocytes	No. of oocytes examined (%)*	No. (%) of normal oocytes**	
			Morphology	PI stain
Fresh	–	20	20 (100)[a]	19 (95.0)[a]
E30S	55	51 (92.7)	30 (58.8)[b]	9 (17.6)[b]
DAP213	40	39 (97.5)	24 (61.5)[b]	2 (5.1)[b]

*The percentages of examined oocytes of vitrified oocytes. **The percentages of normal oocytes of oocytes that were examined for morphology or by PI stain. Values with different superscripts in the same column are significantly different at $P<0.05$.

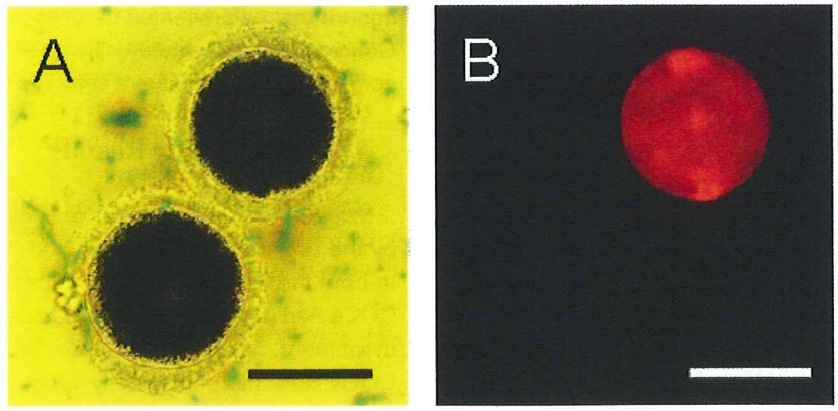

Fig. 2. Morphological appearance of canine oocytes after vitrification by the E30S method. Both oocytes showed morphologically normal appearance under light microscopy (A); however, PI stain revealed that one of the oocytes (upper) was damaged (B). Bar = 100 μm.

of oocytes with integral plasma membrane as measured by PI stain in the E30S group was higher than that in the DAP213 group ($P>0.05$). These results suggest that the integrity of oocytes after cryopreservation can not be evaluated by morphology alone, and it may be necessary to make histochemical examinations such as with PI stain. Further study is required to examine the potential of maturation and subsequent fertilization in vitrified canine oocytes. Canine reproductive physiology is considerably different from other mammalian species. For example, oocytes are ovulated at the germinal vesicle stage (an immature diploid stage) and complete their meiotic maturation in the oviduct. Thus, canine oocytes and embryos spend a long time prior to implantation in the reproductive tract. Due to these singular reproductive features, the actual situation and mechanisms of early development such as oocyte maturation, fertilization and subsequent

embryogenesis are not fully understood in canine species, as compared to many other domestic mammalian species. Thus, the efficiency of in vitro maturation of canine oocytes remains very low compared with that of other domestic animals.

Because vitrification is a non-equilibrium cryopreservation method that needs relatively high concentrations of cryoprotectants, a step-wise addition of cryoprotectants may reduce the toxic effect of cryoprotectants and is considered to minimize damage due to extreme cell-volume expansion [8]. In fact, for vitrification of bovine GV oocytes, three-step exposure to cryoprotectants showed less damage than the single-step procedure [9]. Aono *et al.* [10, 11] reported high survival rates and subsequent production of blastocysts of mouse GV oocytes after vitrification by a step-wise manner with permeable cryoprotectants for treatment of oocytes. They suggested that osmotic injury to cells occurring in the GV oocytes is due to the osmotic stress accompanying treatment with permeable cryoprotectants. On the other hand, the injury to the cells in the process of cryopreservation can be due to osmotic effects accompanying treatment with permeable cryoprotectants [12, 13]. Fuku *et al.* [14] and Kasai *et al.* [15] proposed that the supplementation of saccharides such as sucrose into the vitrification medium would reduce toxicity to the embryos by reducing the extracellular concentration of the cryoprotectant. In our present study, the DAP213 and E30S methods were used with two and four-step exposures to vitrification solutions, respectively. Moreover, sucrose was used as the cryoprotectant only in the E30S method. Thus, it may have been the cause of the difference in membrane integrity between the vitrification methods.

High cooling and warming rates are required to prevent ice crystal formation and reduce severe chilling injury when oocytes are cryopreserved by vitrification. In efforts to increase cooling and warming rates during vitrification, modification of the methods has taken place, especially the development of various containers, such as open-pulled straws [16], cryoloops [17] and cryotops [18]. In the present study, the E30S method using the cryotop required very small volumes of oocyte suspension liquid (less than 1 μl), while the oocytes were suspended in 100 μl of vitrification solution in the DAP213 method. Minimizing the volume of the solution in which oocytes are vitrified might result in higher viability of oocytes after vitrification. Moreover, the lower temperature of the vitrification solution may have decreased the viability of the oocytes in the DAP213 group, since oocytes were treated with vitrification solution at room temperature in the E30S method, and oocytes were kept at 0°C for 10 min in the DAP213 method. However, both methods of vitrification were statistically comparable for morphology for the cryopreservation of canine oocytes.

This study also showed that vitrified-warmed oocytes in both methods were damaged and had decreased integrity of the plasma membrane. The sensitivity of canine oocytes to cryopreservation may be related to their high lipid content and they may become tolerant to cryopreservation if their lipid content is reduced. In porcine, the high sensitivity of oocytes and embryos to cryopreservation has been ascribed to their high cytoplasmic lipid content, and the removal of cytoplasmic lipid droplets improved the survival of porcine oocytes and embryos [19, 20]. Thus, further study seems to be required to develop an optimal cryopreservation method for canine oocytes.

Acknowledgements

This study was supported by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Funds from the Ministry of Health, Labour and Welfare of Japan.

References

1) Rodrigues, Bde A., dos Santos, L.C. and Rodrigues, J.L. (2007): Effect of maturation medium on in vitro cleavage of canine oocytes fertilized with fresh and cooled homologous semen. Zygote, 15, 43–53.
2) Otoi, T., Shin, T., Kraemer, D.C. and Westhusin, M.E. (2004): Influence of maturation culture period on the development of canine oocytes after in vitro maturation and fertilization. Reprod. Nutr. Dev., 44, 631–637.
3) Nakagata, N. (1993): Survival of mouse morulae and blastocysts derived from in vitro fertilization after ultra rapid freezing. Jikken Dobutsu, 42, 229–231.
4) Ishijima, T., Kobayashi, Y., Lee, D.S., Ueta, Y.Y., Matsui, M., Lee, J.Y., Suwa, Y., Miyahara, K. and Suzuki, H. (2006): Cryopreservation of canine ovaries by vitrification. J. Reprod. Dev., 52, 293–299.
5) Fujihira, T., Kishida, R. and Fukui, Y. (2004): Developmental capacity of vitrified immature porcine oocytes following ICSI: effects of cytochalasin B and cryoprotectants. Cryobiology, 49, 286–290.
6) Whittingham, D.G. (1974): Embryo banks in the future of developmental genetics. Genetics, 78, 395–402.
7) Katayama, K.P., Stehlik, J., Kuwayama, M., Kato, O. and Stehlik, E. (2003): High survival rate of vitrified human oocytes results in clinical pregnancy. Fertil. Steril. 80, 223–224.

