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Effects of different extenders on DNA integrity of boar spermatozoa following freezing–thawing ☆

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ABSTRACT

The sperm-rich fraction, collected from eight mature Yorkshire boars, was frozen in an extender containing 9% LDL (w/v), 100 mM trehalose, or 20% yolk (v/v), respectively. Sperm DNA integrity was assessed using the single-cell gel electrophoresis (SCGE). Other sperm quality characteristics such as motility, acrosome and membrane integrity were also monitored. The results showed that freezing–thawing caused an increase in sperm DNA fragmentation, and extender containing 9% LDL could significantly protect sperm DNA integrity ($P < 0.05$) from the damage caused by cryopreservation and decrease DNA damages compared with extender containing 100 mM trehalose and 20% yolk (v/v). No significant difference in damaged DNA was detected between frozen and unfrozen semen samples for extender of 9% LDL and 100 mM trehalose, but cryopreservation could increase the degree of DNA damage ($P < 0.05$), the percentage of damaged DNA degree of grade 2 and 3 was significantly increased. The deterioration in post-thaw sperm DNA integrity was concurrent with reduced sperm characteristics. The data here demonstrated that the cryoprotectant played a fundamental role in reducing boar sperm DNA damage and protecting DNA integrity. It can be suggested that evaluation of sperm DNA integrity, coupled with correlative and basic characteristics such as motility, acrosome integrity and membrane integrity, may aid in determining the quality of frozen boar semen.

Artificial insemination (AI) has been widely used for genetic improvement in cattle. Unlike being widely used in bovine industry, AI in swine industry was limited due to lower fertility of boar spermatozoa after cryopreservation [1–3]. Nevertheless, owing to the increasing value of high quality boars, the interest in freezing of semen is rising. Johnson et al. [4] indicated that the most commonly accepted method of preserving male reproductive capacity was semen cry-preservation, and was generally acknowledged to cause impaired fertility.

The integrity of sperm DNA was very important for the success of fertilization and the development of fetus and offspring [5]. A variety of methods had been developed for detecting DNA strand damage [6–8]. Duty et al. [9] had proven that single-cell gel electrophoresis (SCGE) was a very sensitive method for detecting DNA strand breaks in human sperm. The SCGE, also known as comet assay, since damaged DNA looked like a comet, was first introduced by Ostling and Johanson [10]. In domestic farm animals, the SCGE had been used to detect DNA damage of stallion semen in response to cooling injury [11]. Hamamah et al. [12] observed the damage to sperm DNA in pig. In recent reports, this method has been used to detect DNA damage in boar sperm during liquid storage and cryopreservation [13–16].

Evidence had showed that cryo-damage induced by freeze–thaw could be minimized by cryoprotectants [4]. In the 1970s, Evans et al. [17] indicated that egg yolk low-density lipoprotein contained 75% of lipid and 25% of a residual lipoprotein. The ether-extracted lipid was composed of 75% triglycerides, 7% sterols, 2% mono- and di-glycerides, and 16% phospholipids. Pace et al. [18] had purified egg yolk and observed that the low-density fraction had a cryoprotective function. Watson [19,20] investigated the protection function of low-density lipoprotein fraction (LDF) and demonstrated that bull sperm with LDF had a higher viability and less acrosome damage than those with egg yolk, while ram sperm showed greater acrosome damage with LDF compared with those with egg yolk during freezing and thawing. Subsequently, numerous studies had demonstrated that low-density lipoproteins (LDL) could be largely responsible for the resistance against cold shock and for the improvement of sperm quality in freeze–thaw process [21–23]. We had found that LDL possessed remarkable cryoprotective properties for frozen boar sperm and the extender with 9% LDL concentration significantly enhanced sperm quality after freezing–thawing [15,24]. Since the important function of resisting dehydration or freezing was found,

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trehalose had been widely used as a non-permeating cryoprotectant and significantly protected spermatozoa against freezing damage [25–27]. Recently, we had studied the effects of extender of boar semen supplemented with different amounts of trehalose. Moreover, the optimum concentration of trehalose supplementation had been determined to be 100 mM [28]. The effects of the freeze–thaw process and cryoprotectant on boar sperm were most often assessed in terms of sperm motility, membrane integrity, acrosome integrity and fertilization capacity. However, these parameters provided no information about the integrity of the sperm DNA after cryopreservation.

Nevertheless, little is known about the effects the types of semen extender on the integrity of DNA in frozen boar sperm. The reexamination of currently used the type of cryoprotectants of semen dilution, using a variety of different sperm quality measurement and DNA integrity in particular, may bring new information on how to handle frozen boar semen in a lenient way to minimize damage. Until now, about the effects of different cryoprotectants especially LDL and trehalose on boar sperm DNA integrity are very limited and fewer authors have evaluated the degree of boar spermatozoa DNA damage after freezing. In this study, we employed the modified single-cell gel electrophoresis to examine the effects of LDL and trehalose on DNA integrity of freeze–thaw boar sperm. The aim of present study was to investigate the effects of different extenders on DNA integrity of boar sperm following freezing–thawing. In addition, the basic characteristics of sperm such as motility, acrosome integrity and membrane integrity were assessed. Furthermore, we attempted to provide scientific evidence for the better cryopreservation prosperity of LDL and trehalose.

