Available online at www.jbr-pub.org

Open Access at PubMed Central

The Journal of Biomedical Research, 2017 31(5): 453–461

JBR

Original Article

Effects of difructose dianhydride (DFA)-IV on *in vitro* fertilization in pigs

Young-Joo Yi[1,2,✉], S. Kamala-Kannan[1], Jeong-Muk Lim[1], Byung-Taek Oh[1], Sang-Myeong Lee[1,2,✉]

[1] *Division of Biotechnology, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan 54596, Korea;*
[2] *Safety, Environment and Life Science Institute, Chonbuk National University, Iksan 54596, Korea.*

Abstract

Difructose dianhydride IV (DFA-IV) is produced from levan, which is a natural polysaccharide that belongs to the fructan family, through the activity of levan fructotransferase (LF) derived from microorganisms. Recently, DFA-IV has been expected to have diverse applications in the food and medical industry. Here, we examined the potential application of DFA-IV for *in vitro* fertilization (IVF) in pigs. In the assessment of acrosomal integrity during incubation, intact acrosomal or viable spermatozoa were highly sustained in 0.1% or 0.25% DFA-IV (69.8%-70.8%, $P<0.05$). Reactive oxygen species (ROS) levels during sperm incubation decreased following the addition of DFA-IV, and 0.1%-0.5% DFA-IV in particular significantly decreased ROS production relative to that seen with no addition or 0.75% DFA-IV. Total fertilization (mono + polyspermic oocyte) rate was significantly higher in the addition of 0.1% DFA-IV (94.2%) than with other concentrations (71.8%-86.7%, $P<0.05$). When using reduced IVF times and lower sperm numbers, we found that addition of 0.1%–0.5% DFA-IV significantly increased the fertilization rate ($P<0.05$). Fertilized oocytes treated with 0.1% DFA-IV exhibited higher embryonic development and blastocyst formation than those treated with other concentrations ($P<0.05$). Consequently, the addition of DFA-IV during IVF improved fertilization and embryonic development, suggesting the possible use of novel sugars for enhancement of assisted reproductive technology (ART) in mammals.

Keywords: difructose dianhydride-IV, spermatozoa, *in vitro* fertilization, embryo, pig

Introduction

Sugar is an essential component of the sperm extender because it provides an energy source for sperm viability and maintains the osmotic pressure of the freezing diluent[1–2]. Many types of sperm extenders, with short- or long-term storage capacity, have been established to prolong sperm longevity; however, sperm viability is often not sufficient. Levan, a nonstructural carbohydrate polymer, belongs to the fructan family and is made up of fructose residues[3]. Levan has been reported to have various biomedically advantageous properties such as anti-oxidative, anti-tumorogenic, anti-inflammatory, anti-carcinogenic, and anti-hyperglycemic effects[4–9]; these diverse characteristics may facilitate the application of levan as a

✉Corresponding author: Young-Joo Yi and Sang-Myeong Lee, Division of Biotechnology, College of Environmental and Bioresource Sciences; Safety, Environment and Life Science Institute, Chonbuk National University, Iksan 54596, Korea. Tel/Fax: + 82-63-850-0835/+ 82-63-850-0834, E-mails: yiyj@jbnu.ac.kr, leesangm@jbnu.ac.kr.

Received 12 September 2016, Revised 24 November 2017, Accepted 10 January 2017, Epub 20 April 2017

CLC number: R715, Document code: A

The author reported no conflict of interest.

doi:10.7555/JBR.31.20160115

pharmaceutical and therapeutic agent, contributing to the development of a multifunctional drug.

Difructose anhydride (DFA) is composed of two fructose residues in which the reducing end of each residue is linked to the non-reducing hydroxyl group of the counter residue[10]. Five DFAs have been identified; among them, DFA-II and V are synthesized chemically, whereas DFA-I and DFA-III are produced enzymatically from inulin, a naturally produced polysaccharide found in plants, through catalysis by inulin fructotransferase[11]. DFA-IV (α-D-fructosefuranose-β-D-fructofuranose-2',6:2,6'-dianhydride) is produced from levan through catalysis by levan fructotransferase (LF), which is derived from microorganisms[12]. Chemical synthesis of DFA from natural fructans has been shown to have low reaction selectivity, and the product is difficult to separate and purify. Moreover, such chemical synthesis causes environmental pollution; thus, enzymatic synthesis using microbes or plants has been considered as a favorable alternative for the preparation of DFA[11]. Although levan has been reported to have many applications, the physiologic function of DFA-IV has not been extensively studied to date.

Various assisted reproductive technologies (ARTs) have been developed to optimize fertilization in humans and animals. Above all, *in vitro* fertilization (IVF) using oocyte matured *in vitro* is considered as a good tool to evaluate fertilization competence. Therefore, in this study, we examined the potential application of DFA-IV during IVF to examine whether this carbohydrate could be used as a new alternative sugar in ARTs in mammals.