8) Rall, W.F. (1987): Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology, 24, 387–402.
9) Abe, Y., Hara, K., Matsumoto, H., Kobayashi, J., Sasada, H., Ekwall, H., Rodriguez-Martinez, H. and Sato, E. (2005): Feasibility of a nylon-mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. Biol. Reprod., 72, 1416–1420.
10) Aono, N., Naganuma, T., Abe, Y., Hara, K., Sasada, H., Sato, E. and Yoshida, H. (2003): Successful production of blastocysts following ultrarapid vitrification with step-wise equilibriation of germinal vesicle-stage mouse oocytes. J. Reprod. Dev., 49, 501–506.
11) Aono, N., Abe, Y., Hara, K., Sasada, H., Sato, E. and Yoshida, H. (2005): Production of live offspring from mouse germinal vesicle-stage oocytes vitrified by a modified stepwise method, SWEID. Fertil. Steril., 84 (Suppl. 2), 1078–1082.
12) Kuwayama, M., Fujikawa, S. and Nagai, T. (1994): Ultrastructure of IVM-IVF bovine blastocysts vitrified after equilibration in glycerol 1,2-propanediol using 2-step and 16-step procedures. Cryobiology, 31, 415–422.
13) Mtango, N.R., Varisanga, M.D., Dong, Y.J., Otoi, T. and Suzuki, T. (2001): The effect of prefreezing the diluent portion of the straw in a step-wise vitrification process using ethylene glycol and polyvinylpyrrolidone to preserve bovine blastocysts. Cryobiology, 42, 135–138.
14) Fuku, E.J., Liu, J. and Downey, B.R. (1995): In vitro viability and ultrastructural changes in bovine oocytes treated with a vitrification solution. Mol. Reprod. Dev., 40, 177–185.
15) Kasai, M., Komi, J.H., Takakamo, A., Tsudera, H., Sakurai, T. and Machida, T. (1990): A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. Mol. Biotech., 89, 91–97.
16) Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobsen, H., Greve, T. and Callesen, H. (1998): Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev., 51, 53–58.
17) Lane, M., Bavister, B.D., Lyons, E.A. and Forest, K.T. (1999): Containerless vitrification of mammalian oocytes and embryos. Nat. Biotechnol., 17, 1234–1236.
18) Hochi, S., Terao, T., Kamei, M., Kato, M., Hirabayashi, M. and Hirao, M. (2004): Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. Theriogenology, 61, 267–275.
19) Ushijima, H., Yoshioka, H., Esaki, R., Takahashi, K., Kuwayama, M., Nakane, T. and Nagashima, H. (2004): Improved survival of vitrified in vivo-derived porcine embryos. J. Reprod. Dev., 50, 481–486.
20) Hara, K., Abe, Y., Kumada, N., Aono, N., Kobayashi, J., Matsumoto, H., Sasada, H. and Sato, E. (2005): Extrusion and removal of lipid from the cytoplasm of porcine oocytes at the germinal vesicle stage: centrifugation under hypertonic conditions influences vitrification. Cryobiology, 50, 216–222.

Microbial Pathogenesis 45 (2008) 105–109

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Expression of heme oxygenase-1 is associated with abortion caused by *Brucella abortus* infection in pregnant mice

Masato Tachibana [a], Kenta Watanabe [a], Yuki Yamasaki [a], Hiroshi Suzuki [b], Masahisa Watarai [a,*]

[a] Department of Applied Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro-shi, Hokkaido 080-8555, Japan
[b] Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro-shi, Hokkaido 080-8555, Japan

ARTICLE INFO

Article history:
Received 17 December 2007
Received in revised form 4 March 2008
Accepted 3 April 2008
Available online 16 April 2008

Keywords:
Brucella abortus
Abortion
HO-1
Placenta

ABSTRACT

Brucella abortus is a facultative intracellular pathogen that can survive inside macrophages and trophoblast giant (TG) cells, and the causative agent of brucellosis. In the present study, we found that expression of heme oxygenase-1 (HO-1) in TG cells is correlated with abortion induced by *B. abortus* infection in pregnant mice. Expression of HO-1 in the placenta was decreased by *B. abortus* infection and treatment with cobalt-protoporphyrin (Co-PP), which is known to up-regulate HO-1 expression, inhibited abortion due to the bacterial infection. In TG cells, treatment with Co-PP was shown to up-regulate HO-1, whereas its expression was decreased by *B. abortus* infection. Such down-regulation of HO-1 in the TG cells was enhanced by IFN-γ treatment. HO-1 down-regulation in TG cells due to knockdown or IFN-γ treatment served to induce cell death caused by *B. abortus* infection. These results suggest that down-regulation of HO-1 in TG cells due to *B. abortus* infection is an important event in infectious abortion.

1. Introduction

Brucellosis is a serious debilitating disease in humans and results in abortion and sterility in domestic animals. The etiologic agents of brucellosis are *Brucella* spp., small gram-negative and facultative intracellular pathogens that can multiply within professional and non-professional phagocytes [1,2]. In contrast to other intracellular pathogens, *Brucella* species do not produce exotoxins, antiphagocytic capsules or thick cell walls, resistant forms or fimbriae, and do not show antigenic variation [3]. A key aspect of the virulence of *Brucella* is its ability to proliferate within professional and non-professional phagocytic host cells, thereby successfully bypassing the bactericidal effects of phagocytes, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within host cells [4,5]. Infection in humans is almost exclusively due to zoonosis, either through direct contact with infected animals or from contaminated dairy products [6]. The mouse model, particularly that using the non-pregnant mouse, has been used extensively to study some aspects of the pathogenesis of brucellosis [7]. While brucellosis is known to primarily affect the reproductive tract in the natural host and has been much studied, little is known regarding the cellular and molecular mechanisms of *Brucella* infection in the pregnant mouse [8]. The infectious abortion model using the pregnant mouse is a powerful tool for investigating the mechanisms of *Brucella* pathogenesis and in our previous study we demonstrated that *Brucella abortus* causes abortion in pregnant mice by inoculating bacteria on day 4.5 of gestation [9]. We found that there was a higher degree of bacterial colonization in the placenta than in other organs, that there were many bacteria in trophoblast giant (TG) cells in the placenta and that abortion was not induced in an intracellular replication-defective mutant. Transient interferon-γ (IFN-γ) production induced by infection with *B. abortus* also contributes to infectious abortion and its neutralization served to prevent abortion.

