Elucidation of infertility in crossbred cattle-yak and development of yak semen preservation method

November 2022

Megumi Nagahara

CONTENTS

ABBREVIATION

AcK9	Acetyl-histone H3 Lys9
AI	Artificial Insemination
ANOVA	Analysis of variance
CASA	The computer-assisted sperm analysis
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9
DNA	Deoxyribonucleic acid
FITC-PNA	Fluorescein isothiocyanate-labelled peanut agglutinin
GGTA1	Glycoprotein galactosyltransferase alpha-1,3
LN2	Liquid nitrogen
OEP	Orvus ES Paste
PCNA	Proliferating cell nuclear antigen
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

INTRODUCTION

The yak is a member of the bovine family, characterized by its long hair and adaption to hypoxia, which allows it to inhabit high altitudes of 2000 to 5000 m above the forest limit, where it would be difficult for other animals to live (Wiener et al. 2003, Zhang et al. 2021, Anand et al. 1986, Barsila et al. 2015). The value of yaks as livestock is closely related to nomadic life. Milk products are processed into milk, cheese, and yogurt and consumed as raw milk and milk wine. Meat and blood are boiled, roasted, or preserved as dried meat or sausage. Their long coats can be used as winter clothing and luxury garments, producing their income for nomads. They are also useful as carriers of gels and cargo during migration, and their droppings are used as fuel (Wiener et al. 2003, Robert et al. 2021). In addition, livestock owned by Mongolian nomads include not only yaks but also indigenous cattle, therefore there are also included cattle-yak hybrids. Cattle-yak hybrids are larger than yaks, provide more milk and meat, and are stronger, making them more useful for handling cargo (Wiener et al. 2003). However, cattle-yak bulls lack spermatogenesis and are sterile.

Efficient livestock management is important for farmers to secure a stable food source and income. In Mongolia, where it is very cold in winter, yak reach sexual maturity slowly because of seasonal variations in nutritional status, requiring 2 to 3 years for females (Zi et

al. 2003). Spermatogenesis is not observed in males until they are 2 years old or older (Wiener et al. 2003). In addition, sperm vitality, concentration, and quantity are affected by change in season (Sarker et al. 2009). Therefore, mating should be concentrated in the summer when vegetation is thick and food is abundant. In Mongolia, yak breeding relies on natural mating. Artificial insemination (AI) is not widespread in nomadic herds (Wiener et al. 2003). Although breeding depends on natural mating, it has the advantage of no human involvement. Disadvantages also exist, such as the transmission of sexual diseases and individuals that do not conceive (McGowan et al. 1999). In addition, repeated breeding in a restricted population can increase the inbreeding coefficient (Rokouei et al. 2003, Ríos-Utrera et al. 2020). If outside bloodlines are incorporated, bulls must be moved from one herd to another to coincide with the estrus season. Thus, tremendous effort and time must be spent to ensure mating. However, using frozen semen and AI technology can solve this problem. Therefore, as described in Chapter 1, we addressed the development and usefulness of onsite yak semen cryopreservation methods in Mongolia.

Various factors are key to the success of semen cryopreservation, including sufficient quality sperm, freezing protocols, and freezing extender (Barbas et al. 2009). Extensive research has been conducted on substances that reduce the loss of vitality after freeze-thaw, and the usefulness of glycerol and the surfactant Orvus ES Paste (OEP) or the addition of

Equex STM Paste to protect sperm from frost damage has been demonstrated in pigs, dogs, and cats (Pursel et al. 1978, Axner et al. 2004, Mizutani et al. 2010, Rota et al. 1997). OEP and Equex STM Paste are water-soluble anionic detergents that solubilize activators. The addition of Equex STM Paste has been shown to improve motility after freezing and thawing in cattle (Arriola et al. 1987); however, there have been no reports on frozen semen preservation in yaks.

Sperm for cryopreservation are generally ejaculated by artificial vagina or electrical stimulation. However, there are reports on the usefulness of frozen semen made from epididymis-derived sperm by effectively utilizing testes recovered from accidental death or castration of valuable wild or domestic animals (Martins et al. 2007). In deer, sperm were collected from the epididymis of a hunted buck, frozen, and a doe was artificially inseminated. Fertilized eggs were collected by uterine washing, embryos were frozen, and inseminated in the doe to produce fawns (Zomborszky et al. 1999). Utilizing such a valuable resource preserves biodiversity and contributes to yak production. Therefore, in Chapter 1 of this study, we investigated the usefulness of adding OEP to frozen extenders and examined the effective methods of yak semen cryopreservation using sperm derived from the artificial vaginal ejaculation method and epididymis collection following castration.

We then turned our attention to thawing and refreezing the semen. In recent years, sex-sorted semen in cattle has been effective for sex-controlled breeding, and sex-sorted semen obtained using flow cytometry has been widely used (Johnson et al. 1989, Butler et al. 2014). However, to use imported semen or other precious semen as sex-sorted semen, it necessary to be sorted and refrozen after thawing. The benefits of refreezing include the usefulness of frozen semen from wild animals and prime sires for conservation in genome source banking (Wildt et al. 2000, Saragusty et al. 2009). Double-freezing techniques with ram semen have reported fertile specimens (Hollinshead et al. 2004, Graaf et al. 2007). In addition, it has been reported that conception is possible in cows with semen frozen once in large volumes, thawed, and refrozen into straws (Saragusty et al. 2009, Arav et al. 2002). However, the additional cold stress of refreezing results in the death of 40 to 50% of the sperm (Watson et al. 2000). Therefore, it is important to improve motility and viability after refreezing and thawing. To preserve this valuable frozen semen and utilize it for yak sex determination and reproductive research, it is essential to improve the quality of the frozen semen after refreezing. Therefore, in Chapter 2, we report our investigation of the effects of adding OPE to improve the quality of frozen semen after refreezing in cows.

In addition, normal spermatogenesis requires germ cell proliferation, differentiation, and apoptosis, and the correct execution of these processes results in spermatogenesis. However,

it has been reported that epigenomic abnormalities in each stage and cell can lead to abnormal molecular expression and apoptosis imbalances, resulting in spermatogenesis arrest (Li et al. 2020, Zhang et al. 2020, Gao et al. 2020, Yin et al. 2020). Reports on cattle-yak hybrids have shown that meiosis is altered at the molecular level (Cai et al. 2017, Wu et al. 2020, Liu et al. 2018, Robert et al. 2021). However, no studies on cell proliferation have been reported.

In Chapter 3, we focused on proliferating the cell nuclear antigen (PCNA), a known marker of cell proliferation in seminiferous tubules, and examined cell proliferation in sperm-associated cells in cattle-yak crossbreds. PCNA plays an important role in cell proliferation related to DNA replication, DNA repair, and cell cycle regulation (Elizabeth et al. 2016, Wrobel et al. 1995), and its expression increases during DNA synthesis. For normal spermatogenesis, the balance between cell proliferation and apoptosis is crucial and requires proper functioning. Therefore, we confirmed whether cell proliferation was normal by examining PCNA expression in the seminiferous tubules of hybrids that did not undergo normal spermatogenesis. In addition, because apoptosis is an essential factor in spermatogenesis, we confirmed apoptosis in sperm-related cells in the seminiferous tubules using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay and examined the balance between cell proliferation and apoptosis.

By synthesizing these three studies, we believe we can contribute to developing of a semen preservation method for yaks in Mongolia and elucidating the reason for infertility in cattle-yak crossbreds. We planned these experiments to provide useful information for people who depend on livestock products for their livelihood.

Chapter 1

Effects of Orvus Es Paste (OEP) on the motility and viability of yak (*bos grunniens*) epididymal and ejaculated spermatozoa after freezing and thawing

1. Introduction

In Mongolia, yaks (*Bos grunniens*) inhabit areas above the tree line at 2000 m in the north that extend to 3000 m in the Mongolian Altai and provide meat and milk to indigenous people in these regions in which few other animals can survive. Yak-cattle hybrids (*Bos taurus*) are larger, produce more milk, and are better able to withstand warmer climates at lower altitudes than yak (Wiener et al., 2003). Therefore, crossbreeding with cattle has been desired to increase milk and meat production in these regions. In yak bulls, spermatozoa have not been observed in the epididymal fluid before the age of 2 years (Wiener et al., 2003). Moreover, seasonal effects on the quality of yak semen (i.e., motility, concentration and ejaculate volume of spermatozoa) have been demonstrated (Sarkar et al., 2009). In Mongolia, artificial insemination (AI) programs are not generally used for the production of new generations of yak or other species because mating is performed naturally. Thus, the use of AI with frozen semen seems to have numerous benefits over natural mating that include breeding and selection schedules that contribute to increasing the production of Mongolian yak and hybrids.

The appropriate retrieval and cryopreservation of epididymal spermatozoa following the accidental death of a valuable yak and from wild yak would also greatly aid not only the preservation of biodiversity but also the production of yak and hybrids. The success of

semen cryopreservation depends on several factors that include initial quality of the semen samples, the cryopreservation protocol and the use of freezing extenders (Barbas and Mascarenhas, 2009). Currently, combinations of glycerol and detergents such as Orvus ES Paste (OEP) and Equex STM Paste are widely used, and the benefits of the use of such combination have been documented in several species; e.g., pigs (Pursel et al., 1978), cats (Axner et al., 2004), and dogs (Rota et al., 1997; Mizutani et al., 2010). OEP and Equex STM Paste are known to contain a water-soluble anionic detergent that solubilises active molecules. Arriola and Foote (Arriola and Foote, 1987) reported that a freezing extender supplemented with Equex STM Paste improves the post-thaw motility of bull spermatozoa. However, there are no reports concerning the use of detergents for the cryopreservation of yak spermatozoa. Thus, the present study was conducted to evaluate the potential effects of OEP on the cryopreservation of epididymal and ejaculated yak spermatozoa.

2. Materials and Methods

Animals

Semen collection was performed at a yak farm in the district of Bayandelger, Mongolia. The yak bulls were fed ad libitum with grass in the grassland. Epididymal and ejaculated yak semen samples collected from ten bulls (2.5-5 years old) and one bull (4 years old), respectively.

Collection and dilution of yak epididymal and ejaculated spermatozoa

The epididymis–testicle complexes were dissected by surgical castration (Chase et al., 1995), and the spermatozoa were immediately collected from the epididymides of each pair according to the method described by Kikuchi et al. (Kikuchi et al., 1998) with minor modifications. Briefly, the luminal fluid containing the spermatozoa was flushed from the distal portion of the cauda epididymis with saline solution (approximately 5 mL) using a syringe. The semen samples from 10 yak bulls were combined and centrifuged at 500 × *g* for 5 min at room temperature to remove the supernatant. The precipitated spermatozoa were gently resuspended first in Tris-egg yolk extender, which consisted of 13.63 g Tris [hydroxymethyl] aminomethane (Tris; Sigma-Aldrich, St. Louis, MO, USA), 7.62 g citric acid monohydrate (Wako Pure Chemical Industries Ltd.), 3.75 g fructose (Sigma-Aldrich), 15.0 g lactose (Wako Pure Chemical Industries Ltd.), 27.0 g raffinose (Wako Pure Chemical Industries Ltd.), 0.09 g theophylline (Sigma-Aldrich), 200 mL egg-yolk, 1,000,000 IU penicillin (*Meiji* Seika Co., *Tokyo*, Japan), and 1 g streptomycin (*Meiji* Seika Co.) and was adjusted to 1,000 mL with distilled water. A polystyrene conical tube containing the diluted spermatozoa suspension was transferred to a 500-mL glass beaker containing 350–400 mL of water at room temperature, which was then maintained at approximately 7°C for 24 h.

The ejaculated spermatozoa were collected via an artificial vagina (inside temperature: 43°C) using an oestrous cow (*Bos taurus*). After the semen collection, the spermatozoa were immediately diluted with an equal volume of the first Tris-egg yolk extender and cooled to approximately 7°C for 3 h as described above.

Freezing of the yak spermatozoa

Before freezing, the epididymal and ejaculated spermatozoa were diluted to final concentrations of 4×10^7 cells/mL with the first Tris-egg yolk extender and then divided into 4 aliquots. The extended spermatozoa suspensions were then mixed with a one-half volume of the second extender (i.e., the first Tris-egg yolk extender with 13% [v/v] glycerol) supplemented with various concentrations (0%, 0.75%, 1.5%, and 3% [v/v]) of Orvus ES paste (OEP; Miyazaki-kagaku, Tokyo, Japan). After 5 min at approximately 7°C, an additional one-half volume of the second extender was added to the spermatozoa suspensions to achieve the final concentrations of 0%, 0.375%, 0.75%, and 1.5% (v/v) of OEP and 6.5% glycerol. The concentrations of the spermatozoa were adjusted to one-half (2×10^7 cells/mL). Aliquots of the spermatozoa suspensions were then immediately loaded into 0.5-ml plastic straws (Fujihira Co., Tokyo, Japan), which were placed on a Styrofoam

plate in liquid nitrogen (LN_2) vapour (4 cm above the surface of the LN_2) and frozen. The straws were kept on the plate for 20 min and then plunged into the LN_2 for storage.

Sperm quality assessments

The quality of the frozen spermatozoa was evaluated after the thawing of straws in a 38 °C water bath. Each frozen-thawed sample was diluted ten-fold with the first Tris-egg yolk extender. To assess the motilities and viabilities of the spermatozoa before and after incubation for 3 h, the sample tubes containing the diluted spermatozoa were placed in a 38 °C water bath and incubated for 3 h.

Quantities (approximately 30 μL) of the sperm suspensions were transferred to a pre-warmed glass slides, and the motilities of the spermatozoa were immediately examined under a phase-contrast microscope. Sperm motility is expressed as a motility index that was calculated according to the following method described by Namula *et al.* (Namula et al., 2014).

$$\text{Sperm motility index} = \frac{100\,w + 75\,x + 50\,y + 25\,z}{100}$$

In this equation, w is the percentage of spermatozoa that exhibited rapid progressive motion (relative to all of the spermatozoa in the assay mixture), x indicates the percentage

that exhibited slow progressive motion, *y* signifies the percentage that exhibited rotational or non-progressive motion, and *z* denotes the percentage that exhibited faint or pendulum-like motion. The assessments were made in three fields from each aliquot of each sample using a phase-contrast microscope (100×, Nikon E100; Nikon Corp., Tokyo, Japan).

