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障害保健福祉総合研究事業

優良補助犬の効率的育成と普及に関する生殖工学的研究

平成２１年度　総括研究報告書

研究代表者　鈴木　宏志

平成２２（２０１０）年　４月

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総括研究報告書

優良補助犬の効率的育成と普及に関する生殖工学的研究

主任研究者　鈴木　宏志　帯広畜産大学教授

（研究要旨）障害者の社会参加の促進に資するため、補助犬の人工繁殖技術の開発と実用化および補助犬適性の遺伝子レベルでの診断系の開発を果たし、優秀な補助犬の効率的育成とその啓蒙・普及を達成することを目的に、最終年度では、(I) 盲導犬の人工授精法の普及に関する検討、(II) 盲導犬の卵巣移植法の確立、(III) イヌ胚の凍結保存技術、胚移植技術の開発および(IV) 遺伝子多型の解析による盲導犬適性検査法の開発を行った。

(I) 盲導犬の人工授精法の普及に関する研究：全国５ケ所の盲導犬事業所で継続的人工授精の試行・取り組みがなされるに至っており、これらの活動に対応した親子鑑定サービスを提供した。また、繁殖生産性の維持、向上のためには、感染症の適切なコントロールが重要な課題であるが、ブルセラ症の迅速、的確な摘発のため、血清抗体法およびPCR法による検査系を確立し、全国の盲導犬協会８施設の繁殖犬、育成犬についての検査サービスを継続的に提供した。さらに、イヌ精子の受精能力の評価にマウス卵子を用いた顕微授精系が利用可能であることを示した。(II) 盲導犬の卵巣移植法の確立：移植後の卵巣組織の卵胞数減少抑制に対するアシアロ化エリスロポエチン効果は、全身投与においても局所投与と同様に発揮することを明らかにした。(IV) イヌ胚の凍結保存技術、胚移植技術の開発：これまでに、４頭の受容雌から合計７頭の凍結胚由来の産仔を得ていることから、本研究において開発されたイヌ胚の凍結融解技術は、十分な再現性を確保しているものと考えられる。(V) 遺伝子多型の解析による盲導犬適性検査法の開発：盲導犬および非盲導犬（不合格犬）について、14遺伝子の26多型について解析を行った結果、合計４種類の多型について、盲導犬群と非盲導犬群の遺伝子頻度に有意な差が認められた。これら４種類の多型について、盲導犬群で有意に頻度の高い遺伝子型すべてを持つイヌの合格率は100％であった。しかしながら、最も頻度の低い遺伝子型を持つイヌに盲導犬は含まれていなかった。以上の成績は、性格関連遺伝子の遺伝子多型が、盲導犬適性の指標と成り得ることを示していると思われる。また、進行性網膜委縮症(PRA)摘発のための遺伝子検査サービスを盲導犬事業所に対して継続的に実施した。

A. 研究目的

障害者の社会参加の促進に資するため、補助犬の人工繁殖技術の開発と実用化および補助犬適性の遺伝子レベルでの診断系の開発を果たし、優秀な補助犬の効率的育成とその啓蒙・普及を達成する基礎・応用研究を計画す

る。

身体障害者補助犬の導入によって自立と社会参加を果たし得る障害者は数多く存在しており、その普及には法体系や社会的受け入れ体制の整備とともに、良質な補助犬の育成体制の整備が不可欠である。我が国の盲導犬は、約 1,000 頭が実働しているが、盲導犬希望者は約 4,800 人と推定されている。盲導犬の安定的・効率的繁殖育成は、最も重要な課題のひとつであるが、現在、全国の盲導犬訓練施設では、優れた雌雄の繁殖犬の確保が困難であること、盲導犬の合格率が低いなどの問題を抱えている。事実、我が国の盲導犬普及率は、欧米先進諸国と比較して 1/2～1/10 程度と極めて低く、まったくの「盲導犬後進国」の状況にある。歴史の浅い他の補助犬の育成、利用に至っては、適切な犬種の選定、繁殖システムなどについて、手探りの状態が続いている。さらに、我が国に導入されているラブラドールリトリバーについては、補助犬としては不適格な股関節形成不全症や進行性の網膜委縮症などの重篤な遺伝性疾患のキャリアーが高頻度で存在するといわれており、実態の把握を含めた緊急な対処を要する状況にある。

適切な資質を有する補助犬の安定的な提供は、我が国の身体障害者の経済社会への一層の進出、貢献を促すものである。本研究の成果は、盲導犬や介助犬などの補助犬、あるいは災害救助犬や麻薬探知犬などの資質向上にも寄与することから、国内の安心・安全で快適な社会の構築への寄与のみならず、大きな国際貢献をも果たす。

B. 研究方法

1）盲導犬の人工授精法の普及に関する研究：新規保存液による凍結保存の実用化を図るため、保存液組成の改良を図る。また、他の盲導犬協会への普及を継続するとともに、規模の小さい盲導犬協会に対しては、人工授精サービスを提供するシステムを実現する。

2）盲導犬の卵巣移植法の確立：移植後の卵胞数減少を克服するため、凍結融解卵巣を免疫不全マウスへ異種移植して、抗酸化剤等の投与効果を検討するとともに、卵胞発育、卵子の成熟誘起の可能性について検討し、より効果的な卵巣移植法を見出す。

3）イヌ胚の凍結保存技術、胚移植技術の開発：胚移植による産仔の獲得の効率化のため、凍結融解条件を精査するとともに、非外科的に胚を回収する方法を確立する。

4）遺伝子多型の解析による盲導犬適性検査法の開発：最終年度においても解析対象遺伝子とサンプル数を拡大して、10 遺伝子、600 サンプルの多型解析を実施する。海外（フィンランド）の盲導犬コロニーからのサンプルについても同様の検討を実施して、比較検討する。疾患関連遺伝子については、本年度も盲導犬繁殖コロニーと実働している 950 頭余りの盲導犬について、進行性網膜萎縮症の遺伝子スクリーニングを継続実施することによって、この危険因子を排除するシステムを構築する。

（倫理面への配慮）

ヒトゲノム・遺伝子解析研究、遺伝子組換え実験には該当しない。動物実験に該当するので、研究機関等における動物実験等の実施

に関する基本指針（文部科学省）、厚生労働省における動物実験等の実施に関する基本指針（厚生労働省）、実験動物の飼養及び保管並びに苦痛の軽減に関する基準（環境省）を遵守して動物愛護上の配慮を行う、また、実験に際しては、国立大学法人帯広畜産大学の動物実験指針に沿った実験計画を立案して、計画書を動物実験委員会に提出し、審査、承認を経て実施する。

C. 研究結果

1）盲導犬の人工授精法の普及に関する研究：全国５ケ所の盲導犬事業所で継続的人工授精の試行・取り組みがなされるに至っており、これらの活動に対応した親子鑑定サービスを提供した。これまでの啓蒙、普及活動によって、我が国の盲導犬事業所における人工授精の効果についての理解は確実に進んでいると考えられる。また、繁殖生産性の維持、向上のためには、ネオスポーラ症やブルセラ症などの適切なコントロールが重要な課題であるが、ブルセラ症の迅速、的確な摘発のため、血清抗体法および PCR 法による検査系を確立し、全国の盲導犬協会８施設の繁殖犬、育成犬についての検査サービスを継続的に提供した。

