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

—Research Note—

# Preimplantation Development of Embryos in Labrador Retrievers

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**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
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## 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.

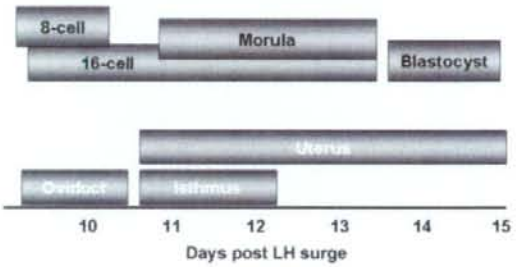


**Fig. 1.** Preimplantation development and localization of embryos in the Labrador Retrievers. The day the plasma concentration of progesterone exceeded 2 ng/ml was considered to be the day of the LH surge (day 0). The embryos developed to the morula and blastocyst stages by 11–13 and 14 days after 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.

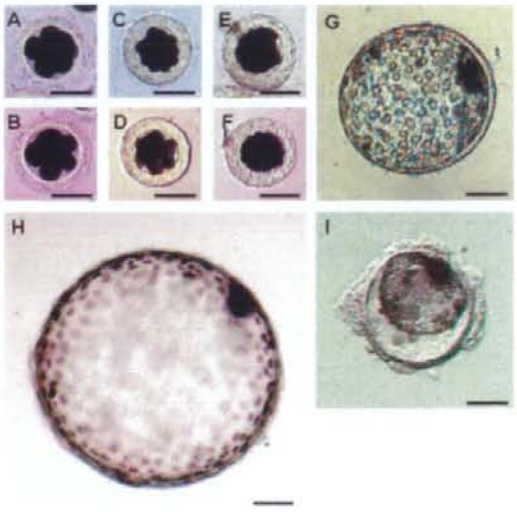


**Fig. 2.** Preimplantation stages of embryos recovered from the female reproductive tract after artificial insemination in the Labrador Retriever. A, B: 8-cell stage. C, D: 16-cell stage. E, F: Morula. G: Expanded blastocyst. H: Large expanded blastocyst. I: Shrunken blastocyst. Bars indicate 100 $\mu$m.

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.

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# Vitrification of Canine Oocytes

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**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
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## 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

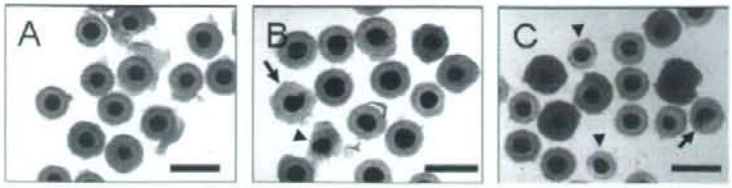


**Fig. 1.** Morphological figures of the cumulus oocyte complexes (COCs). (A) Fresh; (B) vitrified-warmed by the DAP213 method; (C) vitrified-warmed by the E30S method. Some vitrified-warmed COCs had partly dispersed cumulus cells (arrow head) and disrupted cytoplasm (arrow). Bar = 200 $\mu$m.

**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 $P<0.05$.

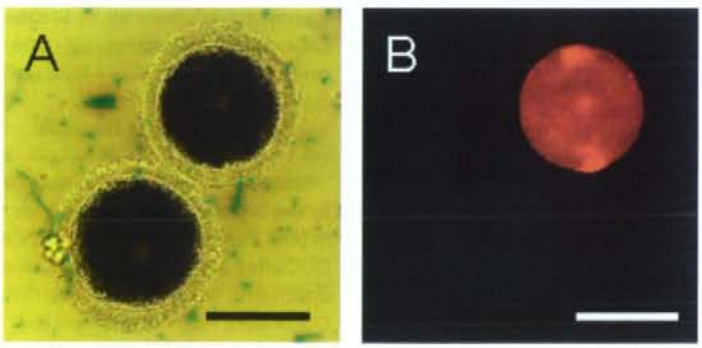


**Fig. 2.** Morphological appearance of canine oocytes after vitrification by the E30S method. Both oocytes showed morphologically normal appearance under light microscopy (A); however, PI stain revealed that one of the oocytes (upper) was damaged (B). Bar = 100 $\mu$m.