Sperm viability was assessed using eosin-nigrosin staining (Bamba, 1988). The staining was performed by mixing an aliquot of spermatozoa with eosin–nigrosin solution (1:1 dilution) for 30 sec before preparing a smear and drying. The live (i.e., membrane-intact) spermatozoa were left unstained, and the dead (i.e., membrane-damaged) spermatozoa were stained pink or red. One hundred spermatozoa were counted over at least two different fields. The sperm viability is expressed as the mean percentage of live spermatozoa.

Statistical analyses

Statistical significance was inferred based on analyses of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) tests that were performed with STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). All percentage data were subjected to arc sin transformation prior to statistical analysis. The data are

expressed as the means ± the SEMs. The differences with probability values (P) of 0.05 or less were regarded as significant.

3. Results

Quality of the epididymal yak spermatozoa that were frozen with OEP

When the motilities and viabilities of the frozen-thawed epididymal spermatozoa were analysed prior to post-thaw incubation, the motility indices of the spermatozoa that were frozen with 0.375 and 0.75% OEP were found to be significantly greater ($P < 0.05$) than those of the control spermatozoa that were frozen without OEP (Table 1). Moreover, the viability rates of the spermatozoa that were frozen with OEP were significantly greater ($P < 0.05$) than those of the control spermatozoa irrespective of the concentration of OEP. Following 3 h of incubation, there were no significant differences in the motility indices of the spermatozoa between the groups. However, the viability rates of the spermatozoa that were frozen with OEP remained significantly greater ($P < 0.05$) than those of the control spermatozoa irrespective of the concentration of OEP.

Table 1. Sperm motilities and viabilities of yak epididymal spermatozoa that were cryopreserved with various concentrations of Orvus Es Paste (OEP) before and after 3 h of post-thaw incubation†

Concentration of	Before incubation		After 3 h of incubation	
OEP (%)	Motility	Viability	Motility	Viability
0	31.7 ± 0.9^{a}	36.2 ± 5.4^{a}	17.3 ± 3.8	28.3 ± 3.2^{a}
0.375	$47.5 \pm 1.0^{b, c}$	75.8 ± 2.0^{b}	27.1 ± 8.6	71.2 ± 3.4^{b}
0.75	55.4 ± 3.3^{b}	78.7 ± 3.1^{b}	39.4 ± 2.2	73.0 ± 6.1^{b}
1.5	$40.0 \pm 4.0^{a, c}$	74.8 ± 2.2^{b}	32.7 ± 10.9	65.5 ± 2.8^{b}

†Three replicate trials were performed. The percentages are presented as the means ± the SEMs.[a-c]The values with different superscripts in the same column are significantly different ($P < 0.05$).

Table 2. Sperm motilities and viabilities of yak ejaculated spermatozoa that were cryopreserved with various concentrations of Orvus Es Paste (OEP) before and after 3 h of post-thaw incubation†

Concentration of	Before incubation		After 3 h of incubation	
OEP (%)	Motility	Viability	Motility	Viability
0	8.4 ± 2.5^{a}	9.3 ± 2.5^{a}	0^{a}	1.7 ± 0.2^{a}
0.375	29.4 ± 0.6^{b}	$15.8 \pm 1.2^{a, b}$	0.6 ± 0.6^{a}	$5.2 \pm 0.4^{a, b}$
0.75	30.8 ± 5.5^{b}	17.5 ± 2.8^{b}	0.2 ± 0.2^{a}	7.3 ± 1.2^{b}
1.5	35.0 ± 10.5^{b}	19.7 ± 2.5^{b}	2.9 ± 1.1^{b}	$5.0 \pm 1.9^{a, b}$

†Three replicate trials were performed. The percentages are presented as the means ± the SEMs.[a-c]The values with different superscripts in the same column are significantly different ($P < 0.05$).

Quality of ejaculated yak spermatozoa that were frozen with OEP

When the motilities and viabilities of the frozen-thawed ejaculated spermatozoa were analysed prior to the post-thaw incubation, the motility indices of the spermatozoa that were frozen with OEP were significantly greater ($P < 0.05$) than those of the control spermatozoa irrespective of the concentration of OEP (Table 2). Moreover, the viability rates of the spermatozoa that were frozen with 0. 75% and 1.5% OEP were significantly greater ($P < 0.05$) than those of the control spermatozoa. After 3 h of incubation, the motility indices and viability rates of the spermatozoa drastically decreased irrespective of the OEP concentration. Compared to the control spermatozoa, greater motility indices and viability rates were observed in the spermatozoa that were frozen with 1.5% and 0.75% OEP, respectively, ($P < 0.05$).

4. Discussion

In this study, we demonstrated that the addition of OEP to the freezing extender significantly improved the mean motility and viability values immediately after thawing in both the epididymal and ejaculated yak spermatozoa. Our results agree with those of previous studies that have reported that the presence of OEP in the freezing extender can sustain the motility and survival of boar and feline spermatozoa (Pursel et al., 1978;

Mizutani et al., 2010). It has been suggested that the use of OEP and glycerol for the cryopreservation of semen protects the acrosome caps of the sperm and thereby increases and maintains post-thaw sperm motility (Pursel et al., 1978; Tsutsui et al., 2000). OEP primarily contains sodium dodecyl sulfate (SDS), which might be act by modifying the structure of egg yolk lipoproteins in the extracellular medium (Arriola and Foote, 1987). The inclusion of SDS in freezing extenders has proven to be beneficial to the cryopreservation of the spermatozoa of a number of domestic and wildlife species (Holt, 2000). OEP has been suggested to only exert its beneficial effects on sperm function in the presence of egg yolk (Hofmo and Almlid, 1991). Therefore, the precise mechanism by which OEP improves sperm cryosurvival is indirect, and its specific action remains unknown.

It has been demonstrated that 0.5-1.5% OEP is the optimum concentration for the preservation of sperm motility and the acrosomes of spermatozoa during the freeze-thaw process (Pursel et al., 1978; Tsutsui et al., 2000; Mizutani et al., 2010). Moreover, epididymal and ejaculated spermatozoa have been suggested to have different susceptibilities to cold shock (White, 1993). In the present study, we found that the addition of OEP at 0.375-1.5% improved the motility and viability of yak spermatozoa immediately after thawing. Although the mean motility and viability values of the frozen-

thawed spermatozoa differed between the epididymal and ejaculated samples, the addition of 0.75% OEP was effective in the preservation of both types of spermatozoa. Therefore, these results indicate that 0.75% OEP is the optimum concentration for freezing extenders for yak spermatozoa.

Alterations in cell membrane properties that occur during the freeze-thaw process change the stability and water permeability of the cell. Membrane fusogenicity and the responses of signal transduction pathways might also be affected by such changes and thus support the notion that post-thaw sperm longevity is reduced by accelerated capacitation (Watson, 1995). In the present study, we found that the beneficial effects of the addition of 0.75% OEP on motility disappeared after 3 h of incubation following thawing in both the ejaculated and epididymal spermatozoa. However, the addition of 0.75% OEP exhibited sustained beneficial effects on the viability of the post-thaw spermatozoa even after incubation. It has been suggested that the SDS in OEP stabilises the sperm membrane and delays capacitation-like changes that eventually lead to the acrosome reaction and subsequent cell death (Pena et al., 2003). Therefore, our findings indicate that sperm longevity following thawing decreased as assessed by motility, but the presence of OEP in the freezing extender might sustain sperm viability by protecting the sperm membrane.

In conclusion, the results presented herein demonstrated that the addition of 0.75% OEP to the freezing extender improved the motilities and viabilities of both epididymal and ejaculated yak spermatozoa. However, in the spermatozoa of both types, the effects of the addition of OEP on motility disappeared after 3 h of incubation, while the effects on viability were sustained. Therefore, further studies are warranted to compare the effectiveness of the addition of OEP in increasing conception rates following artificial insemination with frozen-thawed spermatozoa.

Chapter 2

Effects of Orvus Es Paste (OEP) on the viability of bull spermatozoa after double freezing and thawing

1. Introduction

Controlling the sex ratio permits faster genetic progress and higher productivity due to the elimination of the unwanted sex. The sex pre-selection of semen is beneficial for livestock production because inseminations can be planned to produce a specific sex. Sexed semen using a flow cytometer/sperm sorter is now widely available in dairy industries around the world and has become a commercial application. Flow cytometric sex sorting is only one useable and reasonably accurate method for sexing sperm, in which the sperm are sorted by the equipment on the basis of a 4% difference in DNA content between bovine sperm containing X and Y chromosomes (Butler, Hutchinson, Cromie, & Shalloo, 2014; Johnson, Flook, & Hawk, 1989). On the other hand, the re-freezing of spermatozoa is necessary for flow cytometric sex sorting when using semen from proven bulls that have been already frozen-stored or imported or when the sex-sorting facility is located at a long distance from where the semen are used. If the frozen-thawed semen is sorted and then refrozen and still keeps its functionality to be used for artificial insemination (AI) or other assisted reproductive technologies, it would enable shipping after sorting. To date, some studies in ram semen demonstrate that the double freezing of sex-sorted spermatozoa is possible (de Graaf, Evans, Maxwell, Cran, & O'Brien, 2007; Hollinshead et al., 2004). It is suggested that dead or damaged cells are sorted out, and thus

the sample used for the second freezing is rich in viable, motile cells (Saragusty, Gacitua, Zeron, Rozenboim, & Arav, 2009). However, it remains unclear whether more than one freeze–thaw cycle affects the viability of bull spermatozoa and their functionality.

Freezing conditions interfere with semen quality, and the low fertility of frozen–thawed semen is associated with the damage that occurs during cryopreservation. It is generally assumed that 40–50% of the spermatozoa do not survive the freezing and thawing process, even with optimized protocols (Watson, 2000). Many factors involved in the cryopreservation of semen directly or indirectly interfere with spermatozoa viability after thawing, of which a semen extender plays a significant role in successful semen freezing (Layek, Mohanty, Kumaresan, & Parks, 2016). Currently, combinations of glycerol and detergents, such as Orvus ES Paste (OEP) and Equex STM Paste, are reported to benefit successful freezing in several species; e.g., pigs (Pursel, Schulman, & Johnson, 1978), cats (Axner, Hermansson, & Linde-Forsberg, 2004), and dogs (Mizutani et al., 2010; Rota, Strom, Linde-Forsberg, & Rodriguez-Martinez, 1997). OEP and Equex STM Paste contain a water-soluble anionic detergent that solubilizes active molecules. The supplementation of the detergents to a freezing extender is demonstrated to improve the post-thaw motility of bovine and Yak spermatozoa (Arriola & Foote, 1987; Shimazaki et

al., 2015). Thus, the present study was conducted to evaluate the potential effects of OEP on the refreezing of bull spermatozoa.

2. Materials and methods

Semen

All the semen samples used in the present study were frozen according to the standard procedures that use a modified Tris-egg yolk extender, which consists of 13.63 g Tris [hydroxymethyl] aminomethane (Tris; Sigma-Aldrich, St. Louis, MO, USA), 7.62 g citric acid monohydrate (Wako Pure Chemical Industries Ltd., Osaka, Japan), 3.75 g fructose (Sigma-Aldrich), 15.0 g lactose (Wako Pure Chemical Industries Ltd.), 27.0 g raffinose (Wako Pure Chemical Industries Ltd.), 0.09 g theophylline (Sigma-Aldrich), 200 ml egg-yolk, 1,000,000 IU penicillin (Meiji Seika Co., Tokyo, Japan), and 1 g streptomycin (Meiji Seika Co.) and was adjusted to 1000 ml with distilled water (Takahashi, 2015). The ejaculated samples were collected from six Japanese Black bulls (3-9 years old) raised in the Yamaguchi Prefectural Agriculture & Forestry General Technology Center (Yamaguchi, Japan) that were frozen in the Tris-egg yolk extender,

containing a final concentration of 6.5% glycerol (spermatozoa concentration, 5×10^7 cells/ml) and then were packed in 0.5-ml plastic straws.

Semen refreezing

After thawing the frozen straws (the same batches made from one ejaculate from each bull), the semen was expelled into a polystyrene conical tube containing modified phosphate-buffered saline (m-PBS; Nihonzenyaku, Fukushima, Japan) and then washed by centrifugation at 650 × g for 5 min. The pellet of the spermatozoa was gently resuspended, first, in Tris-egg yolk extender. After the suspension, the spermatozoa were diluted to final concentrations of 6×10^7 cells/ml with the first Tris-egg yolk extender. A polystyrene conical tube containing the spermatozoa suspension was transferred to a 500-ml glass beaker containing 350 ml of water at room temperature, which was then maintained at approximately 5°C for 2 h. The cooled spermatozoa suspensions were then mixed with a one-half volume of the second extender (the first Tris-egg yolk extender with 13% [v/v] glycerol) supplemented with or without the Orvus ES Paste (OEP; Miyazaki-kagaku, Tokyo, Japan). After 5 min at approximately 5°C, an additional one-half volume of the second extender was added to the spermatozoa suspensions to achieve a final concentration of 6.5% glycerol. The concentrations of the spermatozoa were adjusted to one-half (3×10^7

cells/ml). Aliquots of the spermatozoa suspensions were then immediately loaded into 0.25-ml plastic straws (Fujihira Co., Tokyo, Japan), which were placed on a Styrofoam plate in liquid nitrogen (LN_2) vapour (4 cm above the surface of the LN_2) and frozen. The straws were kept on the plate for 20 min and then were plunged into the LN_2 for storage.

Sperm quality assessments

A 300 µl aliquot of each frozen-thawed semen sample was mixed by pipetting and was warmed at 37°C for 10 min before evaluation. Motility analyses were performed using the computer-assisted sperm analysis (CASA) system. Analyses of the viability, acrosome integrity, and plasma membrane integrity were conducted according to the methods described by Wittayarat et al. (2012).