これまでに、卵黄を含む凍結保存液に代わるスキムミルクとグルコースを基礎とする新規のイヌ精子の凍結保存液(SG 液)を開発したが、SG 液にさらに改良を加えた結果、イヌ精子の凍結保存にはトレハロースがより適していることを明らかにし、スキムミルクとトレハロースを基礎とする凍結保存液を用いて凍結保存した精子由来の産仔を得ることに成功した。さらに、凍結融解あるいは凍結乾燥精子の in vitro における受精能力の検査系を開発するために、マウス卵子にイヌ新鮮、凍結融解および凍結乾燥精子を顕微授精して、その後の発生を観察した結果、精子由来の前核形成に至る事を明らかにした。これにより、イヌ精子の受精能力の評価にマウス卵子を用いた顕微授精系が利用可能であることが示唆された。

2）盲導犬の卵巣移植法の確立：卵巣移植術においては、移植後の移植片に対する酸化ストレスあるいは虚血状態によって、移植後の卵巣組織の卵胞の多くが失われるといわれていたが、これがイヌ卵巣においても同様に認められることを明らかにするとともに、この現象の抑制にはアシアロ化したエリスロポエチンの投与が有効であることを示した。加えて、アシアロエリスロポエチンの投与は、全身投与でも局所投与でも同様に機能することも明らかにした。今後は、アシアロエリスロポエチンの投与下でイヌ卵巣の同種移植を試みることが効果的であると考える。

3）イヌ胚の凍結保存技術、胚移植技術の開発：昨年度にエチレングリコールとシュークロースから成るガラス化保存液で回収胚の凍結保存を行い、これをヒト用膀胱鏡を用いて非外科的に移植した結果、世界で初めて、イヌ凍結胚由来の産仔を得ることに成功したが、これまでに、４頭の受容雌から合計７頭の凍結胚由来の産仔を得ていることから、十分な再現性を確保しているものと考えられる。また、卵母細胞を用いた実験から、DAP213 を凍結保存液、クライオトップを支持体とする凍結融解方法(DAP-TOP)が効果的であるとの

成績を得たことから、胚の凍結保存におけるDAP-TOPの有効性について検討中である。

4）遺伝子多型の解析による盲導犬適性検査法の開発：盲導犬および非盲導犬（不合格犬）、それぞれ、約320例のDNAサンプルを血液、口腔粘膜細胞あるいは爪から調整し、14遺伝子の26多型について解析を行った結果、5-HTR1B、DRD4およびGLT-1に存在する合計4種類の多型について、盲導犬群と非盲導犬群の遺伝子頻度に統計学的に有意な差が認められた。これら4種類の多型について、盲導犬群で有意に頻度の高い遺伝子型すべてを持つイヌの合格率は100%（10/10）であった。しかしながら、最も頻度の低い遺伝子型を持つイヌに盲導犬は含まれていなかった(0/4)。以上の成績は、性格関連遺伝子の遺伝子多型が、盲導犬適性の指標と成り得ることを示していると思われる。また、盲導犬事業所に対してPRAの遺伝子検査サービスを継続的に実施した。

D. 考察

1）盲導犬の人工授精法の普及に関する研究：卵黄の代替となる新規の凍結保存液を開発したことは、今後の凍結精子の国際的流通をより容易にする成果であると思われる。今後は、糖とスキムミルクを成分とする新規の凍結保存液が全ての種雄由来の精液に妥当性を有しているか否かの検証が必要である。

2）盲導犬の卵巣移植法の確立：凍結融解卵巣の移植に際して認められる卵胞数の減少抑制には、アシアロ化エリスロポエチンが効果的に作用することを、再現性を持って観察したことから、今後、イヌ同種移植への応用が期待される。また、卵巣移植にアシアロ化エリスロポエチンの投与が有効であることを明らかにしたことは、イヌの卵巣移植だけではなく、ヒトの卵巣移植術の開発にも寄与すると考える。

3）イヌ胚の凍結保存技術、胚移植技術の開発：イヌ胚の凍結保存技術の開発に成功した。しかしながら、移植の成功には再現性はあるものの依然として低率であるため、実用化に向けた一層の研究開発が求められるところである。また、非外科的胚の回収方法の開発には、適切なカテーテルの開発が困難であることから進展が認められていない。胚の凍結保存の実用化を推進する上でも、非外科的回収法の開発が急務である。

4）遺伝子多型の解析による盲導犬適性検査法の開発：これまでの検討によって、盲導犬適性を左右する遺伝子多型が複数同定された。今後は、これらの成績の繁殖業務への応用を試みることが必要である。この技術の応用によって、訓練後の盲導犬の合格率を2倍以上に向上させることが期待できる。本研究の成果は、盲導犬、介助犬等の補助犬のみならず、麻薬探知犬や検疫犬等の他の使役犬の普及や質の向上にも貢献し得ることから、我が国の安全・安心で快適な社会の構築ばかりではなく、国際貢献にも利用し得るものと考える。

E. 結論

研究計画に沿った活動によって、新規のイヌ凍結精子保存液の開発を果たすとともに、人工授精の実用化、普及を進展させた。また、ブルセラ症、進行性網膜委縮症の診断・検査

系を確立し、診断サービスを継続的に提供した。加えて、世界で初めて、凍結胚由来の産仔を、しかも非外科的移植によって得たことは、特筆すべき成果であると考える。今後の本研究の継続的遂行によって、一層の優良補助犬の効率的育成と普及に寄与する成果が得られるものと思われる。

F. 健康危険情報

該当事項なし

G. 研究発表

1. 論文発表

1) Kawase Y, Tachibe T, Hani T, Tateishi H, Jishage K, Suzuki H: Effect of zona incision by Piezo-micromanipulator (ZIP) on the *in vitro* fertilization in 21 transgenic mice lines. *Exp. Anim.* 58: 415-419, 2009.

2) Ishijimja T, Abe Y, Suzuki H: Follicular loss of the cryopreserved canine ovary after xenotransplantation. *J. Mamm. Ova Res.* 26: 61-65, 2009.

3) Suwa Y, Abe Y, Lee DS, Ueta YY, Suzuki H: Individual fertility differences in the frozen-thawed spermatozoa among semen donors in the Labrador Retriever. *Reprod. Med. Biol.* 8: 125-129, 2009.

4) Fukumoto S, Tamaki Y, Igarashi I, Suzuki H, Xuan X: Immunogenicity and growth inhibitory efficiency of the prime-boost immunization regime with DNA followed by recombinant vaccinia virus carrying the P29 gene of *Babesia gibsoni* in dogs. *Exp. Parasitol.* 123: 296-301, 2009.

5) 鈴木宏志：盲導犬の生殖工学、日本生殖内分泌学会雑誌、14：44-47，2009.

6) 鈴木宏志：盲導犬の人工繁殖、日本補助犬科学研究、3：9-16，2009.

7) Watanabe H, Asano T, Abe Y, Fukui Y, Suzuki H: Pronuclear formation of freeze-dried canine spermatozoa microinjected into mouse oocystes. *J. Assist. Reprod. Genet.* 26: 531-536, 2009.

8) Watanabe H, Suzuki H, Fukui Y: Fertilizability, developmental competence, and chromosomal integrity of oocytes microinjected with pre-treated spermatozoa in mice. *Reproduction* 139: 513-521, 2010.

9) Abe Y, Asano T, Ali M, Suzuki H: Vitrification of canine cumulus-oocyte complexes in DAP213 with a cryotop holder. *Reprod. Med. Biol.* In press.