Pregnancy leads to a generalized suppression of the adaptive immune system, typified by significantly decreased cell-mediated immunity and reduced T helper cell (Th) 1 responsiveness [10–12]. This immunosuppressed state prevents maternal rejection of the fetus but has the unfortunate consequence of increasing maternal susceptibility to certain infectious agents [13,14]. Immunity against *B. abortus* is principally mediated by cellular immune responses since it is an intracellular pathogen, and involves antigen-specific T-cell activation of CD4 and CD8 T cells and humoral responses. Protection of the host against *B. abortus* infection is thought to be mediated primarily by a Th1 type of immune response than a Th2 response [15]. For many other intracellular bacterial and protozoan pathogens, it has been shown that IFN-γ is an important component of Th1 immune responses and contributes to control through its ability to stimulate macrophages to kill more microbes. The role

* Corresponding author. Tel.: +81 155 49 5387; fax: +81 155 49 5389.
E-mail address: watarai@obihiro.ac.jp (M. Watarai).

0882-4010/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micpath.2008.04.002

of IFN-γ in the control of *B. abortus* infections has been demonstrated by supplementing BALB/c mice with recombinant IFN-γ, when such treatment resulted in a 10-fold decrease in the number of bacteria at 1 week after infection [16]. It has also been shown that neutralizing endogenous IFN-γ by administering anti-IFN-γ monoclonal antibodies results in a decrease in control [15]. Despite these results, however, the role of other factors in abortion induced by *B. abortus* infection in the pregnant mouse is still unknown.

In the present study, we investigated the role of heme oxygenase (HO)-1 in abortion induced by *B. abortus* infection in the pregnant mouse. Our results suggested that the expression of HO-1 in TG cells was down-regulated by IFN-γ treatment and *B. abortus* infection, and this led to infectious abortion.

2. Results

2.1. Abortion induced by B. abortus infection is dependent on expression of HO-1 in the placenta

Previous studies have reported the presence of HO-1 in the mammalian placenta and postulated a protective role for HO during pregnancy [17–19]. To investigate the role of HO-1 in abortion induced by *B. abortus* infection, the effect of cobalt-protoporphyrin (Co-PP) on infectious abortion was tested. Treatment with Co-PP, which is known to up-regulate HO-1 expression, had a positive effect on long-term graft acceptance [20,21] and helped prevent fetal rejection in pregnant mice [17]. In a previous study [9], we observed that *B. abortus* infection induced abortion. In the present study, treatment with Co-PP was seen to inhibit abortion due to *B. abortus* infection in pregnant mice as compared with non-treated pregnant mice (Fig. 1A). However, there was no significant difference between mice treated with Co-PP and non-treated mice as regards bacterial growth in the spleen (Fig. 1B).

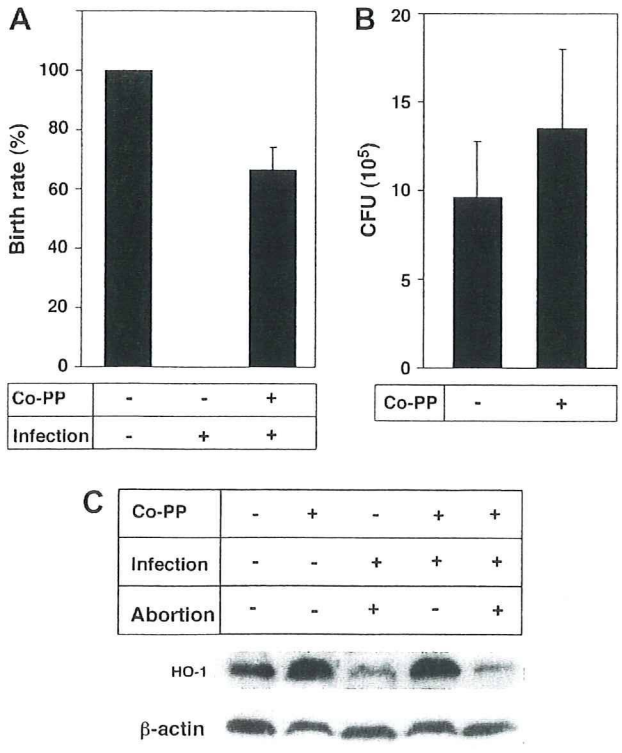

Fig. 1. Abortion prevented by treatment with Co-PP. Pregnant mice inoculated with or without Co-PP were infected with *B. abortus*. The figure shows birth rate (A), bacterial growth in spleen (B), and expression of HO-1 in immunoblotting (C).

To find out if prevention of infectious abortion is dependent on HO-1 expression in the placenta, its amounts in the placenta were determined by immunoblotting. We observed an increase in HO-1 in the placentas of uninfected pregnant mice due to Co-PP treatment (Fig. 1C). Though there was a marked decrease in the amount of HO-1 in the placentas of *B. abortus* infected pregnant mice, the placentas of mice in which abortion had been prevented by Co-PP treatment had greater amounts of HO-1 in the placenta than mice in which abortion had not been prevented by Co-PP treatment (Fig. 1C). These results suggest that abortion induced by *B. abortus* infection is dependent on the expression of HO-1 in the placenta.

2.2. Decrease in HO-1 expression due to B. abortus infection in trophoblast giant cells

B. abortus has been observed to specifically infect trophoblast giant (TG) cells in the placentas of pregnant mice [8,9]. To investigate the protective role of HO-1 further, we used an *in vitro* cell culture system for trophoblast stem (TS) cells and TG cells differentiated from TS cells. Replication of *B. abortus* in TG cells was confirmed by fluorescence microscopy (Fig. 2C). Expression of HO-1 was observed in TG cells and this was decreased by *B. abortus* infection (Fig. 2A). Also, up-regulation of HO-1 expression was observed in both infected and uninfected TG cells treated with Co-PP in a concentration dependent manner (Fig. 2A). However, there was no significant difference between TG cells treated with Co-PP and non-treated TG cells as regards intracellular growth of bacteria (Fig. 2B).

Since a transient increase in IFN-γ brought about by *B. abortus* infection was observed to promote abortion in pregnant mice [9], we investigated the effect of IFN-γ treatment on HO-1 expression in TG cells. HO-1 expression in TG cells was significantly decreased by IFN-γ treatment in a concentration dependent manner, and this down-regulation of HO-1 was further enhanced by *B. abortus* infection (Fig. 3).