Each sample was diluted 10-fold with PBS supplemented with 0.3% BSA and was placed in a warm glass chamber to assess motility. Briefly, approximately 5 µl of the sperm suspension was transferred to a warm chamber (2-chamber slide, 20 microns in depth; Leja Products B.V., Nieuw-Vennep, The Netherlands) and then was placed on a warm plate at 37°C. Sperm motility was evaluated using the CASA system (Sperm Class Analyzer®: SCA® v.4.2 Microptic, Barcelona, Spain). The analysis was based on the examination of 25 consecutive, digitised images obtained from 3 fields using a ×10 phase contrast

objective, and at least 300 spermatozoa per sample were analysed. The analysis time was 1 sec per field, and the images were taken with a time lapse of 1 sec. Therefore, the image capture speed was one every 40 msec. After acquiring the representative fields, the total motile spermatozoa and progressive motile spermatozoa (> 45% of straightness coefficient) were recorded.

The sperm viability was assessed using a live/dead stain combination (SYBR-14/propidium iodide [PI], LIVE/DEAD Sperm Viability Kit; Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's protocol, with minor modifications. Briefly, an aliquot of semen (5 µl) was mixed with 50 µl of a solution containing 3 µl of PI (diluted 1:10 in distilled water) and 3 µl of SYBR-14 (diluted 1:500 in DMSO) and was incubated according to the manufacturer's instructions. One hundred spermatozoa were assessed in each of two duplicate aliquots of each sample and was evaluated using a fluorescence microscope (200×, Nikon Eclipse 80i; Nikon Corp.) with a 488-nm filter. The live sperm nuclei were stained with SYBR-14 and were bright fluorescent green, whereas the dead sperm nuclei exhibited a red (PI) fluorescence.

The plasma membrane integrity of the sperm was assessed using the hypo-osmotic swelling test. Briefly, an aliquot of semen (20 µl) was mixed with 80 µl of a hypo-osmotic

solution (150 mOsm/kg) containing 13.5 mg/ml D-fructose and 7.35 mg/ml trisodium citrate dehydrate in distilled water. The samples were incubated at 37°C for 10 min. Then, 10 µl of each sample was placed on a slide and was overlaid with a coverslip. One hundred spermatozoa in three fields from one aliquot of each sample were assessed using a phase-contrast microscope (400×, Nikon TE300; Nikon Corp.). The plasma membrane integrity of the sperm was expressed as the percentage of sperm with curled tails (intact plasma membrane) out of the total number of spermatozoa.

The acrosomal integrity of the spermatozoa was measured using fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Vector Laboratories, Inc., Burlingame, CA, USA). The samples were spread on slides, air-dried at room temperature, and fixed with absolute ethanol for 10 min at room temperature. After drying, the slides were spread with 30 µl of FITC-PNA (100 µg/ml) in PBS and were incubated in a dark, moist chamber for 30 min at 37°C. The slides were then rinsed with PBS, air-dried, and overlaid with a coverslip. The acrosomal status (intact or reacted) was determined from the FITC-PNA staining pattern observed using fluorescence microscopy (400×, Nikon Eclipse 80i; Nikon Corp.) with a 488-nm filter. In total, 100 spermatozoa were counted over at least three different fields. The spermatozoa were considered acrosome intact if the acrosome stained green, while those with no staining or a single band of green staining at

the equatorial segment were considered to have non-intact acrosomes (a damaged acrosome). The acrosomal integrity was expressed as the mean percentage of spermatozoa with intact acrosomes.

Experimental design

In the first experiment, we examined the effect of the OEP concentration on the quality of the refrozen spermatozoa. The cooled spermatozoa suspensions, derived from a bull, were mixed with the second extender supplemented with OEP and were frozen with final concentrations of 0%, 0.375%, 0.75%, 1.5% or 3% OEP. After thawing, the sperm quality (motility, viability, plasma membrane integrity, and acrosomal integrity) was assessed as described above.

In the second experiment, we tested the effect of OEP supplementation on the quality of refrozen spermatozoa derived from different bulls. The final concentration of OEP (0.375%) that was found to be most suitable for the quality of refrozen spermatozoa in the first experiment was used in this experiment. The cooled spermatozoa suspensions, derived from six bulls, were frozen in the freezing extender supplemented with or without 0.375% OEP.

Statistical analysis

All the experiments were repeated 5 times. For the analysis of the sperm characteristics in Experiment 1, the statistical significance was inferred based on the analyses of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) tests that were performed with STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). All the percentage data were subjected to arc sin transformation prior to the statistical analysis. For the analysis of the sperm characteristics in Experiment 2, the differences in each bull were evaluated using an independent Student's t-test. The data were expressed as the means ± the SEMs. The differences with probability values (P) of 0.05 or less were regarded as significant.

3. Results

Effect of OEP concentration

The mean percentages of total and progressive motility of the spermatozoa refrozen with 0.375% and 0.75% OEP were significantly higher ($P < 0.05$) than those of the control spermatozoa refrozen without OEP (Table 1). Moreover, the percentages of viability and the plasma membrane integrity of spermatozoa refrozen with 0.375% OEP were significantly higher ($P < 0.05$) than those of the spermatozoa refrozen with the other

Table 1. Quality of the bull spermatozoa after refreezing with various concentrations of Orvus Es Paste (OEP)*

Concentration of OEP (%)	Percentages of spermatozoa				
	Total motility	Progressive motility	Viability	Plasma membrane integrity	Acrosomal integrity
Pre-freezing**	67.7 ± 2.7[a]	38.1 ± 5.2[a]	52.1 ± 2.1[a]	45.7 ± 0.7[a]	96.6 ± 0.3[a]
0	14.1 ± 3.7[b,d]	3.3 ± 0.9[b]	9.0 ± 2.6[b]	15.6 ± 0.8[b]	97.5 ± 0.2[b]
0.375	29.3 ± 3.2[c]	14.1 ± 1.6[c]	37.3 ± 1.7[c]	32.6 ± 0.7[c]	97.5 ± 0.2[b]
0.75	23.4 ± 3.1[c,e]	11.7 ± 1.8[c]	25.0 ± 1.1[d]	22.9 ± 1.7[d]	97.2 ± 0.3[a,b]
1.5	17.4 ± 1.1[b,e]	7.0 ± 0.4[b,c]	17.9 ± 2.3[e]	15.7 ± 2.1[b]	97.6 ± 0.1[b]
3	6.0 ± 1.7[d]	0.2 ± 0.2[b]	1.0 ± 0.4[f]	3.4 ± 0.3[e]	96.9 ± 0.3[a,b]

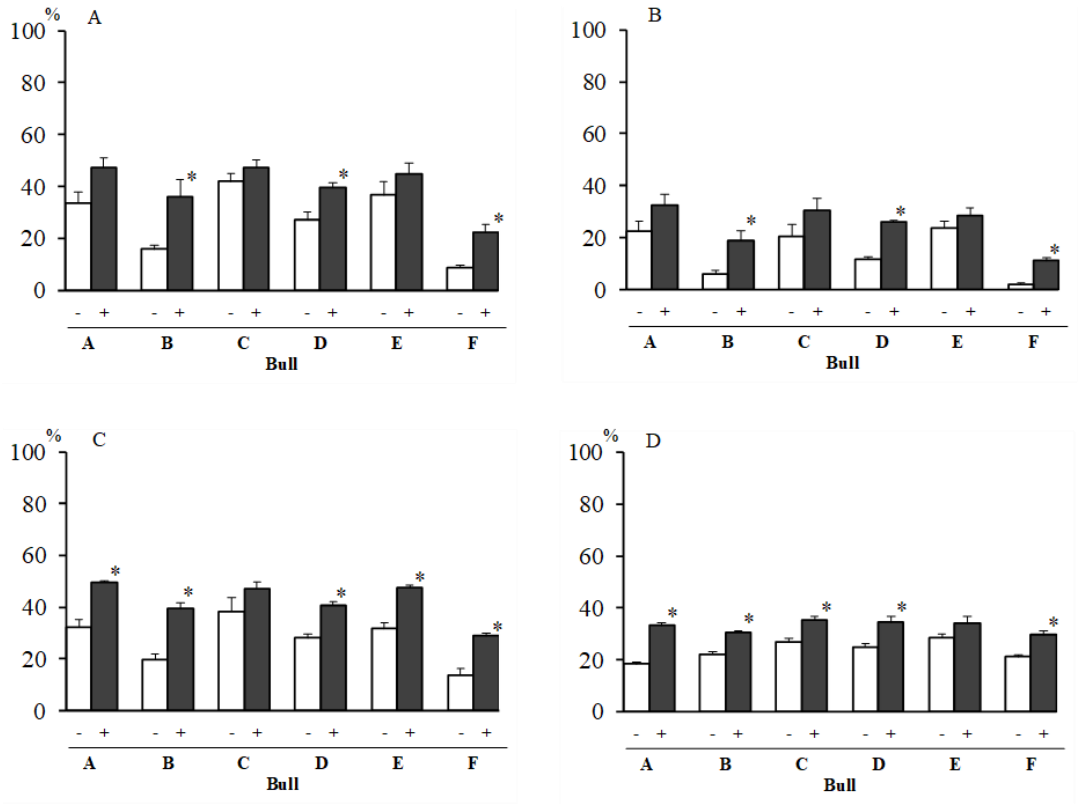
*Five replicate trials were carried out. The data are expressed as the mean ± SEM.

**The quality of spermatozoa was examined before refreezing.

[a-f]Values with different superscripts in the same column are significantly different ($P < 0.05$).

concentrations of OEP. The percentages of the motility, viability and plasma membrane integrity of the refrozen spermatozoa decreased compared with those of the pre-frozen spermatozoa, irrespective of the OEP concentration ($P < 0.05$). However, the acrosomal integrity of the spermatozoa were unaffected by the refreezing-thawing and the OEP concentration.

Figure 2. Total motility (A), progressive motility (B), viability (C), and plasma membrane integrity (V) of the spermatozoa after refreezing with (+) or without (-) 0.375% Orvus ES Pasete (OEP) in six bulls. Each bar represents the mean ± SEM. *denotes a significant difference in each bull ($P<0.05$)



Effect of OEP supplementation

The effect of 0.375% OEP supplementation on the quality of the refrozen-thawed spermatozoa was assessed in six bulls, and of which, the spermatozoa from three bulls had significantly higher percentages of total and progressive motility ($P < 0.05$) (Fig. 2, A and B). Moreover, the spermatozoa from five bulls significantly increased the percentages of viability and plasma membrane integrity by the OEP supplementation ($P < 0.05$) (Fig. 2, C and D). However, the acrosomal integrity of the spermatozoa was unaffected by the OEP supplementation in all the bulls.

4. Discussion

In the present study, we showed that the addition of 0.375% OEP to the freezing extender significantly improved the motility, viability, and plasma membrane integrity of refrozen-thawed spermatozoa. It is demonstrated that 0.5-1.5% OEP is the optimum concentration for the preservation of fresh spermatozoa in other species during the freeze-thaw process (Mizutani et al., 2010; Pursel et al., 1978; Shimazaki et al., 2015). In this study, however, we found that the addition of OEP with a lower concentration to the freezing extender was effective for the preservation of the refrozen-thawed spermatozoa. In general, about half of the spermatozoa cannot survive cryopreservation even with

optimized freezing protocols (Watson, 2000). Our results also showed that the post-thaw viability of the fresh spermatozoa was 52%. Moreover, the percentages of motility, viability, and plasma membrane integrity of the spermatozoa that were refrozen without OEP decreased by less than 20% after thawing. The reduction is suggested to arise from factors affecting the proportion of survivors (e.g., cold shock susceptibility, cooling rate, diluent composition and osmotic stress) and factors influencing the functional status of the survivors (e.g., membrane stability, oxidative damage, membrane receptor integrity, and nuclear structure) (Holt, 2000; Watson, 2000). Alterations in the cell membrane properties that occur during the freeze-thaw process change the stability and water permeability of the cells. Therefore, the optimum concentration of OEP for the refreezing of bull spermatozoa might be different from that for the fresh spermatozoa.

It has been reported that OEP has beneficial effects on sperm function in the presence of egg yolk (Hofmo & Almlid, 1991). OEP contains sodium dodecyl sulfate (SDS), which may act by modifying the structure of the egg yolk lipoproteins in the freezing extender (Arriola & Foote, 1987). Arriola and Foote (1987) demonstrated that when bull spermatozoa were frozen, the presence of SDS was less susceptible to the osmotic shock induced by the addition of glycerol. It is suggested that the SDS in OEP stabilizes the sperm membrane and delays capacitation-like changes that eventually lead to

the acrosome reaction and subsequent cell death (Pena, Lugilde, Barrio, Herradon, & Quintela, 2003). Therefore, the precise mechanism by which OEP improves sperm cryosurvival is indirect. In the present study, we observed that the post-thaw indicators (the motility, viability, and plasma membrane integrity) were higher in the spermatozoa refrozen with 0.375% or 0.75% OEP than in the spermatozoa refrozen without OEP (Table 1). Moreover, the addition of 0.375% OEP increased the percentages of viability and plasma membrane integrity of the post-refrozen spermatozoa compared with 0.75% OEP. The indicators examined in the different bull spermatozoa consistently showed that the protective effect was more pronounced when the spermatozoa were refrozen with 0.375% OEP. Therefore, our findings indicate that the addition of 0.375% OEP exhibited the beneficial effects on the refreezing of bull spermatozoa. On the other hand, previous studies suggest that the use of OEP and glycerol for the cryopreservation of semen in pig, dog, and cat protects the acrosome caps of the sperm and, thereby, increases and maintains post-thaw sperm motility (Mizutani et al., 2010; Pursel et al., 1978; Tsutsui, Hase, Hori, Ito, & Kawakami, 2000). However, our results showed that acrosome integrity of the post-refrozen spermatozoa was unaffected, irrespective of the OEP concentration and the different bulls. These results are in agreement with the experiment of Morton et al. (2010) who reported that post-thaw motility of epididymal alpaca spermatozoa was higher when

the spermatozoa were frozen and thawed in the presence of SDS, while the acrosome integrity was unaffected. In the present study, the acrosomal status was determined by the FITC-PNA staining pattern. This discrepancy about the effect of OEP on the acrosome integrity remains to be explained, but it might result from different classification of the acrosome status or other factors (e.g., species, and freezing protocol).

