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Effects of Alpha-G Rutin Supplementation in Sperm Freezing Extender on Dog Sperm Cryopreservation

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Abstract : This study was designed to investigate the effects of alpha-glucosyl rutin (G-rutin) and its comparative effects with other antioxidants (glutathione: GSH, catalase: CATA and beta-mercaptoethanol : βME) on dog sperm freezing. In the first experiment (E1), the spermatozoa were diluted in freezing extender supplemented with 0 (control), 0.001, 0.01, or 0.1% G-rutin and frozen using liquid nitrogen (LN_2). The progressive motility, reactive oxygen species (ROS) level and apoptosis of spermatozoa were assessed after sperm thawing at 37°C for 25 sec. In the second experiment (E2), 0.1% G-rutin group was compared with 10 mM β-ME, 5 μM GSH and 50 μM CATA groups by assaying progressive motility, viability and gene expression of *Bcl-2* and *SMCP* after sperm freezing and thawing. In E1, 0.1% G-rutin group showed higher ($P < 0.05$) post-thaw progressive motility and lower ($P < 0.05$) ROS levels. In E2, the expressions of SMCP in G-rutin group were higher ($P < 0.05$) than in CATA group while Bcl-2 expression of G-rutin group was higher ($P < 0.05$) than β-ME and CATA groups. However, there were no significant differences in progressive motility and viability. Therefore, we suggest that G-rutin can be used as a potentially antioxidative supplement in dog sperm freezing extender on the basis of gene expression related to motility and apoptosis as well as ROS level.

***Key words* :** antioxidant, dog, freezing extender, gene expression, G-rutin, reactive oxygen species, spermatozoa.

Introduction

Cryopreservation is a widely used assisted reproduction technique in spermatozoa and plays a major role in the biological preservation of animal species (11,13,15,46). By enhancing the storage and propagation of genetic material using sperm cryopreservation, cost of animal transportation management was reduced (13,14).

However, cryopreservation reduces the post-thaw sperm viability, particularly through the generation of reactive oxygen species (ROS) by the spermatozoa (15,37). ROS generation has been associated with a reduction of sperm motility, decreased capacity of sperm-oocyte fusion and infertility. When the ROS generation exceeds the sperm antioxidant defense mechanisms, a state of oxidative stress is induced, This state is characterized by peroxidative damage to the sperm plasma membrane and DNA double strand breakage in the sperm nucleus (5,18,24). Moreover, spermatozoa are considered highly sensitive to lipid peroxidation due to its high content of polyunsaturated fatty acids in their plasma membrane (6,27,37).

Therefore, supplementation of antioxidants in freezing extender could reduce these detrimental effects of oxidative stress and consequently improve the post-thaw sperm quality. There are well-known antioxidants used in sperm freezing. Glutathione (GSH) and catalase (CATA) have anti-oxidative effect and improved sperm motility when they were added to freezing extender (16,30,34). Beta mercaptoethanol (β-ME) has anti-oxidative effect and increased sperm motility by supplementation of β-ME in boar sperm thawing solution (47). Rutin is a kind of polyphenolic flavonoid compounds found mainly in plants such as the budda-plant and the buckwheat. The 'rutin' name is derived from the *Ruta graveolens* which is one of the rutin-containing plants. Chemically, it is a glycoside comprised of flavonolic aglycone quercetin along with disaccharide rutinose (22). Rutin has been shown to have a number of pharmacological effects including antioxidative, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardio-protective effects (20-22,25,41-43). Recently, the antioxidant effect of rutin has been tested on induced lipid peroxidation (LPO)in swim-up selected human spermatozoa with a significant effect on sperm viability and motility through the reduction of lipid peroxidation (31). To the best of our knowledge, up to date, rutin has not been used to reduce the ROS generation during sperm freezing, although it was used during the incubation of thawed spermatozoa (28,29). Therefore, in this study, we investigated the effect of rutin on sperm freezing by rutin supplementation in freezing extender.

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Increased incidence of cell death (apoptosis) in cryopreserved spermatozoa is an important indicator of inadequate or suboptimal in vitro environment and freezing damage of spermatozoa (33). ROS normally function as signaling molecules for many processes involved in fertilization. However, when ROS levels exceed the antioxidants defense systems, they exert damages on spermatozoa such as LPO and apoptosis (2,3,23). Regulation and decision of cells to undergo through an apoptotic pathway are monitored by cell death regulatory genes that belong to the B-cell lymphoma 2 (Bcl-2) gene family (44,45). Assessment of apoptosis and expression level of an apoptosis-related gene has been successfully used to examine the quality of the reproductive cells according to environmental change (36).