Material and methods

Isolation and preparation of DFA-IV

DFA-IV was obtained from Realbiotech Co. Ltd. (Kongju, Chungnam-do, South Korea). DFA-IV was synthesized from sucrose by levansucrase isolated from *Zymomonas mobilis*. Sucrose is hydrolysed in the presence of levansucrase at 10°C for 20 hours in acetic acid buffer (pH 5.0). Levan was purified from the reaction solution and subjected to reaction at 37°C for 40 hours in acidic buffer (pH 3.0-7.0) in the presence of levan fructotransferase (LF) obtained from *Escherichia coli* JUD81 (KCTC 0877BP) to produce DFA-IV. The reaction mixture was allowed to stand in hot water for 5 minutes to inactivate the remaining enzymes and loaded onto a charcoal column. The column was washed with 5% ethanol and 25% ethanol to elute the absorbed DFA-IV. The effluents containing DFA-IV were collected and concentrated to a volume of 30 mL using a rotary evaporator. To the concentrate, 100% ethanol was added to a final ethanol concentration of 95% or higher, facilitating crystallization of DFA-IV. These precipitates were washed many times with pure ethanol and dried to yield 2.5 g of pure DFA-IV.

Characterization of DFA-IV

Fourier transform infrared (FTIR) spectra of the DFA-IV and l-fructose powders were obtained in KBr pellets on a Perkin-Elmer FTIR spectrophotometer (Irvine, CA, USA) in the diffuse reflectance mode at a resolution of 4 cm^{-1}. The absorbance was obtained in the range of 400 to 4,000 cm^{-1}. X-ray diffractograms (XRD) were obtained using a Cu Kα incident beam (λ = 0.1546 nm), monochromated by a nickel filtering wave at a tube voltage of 40 kV and tube current of 30 mA. The scanning was carried out in the region of 2θ from 4° to 60° at 0.04°/minute with a time constant of 2 seconds. High-performance liquid chromatography (HPLC) analysis of DFA-IV was performed on an Alliance 2695 HPLC system (Waters Co., Milford, MA, USA) composed of a quaternary pump, an auto sampler, a corona charged aerosol detector (CAD; Thermo Fisher Scientific, Chelmsford, MA, USA), and a Waters Empower pro data handling system (Waters Co.). A column (4.6 mm id × 250 mm; Shodex, Tokyo, Japan) was employed for the analysis, eluted with a gradient of acetonitrile and water at a flow rate of 1.0 ml/minute, and l-fructose was used as a standard.

Liquid boar semen processing

The present study was performed in accordance with the guidance provided by the Animal Care and Use Committee (ACUC) of Chonbuk National University, South Korea. Semen was collected from proven fertile adult Duroc boars, 15-22 months of age. The boars were placed on a routine collection schedule of one collection per week. The sperm-rich fraction of ejaculate was collected into an insulated vacuum bottle, and fractions with greater than 85% motile spermatozoa were used. Sperm concentrations were estimated with a hemocytometer, and semen was diluted with Beltsville thawing solution (BTS)[13] to a final concentration of 1×10^8 spermatozoa/mL. The diluted semen was stored in a storage unit at 17°C for 5 days, and sperm motility was observed optically under a light microscope at 38.5°C.

Measurement of sperm viability, acrosomal integrity, and intracellular ROS on spermatozoa

Boar spermatozoa (1×10^7 spermatozoa/mL) were incubated in BTS with or without varying concentrations of DFA-IV [final concentration of 0%–0.75% (w/v)] for 2 hours at 37.5°C. Sperm viability was assayed using LIVE/DEAD® Sperm Viability kit (Molecular

Probes, Eugene, OR, USA), following the manufacturer's protocol. For the assessment of acrosomal integrity, spermatozoa were stained with 10 μg/mL lectin PNA-FITC conjugate (PNA) and propidium iodide (PI), then images were acquired on a fluorescence microscope (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Seoul, Korea) with camera (DS-Fi2, Nikon) and imaging software (version 4.30, Nikon). Spermamtozoa were classified as viable (SYBR14) or dead sperm (PI), and intact (PNA +) or damaged acrosomal sperm (PNA-). The level of intracellular reactive oxygen species (ROS) in sperm was assayed using 1 μmol/L carboxy-DCFDA (Invitrogen, Eugene, OR, USA). The fluorescence intensity was measured using a multimode microplate reader (Spark™ 10M, Tekan, Männedorf, Switzerland) with excitation (ex.) at 485 and emission (em.) at 520 nm.

Collection and *in vitro* maturation (IVM) of porcine oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory. Cumulus oocyte complexes (COCs) were aspirated from antral follicles (3-6 mm in diameter), washed three times in HEPES-buffered Tyrode lactate (TL-HEPES-PVA) medium containing 0.01% (w/v) polyvinyl alcohol (PVA), and then washed three times with oocyte maturation medium[14]. A total of 50 COCs were transferred to 500 μl of maturation medium covered with mineral oil in a 4-well multidish equilibrated at 38.5°C in an atmosphere containing 5% CO_2. The medium used for oocyte maturation was tissue culture medium (TCM) 199 (cat. #50-050-PB; Mediatech, Inc., Manassas, VA, USA) supplemented with 0.1% PVA, 3.05 mmol/L d-glucose, 0.91 mmol/L sodium pyruvate, 0.57 mmol/L cysteine, 0.5 μg/mL lutenizing hormone (LH, L5269, Sigma, St. Louis, MO, USA), 0.5 μg/mL follicle-stimulating hormone (FSH, F2293, Sigma), 10 ng/ml epidermal growth factor (E4127, Sigma), 75 μg/mL penicillin G, and 50 μg/mL streptomycin. After 22 hours of culture, the oocytes were cultured in TCM199 without LH and FSH for 22 hours at 38.5°C in an atmosphere containing 5% CO_2 in air.