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 $\mu$l), while the oocytes were suspended in 100 $\mu$l 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.

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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

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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

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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).

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).

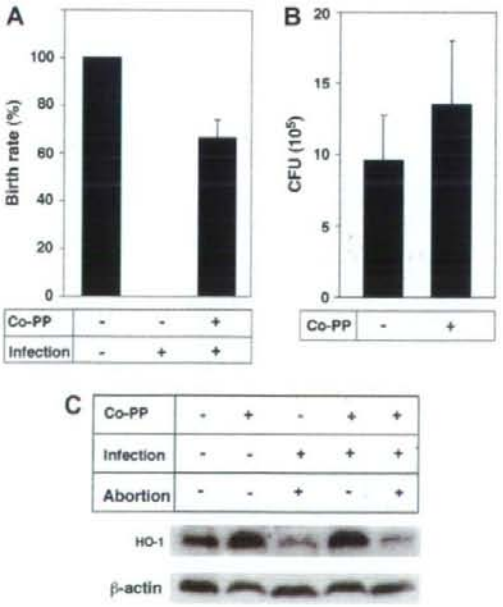


**Fig. 1.** Abortion prevented by treatment with Co-PP. Pregnant mice inoculated with or without Co-PP were infected with *B. abortus*. The figure shows birth rate (A), bacterial growth in spleen (B), and expression of HO-1 in immunoblotting (C).

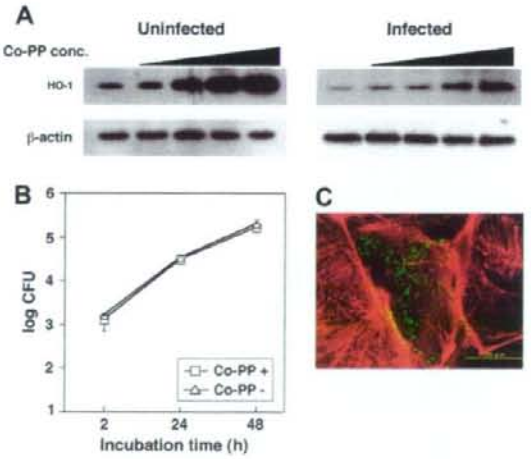


**Fig. 2.** Expression of HO-1 in *B. abortus* infected TG cells pretreated with Co-PP. Expressions of HO-1 and β-actin (control) were examined by means of immunoblotting in TG cells treated with Co-PP (0, 0.75, 2.25, 7.5 or 22.5 μg/ml, respectively) which were infected with or without *B. abortus* (A). 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.

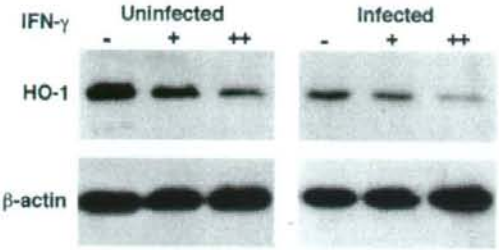


**Fig. 3.** Expression of HO-1 in IFN-γ treated TG cells. IFN-γ (0, 2.5 or 7.5 × $10^3$ units/ml) treated TG cells were infected with or without *B. abortus*, and then the expression of HO-1 and β-actin (control) were examined by means of immunoblotting.