In conclusion, the results presented herein demonstrated that the optimum concentration of OEP for the refreezing of bull spermatozoa is 0.375%, and the addition of OEP to the freezing extender may improve the motility, viability, and plasma membrane integrity of refrozen-thawed spermatozoa. However, almost 70% of the population did not survive after refreezing and thawing. Further studies are necessary to improve the viability of post-refrozen spermatozoa for successful AI using sex-sorted semen that have already been frozen-stored.

Chapter 3

Disruption of cell proliferation and apoptosis balance in the testes of crossbred cattle-yaks affects spermatogenic cell fate and sterility

1. Introduction

In cattle-yak crossbreeds, male hybrids are sterile because of spermatogenesis arrest, but the conservation and maintenance of dominant traits in the next generation can benefit livestock farmers (Wang, Vandepitte, & Wu, 1994). There are several reports regarding the candidate factors related to the infertility of cattle-yak, especially for the disturbance of meiosis (Li et al., 2020; Wang et al., 2012; Yan et al., 2014; Zhang et al., 2009). Furthermore, omics analyses have been recently conducted on cattle-yak testes (Cai et al., 2017; Sun et al., 2017; Xu et al., 2018; Yu et al., 2016). However, the mechanisms underlying interspecies hybrid male sterility are still not well understood currently.

To understand sterility in cattle-yak crossbreeds, we previously focused on changes in DNA methylation and histone acetylation, which can mediate the changes in gene expression of many molecules for spermatogenesis (Phakdeedindan et al., 2021). DNA methylation levels of all testicular cell types in yaks, except for spermatogonia, did not change during maturation; however, hypermethylation occurred in F2 backcross cattle-yaks, indicating that gene expression was highly suppressed (Phakdeedindan et al., 2021). In addition, acetyl-histone H3 Lys9 (AcK9) expression in spermatogonia and testicular somatic cells in F2 backcross cattle-yaks was significantly different from that in yaks of the same age (Phakdeedindan et al., 2021). Therefore, we considered that inappropriate expression levels

of both AcK9 and DNA methylation may be major factors in the induction of infertility in male cattle-yaks following spermatogenesis arrest.

To produce an appropriate amount of sperm for an adequate period of time, the maintenance of cellular homeostasis, which is the balance between germ cell proliferation, differentiation and apoptosis, is required for spermatogenesis. Although there are several reports on the molecules involved in the differentiation of meiosis (Cai et al., 2017; Liu et al., 2011; Robert et al., 2021; Wu et al., 2020), there have been no reports on the levels of proliferation and apoptosis in yak testes and cattle-yak hybrid testes. It is clear that epigenomic changes are important for the control of these events at several stages and cell types in the testes during spermatogenesis (Li, Wang, & Xu, 2020; Liu et al., 2018; Yin et al., 2020). DNA methylation affects genes involved in different cellular pathways, including apoptosis (Gopisetty, Ramachandran, & Singal, 2006), and apoptotic germ cells show different levels of DNA methylation of CCGG sites compared with normal germ cells in mice (Koji, Kondo, Hishikawa, An, & Sato, 2008). Histone acetyl transferase and histone deacetylase regulate histone acetylation and can act as both suppressors and inducers of cell proliferation, depending on the genes that they regulate in cancer cells (Gujral, Mahajan, Lissaman, & Ponnampalam, 2020). Recently, epigenomic studies have been conducted to better understand male cattle-yak infertility. We suspected that inappropriate expression of

AcK9 and DNA methylation may induce different levels of proliferation and apoptosis in the testicular cells of cattle-yaks following spermatogenesis arrest.

Proliferating cell nuclear antigen (PCNA) plays an important role in the mediation of cell cycle control, DNA replication and replication-coupled DNA damage tolerance and repair processes. PCNA expression levels increase during the S phase of DNA replication (Boehm, Gildenberg, & Washington, 2016; Wrobel, Bickel, & Kujat, 1996) and is thought to be a good marker of cell proliferation in both yak and cattle-yak testes.To further investigate the mechanism of infertility in cattle-yaks, this study aimed to determine whether the balance between testicular germ cell proliferation and apoptosis differs between yaks and crossbred yaks at a given age stage by evaluating PCNA expression and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assays in the testicular cells of both yaks and cattle-yaks.

2. Materials and Methods

Animal and tissue preparation

The experimental yaks and cattle were obtained from yak farms in the Khövsgöl province and the Ikhtamir district of the Arkhangai province, Mongolia in July 2012 of breeding season. F2 hybrid cattle-yak was produced via the backcrossing of sire yak and F1 female

cattle-yak. Testicular tissue samples from yaks of various ages (1 year; n = 3, 2 year; n = 3, 3 year; n = 3) and 2-year-old F2 hybrid cattle-yaks (n = 3) were collected via castration using either emasculator or knife, or after slaughtering. The collected samples were immediately fixed in the field with 10% formalin solution for 24 h and taken back to the laboratory. The tissues were rinsed with PBS 3 times for 1 h, then dehydrated with serial alcohol (70% to 100%) for 1 h each and replaced with xylene 3 times for 1 h. Finally, the tissues were impregnated in paraffin 3 times for 1 h and embedded. Paraffin sections (4 μm thick) were prepared from each specimen. The developmental stages of the testes were confirmed using haematoxylin and eosin stains. This study was approved by the Animal Ethics Committee/Institutional Review Board of University of East Asia (#AEC-TOUA-H30-4).

Immunohistochemistry

Sections were deparaffinized by xylene and rehydrated by ethanol, washed with phosphate-buffered saline (PBS) and subjected to 10 mM citric acid buffer (pH 6.0) for antigen retrieval in microwave for 30 min. Sections were then blocked endogenous peroxidase activity in 0.3% H2O2/methanol for 1 h. After preincubation of the sections with normal goat IgG (500 μg/ml; Dako,) dissolved in 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 1 h to block non-specific reactions, primary mouse monoclonal antibody against PCNA (2 μg/ml; SNCL-PCNA, Novocastra™) diluted with 1% BSA in PBS was

applied to the sections and incubated overnight. For the negative control, normal mouse IgG (Dako) was adjusted to a working concentration for the primary antibody. The sections were then incubated with goat anti-mouse IgG (Fab') labelled with an amino acid polymer–peroxidase complex (Histofine Simple Stain MAX PO [M], Nichirei Co.) for 30 min. Then, sections were visualized using 3,3′ -diaminobenzidine (DAB) (Histofine Simple Stain DAB solution, Nichirei Co.). Finally, the nuclei of the sections were counterstained with haematoxylin, dehydrated, and mounted using Eukitt quick-hardening mounting medium (Fluka Analytical) with a glass coverslip. All procedures were performed at 25°.

TUNEL assay

To identify apoptotic germ cells, TUNEL assays were performed according to the method described by Koji et al. (2008) with a slight modification. Paraffin sections (4 μm thick) on silane-coated glass slides were dewaxed and digested with 10 μg/ml proteinase K in PBS at 37°C for 15 min. The sections were then incubated with TdT buffer (825 mM Tris/HCl buffer, pH 6.6, containing 0.2 M potassium cacodylate and 0.25 mg/mL BSA) at 25°C for 30 min. After incubation, the slides were reacted with 400 U/ml TdT (Roche Diagnostics) dissolved in TdT buffer supplemented with 2.5 μM biotin-16-dUTP (Roche Diagnostics), 20 μM dATP, 1.5 mM CoCl2 and 0.1 mM dithiothreitol at 37°C for 90 min. For the negative control, the reaction was conducted without TdT. After washing with Milli-Q water, the sections were

incubated with HRP-conjugated streptavidin (Histofine, Nichirei Co.) for 20 min and visualized using DAB (Histofine Simple Stain DAB solution, Nichirei Co.) for 20 min. Finally, the nuclei of the sections were counterstained with haematoxylin, dehydrated and mounted using Eukitt quick-hardening mounting medium (Fluka Analytical) with a glass cover slip.

Quantification of PCNA and TUNEL staining

All images were obtained using a microscope (Nikon Ecrips NiUTRFLM; Nikon) equipped with a Nikon DS-Fi2-U3 digital camera. The examined fields were chosen randomly by examiners who were blinded to the tissue groups.

The number of PCNA-immunopositive cells in each group of histological sections was visually counted. The spermatogenic cell data are represented as mean percentage values of immunopositive cells compared with the total spermatogenic cells, excluding round spermatids and spermatozoa in the seminiferous tubules. Three seminiferous tubules from each section were randomly selected for examination. Furthermore, the numbers of PCNA immunopositive Leydig cells in hybrid yak testes were represented as PCNA labelling index: mean % values of immunopositive Leydig cells compared with the total Leydig cells in the

interstitial area. Five interstitial area from each section were randomly selected for examination.

To quantify the degree of apoptosis, we also visually counted the number of TUNEL-positive cells in each group of histological sections and randomly examined thirty seminiferous tubules from each section. The data were represented as mean percentage values of seminiferous tubules containing TUNEL-positive cells and as mean values of TUNEL-positive cells in each seminiferous tubule. Furthermore, the numbers of TUNEL-positive Leydig cells in hybrid yak testes were represented as TUNEL index: mean % values of TUNEL-positive Leydig cells compared with the total Leydig cells in the interstitial area. Five interstitial area from each section were randomly selected for examination.

Statistical analysis

All values are expressed as the mean ± standard error. Student's t-test was used to assess the differences between data pairs using Microsoft Excel (Microsoft Corp.). Analysis of variance (ANOVA) and Tukey's multiple comparisons tests were performed to investigate the differences among the 1-, 2- and 3-year-old yaks using JMP soft-ware (SAS Institute Inc.). Differences with a probability value (p) of 0.05 or less were considered statistically significant.

3. Results

PCNA expression in testicular cells during Yak development

The expression of PCNA in testicular cells was observed in the testes of yaks of all ages, from immature to mature stages (Figure 1a–c). 1-year-old immature yak showed PCNA expression in some spermatogonia (Figure 1a), and mature 2- and 3-year-old yaks showed PCNA expression in both spermatogonia and spermatocytes (Figure 1b,c). All somatic cells, including Sertoli cells, myoid cells and Leydig cells, did not show any staining. The percentage of PCNA-immunopositive spermatogenic cells was significantly higher in mature 2- and 3-year-old yaks than in immature 1-year-old yaks ($p < .05$, Figure 2a). The negative control showed no staining for the antibody examined in all cases as shown in Figure 1b inset.

PCNA expression in testicular cells of mature Yak and cattle-yaks

PCNA expression in testicular cells was observed in both 2-year-old yak testes with normal spermatogenesis and hybrid 2-year-old cattle-yak testes with spermatogenic arrest (Figure 1b,d). There was no significant difference in the percentage of PCNA-immunopositive spermatogenic cells between the groups (Figure 2b). There was no staining in Sertoli cells or myoid cells of 2-year-old cattle-yak testes, but staining was seen in Leydig cells (Figure 1d). The Leydig cells of 2-year-old cattle-yak testes showed $55.70 \pm 3.51\%$

PCNA-labelling index. The negative control showed no staining for the antibody examined in all cases as shown in Figure 1b inset.

Apoptosis of testicular cells during Yak development

The percentage of apoptotic cells in the testes was examined using the TUNEL assay. TUNEL-positive cells were observed in the testes of yaks of all ages, from the immature to mature stages (Figure 3a–c). One-year-old immature yaks showed TUNEL-positive cells in some spermatogonia (Figure 3a), and mature 2- and 3-year-old yaks showed TUNEL-positive cells in both spermatogonia and spermatocytes (Figure 3b, c). All somatic cells, including Sertoli, myoid and Leydig cells, did not show any staining. The percentage of TUNEL-positive seminiferous tubules was significantly higher in mature 2-year-old yaks than in immature 1-year-old yaks ($p < .05$, Figure 4a). Furthermore, the number of TUNEL-positive spermatogenic cells in the seminiferous tubules also increased in mature 2-year-old yaks compared with that in immature 1-year-old yaks ($p < .05$, Figure 4b). However, 3-year-old yaks showed lower levels of apoptosis than 2-year-old yaks (Figure 4a,b). The negative control showed no staining for the TUNEL assay examined in all cases as shown in Figure 3b inset.

Apoptosis of testicular cells in mature yak and cattle-yaks

TUNEL-positive spermatogenic cells were observed in both 2-year-old yak testes with normal spermatogenesis and 2-year-old cattle-yak testes with spermatogenic arrest (Figure 3b,d). The percentage of TUNEL-positive seminiferous tubules was significantly lower in 2-year-old cattle-yak testes than in 2-year-old yak testes ($p < .05$, Figure 5a). Furthermore, the number of TUNEL-positive spermatogenic cells in the seminiferous tubules also decreased in 2-year-old cattle-yak testes compared with that in 2-year-old yak testes ($p < .05$, Figure 5b). There was no staining seen in Sertoli cells, myoid cells, but some was seen in Leydig cells of 2-year-old cattle-yak testes (Figure 3d). The Leydig cells of 2-year-old cattle-yak testes showed $31.84 \pm 7.85\%$ TUNEL- index. The negative control showed no staining for the TUNEL assay examined in all cases as shown in Figure 3b inset.