2. 学会発表

1) 阿部靖之、鈴木宏志：イヌ生殖細胞の凍結保存 - 盲導犬の効率的な繁殖を目指して - 第 14 回日本臨床エンブリオロジスト学会 pp32, 2009.

2) 石嶋隆子、阿部靖之、鈴木宏志：凍結融解イヌ卵巣の異種移植におけるアシアロ化エリスロポエチン投与の卵胞数減少抑制効果について　第 50 回日本哺乳動物卵子学会　S45, 2009.

3) Asano T, Abe Y, Suwa, Y, Suzuki H: Cryopreservation of canine spermatozoa in a skim milk/sugar-based extender. International Symposium on Laboratory Animal Science, Bangkok, Thailand, pp106, 2009.

4) Suzuki H, Abe Y, Asano T, Suwa Y: Successful delivery of pups from cryopreserved canine

embryos. International Symposium on Laboratory Animal Science, Bangkok, Thailand, pp98, 2009.

5) 柳本（植田）佳子、松原健一、浅田理央、大嶋なつみ、白砂早織、小林菜苗、諏訪義典、鈴木宏志：セロトニン受容体 1B とグルタミン酸トランスポーターの遺伝子多型が盲導犬適性を左右する 第 32 回日本分子生物学会年会 4P-0905, 2009.

6) 鈴木宏志：精子の凍結乾燥による保存と受精能 第 28 回日本アンドロロジー学会学術大会および第 15 回精子形成・精巣毒性研究会共同開催学会 シンポジウム 2「精子形成 基礎と臨床」pp69, 2009.

7) Abe Y, Ali M, Asano T, Suwa Y, Suzuki H: Improved viability after vitrification of canine oocytes at germinal vesicle stage using DAP213 solution with cryotop holder. 42nd Annual Meeting, Society for the Study of Reproduction, 620, 2009.

8) Suzuki H, Asano T, Suwa Y, Abe Y: Successful delivery of pups from cryopreserved canine embryos. 42nd Annual Meeting, Society for the Study of Reproduction, 619, 2009.

9) 阿部靖之、浅野智由、諏訪義典、鈴木宏志：イヌ胚のガラス化保存法および非外科的な移植法の開発 第 102 回日本繁殖生物学会大会 j91, 2009.

10) Suzuki H: Reproductive bio-technology for the guide dog for the blind in Japan. The 2009 International Symposium, Recent Research Trends using Animal in Biomedical Science. Chungnam National University, Korea. pp18-22, 2009.

11) 鈴木宏志、阿部靖之、浅野智由、諏訪義典：イヌ胚の凍結保存と移植 第 147 回日本獣医学会学術集会 pp265, 2009.

12) 鈴木宏志、阿部靖之、浅野智由、諏訪義典：イヌ胚のガラス化保存 第 148 回日本獣医学会学術集会 pp241, 2009.

13) 鈴木宏志、阿部靖之、浅野智由、諏訪義典：イヌ胚の凍結融解後の産仔への発生 第 56 回日本実験動物学会総会 2PO-099, 2009.

H. 知的財産権の出願・登録状況

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

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雑誌

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鈴木宏志	盲導犬の人工繁殖	日本補助犬科学研究	3	9-16	2009
Watanabe H, Asano T, Abe Y, Fukui Y, Suzuki H	Pronuclear formation of freeze-dried canine spermatozoa microinjected into mouse oocystes	*J. Assist. Reprod. Genet.*	26	531- 536	2009
Kawase Y, Tachibe T, Hani T, Tateishi H, Jishage K, Suzuki H	Effect of zona incision by Piezo-micromanipulator (ZIP) on the *in vitro* fertilization in 21 transgenic mice lines.	*Exp. Anim.*	58	415-419	2009
Fukumoto S, Tamaki Y, Igarashi I, Suzuki H, Xuan X	Immunogenicity and growth inhibitory efficiency of the prime-boost immunization regime with DNA followed by recombinant vaccinia virus carrying the P29 gene of *Babesia gibsoni* in dogs.	*Exp. Parasitol.*	123	296-301	2009
Watanabe H, Suzuki H, Fukui Y	Fertilizability, developmental competence, and chromosomal integrity of oocytes microinjected with pre-treated spermatozoa in mice.	*Reproductio n*	139	513-521	2010
Abe Y, Asano T, Ali M, Suzuki H	Vitrification of canine cumulus-oocyte complexes in DAP213 with a cryotop holder.	*Reprod. Med. Biol.*			In press

—Brief Note—

Follicular Loss of the Cryopreserved Canine Ovary after Xenotransplantation

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Abstract: The effect of cryopreservation and subsequent xenotransplantation on the follicular reserve of the canine ovary by using non obese diabetic-severe combined immunodeficient (NOD-SCID) mice was examined. Vitrified-warmed canine ovarian tissues were placed into the ovarian bursa of mice, and then were removed and subjected to histological examination at 4 weeks after the transplantation. Over 30% of primordial follicles and 65% of early primary follicles survived after cryopreservation. However, regardless of breed or age, percentages of survived primordial follicles and early primary follicles after the transplantation ranged from 0–7% and from 0–15%, respectively. These results indicate that the majority of primordial follicles and early primary follicles in vitrified-warmed canine ovarian tissues disappear after xenotransplantation. Further studies will be required to be able to enhance the survival of transplanted cryopreserved ovarian follicles in canine.

Key words: Canine, Cryopreservation, Ovary, Transplantation

Introduction

Advances in the diagnosis and treatment of cancer have resulted in a growing population of adolescent and adult long-term survivors of malignancies [1] with infertility problems due to induced premature ovarian failure [2]. Although several options are currently available to preserve fertility in cancer patients, cryopreservation of ovarian tissue is the only option available for prepubertal girls and women in need of immediate chemotherapy [3–8]. On the other hand, the cryopreservation of ovarian tissues is a potentially significant technology for the preservation of the genetic resources of working dogs as well as other target animals [9]. However, it has been reported that a large proportion of follicles are lost during the initial ischemia which occurs after transplantation of mouse [10, 11], sheep [12, 13] and human ovaries [14, 15]. Thus, to corroborate the evidence for possible follicular loss after transplantation in canine ovarian tissues, we examined the effect of cryopreservation and subsequent xenotransplantation on the follicular reserve of the canine ovary by using non obese diabetic-severe combined immunodeficient (NOD-SCID) mice.

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Materials and Methods

Female NOD-SCID mice were purchased from a commercial supplier (Charles River Japan, Kanagawa, Japan). All animals were housed in polycarbonate cages, and maintained in a specific pathogen-free environment in light-controlled (lights-on from 07:00 to 19:00) and air-conditioned rooms (temperature: 24 ± 1°C, humidity: 50 ± 10%). They had access to standard laboratory chow (CE-2; CLEA Japan, Tokyo, Japan) and water *ad libitum*. The ovaries from 5-month-old and 6 month-old mixed breeds, a 4-month-old miniature dachshund and a 11-month-old toy poodle were frozen-thawed and transplanted into the ovarian bursa of 8-wk-old NOD-SCID mice. The cryopreservation procedures and ovarian transplantation were performed according to the method of Ishijima *et al.* [9]. Briefly, ovarian tissue was minced into 1.0–1.5 mm cubes, which were immersed in 1 M dimethyl sulfoxide (DMSO) at room temperature for 60 sec and then placed in a 1-ml cryotube (Nalge Nunc International KK, Tokyo, Japan) containing 5 μl of DMSO, and the tube was cooled on ice for 5 min. After addition of DAP 213 (2 M DMSO, 1 M acetamide, 3 M propylene glycol) solution [16] precooled on ice, the tube was cooled on ice for 5 min

then immersed in liquid nitrogen. The grafts were stored in the liquid nitrogen for 2–6 months. For warming, the tube was removed from the liquid nitrogen, the liquid nitrogen in the tube was discarded and then the tube was allowed to stand at room temperature for 60 sec. After the addition of 900 μl of 0.25 M sucrose prewarmed to 37°C into the tube, the suspension was quickly stirred by mild pipetting and washed with PBI [17] five times. A portion of the excised ovaries was fixed with 10% formalin to prepare pre-transplant ovarian tissue samples.