### 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

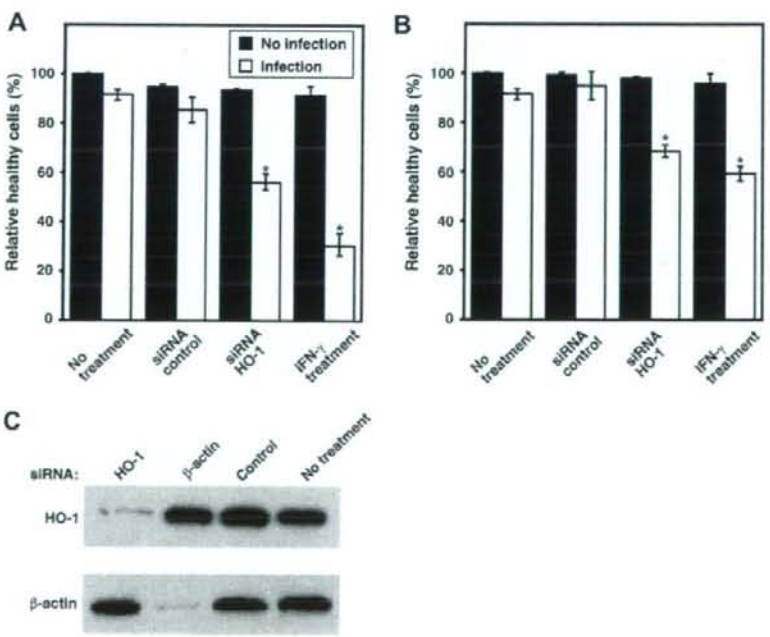


**Fig. 4.** Expression of HO-1 correlated with TG cell death due to *B. abortus* infection. HO-1 depleted and IFN-γ treated TG cells were infected with or without *B. abortus*, and cell death was determined using a JC-1 Mitochondrial Membrane Potential Assay Kit (A) and MEBSTAIN Apoptosis Kit Direct (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.

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*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

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**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
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## 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

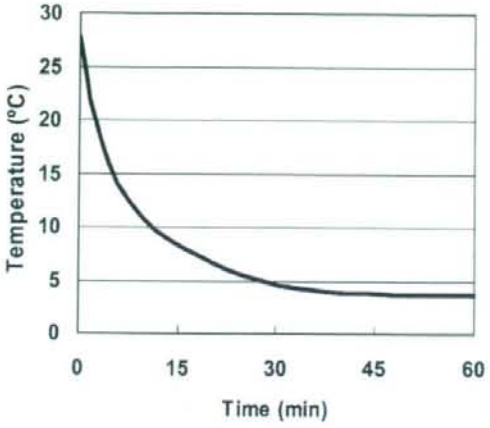


Fig. 1. Cooling rate of 1 ml of the skim milk/glucose-based extender at 4 C in a refrigerator. The temperature of the sample was measured using a thermo recorder.

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 $2 \times 10^8$ 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 $\mu$m/sec; the velocity straight line (VSL) - the average velocity measured in a straight line from the beginning to the end of track in $\mu$m/sec; the curvilinear velocity (VCL) - the average velocity measured over the actual point-to-point track followed by the cell in $\mu$m/sec; the amplitude lateral head (ALH) - amplitude of lateral head displacement in $\mu$m; 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 $\mu$m/sec; Medium, ≥5 $\mu$m/sec, <25 $\mu$m/sec; Slow, >0 $\mu$/sec, <5 $\mu$m/sec; and Static, 0 $\mu$m/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 ($2 \times 10^8$ 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

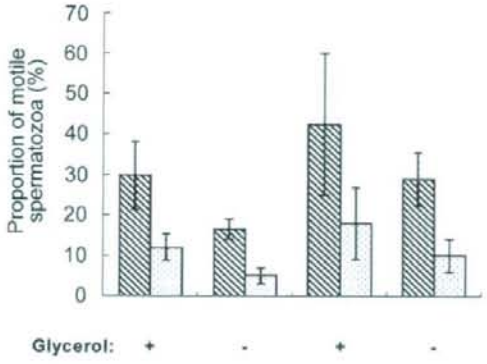


**Fig. 2.** Effect of semen exposure with or without glycerol on the proportion of total (slanted line bar) and progressively (stippled bar) motile spermatozoa after freezing and thawing. Experiments were replicated two or three times to examine the sperm motility using ejaculates of ICH or NAN. There was no significant difference between the groups with and without glycerol or cooling at 4 C for 1 and 3 h ($P>0.05$).

freshly ejaculated semen from different dogs was performed to avoid failure of conception in one bitch. The bitch was inseminated with the cryopreserved and fresh semen on Days 5 and 6, respectively. Another bitch was inseminated with only the cryopreserved semen on Days 6, 7 and 8. Paternity for the delivered pups was examined using microsatellite markers, as described previously [8].