IVF and *in vitro* culture (IVC) of porcine oocytes

After IVM, cumulus cells were removed with 0.1% hyaluronidase in TL-HEPES-PVA medium[14]. Thereafter, oocytes were placed into four 100 μL drops of modified Tris-buffered medium (mTBM) covered with mineral oil in a 35-mm polystyrene culture dish. One milliliter of liquid semen preserved in BTS was washed twice in phosphate-buffered saline (PBS) containing 0.1% PVA (PBS-PVA) at 800 × *g* for 5 minutes. At the end of the washing procedure, spermatozoa were resuspended in mTBM. After appropriate dilution, 1 μL of the sperm suspension was added to medium containing oocytes to give a final sperm concentration of 1×10^5 or 5×10^5 spermatozoa/ml. Different concentrations of DFA-IV [0%–0.75% (w/v)] were added to fertilization drops at the time of sperm addition during IVF. Oocytes were co-incubated with spermatozoa for 2 or 5 hours at 38.5°C in an atmosphere containing 5% CO_2. After IVF, oocytes were transferred into 500 μL porcine zygote medium (PZM-3)[15] containing 0.4% bovine serum albumin (BSA, A0281, Sigma) and cultured for an additional 20, 48, or 144 hours. The IVF and IVC studies were repeated five times for each treatment regimen.

Evaluation of pronuclear formation and embryonic development in embryos

Oocytes/embryos were fixed with 2% formaldehyde for 40 minutes at room temperature (RT), washed thrice with PBS, permeabilized with PBS-Triton X-100 for 30 minutes, and stained with 2.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; DNA staining; Molecular Probes, Eugene, OR, USA) for 40 minutes. The number of sperm bound to the zona pellucida (ZP), fertilization status of the zygotes (unfertilized, fertilized-monospermic, or fertilized-polyspermic), cleaved embryo number, blastocyst formation, and cell number per blastocyst were assessed under a fluorescence microscope (Nikon Eclipse Ci microscope; Nikon Instruments Inc., Seoul, Korea).

Statistical analysis

Values are expressed as the mean±standard error of the mean (SEM). Data analyses were processed using one-way analysis of variance (ANOVA) with SAS package 9.3 (SAS Institute Inc., Cary, NC, USA) in a completely randomized design. Duncan's multiple range test was performed to compare values of individual treatment when the F-value was significant ($P < 0.05$).

Results

Characterization of DFA-IV

The FTIR spectra of l-fructose and DFA-IV are depicted in ***Fig. 1A***. The characteristic bands of l-fructose had specific maxima at 784, 977, 1,055, 1,267, 1,339, and 1,430 cm^{-1}. However, those of DFA-IV had specific maxima at 813, 918, 1,047, 1,137, 1,456, and 1,659 cm^{-1}. The most intense peaks of l-fructose (1,055 cm^{-1}) and DFA-IV (1,047 cm^{-1}) could be

ascribed to C–O and C–OH stretching, and the peaks at 918 (DFA-IV) and 977 cm^{-1} (l-fructose) were assigned to the exocyclic C–O vibration of the sugars. The broad O–H stretching of DFA-IV was observed around 3,415 cm^{-1}, and weak C–H vibration was observed at 2901 cm^{-1}[16]. The peak of DFA-IV at 1659 cm^{-1} could be assigned to the H–O–H scissor of residual water[17] The absorbance at 1137 cm^{-1} indicated the pyranose form of sugars[18]. The XRD profiles of l-fructose and DFA-IV are depicted in ***Fig. 1B***. Pure DFA-IV has a broad diffraction peak between 2θ = 10 and 32°, a characteristic peak of DFA-IV, but no narrow sharp peaks were noticed[19]. However, several sharp narrow peaks were observed for l-fructose between 2θ = 12 and 42°. The sharp narrow peaks indicated the crystalline nature of the fructose[20]. The HPLC chromatogram of DFA-IV exhibited two peaks with retention times of 9.75 and 13.87 min corresponding to the l-fructose (25.8%) and DFA-IV (74.2%; ***Fig. 1C***), respectively.

Sperm viability, acrosomal integrity and ROS level in spermatozoa incubated with DFA-IV

Boar spermatozoa were preserved in BTS with or without varying concentrations of DFA-IV for 5 days (***Fig. 2A***). There was no significant difference in sperm motility among the treatment groups until day 4, but then sperm motility decreased in a dose dependent manner, and higher motility was observed in sperm preserved with 0.1% DFA-IV (77.0%) than those of other concentrations on day 5, (61.0%-71.0%; ***Fig. 2A***). When spermatozoa were incubated in BTS in the presence of DFA-IV for 2 hours, significantly higher sperm viability was seen in the treatment of 0.25% DFA-IV (90.4% *vs.* 82.4%-86.8%), along with lower rate of dead spermatozoa (9.7% *vs*. 13.2%-17.6%, $P<0.05$; ***Fig. 2B***). In the assessment of acrosomal integrity, intact acrosomal or viable spermatozoa (PNA-/PI-) were highly sustained in 0.1 or 0.25% DFA-IV (69.8-70.8%, $P<0.05$), and higher rates of damaged acrosome or dead spermatozoa showed in no treatment or 0.5% and 0.75% DFA-IV (16.2%-19.7%), but no significant differences indicated (***Fig. 2C***). Excessive production of reactive oxygen species (ROS) can induce membrane lipid peroxidation, DNA damage, and fertilization impairment on spermatozoa[21–23]. However, an appropriate level of ROS mediates capacitation and acrosomal exocytosis, which are key processes required for fertilization[24]. Therefore, we next examined intracellular ROS levels after sperm incubation (***Fig. 2D***). ROS levels were significantly decreased in the presence of DFA-IV, and 0.1%–0.5% concentrations in particular significantly decreased ROS production relative to that observed in samples without or with 0.75% DFA-IV ($P<0.05$; ***Fig. 2D***).