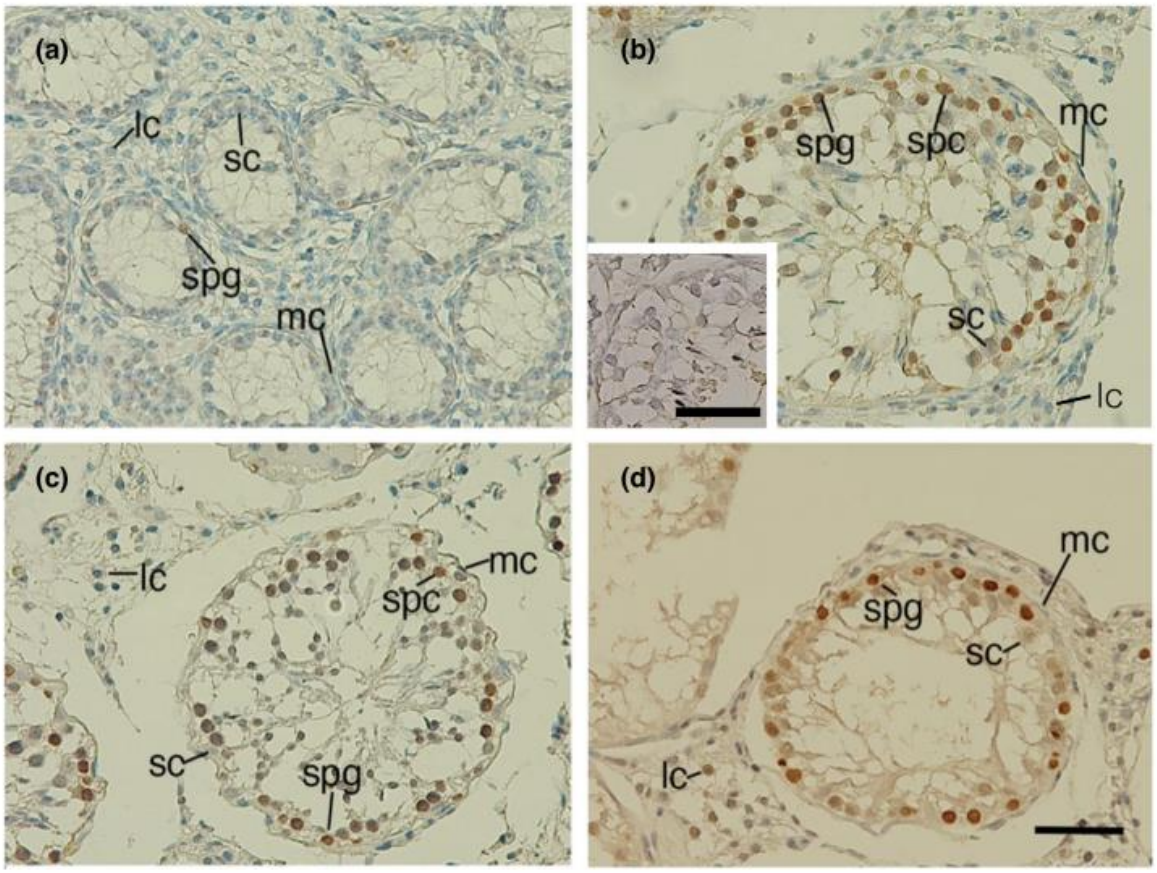


FIGURE 1 Expression of PCNA in testicular cells of yak and cattle-yaks. (a) immature yaks (1-year-old), (b) mature yaks (2-year-old), (c) mature yaks (3-year-old) and (d) crossbred cattle-yaks (F2) (2-year-old). Inset in (b), negative control of mature yaks (2-year-old); PCNA, proliferating cell nuclear antigen; spg, spermatogonia; spc, spermatocyte; sc, Sertoli cell; lc, Leydig cell; mc, myoid cell. Scale bar = 50um.

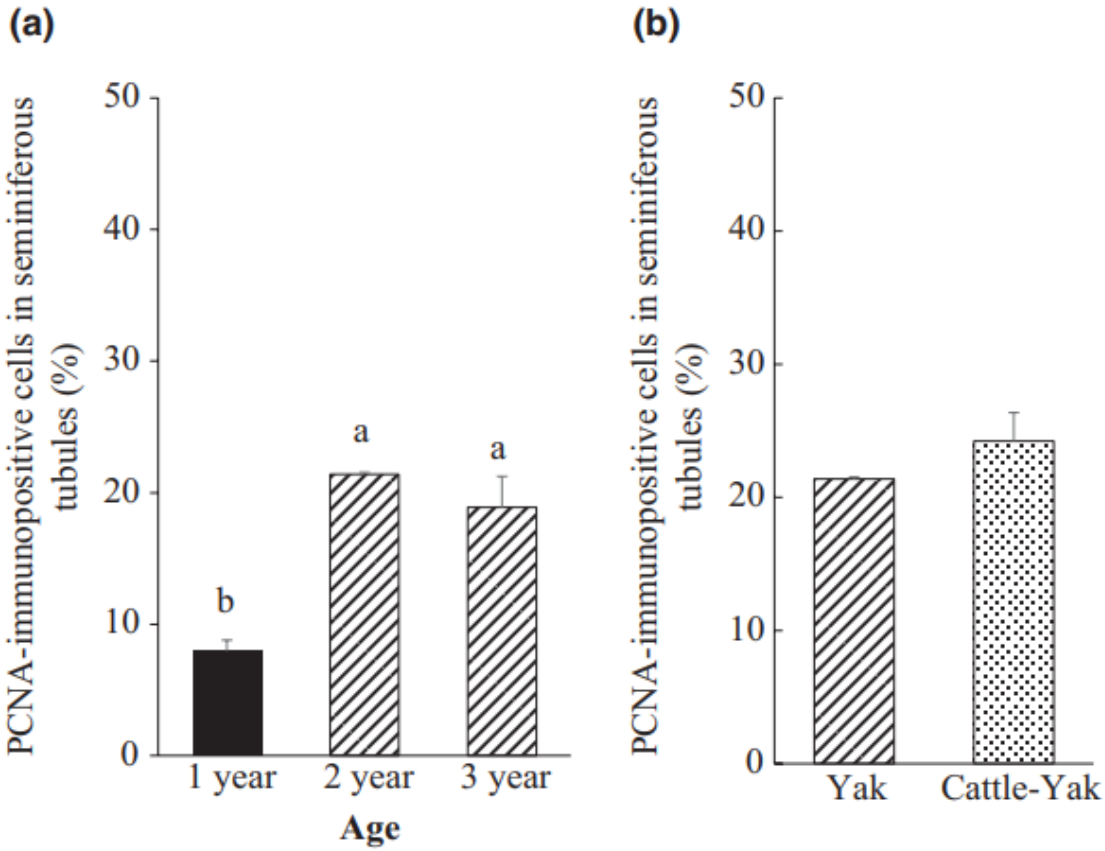


FIGURE 2 Expression of PCNA in testicular cells of yaks and cattle-yaks. a) Developmental proliferation changes in yaks of different ages. (b) Comparison of proliferation changes in 2-year-old yaks and 2-year-old crossbred cattle-yaks (F2). Percentage of PCNA-immunopositive testicular cells. Bars and error bars represent mean ± standard error (1 year; n = 3, 2 years; n = 3, 3 year; n = 3). Different lowercase letters represent significant differences ($p < .05$). PCNA, proliferating cell nuclear antigen

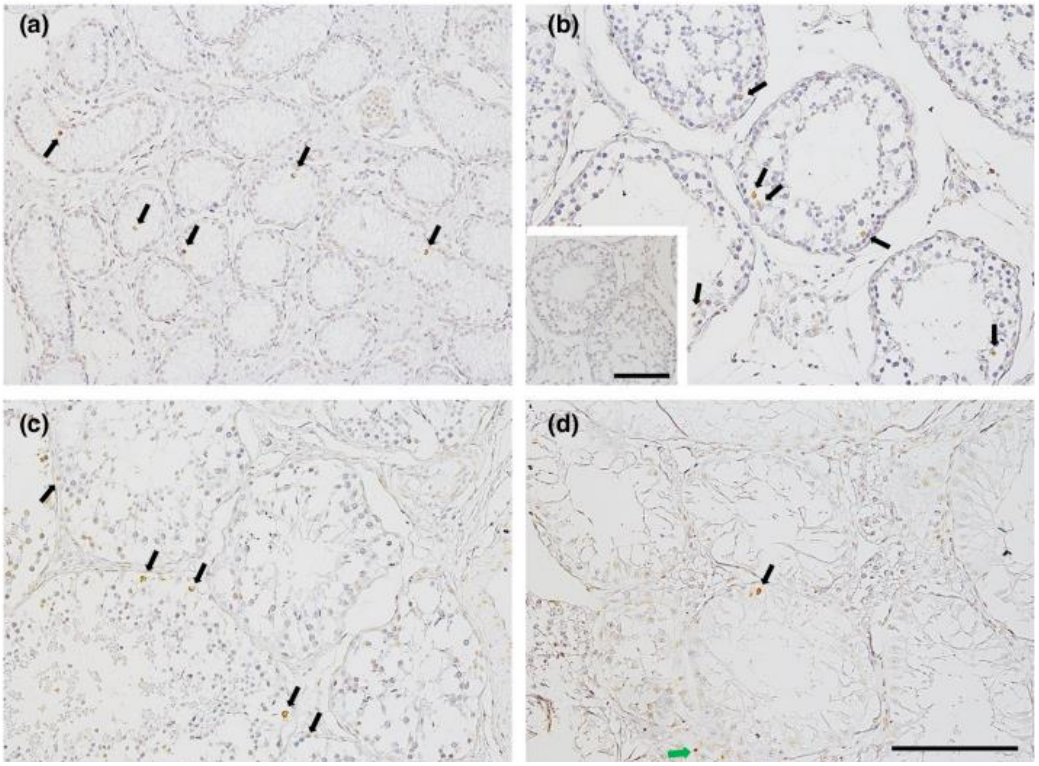


FIGURE 3 TUNEL-positive cells in testes of yaks and cattle-yaks. (a) Immature yaks (1-year-old), (b) mature yaks (2-year-old), (c) mature yaks (3-yearold) and (d) crossbred cattle-yaks (F2) (2-year-old). Inset in (b), negative control of mature yaks (2-year-old); TUNEL, terminal deoxynucleotidyl transferasemediated dUTP nick end labelling; black allow, TUNEL-positive germ cells in seminiferous tubules.; green allow, TUNEL-positive Leydig cells in interstitial area. Scale bar = 100 um

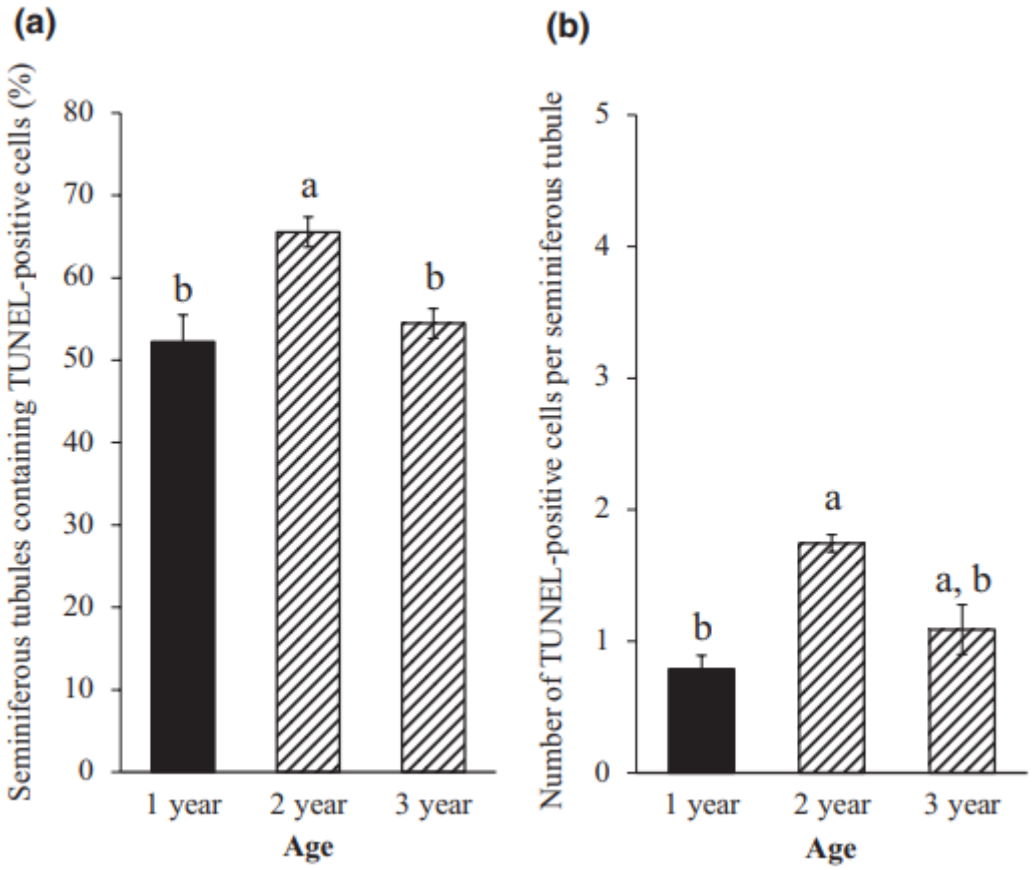


FIGURE 4 Developmental apoptosis changes in yaks of different ages. (a) the percentage of TUNEL-positive seminiferous tubules. (b) the number of TUNEL-positive spermatogenic cells in the seminiferous tubules. Bars and error bars represent mean ± standard error (1 year; n = 3, 2 years; n = 3, 3 year; n = 3). Different lowercase letters represent significant differences ($p < .05$). TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

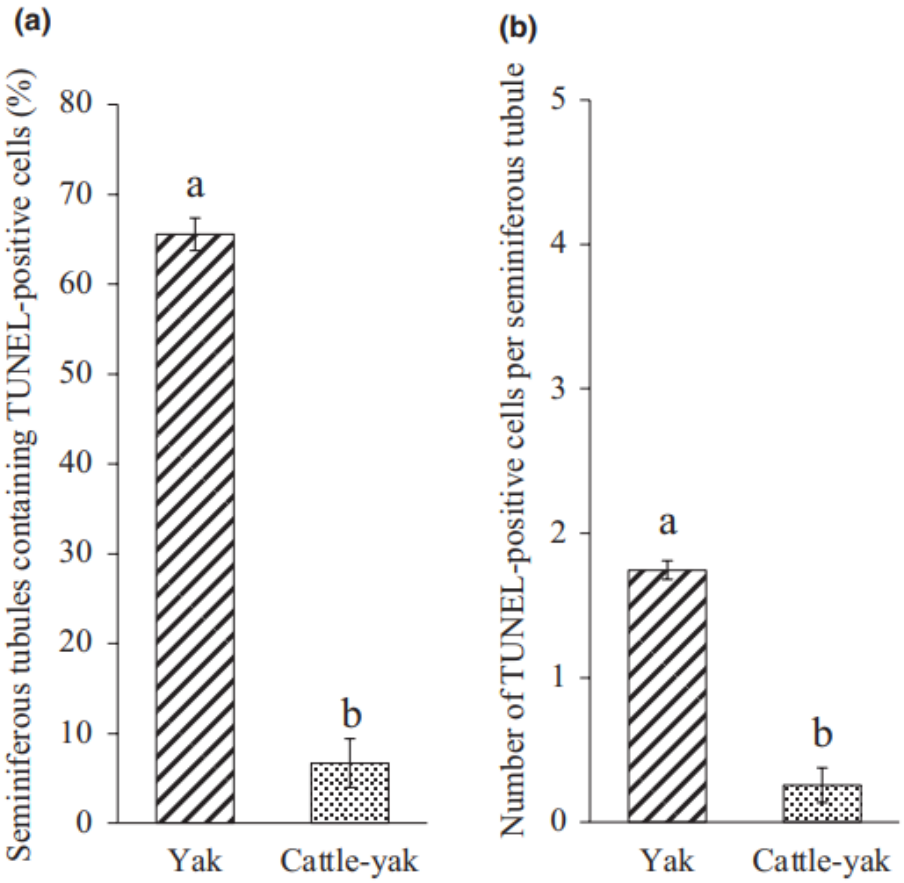


FIGURE 5 Comparison of apoptosis changes in 2-year-old yaks and 2-year-old crossbred cattle-yaks (F2). (a) the percentage of TUNEL-positive seminiferous tubules. (b) the number of TUNELpositive spermatogenic cells in the seminiferous tubules. Bars and error bars represent mean ± standard error (yaks; n = 3, F2 hybrids; n = 3). Different lowercase letters represent significant differences ($p < .05$). TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

4. Discussion

Spermatogenetic cell fates are appropriately selected for proliferation, differentiation, and apoptosis to control sperm production during normal spermatogenesis under natural conditions. It is possible that many conditions disrupt or decrease sperm production by causing changes in normal cell fates. Interspecies hybridization of yaks and cattle results in sterility in males because of spermatogenic arrest. In the present study, we investigated the proliferation and apoptosis status during the development of yaks and hybrid cattle-yak using PCNA immunohistochemistry and TUNEL assay.