NOD-SCID mice (n = 13) were anesthetized by intraperitoneal administration of sodium pentobarbital (5 mg/ml, Nembutal, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), then the dorsal skin was incised to draw out the ovaries. An incision was made in the lateral side of each ovary to remove the mouse ovary in the ovarian bursa, leaving a part to ensure blood flow to the canine ovarian xenograft after transplantation, and a piece of frozen-thawed canine ovarian tissue was introduced into the ovarian bursa. A hemostatic gelatin sponge (Spongel, S022Y01, Astellas, Japan) was also placed in the ovarian bursa. The skin incision was closed with a clip (9-mm auto clip, 427631, Becton Dickinson). The operated mice were placed on a warm plate until sufficient recovery had occurred to allow movement. At 4 weeks after the operation, the transplanted ovaries were removed and fixed with 10% formalin and subjected to hematoxylin and eosin staining together with the pre-transplant ovarian tissue samples. To evaluate the effects of freezing and thawing, and subsequent xenotransplantation, follicles that visibly contained an ovum (oocyte) with a nucleus were counted according to the classification of Oktay *et al.* [18] as follows. Primordial follicles comprise follicles containing an oocyte partially or completely encapsulated by squamous pregranulosa cells; Early primary follicles are follicles in which at least one of the pregranulosa cells had become columnar (enlarged); Primary follicles are follicles in which all of the granulosa cells exhibit enlargement and a single layer of granulosa cells; Transitional follicles comprise follicles containing an oocyte encapsulated by a 1–2 layer of columnar granulosa cells; Preantral follicles are made up of follicles containing an oocyte encapsulated by more than 2 layers of granulosa cells with no antrum formation; Antral follicles are follicles containing an oocyte encapsulated by more than 2 layers of granulosa cells with antrum formation. For pre-transplant ovarian tissues, ten tissue samples were randomly selected and the number of follicles in the ten tissue samples was counted. The number of follicles in a circle of 900 μm in diameter, i. e., a view field of 0.64 mm^2, containing the highest number of follicles in each selected tissue sample was counted (for a total 10 view fields). This number was recorded as the number of follicles before transplantation. For transplanted ovarian tissue, five sections (7 μm in thickness) were sequentially prepared for a tissue specimen (a block). A total of six graft samples were examined for each experimental group. The distance between sections was 40–50 μm. The number of follicles in a circle of 900 μm in diameter, i. e., a view field of 0.64 mm^2, containing the highest number of follicles in each section, was counted (in a total of 5 fields of view). The survival rates of follicles were calculated as the number of follicles in pre-transplant ovarian tissues / number of follicles in fresh ovarian tissue samples × 100, and the number of follicles in transplanted ovarian tissues / number of follicles in pre-transplant ovarian tissue samples × 100.

The tissues and 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.

Results

On autopsy, cryopreserved canine xenografts were distinguishable and were recovered in 13 of 13 mice (100%), and were identified in 20 of 26 (78%) of the transplanted sites. The average number of primordial, early primary, primary, transitional, preantral and antral follicles and the ratio of each developmental stage of follicles in fresh ovarian sections from the four bitches were varied. Namely, the mean numbers of primordial, early primary, primary, and transitional and preantral follicles per 0.64 square millimeter in the fresh ovary of the 6-month-old mixed breed were 38.4, 2.0, 0.2, and 0, respectively. While, for the 5-month-old mixed breed, the mean numbers of primordial, early primary, primary, transitional and preantral follicles per 0.64 square millimeter in fresh ovary of 6-month-old mixed breed were 8.2, 1.0, 2.4, 1.7 and 1.2 respectively. There were individual differences in the developmental stage of oocytes rather than the breed and/or age differences (Table 1). In addition, it seems that follicles are unevenly distributed in ovarian tissues, because the numbers of primordial and early primary follicles in the 5-month-old mixed breed and the 11-month-old toy poodle showed an increase in the number of follicles after cryopreservation. Namely, survival rates of primordial and early primary follicles after the

Table 1. Follicular loss of canine ovary after cryopreservation and subsequent xenotransplantation at 4 weeks after transplantation

Breed and age	Exp. Group	Mean number of follicles (/0.64 mm^2)					
		Pri mordial	Early primary	Primary	Transitional	Pre antral	Antral
Mixed	Fresh	38.4	2.0	0.2	0	0	0
6M	Cryo*	11.6	1.3	0.3	0	0.1	0
	Tp**	0.8	0.2	0.1	0.1	0.1	0.1
Mixed	Fresh	8.2	1.0	2.4	1.7	1.2	0
5M	Cryo	15.5	1.4	1.5	0.7	0.4	0
	Tp	0	0	0	0	0	0
Toy	Fresh	15.3	2.1	3.0	2.0	2.0	0.1
Poodle	Cryo	47.5	2.6	2.9	1.6	1.4	0
11M	Tp	1.5	0.1	0.1	0.1	0.7	0
Miniature	Fresh	14.8	3.3	4.1	3.6	0.8	0
Dachshund	Cryo	13.4	2.3	2.4	0.9	0.4	0
4M	Tp	0.3	0.3	0.1	0	0	0

*: Crypreserved ovarian tissues. **: Transplanted ovarian tissues.