Increased sperm penetration in IVF medium supplemented with DFA-IV

Oocytes were co-incubated with 5×10^5 spermatozoa/ml for 1 hour, fixed, and stained with DAPI, and the number of spermatozoa bound to the ZP was counted under fluorescence microscope (***Fig. 3A&B***). Significantly higher number of spermatozoa was attached to ZP in the presence of 0.1% or 0.25% DFA-IV (297.9-302.4) compared with those in the presence of other concentrations (208.6-253.7, $P<0.05$; ***Fig. 3A***).

Oocytes were inseminated with 5×10^5 spermatozoa/mL for 5 hours in the absence or presence of DFA-IV (***Fig. 3C***). A higher percentage of monospermic oocyte was observed after IVF with 0.75% DFA-IV (33.6%) than after IVF without (21.7%) or with 0.1%–0.5% DFA-IV (17.6%-29.4%), and higher rates of polyspermic oocytes were observed after IVF with 0.1%–0.5% DFA-IV (57.3%-67.1%) than after IVF without (50.1%) or with 0.75% DFA-IV (39.7%); however, no significant differences between monospermic and polyspermic oocyte rates were found among the treatments (***Fig. 3C***). Notably, the total fertilization rate increased significantly after IVF with 0.1% DFA-IV (94.2%) compared to IVF using other concentrations (71.8-86.7%, $P<0.05$; ***Fig. 3C***).

Polyspermy leads to failure of preimplantation embryonic development[25]. In the above IVF results, over 50% polyspermy occurred in all treatments due to high sperm motility and irregular oocyte quality derived from oocytes matured *in vitro*; thus, we used a reduced insemination time (gamete co-incubation time: 2 hours) and lower sperm concentration (1×10^5 spermatozoa/ml; ***Fig. 3D***). The rate of monospermic oocytes increased after IVF with 0.1% DFA-IV (59.8%) compared with that in other groups; however, the differences were not significant (43.8%-51.0%; ***Fig. 3D***). Significantly higher rates of polyspermic oocytes were observed after addition of 0.25% or 0.5% DFA-IV (27.3%-31.7%) compared with those in the control or after addition of other concentrations of DFA-IV (5.9%-17.6%, $P<0.05$; ***Fig. 3D***). The total fertilization rate was significantly increased after IVF with 0.1%-0.5% DFA-IV (75.2%-80.0% *vs.* 49.6%-66.5% for no treatment or 0.75% DFA-IV, $P<0.05$; ***Fig. 3D***).

Improvement of fertilization and embryonic development after IVF with DFA-IV

After IVF with reduced sperm number and co-incubation time (2 hours) with DFA-IV, fertilized oocytes were further cultured for 48 or 144 hours to