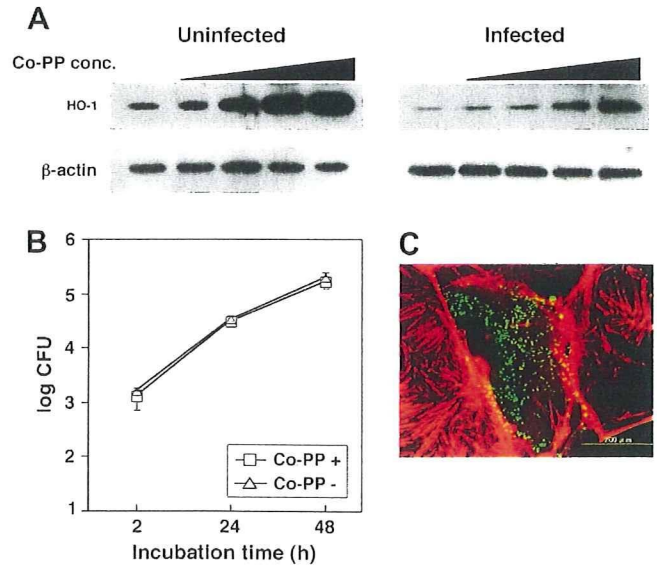

Fig. 2. Expression of HO-1 in *B. abortus* infected TG cells pretreated with Co-PP. Expressions of HO-1 and β-actin (control) were examined by means of immunoblotting in TG cells treated with Co-PP (0, 0.75, 2.25, 7.5 or 22.5 μg/ml, respectively) which were infected with or without *B. abortus* (A). Bacterial growth in TG cells treated with or without Co-PP (22.5 μg/ml) is shown (B). Bacterial replication in TG cells (C). *B. abortus* was deposited onto TG cells which were then incubated for 48 h, fixed and stained for actin filaments with Alexa Fluor 594-phalloidin. The figure shown GFP-expressed bacteria (green) and Alexa Fluor 594 channel (red) merged images.

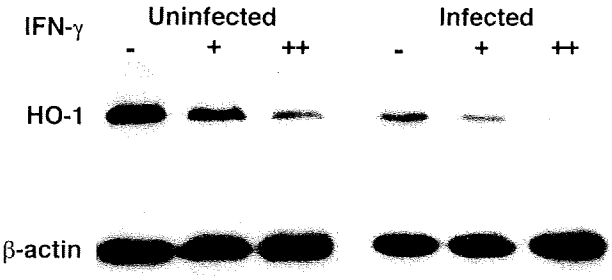

Fig. 3. Expression of HO-1 in IFN-γ treated TG cells. IFN-γ (0, 2.5 or 7.5 × 10^3 units/ml) treated TG cells were infected with or without *B. abortus*, and then the expression of HO-1 and β-actin (control) were examined by means of immunoblotting.

2.3. *HO-1 protects against cell death due to* B. abortus *infection*

Since HO-1 has been reported to have antiapoptotic properties [22,23], we investigated the rate of cell death due to infection with *B. abortus*. To examine the effect of HO-1 on TG cell death, we reduced the amount of endogenous HO-1 by transfecting HO-1-specific small interfering RNA (siRNA) duplexes into the TG cells. After 48 h of transfection with HO-1-specific siRNA, the expression level of HO-1 was no longer detectable, but was not affected by transfection with β-actin or the control siRNA (Fig. 4C). Cell death was not observed in HO-1 knockdown cells created using siRNA, IFN-γ treated cells, or no treatment cells (Fig. 4A and B). Cell death was also not observed in *B. abortus* infected TG cells significantly (Fig. 4A and B). However, infection of *B. abortus* induced cell death in HO-1 knockdown and IFN-γ treated TG cells (Fig. 4A and B).

3. Discussion

It has been proposed that the cytoprotective enzyme HO plays a critical role in graft acceptance [24]. HO is the rate-limiting enzyme in heme catabolism, which generates free iron, biliverdin, and carbon dioxide, the main products of its action. Biliverdin is converted into bilirubin by biliverdin reductase. Accumulation of free heme, which is toxic, leads to tissue inflammation and injuries [25,26]. HO is therefore responsible, at least in part, for preventing such injuries [25,26]. Among the three identified mammalian HO isoforms, HO-1 is a stress-responsive protein, which is implicated in antioxidant defense mechanisms and modulation of vascular tone [26,27]. Much evidence points to an up-regulation of HO-1 allowing the acceptance of mouse and rat allografts [28,29], while its down-regulation or absence is directly related to acute graft rejection [22]. It has been reported that HO-1 levels at the materno–fetal interface of mice undergoing abortion were down-regulated [18,30], and that human miscarriage and pre-eclampsia were associated with diminished placental HO levels [31,32]. In this study, we observed that expression of HO-1 was associated with abortion induced by *B. abortus* infection. *B. abortus* infection caused down-regulation of HO-1 in the placenta and we consider this to be one reason for abortion induced by bacterial infection.

Sollwedel et al. reported that up-regulation of HO-1 by Co-PP during the implantation window could prevent abortion in mice [17]. They also found that induction of HO-1 by Co-PP prevented