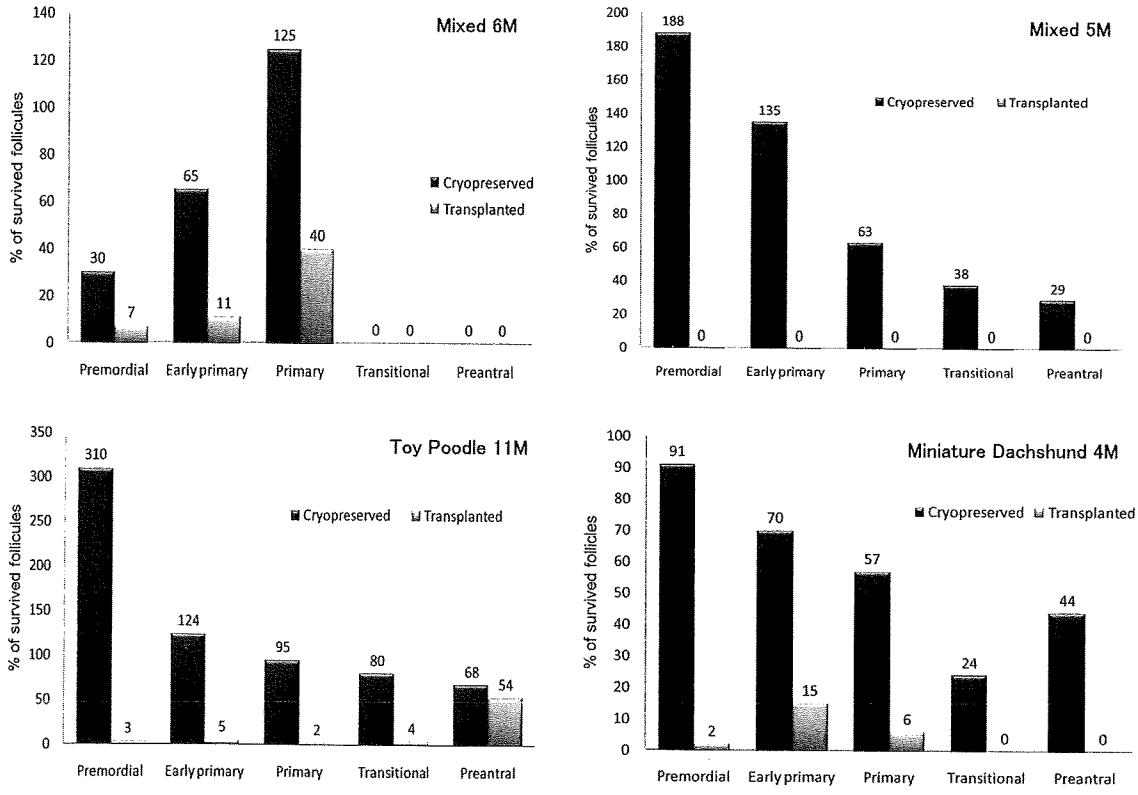

Fig. 1. Percentages of surviving follicles in cryopreserved and subsequently transplanted canine ovaries. The survival rates of follicles were calculated as number of follicles in cryopreserved ovarian tissues / number of follicles in fresh ovarian tissue samples × 100, and number of follicles in transplanted ovarian tissues / number of follicles in cryopreserved ovarian tissue samples × 100.

cryopreservation were 188% and 135% in the 5-month-old mixed breed, and 310% and 124% in the 11-month-old toy poodle, respectively (Fig. 1). However, as shown in Fig. 1, the survival rates of primordial and early primary follicles after the cryopreservation in the 6-month-old mixed breed were 30% and 65%,

respectively. These results indicate that the follicular loss of the canine ovary was not drastically extended by the cryopreservation procedure itself. On the other hand, the detrimental effect of transplantation on the follicular survival was remarkable, even though the follicles were unevenly distributed in the ovarian tissues. The percentages of surviving primordial follicles and early primary follicles ranged from 0–7% and from 0–15%, respectively (Fig. 1).

Discussion

Although the uneven distribution of follicles in the canine ovary make it difficult to interpret the results, it seems that a large proportion of follicles are lost after transplantation of ovaries in canine (Table 1 and Figs.1 and 2) as well as other mammalian species [10–15, 19]. In fact, a high percentage of oocytes as well as granulosa cells survived the cryopreservation and thawing procedure [9, 20–22]. Previously we showed that there was no difference in the morphology and in the average number of primordial and primary follicles between the vitrified-warmed by DAP213 and fresh ovarian canine tissues [9]. It is believed that the reason the primordial follicle is observably resistant to cryoinjury is because the oocyte it contains has a relatively inactive metabolism, as well as the lack of a meiotic spindle, zona-pellucida and cortical granules [2]. Although it has been shown that proliferating cell nuclear antigen was detectable in many of the granulosa cells in the primary follicles of the grafts when canine ovarian tissues were cryopreserved in DAP213 and transferred into the ovarian bursa of NOD-SCID mice [9], the majority of primordial follicles in vitrified-warmed canine ovarian tissues disappeared after transplantation (Table 1 and Fig. 1). The main reason for the follicular loss after cryopreservation and xenografting seems to be the ischemic effect which takes place after transplantation rather than cryopreservation *per se* [11, 23]. Several attempts have been made to prevent the follicular loss of cryopreserved ovarian tissues after transplantation. However, an effective solution has not been found to date. Kim *et al.* [24] showed that a water soluble antioxidant (ascorbic acid) reduces apoptosis in the ovarian cortex by up to 24 h *in vitro*. It has been reported that treatment with vitamin E, a lipid soluble antioxidant, improved the survival of follicles in ovarian grafts by reducing ischemic injury [25]. Further studies will be required to be able to enhance the survival of transplanted cryopreserved ovarian follicles in canine as well as other mammalian species.

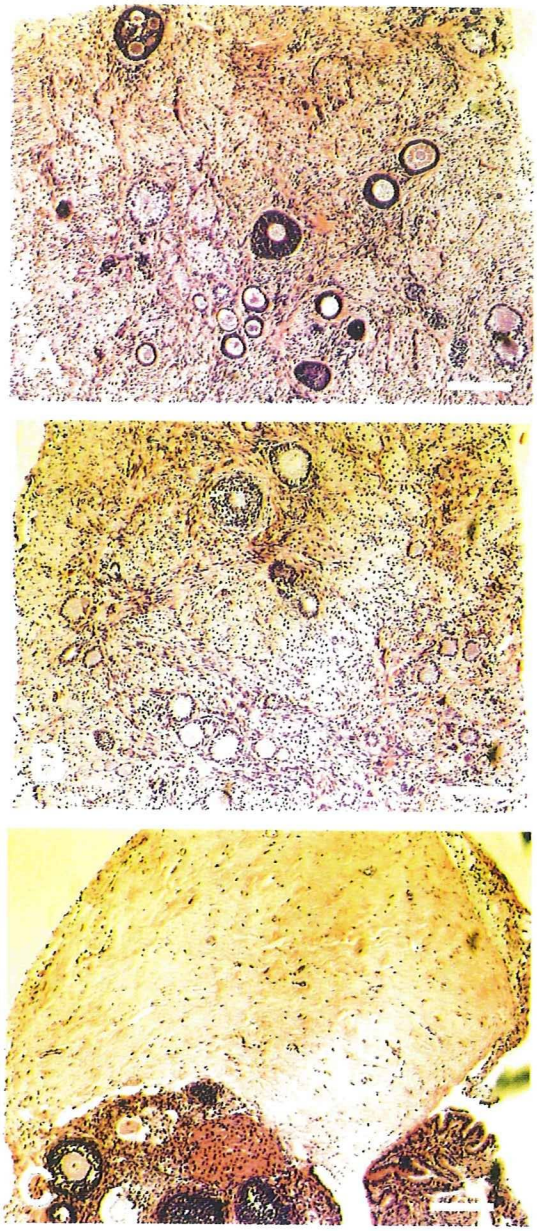

Fig. 2. Hematoxylin and eosin staining of ovarian tissues from a 5-month-old mixed breed. Canine ovaries were cryopreserved by vitrification. The fresh (A) and cryopreserved ovarian tissues (B) are morphologically equivalent. Panel C shows an ovarian tissue recovered from NOD-SCID mice 4 weeks after transplantation of the vitrified-warmed canine ovarian tissues into the bursa. Note the much deeper stain seen in the mouse ovarian tissue (lower) compared to the canine ovary (upper). Canine ovarian grafts (pale stain) successfully adhered to the mouse ovary. Many follicles are seen in canine fresh (A) and cryopreserved (B) ovarian tissues but not in transplanted tissue (C). White bar = 100 μm.