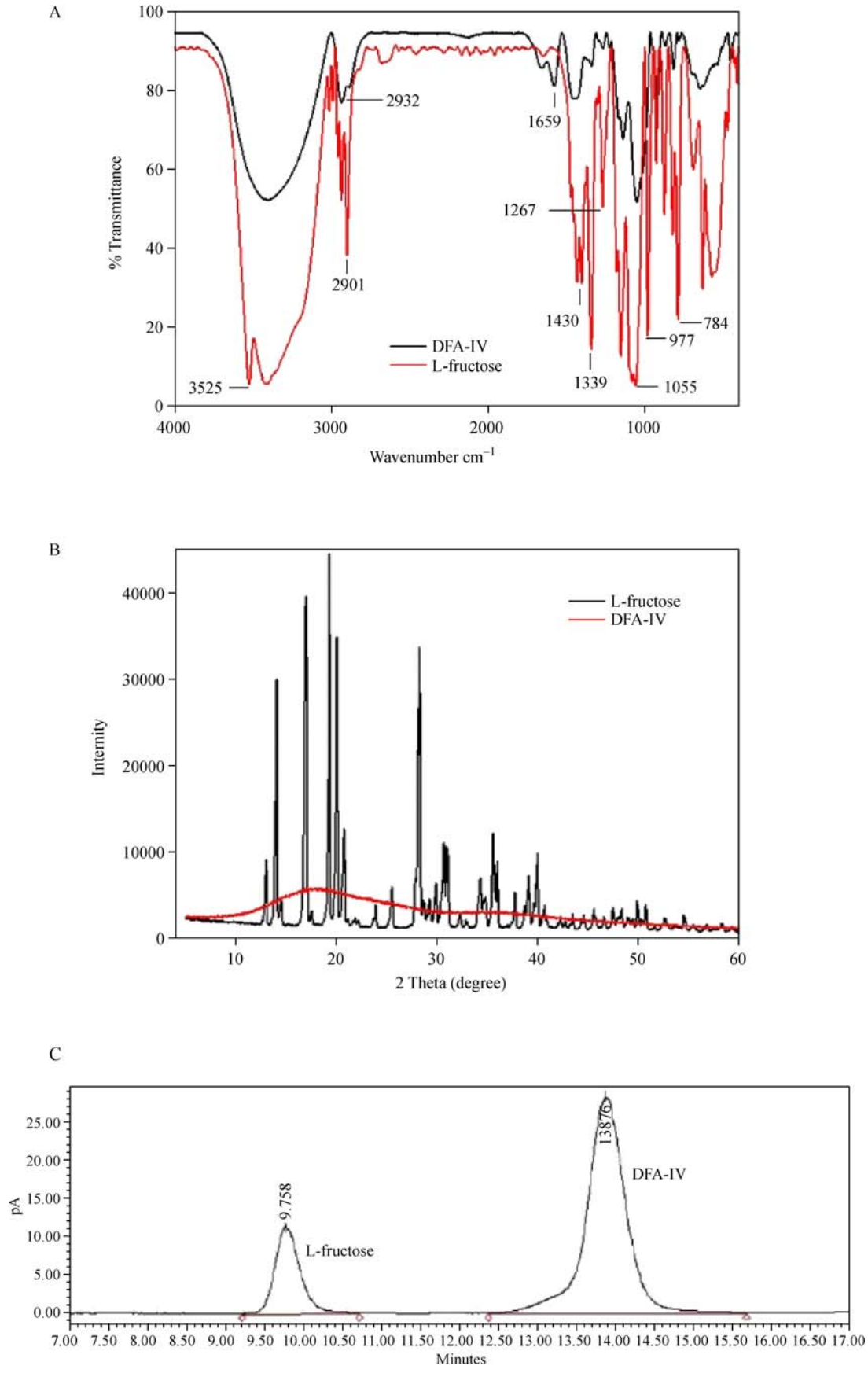

Fig. 1 **FTIR spectrum (A), XRD profile (B), and HPLC analysis (C) of l-fructose and difructose dianhydride IV (DFA-IV).**

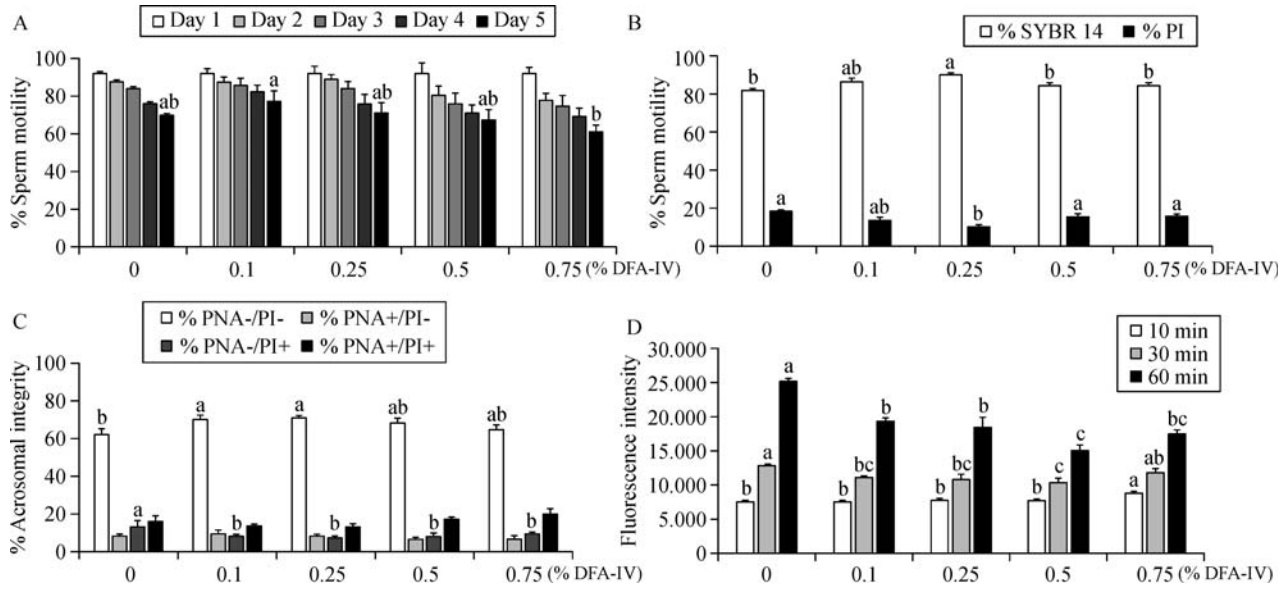

Fig. 2 **Effect of DFA-IV on boar spermatozoa.** Boar sperm were preserved in BTS with DFA IV for 5 days, and sperm motility was observed under a light microscope at 38.5oC (A). Assessment of sperm viability (B) and acrosomal integrity (C) on boar spermatozoa, after 2 hours of incubation. The ROS level was examined in sperm stained with carboxy-DCFDA (D). Experiments were independently repeated five times. Values are expressed as the mean percentage±SEM. The different superscripts (A-C) in each group of columns denote significant differences at $P < 0.05$.