The proliferation levels of spermatogenic cells were higher in mature yaks than in immature yaks; however, all types of somatic cells, including Sertoli cells, myoid cells and Leydig cells, maintained no proliferation at all ages, suggesting that proliferation only became active in spermatogenic cells during maturation in yaks. Anti-Müllerian hormone produced by Sertoli cells in yak testes increases from the fetal stage to the calf stage, then decreases with an increase in age and is thought to play a role in the proliferation of Sertoli cells (Qin et al., 2021). However, in addition to the present study, the proliferation of Sertoli cells in the yaks remained constant during maturation by immunostaining for vimentin, a Sertoli cell marker in our preliminary study. Although we need further examination, it is possible that the different proliferation data of Sertoli cells in yaks in

previous reports (Qin et al., 2021) may be due to cultured cells at different stages (0.5–1 year).

On the contrary, the proliferation levels of spermatogenic cells did not differ between yak testes and hybrid cattle-yak testes at the same age, which suggested that proliferation ability was actively maintained in spermatogenic cells in hybrid cattle-yak testes, although they showed arrest of spermatogenesis. Compared with 2-year-old yak testes, 2-year-old cattle-yak testes rarely contained spermatocytes (Sato et al., 2020), suggesting that the proliferation ability of spermatogonia was higher in crossbred cattle-yak testes. Furthermore, proliferation of Leydig cells in 2-year-old cattle-yak testes was observed, although all types of somatic cells did not show proliferation in yaks of the same age. The important functions of androgens in spermatogenesis are to control the production of spermatocytes, mediate the progress of meiosis and assist in sperm adherence and release from Sertoli cells (De Gendt et al., 2004; O′ Donnell, Nicholls, O’ Bryan, McLachlan, & Stanton, 2011; Wang, Yeh, Tzeng, & Chang, 2009). The testosterone biosynthesis ability of Leydig cells and the number of functional Leydig cell in the testes are thought to be influenced on serum androgen levels (Shan, Bardin & Hardy, 1997). The Leydig cells in 2-year-old cattle-yak testes showed more than 24% higher proliferation index than apoptosis index, suggesting that there is Leydig cell hyperplasia in hybrid cattle-yak testes. In case of

Leydig cell hyperplasia, different levels of serum testosterone level have been reported. When the rat is treated with high concentration of Di-n-octyl phthalate, Leydig cell hyperplasia are observed with increase of serum luteinizing hormone (LH) levels and reduction in testosterone levels (Zhu, Hu, Ji, Huang, Ge, & Wang, 2021). However, LH receptor mutant mouse which same mutation was found in boys with familial male-limited precocious puberty shows the Leydig cell hyperplasia with increase in both serum LH and testosterone levels (McGee, & Narayan, 2013). Although further studies are needed about the serum hormone levels and LH receptor in cattle-yaks, Leydig cell hyperplasia in

2-year-old cattle-yak testes may cause the different levels of testosterone production leading to the infertility. Furthermore, the different functions of steroidogenesis in the individual Leydig cells of cattle-yak hybrid testes (Sato et al., 2020) may be enhanced by an increased number of Leydig cells. Treatment with the histone deacetylation inhibitor trichostatin A increased AcK9 and inhibited PCNA transcription in adipose-derived stem cells (Wang et al., 2016). Our previous study showed lower levels of AcK9 in spermatogonia and Leydig cells in hybrid cattle-yak testes than in yaks of the same age (Phakdeedindan et al., 2021). The increased proliferation of both spermatogonia and Leydig cells in the hybrid cattle-yak testes was thought to be one of the effects caused by lower levels of AcK9.

The present results also showed that apoptosis increased during maturation in yaks; however, all types of somatic cells, including Sertoli cells, myoid cells and Leydig cells, showed no apoptosis at all ages, suggesting that apoptosis only occurs in spermatogenic cells during maturation in yaks with normal spermatogenesis. On the contrary, the apoptosis levels of spermatogenic cells decreased in hybrid cattle-yak testes compared with yak testes of the same age, which suggested that spermatogenic cells could not undergo apoptosis appropriately in hybrid cattle-yak testes. Sirtuin 1 (SIRT1), a histone deacetylase in the testis, is involved in testicular development via SF-1 activation and can inhibit cell apoptosis by deacetylating P53 at the 379th lysine (Gomes et al., 2016; Vaziri et al., 2001); however, SIRT1 does not contribute to SIRT1-mediated apoptosis in cattle-yak testes (Yin et al., 2020). One of the reasons that the spermatogenic cells in hybrid cattle-yak testes could not undergo apoptosis at the same levels as yak testes may be the absence of SIRT1 in cattle-yak testes. Furthermore, apoptosis was observed in Leydig cells in hybrid cattle-yak testes. Our previous study demonstrated that testicular cells, including spermatogonia and Leydig cells, showed higher DNA methylation in cattle-yak testes than in yaks of the same age (Phakdeedindan et al., 2021). Because DNA methylation affects genes involved in various cellular pathways, including apoptosis in cells (Gopisetty et al., 2006), different

levels of apoptosis in cattle-yak spermatogenic cells and Leydig cells may be induced by different levels of DNA methylation.

In conclusion, we found that proliferation and apoptosis were activated only in spermatogenic cells and not in other somatic cells during maturation in yak testes. Furthermore, hybrid cattle-yak testes maintained proliferation ability but had less apoptotic ability in spermatogenic cells when compared to yaks of the same age, suggesting that normal spermatogenic cell fate control is disrupted by changes in the balance between proliferation and apoptosis. In addition to the change in spermatogenic cell fate, Leydig cell proliferation was higher than apoptosis in the hybrid cattle-yak testes, indicating an increased total number of Leydig cells, which may affect spermatogenesis through changes in steroidogenesis. Although there is a possible incorporation of epigenetic changes on crossbred cattle-yak testes, future studies are needed to clarify the modulation of proliferation and apoptosis to elucidate the mechanisms of infertility in hybrid cattle-yak males. If it becomes possible to restore normal cellular homeostasis in cattle-yak, which is different from yak, based on the regulatory mechanism on proliferation and apoptosis, it will be a step towards inducing fertility in cattle-yak and will be useful for livestock farmers in the future.

OVERALLDISCUSSION

Yaks are important livestock for farmers in high mountainous areas regarding livelihood and income (Wiener et al., 2003). Because reproductive management of these livestock leads to stable livelihoods and income, semen cryopreservation and AI techniques are beneficial. In Chapter 1, we demonstrated that developing a method for yak semen cryopreservation and adding OEP to the frozen extender is useful for improving motility and viability immediately after freezing and thawing.

OEP contains sodium dodecyl sulfate (SDS), which modifies the lipoprotein structure of egg yolk (Arriola et al. 1987), prevents membrane denaturation, and delays acrosome reaction and apoptosis, thereby maintaining motility and viability (Pursel et al. 1975, Tsutui et al. 2000, Hofmo et al. 1991, Pena et al. 2003). The optimal concentration of OEP was 0.75% in both epididymal- and ejaculate-derived frozen sperm. The results showed a significant difference in motility and viability compared with the control group immediately after thawing. This agrees with previous reports on the effect of OEP addition to the semen of other animals, which resulted in 0.5 to 1.5% (Pursel et al. 1978, Mizutani et al. 2010, Tsutui et al. 2000). However, this effect was lost at 3 h after thawing. Freezing and thawing denature cells, which changes the permeability of the cell membrane and

reduces its stability. This also affects membrane fusion and the response of signaling pathways, which in turn reduces the lifespan of sperm after freezing and thawing by accelerating sperm capacitation (Watson et al. 1995). Therefore, sperm after freeze-thawing might have died more quickly than fresh sperm. These results suggested that the addition of OEP significantly improved the motility and viability of sperm after freezing and thawing, and was useful in improving sperm quality.

We then focused on refreezing frozen semen. Double freezing of semen is an essential technology for genome resource banking for the conservation of yaks and other rare species, effective utilization of superior sire semen, and the creation of sex-sorted semen (Saragusty et al. 2009, Wildt et al. 2000). Layek et al. (2016) stated various factors that determine survival after double freezing; however, the freezing extender is vital. Studies on refreezing have reported successful conception in rams and cows, but their conception rates are low (Hollinshead et al. 2004, de Graaf et al. 2007, Arav et al. 2002, Saragusty et al. 2009). Therefore, in Chapter 2, we aimed to improve semen quality after double freezing in bulls. The results showed that adding OPE to the semen extender improved the quality of semen after refreezing. The results showed that the addition of 0.375% OEP was effective in improving the motility, viability, and plasma membrane integrity of cattle after refreezing. However, it did not affect acrosomal integrity. In Chapter 2, the most effective

concentration for refreezing was 0.375%, whereas the effective concentration for adding OEP to the semen extender, shown in Chapter 1, was 0.75%. Refreezing, with the addition of further cold stress, reduces viability by half and results in low conception rates (Watson et al. 2000). Reduced viability is primarily caused by the effect on the morphology and function of viable sperm (Holt et al. 2000, Watson et al. 2000). In other words, the plasma membrane may have already changed in morphology after thawing, resulting in different concentrations after refreezing caused in impaired membrane stability and permeability. However, although OEP and glycerol have been shown to protect the acrosome cap and maintain its motility (Pursel et al. 1975, Tsutui et al. 2000, Hofmo et al. 1991, Pena et al. 2003). The present experiments showed no effect on acrosome integrity. The OEP concentration and bull differences were not relevant, which is consistent with the results reported for SDS (Morton et al. 2010). The effect of OEP on acrosome integrity is unknown but may be caused by other factors.

These results suggest that adding OEP to the freezing semen extender for refreezing effectively improves the quality of thawed sperm. However, even with the addition of OEP, 70% of refrozen sperm died compared with fresh sperm. Further studies are warranted to improve survival after refreezing and thawing. In current research on freezing extenders, the Tris egg yolk extender was the most popular semen extender in cattle because of its

protective effect on egg yolk against early frost damage. However, the risk of introducing exotic diseases through the transportation of egg yolk-based products has been recognized, and an alternative to conventional commercial semen extenders is needed. Alternatives have been reported, such as those based on soybean extract containing lecithin instead of egg yolk (Layek et al. 2016) and those using cholesterol cyclodextrin complex and Tris-glycerol (Anzar et al. 2019). Studies have also been conducted on additives, such as the addition of relaxin to improve sperm motility and blastocyst rate (Elkhawagah et al. 2020) and the addition of caffeic acid to freeze dilutions in buffalo to improve post-thaw sperm quality (Soleimanzadeh et al. 2020). Thus, there is still room for further research on enhancing the freezing extenders. Furthermore, what has been demonstrated in cattle and other species must be validated for the cryopreservation of yak semen.

However, the development of yak semen cryopreservation and artificial insemination result in the sterility found in cattle-yak crossbred males. This has been well studied, but the answer is yet to be elucidated. Normal spermatogenesis requires the proper control of cell proliferation, differentiation, and apoptosis in all sperm-related cells. Genes determine them, and it is also important to know when and where the molecular expression levels of those genes change because of modifications by DNA methylation and histone acetylation. Previous studies have identified DNA hypermethylation and the expression of different

histone acetylation levels in germ and somatic cells in cattle-yak testes (Phakdeedindan et al. 2021). In addition, although there have been various reports of disruption of the apoptosis balance because of abnormal epigenomic changes (Cai et al. 2017, Wu et al. 2020, Liu et al. 2018, Robert et al. 2021), until now, no studies have focused on cell proliferation. Therefore, in Chapter 3, we used PCNA (Boehm et al. 2016, Wrobel et al. 1996), which is essential for damage resistance during DNA replication and synthesis, as a marker of cell proliferation. To investigate cell proliferation and apoptosis in various cell types of the cattle-yak testis, cell proliferation and apoptosis in different cell types of cattle-yak testes were investigated.