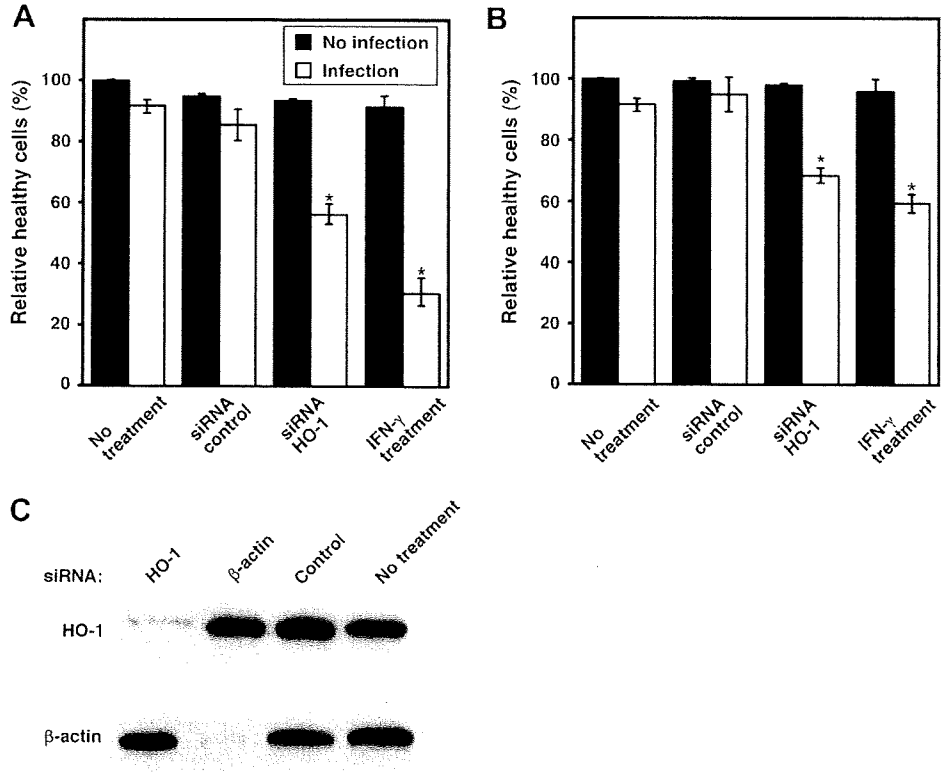

Fig. 4. Expression of HO-1 correlated with TG cell death due to *B. abortus* infection. HO-1 depleted and IFN-γ treated TG cells were infected with or without *B. abortus*, and cell death was determined using a JC-1 Mitochondrial Membrane Potential Assay Kit (A) and MEBSTAIN Apoptosis Kit Direct (B). Quantitative analysis of cell death (A and B). One hundred TG cells per coverslip were examined to determine the total number of live or dead TG cells. Data are the average of triplicate samples from three identical experiments, and the error bars represent the standard deviation. Statistically significant differences in live cell numbers between TG cells depleted in HO-1 (siRNA HO-1) and without HO-1 (siRNA control), and IFN-γ treated and non-treated TG cells are indicated by asterisks (*, $P < 0.01$). TG cells were treated for 48 h with siRNA targeting HO-1, received no treatment (no treatment), or were treated with β-actin or the control (QIAGEN AllStars Negative Control) (C). Expression of the indicated proteins was examined by means of immunoblotting. β-actin was used as an internal control.

fetal rejection, and the down-regulation of HO-1 by zinc-protoporphyrin increased abortion [17]. In the present study, we also observed that induction of HO-1 by Co-PP prevented abortion induced by *B. abortus* infection. Previous studies have reported that during pregnancy, different types of trophoblast cells are important sources of HOs [18,31–33] and participate in the catabolism of the heme protein, avoiding accumulation or recirculation of free heme which could be extremely toxic for the mother and fetus. Down-regulation of HO-1 is potentially very harmful at the materno–fetal interface since large amounts of free heme readily incorporate into endothelial cells, leading to oxidative injury and enhanced adhesion molecule expression [25,34], and allowing migration of inflammatory lymphocytes into the materno–fetal interface from mice undergoing abortion [18]. Therefore, up-regulation of HO-1 is important in protection against infectious abortion.

HOs are expressed in cultured human trophoblast cells [35] and our study showed that HO-1 is also detected in cultured murine TG cells. *B. abortus* infects TG cells in the placenta of pregnant mice specifically [8,9], and this may be a reason for the down-regulation of HO-1 expression in placenta. However, the mechanism of down-regulation of HO-1 in TG cells by *B. abortus* infection remains unknown. Though the Th1/Th2 cytokines paradigm proposes that the up-regulation of proinflammatory cytokines, such as IFN-γ, TNF-α, and IL-6, would lead to fetal rejection [12], high levels of Th2 cytokines would be associated with a successful pregnancy [36]. Further, increased Th1 levels associated with low HO levels could be observed in rejected allografts [21]. The results in this study showed that HO-1 expressed in TG cells was down-regulated by IFN-γ, and that the effect of this cytokine was enhanced by *B. abortus* infection. Th1 cytokines may therefore play an important role in the regulation of HO-1 expression in TG cells.

HO-1 is also believed to have antiapoptotic and tissue-protective properties. Induction of HO-1 expression by heme protects endothelial cells from TNF-α-mediated apoptosis [22,37], but the mechanisms by which HO-1 prevents cells from undergoing apoptosis are still unclear. Since *B. abortus* infection was seen to induce cell death in HO-1 knockdown or IFN-γ treated TG cells, HO-1 expression in TG cells would be associated with the induction of TG cell death by *B. abortus* infection. TG cell death in the placenta would be induced by bacterial infection, and then abortion might occur. Although the immunological mechanisms that govern the success of pregnancy in mammals are highly complex and many factors should participate in infectious abortion, HO-1 may be a putative therapeutic target in abortion by *B. abortus* infection.

4. Materials and methods

4.1. Bacterial strain and mice

B. abortus 544 (ATCC23448), a smooth virulent *B. abortus* biovar 1 strain, was used. The *B. abortus* strain was maintained as frozen glycerol stocks and cultured on Brucella broth (Becton Dickinson) or Brucella broth containing 1.5% agar [38].

Six- to ten-week-old ICR female mice were individually mated to 6- to 10-week-old ICR male mice. All of these mice were obtained from CLEA Japan. The normal gestational time for these mice is 19 days and the vaginal plug was observed on day 0.5 of gestation.

4.2. Virulence in pregnant mice

Groups of five pregnant mice were infected intraperitoneally with approximately 10^4 CFU of brucellae in 0.1 ml saline with or without Co-PP (5 μg/g, SIGMA) at 4.5 days of gestation [9]. On day 18.5 of gestation, their fetuses, placentas, and spleens were removed and homogenized in saline. Tissue homogenates were serially diluted with PBS and plated on Brucella agar to count the number of CFU in each organ. Fetuses were determined to be alive if there was a heartbeat, and dead if there was no heartbeat [9]. The animal experiments were permitted by Animal Research Committee of Obihiro University of Agriculture and Veterinary Medicine.

4.3. Cell culture

Trophoblast stem (TS) cells were cultured in TS medium in the presence of FGF4, heparin and mouse embryonic fibroblast (MEF)-conditioned medium as described previously [39]. The TS medium was prepared by adding 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, and 2 mM L-glutamine to RPMI 1640. To induce differentiation to trophoblast giant (TG) cells, the cells were cultured in TS medium alone. The TG cells were seeded ($1–2 \times 10^5$ per well) in 48 well tissue culture plates for all assays.

4.4. Efficiency of bacterial replication within cultured cells

Bacterial infection and intracellular survival assays were performed according to a modified version of the method of Kim et al [40]. *B. abortus* strains were deposited onto TG cells at a multiplicity of infection (MOI) of 10 by centrifugation at 150 × g for 10 min at room temperature. To measure intracellular replication efficiency, the infected cells were incubated at 37 °C for 30 min, washed once with TS medium and then incubated with TS medium containing gentamicin (30 μg/ml) for 2, 24, 48 and 72 h. Next, the cells were washed three times with PBS and lysed with cold distilled water. CFU values were determined by serial dilution on Brucella plates. Percentage protection was determined by dividing the number of bacteria surviving by the number in the infectious inoculum. Co-PP or recombinant IFN-γ (Cedarlane Laboratories) was added to the TS medium at the indicated concentrations 12 h before infection.

4.5. Immunoblotting

Placenta or cell lysates were separated on 12% polyacrylamide gels and transferred to a PVDF membrane, which was incubated for 1 h at room temperature with primary antibody (anti-HO-1, Stressgen) in 5% skim milk. They were then washed three times in Tris buffered saline (TBS) with 0.02% Tween 20, incubated for 30 min with a horseradish peroxidase (HRP)-conjugated secondary antibody and then washed again. Immunoreactions were visualized by ECL (GE Healthcare Life Sciences). Antibody for β-actin was purchased from SIGMA.