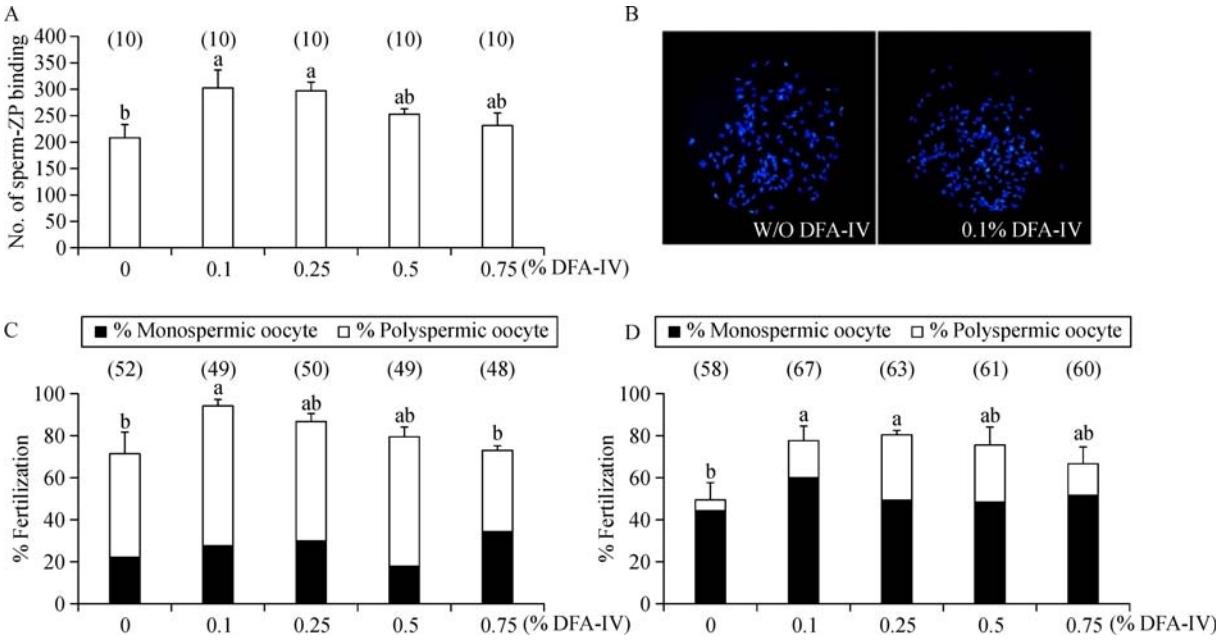

Fig. 3 **Effects of DFA-IV on sperm penetration during IVF.** Oocytes were co-incubated with 5×10^5 spermatozoa/ml for 1 hour. Sperm number bound to the zona pellucida (ZP) was counted (A) after fixation and DAPI staining (B). Different concentrations of DFA-IV were added to the fertilization medium. Oocytes were co-incubated with 5×10^5 spermatozoa/mL for 5 hours (C). Reduced gamete co-incubation time (IVF 2 hours) and lower sperm number (1×10^5 spermatozoa/mL) were employed (D). Numbers of inseminated oocytes are indicated in parentheses. Experiments were independently repeated five times. Values are expressed as the mean percentage or number±SEM. The different superscripts (a-c) in each group of columns denote significant differences at $P < 0.05$.

evaluate subsequent embryonic development (***Fig. 4***). Significantly higher rates of cleaved oocytes were observed in oocytes fertilized in the presence of 0.1% DFA-IV (80.0% *vs.* 68.8%-78.8% for no treatment or 0.25%-0.75% DFA-IV, $P < 0.05$; ***Fig. 4A***). The blastocyst formation rates (36.8%) and cell number per

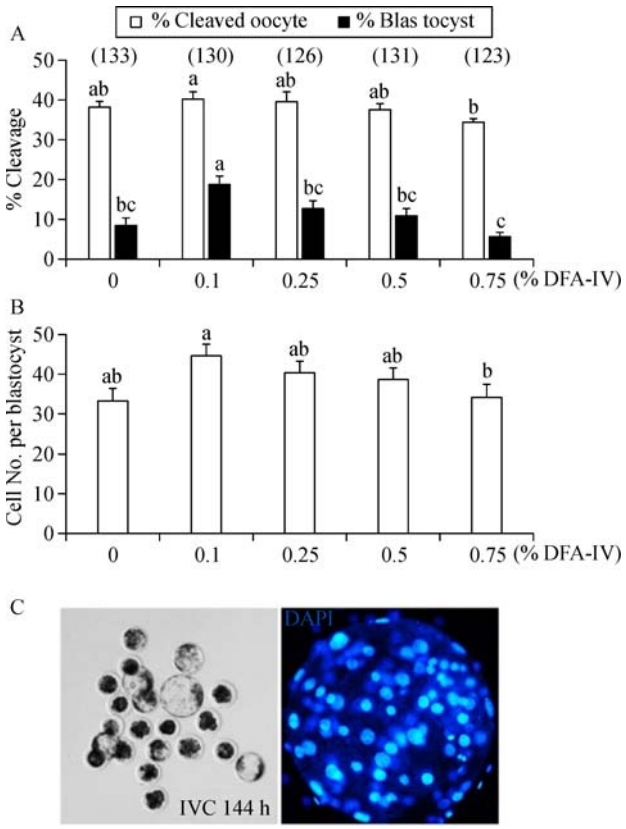

Fig. 4 **Effects of DFA-IV on pig embryo development *in vitro*.** A: Development of embryos derived from oocytes fertilized in the absence or presence of DFA-IV. B: Comparison of average cell number per blastocyst. C: Fertilized oocytes in the presence of 0.1% DFA-IV were cultured in PZM-3 medium for 144 hours, and blastocysts were then fixed and stained with DAPI prior to cell counting (C). Numbers of fertilized oocytes are indicated in parentheses. Experiments were independently repeated five times. Values are expressed as the mean percentage or number±SEM. The different superscripts (A-C) in each group of columns denote significant differences at $P<0.05$.

blastocyst (44.5) increased when oocytes were fertilized in the presence of 0.1% DFA-IV ($P<0.05$; ***Fig. 4B&C***).