*Statistical analysis*

Data were compared using the *t*-test and the StatView software (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant at a level of $P<0.05$.

## Results

When 1-ml of the SG-based extender was cooled to 4 C, the temperature of the sample reached 4 C within 60 min. However, there was a tendency for the proportion of total motile spermatozoa in each of the samples exposed for 3 h in the case of the SG-based extender to be higher than that in the samples after 1 h, but the difference was not significant (Fig. 2). Addition of glycerol to the SG-based extender was remarkable in that in terms of the resulting motility of the cryopreserved canine semen. The results for the glycerol added groups were higher in both the proportion of total motile spermatozoa and progressive motility of spermatozoa compared with the no glycerol groups, although the difference was not significant (Fig. 2).

When 8 ejaculates from 5 dogs were frozen-thawed after 3 h exposure to the SG and EY extenders containing glycerol, as shown in Fig. 3, the proportion of total motile spermatozoa of the samples in the SG and EY extenders ranged from 25–89% (average: 58.8 ± 8.6) and 13–90% (average: 57.1 ± 10.1), respectively. Thus, the effect of the SG extender for cryopreservation of canine

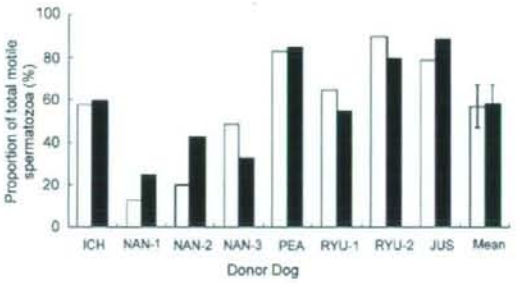


**Fig. 3.** The proportion of total motile spermatozoa frozen with the skim milk/glucose (SG)-based extender (black bar) and egg yolk (EY)-based extender (white bar).

spermatozoa was similar to those obtained with the EY extender (Fig. 3). Other parameters of motility for frozen-thawed spermatozoa in the SG extender were similar to the corresponding parameters in the EY extender (Fig. 4).

Spermatozoa frozen in the SG-based extender containing glycerol after 3 h exposure were transcervically inseminated, resulting in the delivery of 6 pups from 2 recipient bitches (Table 1).

## Discussion

Although egg yolk extender is the most commonly used extender for freezing canine sperm, the process for preparing the extender involves complicated procedures including storage for one to four days prior to use in addition to microbiological problems. Moreover, there are considerable individual differences in the fertilizing capacity of cryopreserved canine spermatozoa frozen in egg yolk-based extender. Thus, an improved system for cryopreservation of canine spermatozoa is required for successful breeding programs in companion and working dog colonies. Although skim milk is itself an extract from biological products (similar to egg yolk), skim milk is commercially available as a reagent and widely used as a cryoprotective additive in mouse [3] and goat [4] spermatozoa. In our preliminary experiment, in which the most suitable concentration of skim milk in terms of the effect on canine sperm motility was determined after freezing and thawing, 30 mg/ml was found to be the most effective concentration in terms of the kinematic parameters from among concentrations of 15, 30 and 60 mg/ml (data not shown). The results in Figs. 1 and 2 suggested that it may be necessary to expose the SG-based extender for 3 h at 4 C, although the temperature of the sample reached 4 C within 60 min. In addition, sufficient exposure time to the cryoprotectants may be a critical factor for the viability of frozen canine spermatozoa. Thus, we utilized 30 mg/ml of skim milk as a component of semen extender and 3 h as the exposure time for the SG extender. When 30 mg/ml of skim milk, 0.3 M glucose and 7% (v/v) of glycerol were provided as cryoprotectants, the motility and other related sperm viability parameters of canine spermatozoa after thawing were similar to those obtained with the EY-based extender (Figs. 3 and 4). Rota *et al.* [9] have similarly shown that