Acknowledgements

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References

1) Blatt, J. (1999): Pregnancy outcome in long-term survivors of childhood cancer. Med. Pediatr. Oncol., 33, 29–33.
2) Donnez, J., Matinez-Madrid, B., Jadoul, P., Van Langendonckt, A., Demylle, D. and Dolmans, M.M. (2006): Ovarian tissue cryopreservation and transplantation: a review. Hum. Reprod. Update, 12, 519–535.
3) Gosden, R.G., Baird, D.T., Wade, J.C. and Webb, R. (1994): Restoration of fertility to oophorectomized sheep by ovarian autografts stored at –196°C. Hum. Reprod., 9, 597–603.
4) Donnez, J. and Bassil, S. (1998): Indications for cryopreservation of ovarian tissue. Hum. Reprod. Update, 4, 248–259.
5) Meirow, D., Ben Yehuda, D., Prus, D., Poliak, A., Schenker, J.G., Rachmilewitz, E.A. and Lewin, A. (1998): Ovarian tissue banking in patients with Hodgkin's disease: is safe? Fertil. Steril., 69, 996–998.
6) Oktay, K., Newton, H., Aubard, Y., Salha, O. and Gosden, R.G. (1998): Cryopreservation of immature human oocytes and ovarian tissue: an emerging technology? Fertil. Steril., 69, 1–7.
7) Donnez, J., Godin, P.A., Qu, J. and Nisolle, M. (2000): Gonadal cryopreservation in young patient with gynaecological malignancy. Curr. Opin. Obstet. Gynecol., 12, 1–9.
8) Donnez, J., Dolmans, M.M., Matinez-Madrid, B., Demylle, D. and Van Langendonckt, A. (2005): The role of cryopreservation for women prior to treatment of malignancy. Curr. Opin. Obstat. Gynecol., 17, 333–338.
9) 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.
10) Candy, C.J., Wood, M.J. and Whittingham, D.G. (1997): Effect of cryoprotectants on the survival of follicles in frozen mouse ovaries. J. Reprod. Fertil., 110, 11–19.
11) Liu, J., Van der Elst, J., Van den Broecke, R. and Dhont, M. (2002): Early massive follicle loss and apoptosis in heterotopically grafted newborn mouse ovaries. Hum. Reprod., 17, 605–611.
12) Aubard, Y., Piver, P., Cognie, Y., Fermeaux, V., Poulin, N. and Driancourt, M.A. (1999): Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. Hum. Reprod., 14, 2149–2154.
13) Baird, D.T., Webb, R., Campbell, B.K., Harkness, L.M. and Gosden, R.G. (1999): Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at –196C. Endocrinology, 140, 462–471.
14) Newton, H., Aubard, Y., Rutherford, A., Sharma, V. and Gosden, R.G. (1996): Low temperature storage and grafting of human ovarian tissue. Hum. Reprod., 11, 1487–1491.
15) Oktay, K., Newton, H. and Gosden, R.G. (2000): Transplantation of cryopreserved human ovarian tissue results in follicle growth initiation in SCID mice. Fertil. Steril., 73, 599–603.
16) Nakagata, N. (1993): Survival of mouse morulae and blastocysts derived from in vitro fertilization after ultra rapid freezing. Jikken Dobutsu, 42, 229–231.
17) Whittingham, D.G. (1974): Embryo banks in the future of developmental genetics. Genetics, 78, 395–402.
18) Oktay, K., Schenken, R.S. and Nelson, J.F. (1995): Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. Biol. Reprod., 53, 295–301.
19) Nisolle, M., Godin, P.A., Casanas-Roux, F., Qu, J., Motta, P. and Donnez, J. (2000): Histological and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. Fertil. Steril., 74, 122–129.
20) Seibzehnrubl, E., Kohl, J., Dittrich, R. and Wildt, L. (2000): Freezing of human ovarian tissue-not the oocytes but the granulose is the problem. Mol. Cell Endocri., 169, 109–111.
21) Martinez-Madrid, B., Dolmans, M. M., Van Langendonckt, A., Defrere, S. and Donnez, J.(2004): Freezing-thawing intact human ovary with its vascular pedicle with a passive cooling device. Fertil. Steril., 82, 1390–1394.
22) Maltaris, T., Beckmann, M.W., Binder, H., Mueller, A., Hoffmann, I., Koelbl, H. and Dittrich, R. (2007): The effect of a GnRH agonist on cryopreserved human ovarian graft in severe combined immunodeficient mice. Reproduction, 133, 503–509.
23) Maltaris, T., Dimmler, A., Muller, A., Binder, H., Hoffmann, I., Kohl, I., Siebzehnrubl, E., Beckmann, M.W. and Dittrich, R. (2005): The use of an open-freezing system with self-seeding for cryopreservation of mouse ovarian tissue. Reprod. Dom. Anim., 40, 250–254.
24) Kim, S.S., Yang, H.W., Kang, H.G., Lee, H.H., Lee, H.C., Ko, D.S. and Gosden, R.G. (2004): Quantitative assessment of ischemic tissue damage in ovarian cortical tissue with or without antioxidant (ascorbic acid) treatment. Fertil. Steril., 82, 679–685.
25) Nugent, D., Newton, H., Gallivan, L. and Gosden, R. G. (1998): Protective effect of vitamin E on ischemia-reperfusion injury in ovarian grafts. J. Reprod. Fertil., 114, 341–346.

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RESEARCH NOTE

Individual fertility differences in the frozen-thawed spermatozoa among semen donors in the Labrador Retriever

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Abstract
Purpose We aimed to validate and determine the possible application of transcervical insemination of frozen semen for improved breeding in guide dogs for the blind in Japan.
Methods From February 2004 to March 2007, a total of 53 Labrador Retriever bitches, used for the breeding of guide dogs for the blind, were transcervically inseminated with frozen-thawed semen from 13 males by means of a cystoscope.
Results The overall whelping and pregnancy rate with the frozen semen was 42%. Pregnancy rates ranged widely from 0 to 100% depending on the semen donor male. Of 13 males, 6 males exhibited severely poor fertility (less than 20% pregnancy rate) and 3 males exhibited high fertility (over 70% pregnancy rate) on artificial insemination. However, the spermatozoa motility after thawing was not significantly different among these dogs. In addition, heterospermic insemination revealed the optimal timing for transcervical insemination with frozen-thawed semen to be by day 6 after the LH surge.
Conclusions Although transcervical insemination of frozen-thawed semen is effective for breeding of guide dogs for the blind, some modification of freeze-thawing procedures might be required to overcome individual fertility differences in the frozen-thawed spermatozoa among semen donor dogs,. In addition, the motility of spermatozoa after thawing might not be an appropriate indicator of the relative fertility of frozen-thawed spermatozoa in dogs.

Keywords Artificial insemination · Cryopreservation · Dog · Heterospermic insemination · Spermatozoa

Introduction

Guide dogs make a remarkable contribution to the quality of life of the blind people, but many countries suffer from an acute shortage of guide dogs such that not all those who need them are able to be supplied. Approximately 1,000 guide dogs for the blind are presently at work in Japan. However, it is estimated that the demand for such guide dogs is approximately 7,800, including latent demand. Although about 120 trained dogs are provided to the society for the blind annually, the actual number of increase is only 20–30 dogs, since 90–100 of them retire due to ageing every year. This is far from an adequate supply of these dogs for the blind in Japan. In addition, only about 30% of the dogs that are trained in fact work out as guide dogs [1]. One of the important challenges from the point of view of animal and veterinary science, as well as social welfare, is to establish a stable and effective breeding and reproduction system for guide dogs for the blind. However, only about 150 breeding dogs, counting both males and females, are currently available in Japan. In addition, it is known that the Labrador Retriever, which is extensively used as a guide dog for the blind in Japan, is a carrier of certain severe, high frequency genetic defects, including some conditions that are specifically problematic for guide dogs such as hip dysplasia, elbow joint dysplasia, cataracts and retinopathy.