4.6. siRNA experiment

The siRNA duplexes used for silencing mouse HO-1 (target sequence: AACAAGTAACATGGAATAATA), and β-actin (target sequence: CACTGACTTGAGACCAATAAA) and AllStars Negative Control siRNA were purchased from QIAGEN. TG cells were transiently transfected using oligofectamine (Invitrogen) with or without a final concentration of 10 nM for siRNAs.

4.7. Detection of cell death

Cell death was determined by means of a JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical) according to the instructions of the manufacturer. Mitochondrial membrane potential, $\Delta\Psi m$, is an important parameter of mitochondrial function used as an indicator of cell health. Healthy cells with a high mitochondrial $\Delta\Psi m$ have red fluorescence while apoptotic or unhealthy cells have a low $\Delta\Psi m$ and green fluorescence [41]. Cell death was confirmed by MEBSTAIN Apoptosis Kit Direct (MBL) according to the instructions of the manufacturer.

4.8. Statistical analysis

All statistical analysis was conducted using the Student's *t* test.

Acknowledgments

We thank Dr. Alexander Cox for critical reading of the manuscript. This work was supported, in part, by grants from the Program for Promotion of Basic Research Activities for Innovative Bioscience, and from Scientific Research (19041006 and 18790298), Japan Society for the Promotion of Science, and from the Institute for Fermentation, Osaka.

References

[1] Delrue RM, Martinez-Lorenzo M, Lestrate P, Danese I, Bielarz V, Mertens P, et al. Identification of *Brucella* spp. genes involved in intracellular trafficking. Cell Microbiol 2001;3:487–97.
[2] Detileux PG, Deyoe BL, Cheville NF. Entry and intracellular localization of *Brucella* spp. in Vero cells: fluorescence and electron microscopy. Vet Pathol 1990;27:317–28.
[3] Finlay B, Falkow S. Common themes in microbial pathogenicity. Microbiol Mol Biol Rev 1997;61:136–69.
[4] Pizarro-Cerda J, Moreno E, Sanguedolce V, Mege JL, Gorvel JP. Virulent *Brucella abortus* prevents lysosome fusion and is distributed within autophagosome-like compartments. Infect Immun 1998;66:2387–92.
[5] Ugalde RA. Intracellular lifestyle of *Brucella* spp. common genes with other animal pathogens, plant pathogens, and endosymbionts. Microbes Infect 1999;1:1211–9.
[6] Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. Lancet Infect Dis 2006;6:91–9.
[7] Enright FM. The pathogenesis and pathobiology of *Brucella* infection in domestic animals. In: Nielsen K, Duncan JR, editors. Animal brucellosis. Boca Raton, FL: CRC Press; 1990. p. 301–20.
[8] Tobias L, Cordes DO, Schurig GG. Placental pathology of the pregnant mouse inoculated with *Brucella abortus* strain 2308. Vet Pathol 1993;30:119–29.
[9] Kim S, Lee DS, Watanabe K, Furuoka H, Suzuki H, Watarai M. Interferon-γ promotes abortion due to *Brucella* infection in pregnant mice. BMC Microbiol 2005;5:22.
[10] Weinberg ED. Pregnancy-associated immune suppression: risks and mechanisms. Microb Pathog 1987;3:393–7.
[11] Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal–fetal relationship: is successful pregnancy a Th2 phenomenon? Immunol Today 1993;14:353–6.
[12] Raghupathy R. Th1-type immunity is incompatible with successful pregnancy. Immunol Today 1997;18:478–82.
[13] Sano M, Mitsuyama M, Watanabe Y, Nomoto K. Impairment of T cell-mediated immunity to *Listeria monocytogenes* in pregnant mice. Microbiol Immunol 1986;30:165–76.
[14] Krishnan L, Guilbert LJ, Russell AS, Wegmann TG, Mosmann TR, Belosevic M. Pregnancy impairs resistance of C57BL/6 mice to *Leishmania major* infection and causes decreased antigen-specific IFN-gamma response and increased production of T helper 2 cytokines. J Immunol 1996;156:644–52.
[15] Zhan Y, Cheers C. Endogenous gamma interferon mediates resistance to *Brucella abortus* infection. Infect Immun 1993;61:4899–901.
[16] Stevens MG, Pugh Jr GW, Tabatabai LB. Effects of γ-interferon and indomethacin in preventing *Brucella abortus* infections in mice. Infect Immun 1992;60:4407–9.
[17] Sollwedel A, Bertoja AZ, Zenclussen ML, Gerlof K, Lisewski U, Wafula P, et al. Protection from abortion by heme oxygenase-1 up-regulation is associated with increased levels of Bag-1 and neuropilin-1 at the fetal–maternal interface. J Immunol 2005;175:4875–85.
[18] Zenclussen AC, Sollwedel A, Bertoja AZ, Gerlof K, Zenclussen ML, Woiciechowsky C, et al. Heme oxygenase as a therapeutic target in immunological pregnancy complications. Int Immunopharmacol 2005;5:41–51.
[19] Zenclussen ML, Anegon I, Bertoja AZ, Chauveau C, Vogt K, Gerlof K, et al. Over-expression of heme oxygenase-1 by adenoviral gene transfer improves pregnancy outcome in a murine model of abortion. J Reprod Immunol 2006; 69:35–52.
[20] Tullius SG, Nieminen-Kelhä M, Buelow R, Reutzel-Selke A, Martins PN, Pratschke J, et al. Inhibition of ischemia/reperfusion injury and chronic graft deterioration by a single-donor treatment with cobalt-protoporphyrin for the induction of heme oxygenase-1. Transplantation 2002;74:591–8.
[21] Woo J, Iyer S, Mori N, Buelow R. Alleviation of graft-versus-host disease after conditioning with cobalt-protoporphyrin, an inducer of heme oxygenase-1. Transplantation 2000;69:623–33.
[22] Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, et al. Expression of heme oxygenase-1 can determine cardiac xenograft survival. Nat Med 1998;4:1073–7.
[23] Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, et al. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. J Exp Med 2000;192:1015–26.
[24] Soares MP, Lin Y, Sato K, Stuhlmeier KM, Bach FH. Accommodation. Immunol Today 1999;20:434–7.
[25] Balla G, Vercellotti GM, Muller-Eberhard U, Eaton J, Jacob HS. Exposure of endothelial cells to free heme potentiates damage mediated by granulocytes and toxic oxygen species. Lab Invest 1991;64:648–55.
[26] Balla J, Jacob HS, Balla G, Nath K, Eaton JW, Vercellotti GM. Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. Proc Natl Acad Sci U S A 1993;90:9285–9.
[27] Applegate LA, Luscher P, Tyrrell RM. Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. Cancer Res 1991;51: 974–8.
[28] Woo J, Iyer S, Cornejo MC, Mori N, Gao L, Sipos I, et al. Stress protein-induced immunosuppression: inhibition of cellular immune effector functions following overexpression of haem oxygenase (HSP 32). Transpl Immunol 1998;6: 84–93.
[29] Chauveau C, Bouchet D, Roussel JC, Mathieu P, Braudeau C, Renaudin K, et al. Gene transfer of heme oxygenase-1 and carbon monoxide delivery inhibit chronic rejection. Am J Transplant 2002;2:581–92.
[30] Zenclussen AC, Joachim R, Hagen E, Peiser C, Klapp BF, Arck PC. Heme oxygenase is downregulated in stress-triggered and interleukin-12-mediated murine abortion. Scand J Immunol 2002;55:560–9.
[31] Barber A, Robson SC, Myatt L, Bulmer JN, Lyall F. Heme oxygenase expression in human placenta and placental bed: reduced expression of placenta endothelial HO-2 in preeclampsia and fetal growth restriction. FASEB J 2001;15: 1158–68.
[32] Zenclussen AC, Lim E, Knoeller S, Knackstedt M, Hertwig K, Hagen E, et al. Heme oxygenases in pregnancy II: HO-2 is downregulated in human pathologic pregnancies. Am J Reprod Immunol 2003;50:66–76.
[33] Ihara N, Akagi R, Ejiri K, Kudo T, Furuyama K, Fujita H. Developmental changes of gene expression in heme metabolic enzymes in rat placenta. FEBS Lett 1998;439:163–7.
[34] Vachharajani TJ, Work J, Issekutz AC, Granger DN. Heme oxygenase modulates selectin expression in different regional vascular beds. Am J Physiol Heart Circ Physiol 2000;278:H1613–7.
[35] Newby D, Cousins F, Myatt L, Lyall F. Heme oxygenase expression in cultured human trophoblast cells during in vitro differentiation: effects of hypoxia. Placenta 2005;26:201–9.
[36] Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S. Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. Nat Med 1998;4:1020–4.
[37] Soares MP, Usheva A, Brouard S, Berberat PO, Gunther L, Tobiasch E, et al. Modulation of endothelial cell apoptosis by heme oxygenase-1-derived carbon monoxide. Antioxid Redox Signal 2002;4:321–9.
[38] Watarai M, Makino S-I, Shirahata T. An essential virulence protein of *Brucella abortus*, VirB4, requires an intact nucleoside-triphosphate-binding domain. Microbiology 2002;184:1439–46.
[39] Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J. Promotion of trophoblast stem cell proliferation by FGF4. Science 1998;282:2072–5.
[40] Kim S, Watarai M, Kondo Y, Erdenebaatar J, Makino S, Shirahata T. Isolation and characterization of mini-Tn5Km2 insertion mutants of *Brucella abortus* deficient in internalization and intracellular growth in HeLa cells. Infect Immun 2003;71:3020–7.
[41] Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 1997;411:77–82.