The results showed that the level of spermatogenic cell proliferation in yaks increased with growth, but the cell proliferation levels were similar when comparing 2-year-old yaks and cattle-yaks. This indicated that the ability of spermatogenic cells to proliferate is maintained even when mature cattle-yaks are spermatogenesis arrest. In contrast, apoptosis of spermatogenic cells was observed at all ages in both yaks and cattle-yaks, but the level of apoptosis in 2-year-old cattle-yaks was significantly lower than in yaks. Leydig cell proliferation and apoptosis were observed in mature cattle-yaks but not in yaks. The cell proliferation index in the cattle-yak was more than 24% higher than the apoptosis index, suggesting Leydig cell hyperplasia. The ability of Leydig cells to produce testosterone and

the number of Leydig cells affect blood androgen levels (Shan et al. 1997). There are also reports that Leydig cell hyperplasia leading to changes in testosterone levels (Zhu et al. 2021, McGee et al. 2013). In our previous studies, we found lower androgen receptor (AR) expression, higher 3β-hydroxysteroid dehydrogenase (3βHSD) expression, and lower AcK9 expression levels in Leydig cells in mature cattle-yaks. (Sato et al. 2020, Phakdeedindan et al. 2021) These findings suggest that abnormal molecular expression and imbalance between cell proliferation and apoptosis in Leydig cells cause hyperplasia and alter testosterone production.

Overall, normal spermatozoa are formed in yaks by precisely eliminating of unwanted cells through apoptosis and proliferation of spermatogenic cells as they mature. However, cell proliferation occurs in the spermatogenic cells of the cattle-yak, but apoptosis is low, indicating that the balance is disrupted. In addition to spermatogenic cells, Leydig cells in cattle-yaks also undergo hyperplasia because of an imbalance between cell proliferation and apoptosis, which may cause changes in steroid production and inhibit spermatogenesis. These changes are possibly caused by the concordance of epigenetic changes. Further research is needed in this field.

Recently, revolutionary gene modification technology has become easily and inexpensively available. Research on genome-editing technology with clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9 system, the hottest technology currently available, which was and highlighted by the Nobel Prize in 2020, is advancing daily (Barrangou et al. 2016, Brinkman et al. 2018, Niu et al. 2014). The CRISPR/Cas9 system is founded on the immune response system inherent in bacteria and archaea to protect against viruses and plasmids (Jinek et al. 2012), and this system is a technique that could be used to knock out or insert specific genes. This technology is expected to be applied to elucidate of gene function and treatment of human cancer, leading to additional advances in human medicine.

One such application is the use in pigs, whose organs are similar to humans, as xenotransplant donors for organ transplants, which are in short supply (Tonelli et al. 2014, Cooper et al. 2015). However, acute rejection is a major obstacle in xenotransplantation (Byrne et al. 2015, Shimizu et al. 2000). Therefore, pigs lacking the GGTA1 gene, a major cause of hyperacute rejection, have been created and studied by somatic cell transplantation (SCNT) (Cheng et al. 2016, Phelps et al. 2003, Xin et al. 2013, Hauschild et al. 2011). Tanihara et al. (2020) have successfully generated GGTA1-deficient pigs using the CRISPR/Cas9 system. However, genetic modification using the CRISPR/Cas9 system

requires biallelic mutation of the target gene, and there is a risk of failure to incorporate the target gene or mosaicism; thus, improvement is still needed. Therefore, we are studying gene editing techniques using the CRISPR/Cas9 system with porcine zygotes to improve the biallelic mutation and blastocyst rates by combining microinjection, electrofusion, and membrane fusion (Tanihara et al. 2016, Le et al. 2021, Takebayashi et al. 2022). Thus, as research on rejection progresses, a barrier to organ transplantation will diminish, and xenotransplantation to humans utilizing pig cells and organs will become safer and less expensive, with unlimited potential to contribute to human medicine.

In conclusion, we believe that research on genome-editing technology using CRISPR/Cas9 has just begun and will create new possibilities as this technology becomes more reliable. We have visited Mongolia several times for yak experiments and sampling. This technology can be applied to cows or pigs to create spermatogenesis-related genome-edited animals that could serve as models for yaks in the future. There is still much to be elucidated about yaks. We hope that applying these technologies will further explain male sterility in cattle-yaks and the advancement of semen preservation technology.

SUMMARY

Yaks are long-haired members of the cattle family adapted to living in high mountains beyond the forest limit and are important domestic animals in the lives of Mongolian nomads. However, their reproduction relies on natural mating by males, and artificial insemination is not widespread. Managing yak reproduction affects productivity and income; hence, we believe that introducing AI technology will help stabilize livelihoods. Therefore, in Chapter 1, we describe the development of a semen cryopreservation method for yaks. The semen used for freezing was provided by spermatozoa derived from ejaculation and the epididymis following castration. The usefulness of adding the OEP to the semen extender was investigated to improve motility and viability after freezing and thawing. OEP is a surfactant that, together with egg yolk, protects against frost damage. The results showed that adding 0.75% OEP significantly improved the quality of semen from both sources.

Having confirmed the usefulness of OEP addition, we turned our attention to the refreezing techniques. Using sex-sorted semen in cattle has become common in recent years, and efficient sexing is important for reproductive management. In Chapter 2, we investigated the effect of adding OEP to the semen extender for refreezing to improve

semen quality after double freezing in cows. The results showed that adding 0.375% OEP improved motility, viability, and membrane integrity. However, despite the quality improvement, nearly 70% of sperm did not survive, indicating that more research is warranted.

Concurrently with frozen semen, we examined infertility in male cattle-yaks. Mongolian nomadic herds contain not only yaks but also domestic cattle. Although yaks are members of the cattle family and there are crossbreeds between yaks and cattle, male cattle-yaks are sterile. Although male sterility has been elucidated at the epigenomic, genetic, and molecular expression levels, we examined the balance between cell proliferation and apoptosis using PCNA as a marker for cell proliferation and TUNEL staining for apoptosis. In Chapter 3, these indices were examined in germ cells and spermatogenesis-related somatic cells by comparing yaks and cattle-yaks. The results showed that spermatogenic cells proliferate both in the yak and the cattle-yak. However, apoptosis in spermatogenic cells was significantly lower in cattle-yaks than in yaks, suggesting that an imbalance between cell proliferation and apoptosis may result in spermatogenesis arrest. In addition, although neither cell proliferation nor apoptosis in Leydig cells was observed in yaks, both were in cattle-yaks. However, the apoptosis index was lower than the cell proliferation index, suggesting that hyperplasia occurred. This

phenomenon may contribute to infertility. In recent years, there has been a revolution in gene modification technology, which continues to advance. We hope that the creation of genetically modified animal models will advance research and explain infertility in Japan. Many issues are yet to be investigated to improve semen freezing technology and elucidate infertility issues in cattle-yak hybrids, further research is required to integrate these technologies.

ACKNOWLEDGEMENT

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Dr. Takeshige Otoi for his continuous support, patience, encouragement, his flexible mindset and immense knowledge. His guidance helped me during the conduct of this research and the writing of this dissertation.

I would like to thank the rest of my thesis committee: Prof. Dr. Taro Mito and Prof. Dr. Yoshihiro Uto for their insightful comments and encouragement me.

My sincere thanks also to Prof. Dr. Mitsuhiro Takagi, Associate Prof. Dr. Masayasu Taniguchi and my fellow labmates for their support, assistance and cooperation. Without them, this research could not have been carried out.

Finally, I would also like to my sincere appreciation to Prof. Dr. Yoko Sato for her practical and mental support, guidance, encouragement, and cooperation.

REFERENCES

Anand, I.S., Harris, E., Ferrari, R., Pearce, P., & Harris, P. (1986). Pulmonary haemodynamics of the yak, cattle, and cross breeds at high altitude. Thorax, 41(9), 696-700.

Anzar, M., Rajapaksha, K., Boswall, L. (2019). Egg yolk-free cryopreservation of bull semen. PLoS One, 14(10), e0223977.

Arav, A., Zeron, Y., Shturman, H., Gacitua, H.. (2002). Successful pregnancies in cows following double freezing of a large volume of semen. Reprod Nutr Dev, 42(6), 583-6.

Arriola, J., & Foote, R. H. (1987). Glycerolation and thawing effects on bull spermatozoa frozen in detergent-treated egg yolk and whole egg extenders. *J Dairy Sci, 70*(8), 1664-1670.

Axner, E., Hermansson, U., & Linde-Forsberg, C. (2004). The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. *Anim Reprod Sci, 84*(1-2), 179-191.

Bamba, K. (1988). Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology, 29*(6), 1245-1251.

Barbas, J. P., & Mascarenhas, R. D. (2009). Cryopreservation of domestic animal sperm cells. *Cell Tissue Banking, 10*, 49-62.

Barrangou, R., & Doudna, J. A. (2016). Applications of CRISPR technologies in research and beyond. *Nature Biotechnology, 34*(9), 933–941.

Barsila, S.R., Devkota, N. R., Kreuzer, M., & Marquardt, S. (2015). Effects of different stocking densities on performance and activity of cattle x yak hybrids along a transhumance route in the Eastern Himalaya. *SpringerPlus, 4*, 398.

Boehm, E. M., Gildenberg, M. S., & Washington, M. T. (2016). The many roles of PCNA in eukaryotic DNA replication. *Enzymes, 39,* 231-254.

Brinkman, E. K., Chen, T., Amendola, M., & Van Steensel, B. (2014). Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Research, 42*(22), 1–8.

Butler, S. T., Hutchinson, I. A., Cromie, A. R., & Shalloo, L. (2014). Applications and cost benefits of sexed semen in pasture-based dairy production systems. *Animal, 8 Suppl 1*, 165-172.

Byrne, Guerard W., McGregor, C. G. A., & Breimer, M. E. (2015). Recent investigations into pig antigen and anti-pig antibody expression. *International Journal of Surgery, 23*, 223–228.

Cai, X., Yu, S., Mipam, T., Yang, F., Zhao, W., Liu, W., … Wu, S. (2017). Comparative analysis of testis transcriptomes associated with male infertility in cattleyak. *Theriogenology, 88,* 28–42.

Chase, C. C., Jr., Larsen, R. E., Randel, R. D., Hammond, A. C., & Adams, E. L. (1995). Plasma cortisol and white blood cell responses in different breeds of bulls: a comparison of two methods of castration. *J Anim Sci, 73*(4), 975-980.

Cheng, W., Zhao, H., Yu, H., … Wei, H. J. (2016). Efficient generation of GGTA1-null Diannan miniature pigs using TALENs combined with somatic cell nuclear transfer. *Reprod Biol Endocrinol. 14*, 77.

Cooper, D. K. C. (2015). The case for xenotransplantation. *Clinical Transplantation, 29*(4), 288–293.

de Graaf, S. P., Evans, G., Maxwell, W. M., Cran, D. G., & O'Brien, J. K. (2007). Birth of offspring of pre-determined sex after artificial insemination of frozen-thawed, sex-sorted and re-frozen-thawed ram spermatozoa. *Theriogenology, 67*(2), 391-398.

Elkhawagah, A. R., Martino, N. A., Ricci, A., Storti, V., Rumbolo, F., Lange-Consiglio, A., Vincenti, L. (2020). Effect of relaxin on cryopreserved beef bull semen characteristics. *Cryobiology, 95*, 51-59.

Gao, Y., Wu, F., Ren, Y., Zhou, Z., Chen, N., Huang, Y., … Dang, R. (2020). MiRNAs Expression Profiling of Bovine (*Bos taurus*) Testes and Effect of bta-miR-146b on Proliferation and Apoptosis in Bovine Male Germline Stem Cells. *International Journal of Molecular Sciences, 21,* 3846.

Gendt, K. D., Swinnen, J. V., Saunders, P. T. K., Schoonjans, L., Dewerchin, M., Devos, A., … Verhoeven, G. (2004). A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proceedings of the National Academy of Sciences of the United States of America, 101,* 1327-1332.

Gomes, A. R., Yong, J.S., Kiew, K.C., Aydin, E., Khongkow, M., Laohasinnarong, S., & Lam, E., W. (2016). Sirtuin1 (SIRT1) in the acetylation of downstream target proteins. *Methods in Molecular Biology, 1436,* 169-188.

Gopisetty, G., Ramachandran, K., & Singal, R. (2006). DNA methylation and apoptosis. *Molecular Immunology, 43,* 1729-40.

Gujral, P., Mahajan, V., Lissaman, A.C., & Ponnampalam, A.P. (2020). Histone acetylation and the role of histone deacetylases in normal cyclic endometrium. *Reproductive Biology and Endocrinology, 18,* 84.

Haldane, J. B. S. (1922). Sex ratio and unisexual sterility in hybrid animals. *Journ of Gen, 12*, 101–109.

Hofmo, P. O., & Almlid, T. (1991). Recent developments in freezing of boar semen with special emphasis on cryoprotectants. Boar Semen Preservation. *Proceedings of the Second International Congress on Boar Semen Preservation, vol. 2. Reprod. Domest. Anim., 1*, 111-122.

Hollinshead, F. K., Evans, G., Evans, K. M., Catt, S. L., Maxwell, W. M., & O'Brien, J. K. (2004). Birth of lambs of a pre-determined sex after in vitro production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa. *Reproduction, 127*(5), 557-568.

Holt, W. V. (2000). Basic aspects of frozen storage of semen. *Anim Reprod Sci, 62*(1-3), 3-22.

Hauschild, J., Petersen, B., Santiago, Y., Queisser, A. L., … Niemann H. (2011). Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. *Proc Natl Acad Sci U S A, 108*, 12013–7.

Jinek, M., East, A., Cheng, A., Lin, S., Ma, E., & Doudna, J. (2013). RNA-programmed genome editing in human cells. *Elife, 2*, e00471.

Johnson, L. A., Flook, J. P., & Hawk, H. W. (1989). Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. *Biol Reprod, 41*(2), 199-203.

Kikuchi, K., Nagai, T., Kashiwazaki, N., Ikeda, H., Noguchi, J., Shimada, A., . . . Kaneko, H. (1998). Cryopreservation and ensuing in vitro fertilization ability of boar spermatozoa from epididymides stored at 4 degrees C. *Theriogenology, 50*(4), 615-623.

Koji, T., Kondo, S., Hishikawa, Y., An, S., & Sato, Y. (2008). In situ detection of methylated DNA by histo endonuclease-linked detection of methylated DNA sites: a new principle of analysis of DNA methylation. *Histochemistyry and Cell Biology*, *130*, 917-925.

Layek, S. S., Mohanty, T. K., Kumaresan, A., & Parks, J. E. (2016). Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. *Anim Reprod Sci, 172*, 1-9.