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Thus, urgent countermeasures are required for a stable supply of guide dogs of high quality and in high quantity in Japan. Although the development and extension of assisted reproductive techniques in canines, such as artificial insemination, embryo transfer and in vitro fertilization has been remarkable, it is not as successful as in other mammalian species such as mice, pigs, cows and humans. However, successful artificial insemination with frozen canine semen has been well documented [2] since the first conception in 1969 [3]. Thus, in terms of the diffusion of the guide dogs for the blind, one of the solutions associated with these problems seems to be an application of artificial insemination by using frozen-thawed semen imported from developed countries such as the USA, UK and Australia. The transcervical insemination technique seems to be readily applicable to artificial insemination with frozen-thawed spermatozoa for large sized canines such as the Labrador Retriever, which is one of most popular breeds for the guiding dog [4]. Here we report successful delivery, with individual differences in fertility of frozen semen among the dogs, after transcervical artificial insemination with frozen-thawed spermatozoa of the Labrador Retriever.

Materials and methods

Animals and timing of artificial insemination

Both male and female Labrador Retrievers used as breeding dogs for guide dogs for the blind in the Hokkaido Guide Dog Association, Japan, were used in this study in the period from February 2004 to March 2007. Plasma progesterone concentrations of bitches were measured by enzyme linked fluorescent assay (SV-5010, SPOTCHEM VIDAS, Arkray, Kyoto, Japan) daily after the appearance of a blood-tinged vaginal discharge and vaginal swelling. The day when the plasma concentration of progesterone exceeded 2 ng/ml was estimated as the occurrence of the LH surge (Day 0) [5]. Bitches were inseminated with frozen-thawed semen 4–8 days after the estimated LH surge with some exceptions. A total of 13 semen donors with proven fertility in natural mating were used in this study. Unsuccessful pregnancy of the bitches after the experimental artificial insemination by frozen-thawed spermatozoa would considerably influence planning for the production and providing of the guide dogs in the guide dog association. Thus, in some cases, heterospermic insemination using both frozen-thawed and freshly ejaculated semen from different dogs was performed to avoid the failure of conception of the bitches. Paternity for the delivered pups was examined by using micro-satellite markers, as previously described [6].

Freezing and thawing of canine spermatozoa

The method of freezing and thawing of the spermatozoa was similar to that described by Christiansen and Schmidt [7] with diluent developed by Rota et al. [8]. Semen was collected by digital manipulation. The first fraction (seminal plasma) of the ejaculate was discarded, and 3–4 ml of the second (sperm-rich fraction) and third (seminal plasma) fractions mixture were pooled and then centrifuged at 700 g for 10 min. The sperm pellet was resuspended with recovered supernatant by gentle mixing at a concentration of 1×10^9 cells/ml. The sperm suspension was diluted with an egg yolk-Tris-citrate-glucose extender [8] at a concentration of 2×10^8 cells/ml at room temperature and then cooled to 4°C in a refrigerator for 4 h. The sample was further diluted with a second diluent, consisting of the first diluent supplemented with 16% (v/v) of glycerol [8], at a final concentration of 1×10^8 cells/ml. 0.5 ml of the diluted sperm suspension was loaded into a 0.5 ml plastic straw (Type 133, NFA101, Fujihira, Tokyo, Japan). Straws were placed in an atmosphere of liquid nitrogen vapor, i.e. placed horizontally 6 cm above the surface of the liquid nitrogen with a height of 8 cm in a styrene foam box (17.5 cm × 24.5 cm × 17.5 cm), and were kept there for 15 min, and then were plunged into the liquid nitrogen. For thawing, straws were put into a water bath at 38°C for 1 min. After examination of motility under light microscopy at a magnification of ×100, the frozen-thawed spermatozoa were subjected to transcervical insemination [4]. The motility of spermatozoa was classified into the following grades: +++, progressively motile at a high speed; ++, progressively motile at a moderate speed; +, progressively motile at a low speed; ±, motile without progression; −, immotile. The proportion of spermatozoa exhibiting a motility grade of +++ or ++ was used as the parameter for sperm motility.

Transcervical artificial insemination and diagnosis of pregnancy

Inseminations were performed on standing and non-sedated bitches. A catheter (8 Fr, Nippon Sherwood, Tokyo, Japan) equipped with a cystoscope for human use (Karl Storz, Germany) was inserted into the *corpus uteri* through the cervical canal [4]. And then 2×10^8 spermatozoa were inseminated through the catheter. Care was then 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).

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

Research Animals established by Obihiro University of Agriculture and Veterinary Medicine, Japan.

Results and discussion

As shown in Table 1, of the 53 bitches that were inseminated, 22 (42%) gave birth to a litter derived from frozen-thawed spermatozoa. All of the live pups exhibited normal appearance and growth. The motility of frozen-thawed spermatozoa from 13 dogs ranged from 55 to 75%. However, the delivery rates after insemination were considerably different between the dogs, ranging from 0 to 100%. Of 13 males, 6 males exhibited severely poor fertility (less than 20% pregnancy rate) and 3 males exhibited high fertility (over 70% pregnancy rate) on artificial insemination. Since the optimal time for insemination with frozen semen theoretically appeared to be around day 4 after the LH surge [9], and it has been suggested that artificial insemination with frozen semen is successful on days 4 and 6 [10], and days 5 and 7 [11, 12] after the LH surge in canine species, we thus performed transcervical insemination between days 4 and 8 after the estimated LH surge in the Labrador Retriever. However, as shown in Table 2, heterospermic insemination in the present study revealed the optimal timing for transcervical artificial insemination with frozen-thawed semen to be by day 6 after the estimated LH surge. When frozen-thawed spermatozoa were inseminated into the bitches by day 6 after the LH surge in heterospermic insemination, only the frozen-thawed spermatozoa contributed to conception in many of the cases (mating no. 1, 2, 3, 4, 6 and 10 in Table 2). When frozen-thawed spermatozoa from RYU were inseminated into bitches on day 4 or 5, however, those spermatozoa did not participate in the pregnancy (mating no. 11 and 12 in Table 2). Although 3 of 11 inseminations from RYU did not eventuate in pregnancy (Table 1), these failures of pregnancy included the above 2 cases (mating no. 11 and 12) and one abortion which was inseminated with frozen semen on LH 6 and 7. These results indicate that the 'hot spot' for the successful conception might be on day 6 after the LH surge. On the other hand, day 7 after the LH surge seems to be still fertile. Because when the frozen-thawed and freshly ejaculated semen were inseminated into the bitches on days 6 and 7, respectively, both frozen-thawed and freshly ejaculated semen successfully contributed to the conception, (mating no. 5, 7 and 13; Table 2). In mating no. 14, spermatozoa from NUR did not contribute to the conception in spite of insemination on day 6 after the estimated LH surge, but the cause of this fertilization or implantation failure appeared to be an individual difference rather than the timing of insemination. This is because the frozen-thawed spermatozoa from NUR never participated in pregnancy in any of the 7 trials in this study (Table 1).