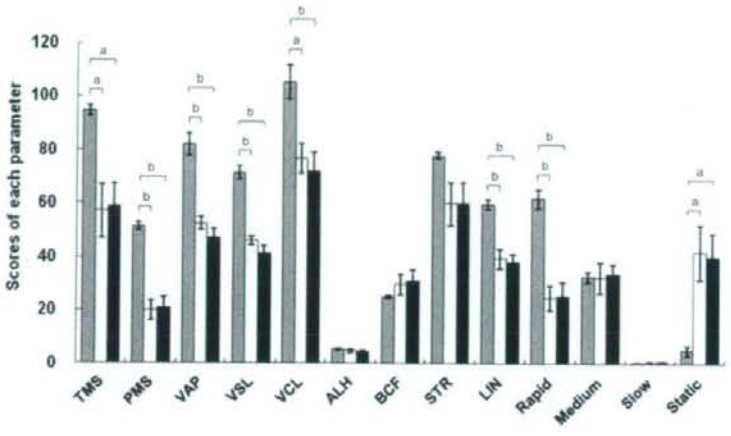


**Fig. 4.** Sperm motility parameters of fresh spermatozoa (gray bars) and spermatozoa frozen with skim milk/glucose-based (black bars) and egg yolk-based extender (white bars). TMS: The proportion of total motile spermatozoa. PMS: the proportion of progressively motile spermatozoa. VAP: velocity average pathway. VSL: velocity straight line. VCL: curvilinear velocity. ALH: amplitude lateral head. BCF: beat cross frequency. STR: straightness. LIN: linearity. The overall sperm population was subdivided into four categories: Rapid, ≥25 μm/sec; Medium, ≥5 μm/sec, <25 μm/sec; Slow, >0 μm/sec, <5 μm/sec; and Static, 0 μm/sec. Experiments were replicated four or eight times to examine the sperm motility in the ejaculates of two (ICH and NAN) or five (ICH, NAN, PEA, RYU and JUS) male Labrador Retrievers in the fresh or cryopreserved groups, respectively. Significantly different between the three groups at $P<0.05$ (a) and $P<0.01$ (b).

**Table 1.** Results of artificial insemination with canine spermatozoa frozen in a skim milk/glucose-based extender

| Bitch no. | No. of pups Delivered | Treatment | |
|---|---|---|---|
| | | Cryopreserved | Fresh |
| 1* | 5 | 1 | 4 |
| 2** | 5 | 5 | – |

*Insemination with cryopreserved and fresh semen on Days 5 and 6, respectively. **Insemination with cryopreserved semen on Days 6, 7 and 8.

the use of skim milk in extenders for freezing canine semen results in sperm motility and viability after thawing comparable to that obtained using a Tris-based buffer with egg yolk, although they did not reportedly determine the fertilizing capacity of the cryopreserved spermatozoa by artificial insemination.

In the bull, it has been reported that egg yolk protects sperm function by preventing the binding of sperm to the major seminal plasma proteins, thereby preventing seminal plasma protein-mediated stimulation of lipid loss from the plasma membrane [10]. As in the case of egg yolk, skim milk prevents the binding of seminal plasma protein to bull sperm and reduces sperm lipid loss while also maintaining sperm motility and viability during storage at 4 C [11]. On the other hand, since it has been shown that the fertilizing lifespan of sperm stored in milk or milk-based extenders dose not exceed 12 h in the goat [12], further modification, such as antioxidant supplementation [13] or shortening of the exposure time in the extender before cryopreservation, might be required to prolong the survival and fertilizing ability of frozen canine spermatozoa.

In conclusion, the results presented here clearly demonstrate that an effective, simple extender composed of skim milk, glucose and glycerol is available for the cryopreservation of canine spermatozoa as an alternative to extenders containing egg yolk, and this may contribute to both efficient exchange of genetic materials and production of guide dogs for the blind.