Journal of Reproduction and Development, Vol. 54, No. 4, 2008

—Technical Note—

Artificial Insemination with Canine Spermatozoa Frozen in a Skim Milk/Glucose-Based Extender

Yasuyuki ABE[1)], Dong-Soo LEE[1)], Hikaru SANO[1)], Koji AKIYAMA[1)], Yoshiko YANAGIMOTO-UETA[1)], Tomoyoshi ASANO[1)], Yoshinori SUWA[2)] and Hiroshi SUZUKI[1,3)]

[1)]Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, [2)]Hokkaido Guide Dogs for the Blind Association, Hokkaido 005-0030 and [3)]Department of Developmental and Medical Technology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

Abstract. Due to the recent outbreak of avian influenza, transportation of frozen canine semen with egg yolk has been sharply restricted. Thus, there is urgent need to develop a novel egg yolk-free extender for freezing canine spermatozoa. In the present study, the effect of using skim milk/glucose (SG)-based extender without egg yolk on the motility and fertilizing capacity of canine spermatozoa frozen-thawed in the presence of glycerol was examined. There was a tendency for the proportion of motile spermatozoa exposed to SG-based extender for 3 h to be higher than that exposed for 1 h, but the difference was not significant. The motility and other viability parameters of canine spermatozoa after thawing were similar to those obtained with an egg yolk-based extender. When spermatozoa frozen with SG-based extender containing glycerol after 3 h exposure were transcervically inseminated into 2 recipient bitches, a total of 6 pups were obtained. These results suggest that a simple extender composed of skim milk, glucose and glycerol is useful for cryopreservation of canine spermatozoa, which may contribute to improved exchange of genetic material and efficient production of companion and working dogs, such as guide dogs for the blind.
Key words: Canine, Cryopreservation, Skim milk/glucose-based extender, Spermatozoa

(J. Reprod. Dev. 54: 290–294, 2008)

Although freezing of canine semen and insemination of canine bitches with frozen-thawed semen is not as commonly used as in bovine and equine animals, successful artificial insemination with frozen canine semen has been well documented [1] since the first conception in 1969 [2]. Cryopreservation of canine spermatozoa offers potential exchange of genetic material, and thus may lead to improvement in the breeding management programs used to produce working dogs. In particular, in guide dog colonies, application of transcervical artificial insemination using frozen canine semen is anticipated to assist with meeting the demand for adequate supply of guide dogs for the blind. Egg yolk is the most commonly used compound in canine semen extenders for protection of spermatozoa from cold shock and disruption during the freezing and thawing process [1]. However, due to a recent outbreak of avian influenza and its triggering of growing concern throughout the world, transportation of frozen or chilled semen exposed to egg yolk has become extremely difficult. Several countries have, in fact, prohibited export and import of canine frozen semen that contains egg yolk. Thus, it is an urgent matter to develop a novel semen extender without egg yolk for use in freezing of canine spermatozoa. As an alterative compound to egg yolk, skim milk seems to be especially suitable as a semen extender in canine species, since a skim milk extender is the most commonly used extender for mouse [3] and goat [4] sperm. We report here successful artificial insemination with canine spermatozoa frozen in a solution containing skim milk, glucose and glycerol.

Accepted for publication: April 7, 2008
Published online in J-STAGE: May 13, 2008
Correspondence: H. Suzuki (e-mail: hisuzuki@obihiro.ac.jp)

Materials and Methods

Collection of ejaculated semen

The ejaculates from a total of five male Labrador Retrievers with proven fertility in natural mating were collected by digital manipulation into sterile tubes (Corning, Corning, NY, USA). The first and third fractions (seminal plasma) of the ejaculate were discarded. Only 3–4 ml of the sperm-rich second fraction of the ejaculates was collected for the experiments.

The animals used in this study were treated and received care under the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine.

Preparation of semen extenders

For a skim milk/glucose (SG)-based extender, 30 mg/ml of skim milk (232100; Difco, Le Pont de Claix, France) and 0.3 M glucose (041-00595; Wako, Osaka, Japan) were dissolved in water for embryo transfer (W1503; Sigma-Aldrich, St. Louis, MO, USA) at 60 C, and then the solution was centrifuged at 10,000 g for 15 min at room temperature. The supernatants were filtered (25CS045AS; Advantec, Tokyo, Japan) and used as the SG-based extender.