Discussion

In this study, we performed FTIR and XRD analyses to characterize DFA-IV. Significant shifts and/or absence of O–H, C–H, C–OH, H–O–H, and C–O absorption peaks in l-fructose confirmed the differences between the sugars. This was further supported by results from XRD studies, which confirmed an amorphous and/or microcrystalline nature of DFA-IV compared to the crystalline nature of l-fructose. Moreover, in pig IVF, sperm penetration increased when oocytes were fertilized in IVF medium supplemented with DFA-IV, and the fertilization rate was enhanced in the presence of 0.1%–0.5% concentrations, resulting in increased blastocyst formation after IVC at 144 hours. Deleterious effects to sperm motility and oocytes were observed at DFA-IV concentrations of 0.8% or higher. Additionally, beneficial effects were not observed when 0.1%-2% DFA-IV was added to oocyte maturation medium or embryo culture medium (data not shown).

Sugars function to supply energy and act as a cryoprotectant due to their capacity to reduce dehydration, intracellular ice formation, and osmotic stress during the freezing and thawing of semen[26–27]. Therefore, various sugar types have been examined in an attempt to optimize the freezing diluent of mammalian spermatozoa, and lactose, fructose, and glucose have been shown to have favorable effects on boar semen freezing, whereas sorbitol does not, suggesting that certain enzymes with functions in the metabolism of sorbitol may not be present in boar spermatozoa[28]. Although monosaccharides have been shown to function in liquid boar semen extenders, disaccharides are primarily used in freezing extenders because they stabilize membrane lipids and promote the membrane integrity of spermatozoa[28–32]. On the other hands, DFA-IV is a small cyclic disaccharide consisting of two fructose residues and having nondigestible and non-absorbable oligosaccharides with half the sweetness of sucrose[10,33]. Oligosaccharides on the sperm cell membrane interact with lectins on the oocyte surface to initiate fertilization *via* hydrogen bonding between hydroxyl groups of sugars and amino acids of

lectins[34]. Therefore, such oligosaccharides (e.g., DFA-IV) may promote improved sperm-oocyte binding associated with increases in the numbers of sperm bound to the ZP and enhancement of the fertilization rate. While levan had viscosity and solidifying in fluids, DFA-IV did not show such characteristics in fluids with higher concentrations that facilitated practical use during IVF. Modified TBM medium, a fertilization medium used in this study, is known to mediate sperm penetration and fertilization in pig IVF[35]. Although this medium is chemically defined and has been shown to be beneficial, the efficiency of fertilization using TBM has been shown to be low due to factors such as polyspermy; thus, a new approach is required for pig IVF[36].

In conclusion, the addition of DFA-IV to IVF medium improved normal fertilization and development of preimplantation embryos. Moreover, ROS production significantly decreased, suggesting that supplementation with DFA-IV may improve conditions during IVF or may mediate ROS levels and sperm-oocyte binding during IVF. The present study confirmed that DFA-IV had favorable effects on boar spermatozoa, which probably enable it to apply to different ARTs, such as sperm freezing; therefore, it requires further study in future. In summary, supplementation of DFA-IV in IVF medium was beneficial for pig IVF, suggesting that DFA-IV may be a novel alternative sugar for enhancement of ARTs in mammals.

Acknowledgements

We thank Hee-Jung Lee for assistance with the experiments, and Agency for Korea National Food Cluster for HPLC analysis. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A6A3A-04063769).

References

[1] Ponglowhapan S, Essén-Gustavsson B, Linde Forsberg C. Influence of glucose and fructose in the extender during long-term storage of chilled canine semen[J]. *Theriogenology*, 2004, 62(8): 1498–1517.

[2] Watson P. The preservation of semen in mammals[J]. *Oxf Rev Reprod Biol*, 1979, 1: 283–350.

[3] Cerning J. Exocellular polysaccharides produced by lactic acid bacteria[J]. *FEMS Microbiol Rev*, 1990, 7(1-2): 113–130.

[4] Dahech I, Belghith KS, Hamden K, et al. Antidiabetic activity of levan polysaccharide in alloxan-induced diabetic rats[J]. *Int J Biol Macromol*, 2011, 49(4): 742–746.

[5] Dahech I, Belghith KS, Hamden K, et al. Oral administration of levan polysaccharide reduces the alloxan-induced oxidative stress in rats[J]. *Int J Biol Macromol*, 2011, 49(5): 942–947.

[6] Kang SA, Jang KH, Seo JW, et al. Levan: applications and perspectives. Microbial production of biopolymers and polymer precursors[J]. *Caister Academic Press, Norwich* 2009: 145–161.

[7] Abdel-Fattah AM, Gamal-Eldeen AM, Helmy WA, et al. Antitumor and antioxidant activities of levan and its derivative from the isolate Bacillus subtilis NRC1aza[J]. *Carbohydr Polym*, 2012, 89(2): 314–322.

[8] Esawy MA, Amer H, Gamal-Eldeen AM, et al. Scaling up, characterization of levan and its inhibitory role in carcinogenesis initiation stage'[J]. *Carbohydr Polym*, 2013, 95(1): 578–587.

[9] Lima RC, de França FP, Lopes CE, and the Calazans GMT. Molecular weight and antitumour activity of Zymomonas mobilis levans[J]. *Int J Biol Macromol*, 2000, 27(4): 245–247.