## 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.

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FULL PAPER *Bacteriology*

# Regulated upon Activation Normal T-Cell Expressed and Secreted (RANTES) Contributes to Abortion Caused by *Brucella abortus* Infection in Pregnant Mice

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(Received 17 December 2007/Accepted 25 March 2008)

ABSTRACT. *Brucella abortus (B. abortus)* is a facultative intracellular pathogen that can survive inside macrophages and trophoblast giant cells, and the causative agent of brucellosis. In the present study, we found that production of regulated upon activation normal T-cell expressed and secreted (RANTES) due to *B. abortus* infection contributes to abortion in pregnant mice. *B. abortus* infected pregnant interferon-γ (IFN-γ) knockout mice died within 15 days of infection, but non-pregnant IFN-γ knockout mice were still alive. With infection by wild type *B. abortus*, a large amount of RANTES production was observed in pregnant IFN-γ knockout mice, and induction of RANTES was also observed in normal pregnant mice infected with the wild type, but not in those infected with the intracellular replication-defective mutant. Production of RANTES and IFN-γ were inhibited in mice inoculated with the respective RANTES or IFN-γ antibody. Neutralization of RANTES, induced by *B. abortus* infection, served to prevent abortion. These results indicate that the production and function of RANTES are correlated with IFN-γ in pregnant mice infected with *B. abortus*.

KEY WORDS: abortion, *Brucella abortus*, IFN-γ, RANTES.

*J. Vet. Med. Sci.* 70(7): 681–686, 2008

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 [8, 9]. 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 [14]. 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 [20, 28]. Infection in humans is almost exclusively due to zoonosis, either through direct contact with infected animals or from contaminated dairy products [19]. The mouse model, particularly that using the non-pregnant mouse, has been used extensively to study some aspects of the pathogenesis of brucellosis [11]. 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 [27]. 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 *B. abortus* causes abortion in pregnant mice by inoculating bacteria on day 4.5 of gestation [15]. 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 an intracellular replication-defective mutant did not induce abortion. Transient interferon-γ (IFN-γ) production induced by infection with *B. abortus* also contributes to infectious abortion, and its neutralization served to prevent abortion.

It has been suggested that during normal pregnancy a shift from a Th1- to a Th2-polarized immune response allows the survival of the fetus [21]. Th2 cytokines such as TGF-β, IL-4 and IL-10 are not only able to prevent immunopathologic events, but also have beneficial effects in successful pregnancy [7]. It has also been suggested that cytokines and chemokines play a role in the pathogenesis of idiopathic recurrent spontaneous abortion [7], but their role in abortion induced by *B. abortus* infection is still unclear.

Regulated upon activation normal T-cell expressed and secreted (RANTES) is a chemokine that has recently been implicated in trophoblast and spermatozoa migration in view of its well-established chemoattractant properties [17, 26]. In the present study, we investigated the pathogenesis of *B. abortus*-induced abortion in pregnant IFN-γ knockout mice and noted that RANTES plays an important role in this process.

## MATERIALS AND METHODS

*Bacterial strains*: All *B. abortus* derivatives were from 544 (ATCC23448) smooth virulent *B. abortus* biovar 1 strains. Ba598 (544Δ*virB4*) was also used in this study [30, 31]. *B. abortus* strains were maintained as frozen glycerol stocks and cultured on Brucella broth (Becton Dickinson, NJ, U.S.A.) or Brucella broth containing 1.5% agar.

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*Mice*: Six to ten-week-old BALB/c female mice were individually mated to 6- to 10-week-old BALB/c male mice. All of these mice were obtained from CLEA Japan (Tokyo, Japan). The normal gestational time for these mice is 19 days, and the vaginal plug was observed on day 0.5 of gestation. In addition, six to eight-week-old IFN-γ knockout mice on the BALB/c background were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). The animal experiments were permitted by Animal Research Committee of Obihiro University of Agriculture and Veterinary Medicine.

*Virulence in pregnant mice*: Groups of three or five pregnant mice were infected intraperitoneally with approximately $10^4$ CFU of brucellae in 0.1 m*l* saline at the indicated days of gestation [15]. On day 18.5 of gestation or when mice died, their fetus, placenta, and spleen 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.