As a control, an egg yolk-Tris-citrate-glucose (EY) extender composed of 20% [v/v] egg yolk, 24 mg/ml Tris(hydroxymethyl)aminomethane (252859; Sigma), 14 mg/ml citric acid monohydrate (035-03495; Wako), 0.8 mg/ml glucose, 0.65 mg/ml

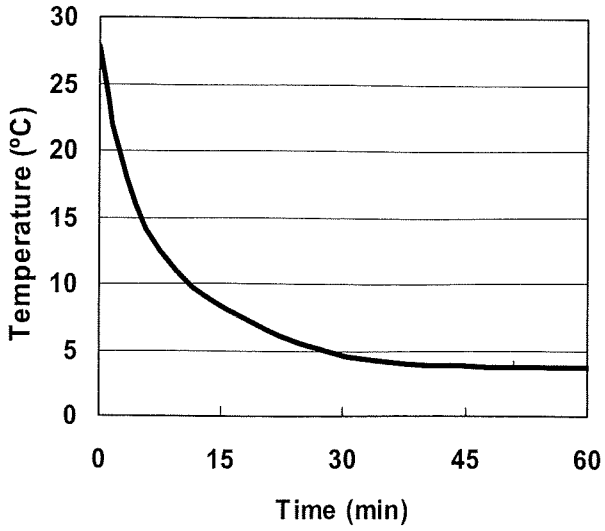

Fig. 1. Cooling rate of 1 ml of the skim milk/glucose-based extender at 4 C in a refrigerator. The temperature of the sample was measured using a thermo recorder.

penicillin G potassium (Meiji Seika, Tokyo, Japan) and 1 mg/ml streptomycin sulphate (Meiji Seika) was prepared as described previously by Rota *et al.* [5]. For the egg yolk preparation, the egg shell was cracked, and the egg yolk was passed from one shell to the other to remove most of the egg white, allowing the egg white to fall. The yolk was slowly transferred from the shell onto a paper towel and then was allowed to roll down the paper towel until it was dry and there was no remnant of egg white left. The yolk lost its glossy appearance and stuck to the paper towel when all the egg white had been removed. When the egg membrane was broken during the procedures described above, the entire egg was discarded. While holding the paper towel back, the egg membrane was punctured with a surgical blade (Feather safety razor, Osaka, Japan), and the contents were drained into a flask (Duran, Mainz, Germany). The flask was stored in a refrigerator for one to four days before use.

Semen dilution and freezing

The collected ejaculates were diluted with the SG or EY extender to give a sperm concentration of 2×10^8 sperm/ml at room temperature and then cooled to 4 C in a refrigerator. The kinetic temperature of 1 ml of SG-based extender in a 1.5-ml microtube (Nippon Genetics, Tokyo, Japan) was monitored with a thermometer (EB22005; Chino, Tokyo, Japan). After addition of extender (total 0.5–1.0 ml) and cooling in the refrigerator (4 C) for 1 or 3 h, the equivalent volume of the second extender, which was the first extender supplemented with or without 14% (v/v) of glycerol (075-00616; Wako, Kanagawa, Japan) at 4 C, was added to the semen aliquots, and the semen samples were left at 4 C for 15 min. The diluted sperm suspension was loaded into a 0.25-ml straw (Fujihira, Tokyo, Japan). The straws were placed in an atmosphere of liquid nitrogen (LN_2) vapor, i.e., placed horizontally 6 cm above the surface of LN_2 in a closed styrene foam box (24.5 cm × 17.5 cm × 17.5 cm), retained there for 15 min and then plunged into the LN_2.

Post-thaw parameters examined

After thawing by immersing the straws in a water bath at 37 C for 60 sec, the content of each straw was expelled into a 1.5-ml microtube, and each sample was evaluated for the following parameters using a light microscope with the aid of a Computer Assisted Sperm Analysis (CASA) system (HTM-CEROS-S; Hamilton Thorne Research, Danvers, MA, USA): the proportion of total motile spermatozoa (TMS); the proportion of progressive motile spermatozoa (PMS); velocity average pathway (VAP) - the average velocity of the smoothed cell path in μm/sec; the velocity straight line (VSL) - the average velocity measured in a straight line from the beginning to the end of track in μm/sec; the curvilinear velocity (VCL) - the average velocity measured over the actual point-to-point track followed by the cell in μm/sec; the amplitude lateral head (ALH) - amplitude of lateral head displacement in μm; the beat cross frequency (BCF) - frequency of sperm heads crossing the sperm average path in Hertz; the straightness (STR) - the average value of the ratio VSL/VAP in percentage form (straightness estimates the proximity of the cell path to a straight line, with 100% corresponding to optimal straightness); and the linearity (LIN) - the average value of the ratio of VSL/VCL in percentage form (linearity estimates the proximity of the cell track to a straight line). The overall sperm population was subdivided into four categories: Rapid, ≥25 μm/sec; Medium, ≥5 μm/sec, <25 μm/sec; Slow, >0 μ/sec, <5 μm/sec; and Static, 0 μm/sec.

Artificial insemination

To demonstrate the fertilizing capacity of the spermatozoa frozen in the SG-based extender containing glycerol after 3 h exposure, the post-thaw spermatozoa were transcervically inseminated into the uteri of two bitches (Labrador Retrievers). To estimate the LH surge, the plasma progesterone concentrations of the bitches were measured daily by enzyme-linked fluorescent assay (SV-5010, Spotchem Vidas; Arkray, Kyoto, Japan) after the appearance of a blood-tinged vaginal discharge and vaginal swelling. One ml of blood was collected from the anterior brachiocephalic vein and then was centrifuged to separate the plasma. The day when the plasma concentration of progesterone exceeded 2 ng/ml was estimated as the occurrence of the LH surge (defied as to Day 0) [6]. The bitches were inseminated with frozen-thawed semen on Days 5–8. Inseminations were performed on the bitches while they were standing and non-sedated. A catheter (8 Fr; Nippon Sherwood, Tokyo, Japan) equipped with a cystoscope for human use (Karl Storz, Tuttlingen, Germany) was inserted into the *corpus uteri* through the cervical canal [7]. Then, 2 ml of the post-thaw semen (2×10^8 spermatozoa) was inseminated through the catheter. Care was taken to avoid backflow of the semen, so the catheter was withdrawn one minute after the insemination and the hind quarters of the bitch were kept elevated for 5 min. Conception was diagnosed by Doppler ultrasonography (VPU-011A; Toshiba, Tokyo, Japan). Unsuccessful pregnancy after experimental artificial insemination by frozen-thawed spermatozoa exerts considerable influence on both the planning for and production of guide dogs in guide dog associations. Thus, insemination of