[10] Saito K, Tomita F. Difructose anhydrides: their mass-production and physiological functions[J]. *Biosci Biotechnol Biochem*, 2000, 64(7): 1321–1327.

[11] Kim CH, Jang EK, Kim SH, et al. Molecular cloning of levan fructotransferase gene from Arthrobacter ureafaciens K2032 and its expression in Escherichia coli for the production of difructose dianhydride IV[J]. *Lett Appl Microbiol*, 2005, 40(3): 228–234.

[12] Jang KH, Ryu EJ, Park BS, et al. Levan fructotransferase from Arthrobacter oxydans J17-21 catalyzes the formation of the di-D-fructose dianhydride IV from levan[J]. *J Agric Food Chem*, 2003, 51(9): 2632–2636.

[13] Pursel VG, Johnson LA. Frozen boar spermatozoa: methods of thawing pellets[J]. *J Anim Sci*, 1976, 42(4): 927–931.

[14] Abeydeera LR, Wang WH, Prather RS, et al. Maturation in vitro of pig oocytes in protein-free culture media: fertilization and subsequent embryo development in vitro[J]. *Biol Reprod*, 1998, 58(5): 1316–1320.

[15] Yoshioka K, Suzuki C, Tanaka A, et al. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium [J]. *Biol Reprod*, 2002, 66(1): 112–119.

[16] Barone JR, Medynets M. Thermally processed levan polymers [J]. *Carbohyd Polym*, 2007, 69: 554–561.

[17] Jathore NR, BuleMV, Tilay AV, Annapure US. Microbial levan from Pseudomonas fluorescens: Characterization and medium optimization for enhanced production[J]. *Food Sci Biotechnol*, 2012, 21: 1045–1053.

[18] Zhao G, Kan J, Li Z, Chen Z. Structural features and immunological activity of a polysaccharide from Dioscorea opposita thunb roots[J]. *Carbohyd Polym*, 2005, 61: 125–131.

[19] Chen X, Gao H, Ploehn HJ. Montmorillonite-levan composite with improved thermal and mechanical properties[J]. *Carbohyd Polym*, 2014, 101: 565–573.

[20] Wang JW, Kuo YM. . Preparation of fructose mediated (polyethylene glycol/chitosan) membrane and adsorption of

heavy metal ions[J]. *J Appl Polym Sci*, 2007, 105: 1480–1489.

[21] Awda BJ, Mackenzie-Bell M, Buhr MM. Reactive oxygen species and boar sperm function[J]. *Biol Reprod*, 2009, 81(3): 553–561.

[22] Silva PF, Gadella BM. Detection of damage in mammalian sperm cells[J]. *Theriogenology*, 2006, 65(5): 958–978.

[23] Buhr M. Preservation of boar sperm alters membrane molecular dynamics[A]. Boar semen preservation II Proceedings of the Second International Conference on Boar Semen Preservation [C]. Beltsville, Maryland, USA, August 1990: 81–93.

[24] Oehninger S, Blackmore P, Mahony M, et al. Effects of hydrogen peroxide on human spermatozoa[J]. *J Assist Reprod Genet*, 1995, 12(1): 41–47.

[25] McCauley TC, Mazza MR, Didion BA, et al. Chromosomal abnormalities in Day-6, in vitro-produced pig embryos[J]. *Theriogenology*, 2003, 60(8): 1569–1580.

[26] Watson PF. The causes of reduced fertility with cryopreserved semen[J]. *Anim Reprod Sci*, 2000, 60-61: 481–492.

[27] Malo C, Gil L, Gonzalez N, et al. Comparing sugar type supplementation for cryopreservation of boar semen in egg yolk based extender[J]. *Cryobiology*, 2010, 61(1): 17–21.

[28] Chanapiwat P, Kaeoket K, Tummaruk P. Cryopreservation of boar semen by egg yolk-based extenders containing lactose or fructose is better than sorbitol[J]. *J Vet Med Sci*, 2012, 74(3): 351–354.

[29] Medrano A, García-Gil N, Ramió L, et al. Hexose-specificity of hexokinase and ADP-dependence of pyruvate kinase play important roles in the control of monosaccharide utilization in freshly diluted boar spermatozoa[J]. *Mol Reprod Dev*, 2006, 73 (9): 1179–1194.

[30] Purdy P. A review on goat sperm cryopreservation[J]. *Small Rumin Res*, 2006, 63: 215–225.

[31] De Leeuw FE, De Leeuw AM, Den Daas JH, et al. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing[J]. *Cryobiology*, 1993, 30(1): 32–44.

[32] Gómez-Fernández J, Gómez-Izquierdo E, Tomás C, et al. Effect of different monosaccharides and disaccharides on boar sperm quality after cryopreservation[J]. *Anim Reprod Sci*, 2012, 133 (1-2): 109–116.

[33] Saito K, Hira T, Suzuki T, et al. Effects of DFA IV in rats: calcium absorption and metabolism of DFA IV by intestinal microorganisms[J]. *Biosci Biotechnol Biochem*, 1999, 63(4): 655–661.

[34] Osborn HM, Khan TH. Oligosaccharides: their synthesis and biological roles: Oxford University Press on Demand[J], 2000.

[35] Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium[J]. *Theriogenology*, 1997, 48(4): 537–544.

[36] Romar R, Funahashi H, Coy P. In vitro fertilization in pigs: New molecules and protocols to consider in the forthcoming years[J]. *Theriogenology*, 2016, 85(1): 125–134.