Materials and methods

Semen collection

Semen was collected from eight mature Yorkshire boars once a week by the gloved hand technique and filtered through four layers of sterile cotton gauze to remove the gel particles, and then transferred into an insulated vacuum bottle. The semen samples of the sperm-rich fraction were assessed for volume, sperm concentration and percentage of motile spermatozoa. The sperm-rich fraction of ejaculates with greater than a minimum of 75% motile and of 80% morphologically normal spermatozoa was used.

Diluents preparation

The component of basic diluent, TCF, consists of 200 mM Tris, 77 mM citric acid and 61 mM fructose. The cooling extender of control was composed of basic diluent plus 25 mg gentamicine, 50,000 IU penicillin and 20 ml egg yolk for 100 ml distilled nonpyrogenic water. For the cooling extender of treatment I, 20% egg yolk of the extender was replaced by 9% (w/v) LDL in TCF (control). LDL was extracted from egg yolks according to the method described by Moussa et al. [22]. For the basic freezing diluent of treatment II, 100 mM trehalose was added into the TCF. For the cooling extender, it was composed of 25 mg gentamicin, 50,000 IU penicillin and 20 ml egg yolk for 100 ml basic freezing diluent. The freezing extenders of control and treatments were composed of the cooling extender and 9% (v/v) glycerol, respectively.

Semen freezing

The semen was processed and frozen by the straw freezing method. The freshly collected semen was randomly divided and transferred into 15 ml pre-warmed tubes, and held for 30 min at room temperature, and subsequently centrifuged at room temperature for 10 min at 500g to remove the seminal plasma. After elimination of seminal plasma, about 12 ml pre-warmed addition of Beltsville thawing solution [4] was added to all of the above tubes, respectively. The tubes were wrapped by 12–15 layers of sterile gauze, and the sperm suspension was slowly cooled to 17 °C, and subsequently centrifuged at 17 °C for 10 min at 800g. The supernatant was then discarded.

The concentrated semen was diluted with the cooling extenders of control and treatment to obtain 1.5×10^9 sperm/ml [29], respectively. The diluted semen was gently mixed, and all of the tubes were wrapped by 12–15 layers of sterile gauze. They were slowly cooled to 5 °C, and equilibrated for 1.5–3 h.

The semen was further diluted (2:1, two parts semen to one part extender) with different freezing extenders, respectively. The final sperm concentration was 1.0×10^9 sperm/ml, and the final glycerol concentration was 3%. The sperm suspension was loaded into 0.25 ml straws immediately, and straws were sealed manually using polyvinyl alcohol. Then the straws were horizontally placed on an aluminum rack and maintained at 5 °C for 2–3 h. The steps of freezing program were as follows: from +5 °C to −5 °C with 1 °C/min by programmable freezing device (Mini Digitcool 1400, IMV, France). All of the straws were then placed in contact with nitrogen vapor for 15 min, about 2–3 cm (−120 °C) above the liquid nitrogen level, and then immersed into the liquid nitrogen (−196 °C) for storage.

Thawing semen

For each treatment, 10 straws were thawed separately by immersing in a water bath at 37 °C for 45 s. Immediately, all of the thawed samples were then transferred into a plastic tube containing 9.5 ml of BTS solution and 0.5 ml of the relevant cooling extender (pre-warmed). Subsequently, all semen samples were incubated at 37 °C for the assessment of sperm parameters.

Assessment of sperm DNA integrity by the neutral comet assay

The procedure of the SCGE described by Morris et al. [30], with few modifications, and was applied to boar semen in the experiment. The frozen-thawed sperm samples were washed by centrifugation (800g, 5 min) at room temperature at first and subsequently were diluted with phosphate buffered saline (PBS, 0.132 g/l $CaCl_2{\cdot}2H_2O$, 0.2 g/l KCl, 8.0 g/l NaCl, 0.121 g/l $MgSO_4{\cdot}7H_2O$, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4) until a final concentration of 2×10^6 sperm/ml. Frozen sperm was prepared immediately after thawing to prevent spermatozoa degradation.

Slide preparation

Agarose solutions of regular and low gelling points were prepared in advance by heated dissolving 0.75% (w/v) normal-melting agarose (Promega Corporation, Madison, U.S.A) and 1% low melting agarose (Promega Corporation, Madison, U.S.A) powder in distilled water, respectively. Slides were prepared by first applying a thin layer of 100 μl 0.75% of the regular agarose and spreading it across the length of the slide using a pipette tip, eliminating agarose in excess. The slides were air-dried and stored at room temperature, and protected from dust and light. For the second agarose layer, 200 μl 1% (w/v) of low melting point agarose was added to 10 μl of each sperm suspension in an Eppendorf tube. An aliquot of 100 μl of this suspension was spread on one slide with a coverslip. This was the second slide layer and was left to solidify at 4 °C for 15 min. After solidification completed, the coverslip was removed and peripheries of the second agarose layer was coated with 1% low melting point agarose to avoid the loss of semen sample during the next procedure. Then all slides were left to solidify at 4 °C for 15 min. Six slides were prepared for each semen sample.