Table 1 Results of transcervical artificial insemination with frozen-thawed spermatozoa in the Labrador Retriever guide dog

Semen donor	Motility of sperm after thawing (%)	No. of parturition/no. of bitches inseminated (%)	No. pups delivered	Litter size
ODY	65–75	4/5 (80)	13	3.3
RYU	70–75	8/11 (73)	28	3.7
BUZ	75	4/4 (100)	19	4.8
ERI	75	1/1 (100)	7	7.0
MAX	70	1/1 (100)	2	2.0
LEG	70	1/3 (33)	6	6.0
JAS	75	2/6 (33)	8	4.0
PEA	70	1/7 (14)	1	1.0
NUR	55–65	0/7 (0)	0	–
MAR	70	0/3 (0)	0	–
QUI	75	0/2 (0)	0	–
ATO	70	0/1 (0)	0	–
KEN	70	0/2 (0)	0	–
Total		22/53 (42)	84	3.8

Motility of spermatozoa was classified into the following grades: +++ progressively motile at a high speed, ++ progressively motile at a moderate speed, + progressively motile at a low speed; ± motile without progression, − immotile. The proportion of spermatozoa exhibiting a motility grade of +++ or ++ was used as the parameter for sperm motility

It has been reported that whelping rates after transcervical insemination with frozen-thawed spermatozoa using a stainless steel catheter (the Norwegian method) in canines ranged from 60 to 90% in several breeds which were inseminated on the second and third day after estimated ovulation [13]. However, the Norwegian method seems to require considerable skill for successful insemination through the cervix in large breeds such as the Labrador Retriever. The catheter could not be inserted through the cervix in approximately 3% of any sized bitches by the Norwegian method [13]. And less than 10% of the bitches of both large and giant breeds required sedation to enable abdominal fixation of the cervix [13]. In the present study, the catheter was successfully inserted into the uterus through the cervix without any sedation required in any of the trials. Thus, transcervical insemination using a stereoscope with a long sheath as is used for human cystoscopy might be an effective technique for artificial insemination of large sized bitches such as the Labrador Retriever. This technique enables spermatozoa to be deposited non-surgically into the uterus in standing, non-sedated bitches. Since the number of fertile spermatozoa and the longevity of the spermatozoa seem to be reduced, it would be more effective to make the deposit closer to the site of fertilization rather than intra-vaginanally. Since a fiber optic endoscope

Table 2 Results of heterospermic insemination with frozen-thawed and freshly ejaculated spermatozoa in the Labrador Retriever guide dog

Mating no.	Bitch	Dogs	Days after LH surge at insemination					No. of live pups
			4	5	6	7	8	
1	ERZ	BUZ		FT	FT			2
		MAR				NM		0
2	SHI	BUZ		FT	FT			3
		MAR			NM			0
3	PAG	BUZ		FT	FT			5
		RYU				EJ		0
4	BEG	BUZ		FT	FT			7
		FUN					NM	0
5	HAN	RYU			FT	FT		4
		PEA				EJ		4
6	BEL	RYU		FT	FT			6
		PEA				EJ		0
7	IND	RYU			FT	FT		5
		MAR				EJ		2
8	GUC	RYU		FT	FT	FT		5
		PEA				EJ		0
9	DRE	RYU		FT	FT	FT		1
		KEI				NM		0
10	BEL	RYU		FT	FT			1
		PEA				EJ		0
11	ION	RYU	FT					0
		PEA		NM				4
12	KER	RYU		FT				0
		TOR		NM		NM		7
13	HAN	RYU			FT	FT		4
		PEA				EJ		4
14	BEL	ODY	FT					0
		NUR			FT			0
		PEA			EJ		EJ	7
15	CHR	LEG	FT	FT	FT			6
		PAL			EJ	EJ		1

FT frozen-thawed spermatozoa, *EJ* freshly ejaculated spermatozoa, *NM* natural mating

and expensive equipment is required to achieve transcervical insemination, the spread of intrauterine insemination for the breeding of guide dogs, as well as other working dogs, may still face certain difficulties. However, in a view of the urgent demand of the increasing numbers of handicapped people, it is necessary to develop and diffuse new assisted reproductive techniques, including artificial insemination, to improve the effective use of genetic resources in dogs. Although the cause of the lower conceptus number and litter size in pregnancy is still unclear in our present study, it might be influenced by the quality of the frozen-thawed semen, the age and gravidity of the bitches used, and/or the optimal timing of insemination. Further studies would be required to develop a novel cryopreservation method which is available to male dogs proven fertile in natural matings.

The total of 15 homospermic and 38 heterospermic inseminations yielded 84 pups from frozen-thawed semen and 91 pups from freshly ejaculated semen, respectively. Heterospermic insemination in combination with cryopreserved spermatozoa and ejaculated semen appeared to be effective to avoid a shortage of production in a colony of guide dogs.

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References

1. Fuyuno I. Biobank provides leads for selecting guide dogs. Nature. 2007;446:119. doi:10.1038/446119a.
2. Johnston SD, Kustritz MVR, Olson PS. Canine pregnancy. In: Canine and feline theriogenology. Philadelphia: WB Saunders; 2001. p. 66–104.
3. Seager SW. Successful pregnancies utilizing frozen dog semen. Artif Insem Dig. 1969;17:6–7.
4. Wilson MS. Transcervical insemination techniques in the bitch. Vet Clin North Am Small Anim Pract. 2001;31:291–304.
5. Johnston SD, Kustritz MVR, Olson PS. Breeding management and artificial insemination of the bitch. In: Canine and feline theriogenology. Philadelphia: WB Saunders; 2001. p. 41–65.
6. Ichikawa Y, Takagi K, Tsumagari S, Ishihama K, Morita M, Kanemaki M, et al. Canine parentage testing based on microsatellite polymorphism. J Vet Med Sci. 2001;63:1209–13. doi:10.1292/jvms.63.1209.
7. Christiansen IJ, Schmidt M. Deep freezing of dog semen: Klg Vetog Landbohjsk, Inst Sterilitetsforskin. Arsbertn. 1980;23:69–75. in Danish with English abstract.
8. Rota A, Strom B, Linde-Forsberg C, Rodriguez-Martinez H. Effects of Equex STM paste on viability of frozen-thawed dog spermatozoa during in vitro incubation at 38°C. Theriogenology. 1997;47:1093–101. doi:10.1016/S0093-691X(97)00066-6.
9. Linde-Forsberg C. Achieving canine pregnancy by using frozen or chilled extended semen. Vet Clin North Am Small Anim Pract. 1991;21:467–85.
10. Linde-Forsberg C. Artificial insemination with fresh, chilled, extended, and frozen-thawed semen in the dog. Semin Vet Med Surg (Small Anim). 1995;10:48–58.
11. Nishiyama T, Kinugasa T, Kimura T, Watanabe G, Taya K, Tsumagari S, et al. Determination of optimal time for mating by artificial insemination with chilled semen using luteinizing hormone surge as an indicator in beagles. J Am Anim Hosp Assoc. 1999;35:348–52.
12. Tsumagari S, Ichikawa Y, Toriumi H, Ishihara K, Morita M, Kanamaki M, et al. Optimal timing for canine artificial insemination with frozen semen and parentage testing by microsatellite markers in superfecundency. J Vet Med Sci. 2003;65:1003–5. doi:10.1292/jvms.65.1003.
13. Thomassen R, Sanson G, Krogenaes A, Fougner JA, Anderson Berg K, Farstad W. Artificial insemination with frozen semen in dogs: a retrospective study of 10 years using a non-surgical approach. Theriogenology. 2006;66:1645–50. doi:10.1016/j.theriogenology.2006.01.022.