Le, Q. A., Tanihara, F., Wittayarat, M., Namula, Z., Sato, Y., Lin, Q., Takebayashi, K., Hirata, M., & Otoi, T. (2021). Comparison of the effects of introducing the CRISPR/Cas9 system by microinjection and electroporation into porcine embryos at different stages. *BMC Res Notes, 14*(1), 7.

Li, U.-C., wang, G.-W, Xu, S.-R., Zhang, X.-N., & Yang, Q.-E. (2020). The expression of histone methyltransferases and distribution of selected histone methylations in testes of yak and cattle-yak hybrid. *Theirogenology, 144*, 164-173.

Liu, Z., Li, Q., Pan, Z., Qu, X., Zhang, C., & Xie, Z. (2011). Comparative analysis on mRNA expression level and methylation status of DAZL gene between cattle-yaks and their parents. *Animal Reproduction Science, 126*, 256-264.

Liu, M., Chen, B., Pei, L., Zhang, Q., Zou Y., Xiao, H., … Wang, H. (2018). Decreased H3K9ac level of StAR mediated testicular dysplasia induced by prenatal dexamethasone exposure in male offspring rats. *Toxicology, 408,* 1-10.

Martins, C. F., Báo, S. N., Dode, M. N., Correa, G. A., Rumpf, R. (2007). Effects of freeze-drying on cytology, ultrastructure, DNA fragmentation, and fertilizing ability of bovine sperm. *Theriogenology, 67*(8), 1307-15.

McGee, S. R., & Narayan, P. (2013). Precocious puberty and Leydig cell hyperplasia in male mice with a gain of function mutation in the LH receptor gene. *Endocrinology, 154*, 3900–3913.

McGowan, A. C., Murray, R. D. (1999). Health status of bulls used for natural breeding on farms in south west Scotland. *Zentralbl Veterinarmed B, 46*(5), 311-21.

Mizutani, T., Sumigama, S., Nagakubo, K., Shimizu, N., Oba, H., Hori, T., & Tsutsui, T. (2010). Usefulness of addition of Orvus ES paste and sodium lauryl sulfate to frozen feline semen. *J Vet Med Sci, 72*(1), 23-27.

Morton, K. M., Evans, G., & Maxwell, W. M. (2010). Effect of glycerol concentration, Equex STM supplementation and liquid storage prior to freezing on the motility and acrosome integrity of frozen-thawed epididymal alpaca (Vicugna pacos) sperm. *Theriogenology, 74*(2), 311-316.

Namula, Z., Kodama, R., Tanihara, F., Morita, Y., Sato, Y., Wittayarat, M., . . . Otoi, T. (2014). Effects of skim-milk supplementation on the quality and penetrating ability of boar semen after long-term preservation at 15 degrees C. *Acta Vet Hung, 62*(1), 106-116.

Niu, Y., Shen, B., Cui, Y., Chen, Y., Wang, J., Wang, L., … Sha, J. (2014). Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell, 156*(4), 836–843.

O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., McLachlan, R. I., & Stanton, P. G. (2011). Spermiation The process of sperm release. *Spermatogenesis, 1,* 14-35.

Pena, A. I., Lugilde, L. L., Barrio, M., Herradon, P. G., & Quintela, L. A. (2003). Effects of Equex from different sources on post-thaw survival, longevity and intracellular Ca2+ concentration of dog spermatozoa. *Theriogenology, 59*(8), 1725-1739.

Phakdeedindan, P., Wittayarat, M., Tharasanit, Y., Techakumphu, M., Shimazaki, M., Sambuu, R., … Sato, Y. (2021). Aberrant levels of DNA methylation and H3K9 acetylation in the testicular cells of crossbred cattle-yak showing infertility. *Reproduction in Domestic Animal,* (in press)

Phelps, C. J., Koike, C., Vaught, T. D., Boone, J., … Ayares, D.L. (2003). Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science, 299*, 411–4.

Piolat, C., Dyon, J.-F., Chavanis, N., Jacquier, C., Durand, C., Pasquier, D. (2003). Testicular Leydig cell hyperplasia in children. *Progrès en Urologie, 13*, 110-114.

Pursel, V. G., Schulman, L. L., & Johnson, L. A. (1978). Effect of Orvus ES Paste on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar sperm. *J Anim Sci, 47*(1), 198-202.

Qin, W., Wang, B., Yang, L., Yuan, Y., Xiong, X., Li, J., & Yin, S. (2021). Molecular cloning, characterization, and function analysis of the AMH gene in Yak (*Bos grunniens*) Sertoli cells. *Theriogenology, 163*, 1-9.

Ríos-Utrera, Á., Montaño-Bermúdez, M., Vega-Murillo, V. E., Martínez-Velázquez, G., Baeza-Rodríguez, J. J., Román-Ponce, S. I. (2021). Genetic diversity evolution in the Mexican Charolais cattle population. *Anim Biosci, 34*(7), 1116-1122.

Robert, N., Yan, C., Si-Jiu, Y., Bo, L., He, H., Pengfei, Z., … Qian, Z. (2021) Expression of Rad51 and the histo-morphological evaluation of testis of the sterile male cattle-yak. *Theriogenology, 172,* 239-254.

Rokouei, M., Vaez, T. R., Moradi, S. M., Sargolzaei, M., Sørensen, A. C. (2010). Monitoring inbreeding trends and inbreeding depression for economically important traits of Holstein cattle in Iran. *J Dairy Sci, 93*(7), 3294-302.

Rota, A., Strom, B., Linde-Forsberg, C., & Rodriguez-Martinez, H. (1997). Effects of equex STM paste on viability of frozen-thawed dog spermatozoa during in vitro incubation at 38 degrees C. *Theriogenology, 47*(5), 1093-1101.

Sarkar, M., Dutta Borah, B. K., Bandopadhayay, S., Meyer, H. H., & Prakash, B. S. (2009). Season of the year influences semen output and concentrations of testosterone in circulation of yaks (Poephagus grunniens L.). *Anim Reprod Sci, 115*(1-4), 300-305.

Sato, Y., Kuriwaki, R., Hagino, S., Shimazaki, M., Sambuu, R., Hirata, M., …Otoi, T. (2020). Abnormal functions of Leydig cells in crossbred cattle–yak showing infertility. *Reproduction in Domestic Animal, 55,* 209-216.

Saragusty, J., Gacitua, H., Zeron, Y., Rozenboim, I., & Arav, A. (2009). Double freezing of bovine semen. *Anim Reprod Sci, 115*(1-4), 10-17.

Shan, L. X., Bardin, C. W., & Hardy, M. P. (1997). Immunohistochemical analysis of androgen effects on androgen receptor expression in developing Leydig and Sertoli cells. *Endocrinology, 138*, 1259–1266.

Shimazaki, M., Sambuu, R., Sato, Y., Kim Do, L. T., Tanihara, F., Taniguchi, M., & Otoi, T. (2015). Effects of Orvus Es Paste on the Motility and Viability of Yak (Bos Grunniens) Epididymal and Ejaculated Spermatozoa after Freezing and Thawing. *Cryo Letters, 36*(4), 264-269.

Shimizu, A., Meehan, S. M., Kozlowski, T., Sablinski, T., Ierino, F. L., Cooper, D. K. C., … Colvin, R. B. (2000). Acute humoral xenograft rejection: Destruction of the microvascular capillary endothelium in pig-to-nonhuman primate renal grafts. *Laboratory Investigation, 80*(6), 815–830.

Soleimanzadeh, A., Talavi, N., Shafiepour, Y. V., Bucak, M. N. (2020). Caffeic acid improves microscopic sperm parameters and antioxidant status of buffalo (Bubalus bubalis) bull semen following freeze-thawing process. *Cryobiology, 95*, 29-35.

Sun, L., Mipam, T. D., Zhao, F., Liu, W., Zhao, W., Wu, S., Xu, C., Yu, S., & Cai, X. (2017). Comparative testis proteome of cattleyak from different developmental stages. *Animal, 11*, 101–111.

Takahashi, Y. (2015). Preservation of semen. *In: Artificial Insemination Association of Japan (eds.), Textbook for the Course of Artificial Insemination in Domestic Animals.* , 304-318 (In Japanese).

Takebayashi, K., Wittayarat, M., Lin, Q., Hirata, M., Yoshimura, N., Torigoe, N., Nagahara, M., Do, L. T. K., Tanihara, F., & Otoi, T. (2022). Gene editing in porcine embryos using a combination of electroporation and transfection methods. *Reprod Domest Anim,* Jun 14.

Tanihara, F., Hirata, M., Nguyen, N. T., Sawamoto, O., Kikuchi, T., Doi, M., & Otoi, T. (2020). Efficient generation of GGTA1-deficient pigs by electroporation of the CRISPR/Cas9 system into in vitro-fertilized zygotes. *BMC Biotechnol, 20*(1), 40.

Tanihara, F., Takemoto, T., Kitagawa, E., Rao, S., Do, L. T. K., Onishi, A., & Otoi, T. (2016). Somatic cell reprogramming-free generation of genetically modified pigs. *Science Advances, 2*(9), 1–9.

Tonelli, M., & Riella, M. (2014). Chronic kidney disease and the aging population. *J Bras Nefrol, 36*(1), 1–5.

Tsutsui, T., Hase, M., Hori, T., Ito, T., & Kawakami, E. (2000). Effects of orvus ES paste on canine spermatozoal longevity after freezing and thawing. *J Vet Med Sci, 62*(5), 533-535.

Vaziri, H., Dessain, S. K., Eaton, E. N., Imai, S.-I., Frye, R. A., Pandita, T. K., … Weinberg, R. A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell*, *107,* 149-159.

Wang, N., Vandepitte, W., & Wu, J. C. (1994). Crossbreeding yak (Bos Grunniens) and yellow cattle (Bos Taurus): Stimulation of a rotational systema and estimation of crossbreeding means and heterosis effects. *Journal of Applied Animal Research, 6*, 1–12.

Wang, R. S., Yeh, S., Tzeng, C. R., & Chang, C. (2009). Androgen receptor roles in spermatogenesis and fertility: Lessons from testicular cell-specific androgen receptor knockout mice. *Endocrine Reviews, 30*, 119–132.

Wang, S., Pan, Z., Zhang, Q., Xie, Z., Liu, H., & Li, Q. (2012). Differential mRNA expression and promoter methylation status of SYCP3 gene in testes of yaks and cattle-yaks. *Reproduction in Domestic Animals, 47*, 455–462.

Watson, P. F. (2000). The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci, 60-61*, 481-492.

Wang, X., Zhang, F.X., Wang, Z.M., Wang, Q., Wang, H.F., Ren, D.P., … Liu, D.J. (2016). Histone H3K9 acetylation influences growth characteristics of goat adipose-derived stem cells *in vitro*. *Genetics and Molecular Research, 15*, gmr15048954

Watson, P. F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev, 7*(4), 871-891.

White, I. G. (1993). Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. *Reprod Fertil Dev, 5*(6), 639-658.

Wiener, G., Jianlin, H., & Ruijun, L. (2003). The Yak. *The Regional Office for Asia and the Pacific. Food and Agriculture Organization of the United Nations, Bangkok, Thailand*.

Wildt, D. E. (2000). Genome resource banking for wildlife research, management, and conservation. *ILAR J, 41*(4), 228-34.

Wittayarat, M., Kimura, T., Kodama, R., Namula, Z., Chatdarong, K., Techakumphu, M., . . . Otoi, T. (2012). Long-term preservation of chilled canine semen using vitamin C in combination with green tea polyphenol. *Cryo Letters, 33*(4), 318-326.

Wrobel, K. H., Bickel, D., Kujat, R. (1995) Immunohistochemical study of seminiferous epithelium in adult bovine testis using monoclonal antibodies against Ki-67 protein and proliferating cell nuclear antigen (PCNA). *Cell & Tissue Research, 283,* 191-201.

Wu, S., Mipam, T. D., Xu, C., Zhao, W., Shah, M. A., Yi, C., … Zhong, J. (2020). Testis transcriptome profiling identified genes involved in spermatogenic arrest of cattleyak. *PLOS ONE, 15*(2), e0229503.

Xin, J., Yang, H., Fan, N., Zhao, B., … Lai, L. (2013). Highly efficient generation of GGTA1 biallelic knockout inbred mini-pigs with TALENs. *PLoS One, 8*, e84250.

Xu, C., Wu, S., Zhao, W., Mipam, T., Liu, J., Liu, W., Yi, C., Shah, M. A., Yu, S., & Cai, X. (2018). Differentially expressed microRNAs between cattleyak and yak testis. *Scientific Reports, 8*, 592.

Yan, P., Xiang, L., Guo, X., Bao, P. J., Jin, S., & Wu, X. Y. (2014). The low expression of Dmrt7 is associated with spermatogenic arrest in cattle-yak. *Molecular Biology Reports, 41*, 7255–7263.

Yin, S., Qin, W., Wang, B., Zhou, J., Yang, L., Xiong, X., & Li, J. (2020). Absence of Sirtuin 1 impairs the testicular development in cattleyak by inactivating SF-1. *Reproduction in Domestic Animal, 55,* 1054–1060.

Zhang, L., Ma, T., Tao, Q., Tan, W., Chen, H., Liu, W., … Tang, K. (2020). Bta-miR-34b inhibits proliferation and promotes apoptosis via the MEK/ERK pathway by targeting MAP2K1 in bovine primary Sertoli cells. *Journal of Animal Science, 98, 10,* 1–15.

Zhu, X., Hu, M., Ji, H., Huang, T., Ge, R. S., & Wang, Y. (2021). Exposure to di-n-octyl phthalate during puberty induces hypergonadotropic hypogonadism caused by Leydig cell hyperplasia but reduced steroidogenic function in male rats. *Ecotoxicology and Environmental Safety, 208*, 111432.

Zi, X. D. (2003). Reproduction in female yaks (Bos grunniens) and opportunities for improvement. *Theriogenology, 59*(5-6), 1303-12.

Zomborszky, Z., Zubor, T., Tóth, J., Horn, P. (1999). Sperm collection from shot red deer stags (Cervus elaphus) and the utilisation of sperm frozen and subsequently thawed. *Acta Vet Hung, 47*(2), 263-70.