*Cytokine measurement*: Serum levels of RANTES, IFN-γ and MCP-1 were measured for infected and uninfected virgin and pregnant mice. To achieve infection, groups of five mice were inoculated intraperitoneally with approximately $10^4$ CFU of brucellae in 0.1 m*l* saline on day 4.5 of gestation, and blood was collected at 1, 3, 5 or 7 days after infection. Blood was collected at the same times for uninfected mice. On day 18.5 of gestation, uteri were removed, and a judgment was made as to whether mice were pregnant or not. Serum levels of RANTES, IFN-γ and MCP-1 were measured with an enzyme linked immunosorbent assay (ELISA) kit (PIERCE Endogen, Rockford, IL, U.S.A.) according to the instructions of the manufacturer. The cytokine antibody array used was obtained from RayBiotech (Norcross, GA, U.S.A.).

*In vivo depletion of RANTES and IFN-γ*: RANTES and IFN-γ were neutralized in the mice through the use of anti-mouse RANTES monoclonal antibodies (clone 53405) or IFN-γ monoclonal antibodies (clone HB170), administering 200 μg of the respective antibody in a volume of 0.1 m*l* intraperitoneally 24 hr before infection. As a control, mice were injected with 200 μg of normal rat IgG in 0.1 m*l* according to the same schedule as for the mice treated with anti-RANTES or anti-IFN-γ monoclonal antibodies. Bacterial infection was achieved as described above. Blood was collected at 1, 3, 5 or 7 days after infection, and serum levels of RANTES and IFN-γ were measured with an ELISA kit as described above. On day 18.5 of gestation, a judgment was made as to whether mice were pregnant or not as described above.

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

## RESULTS

*B. abortus infection in IFN-γ knockout mice*: We previously reported that transient induction of IFN-γ production is a key event in abortion induced by *B. abortus* infection [15]. In order to clarify the contribution of IFN-γ to infectious abortion, pregnant IFN-γ knockout mice were infected with *B. abortus*, and abortion rates were examined. As reported previously, pregnant normal mice remained alive after *B. abortus* infection (Fig. 1A), but all *B. abortus* infected pregnant IFN-γ knockout mice died within 15 days of infection (Fig. 1A). Bacterial growth was examined in the spleens of infected pregnant IFN-γ knockout mice to determine if bacterial colonization was predominantly in the spleen. Colonization by *B. abortus* was much greater in the spleens of pregnant IFN-γ knockout mice than in those of pregnant normal mice (Fig. 1B). *B. abortus* induced splenomegaly as a consequence of the host inflammatory response in the pregnant normal mice, but splenomegaly was not induced in the pregnant IFN-γ knockout mice (Fig. 1C).

*Transient increase in RANTES in pregnant IFN-γ knock-*

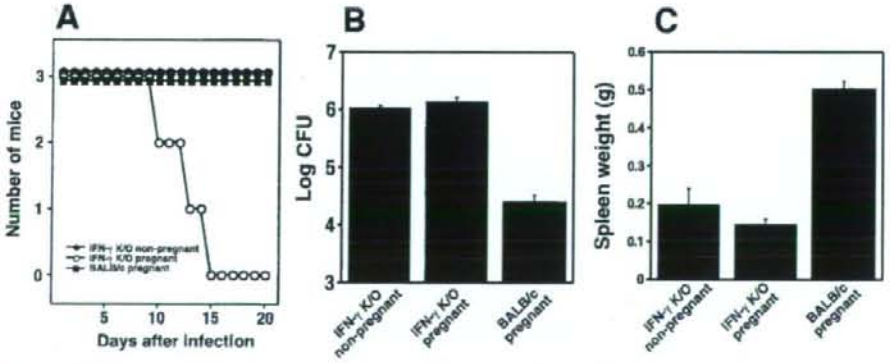


Fig. 1. *B.abortus* infection in pregnant IFN-γ knockout mice. Pregnant (IFN-γ K/O pregnant) and non-pregnant (IFN-γ K/O non-pregnant) IFN-γ knockout mice and pregnant normal mice (BALB/c pregnant) were infected with *B. abortus*. The figure shows number of mice (A), bacterial growth in spleen (B) and spleen weight (C).