The sperm mitochondrial cysteine-rich protein (SMCP) is a rapidly evolving cysteine- and proline-rich protein that is mainly localized in the mitochondrial capsule and considered to be responsible for the enhancement of sperm motility (19). Sperm motility impaired results in the failure of sperm migration in the female reproductive tract and sperm penetration to oocyte membranes during fertilization process (33). Therefore, SMCP gene expression level could be used as indicator of sperm damage due to freezing-thawing.

The aim of this study was first, to investigate the antioxidant properties of G-rutin against ROS in dog spermatozoa and its subsequent effect on sperm motility and apoptosis. Secondly, the present study aimed to evaluate the comparative antioxidant effect of G-rutin with other antioxidants (GSH, CATA and β-ME) on sperm motility, viability and gene expression related to motility and apoptosis.

Materials and Methods

Chemicals

Unless otherwise stated, all chemicals that were used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA). Equex STM paste was attained from Nova Chemical Sales Inc. (Scituate, MA, USA). G-rutin was obtained from DAMY chemical (AA1316600, Seoul, Korea). The solutions were prepared with high purity water (6114VF, Sartorius AG, Göttingen, Germany).

Collection of semen

Semen was collected from five healthy mature dogs (one Poodle mix, one Shih-Tzu, one Lancashire Heeler mix, one Pomeranian and one Jindo) aging from 3 to 5 years. The dogs used in this study were treated and received care under the Guiding Principles for the Care and Use of Research Animals, as established by Jeonbuk National University. The dogs were caged individually and provided with water ad libitum and formulated dog food (Jindo®, Purina) twice daily throughout the experiment (38).

One ejaculate (sperm-rich second fraction) from each dog was collected by digital manipulation. Normal ejaculates with sperm concentrations $\geq 200 \times 10^6$ spermatozoa/ml, progressive motility $\geq 70\%$ and normal morphology $\geq 80\%$ were included in this study. For each replicate, ejaculates from the five dogs were pooled to minimize the individual differences (38,39).

Table 1. Composition of freezing extenders for dog sperm freezing

Constituents	Freezing extenders	
	Extender 1	Extender 2
Tris (g)	2.4	2.4
Citric acid (g)	1.1	1.1
Glucose (g)	4	4
Na-benzyl penicillin (g)	0.8	0.8
Streptomycin sulfate (g)	0.06	0.06
Egg yolk (ml)	20	20
Equex STM paste (ml)	-	1
Glycerol (M)	-	1
Distilled water (ml)	Up to 100	Up to 100
pH	6.53	6.48
Osmolality (mOsm)	740 mOsm	1370 mOsm

Sperm freezing and thawing

The components and characteristics of the freezing extender are presented in Table 1. Ejaculates were centrifuged at 300 x g for 12 min at room temperature. After discarding supernatants, semen pellets were pooled to overcome the individual variations. The pooled pellets were diluted to 2×10^8 sperm/ml with extender 1 and diluted spermatozoa were cooled to 4ºC. The cooled spermatozoa were diluted further with extender 2 (1 : 1, V : V), loaded in 0.5 ml straw (500 µl) and then kept at 4ºC for an additional 30 min. The final sperm concentration was 1×10^8 sperm/ml.

A Styrofoam box ($29.5 \times 18.7 \times 24$ cm^3) was filled with LN_2 to a depth of 5 cm and a rack with two bars was set at 7 cm from the surface of the LN_2. The straws were then aligned horizontally for 20 min on the rack in the LN_2 vapor. Following LN_2 vapor freezing, the straws were then plunged into LN_2 (38). Frozen spermatozoa were thawed at 37ºC for 25 sec.

Sperm progressive motility

The thawed semen (10 µl) was placed on a slide and cover slipped. The percentage of progressively motile spermatozoa was estimated under microscopic examination (Leica DM 2500, Leica Microsystems, USA) at 400 x magnification. Spermatozoa were considered to be motile only if they showed progressive motility with a score of at least 2 or 3 on a scale of 0-4 (32). Sample identity was hidden from the operator and the samples were randomly numbered to avoid the operator bias in subjective evaluation. The samples in each group were assessed in duplicates. The mean of eight successive estimates was recorded as the final motility score (40).

Sperm viability

Viability based on the integrity of sperm plasma membranes was measured as described previously (49). In short, 5 µl SYBR-14 was added to 50 µl spermatozoa and the mixture was incubated for 5 min at room temperature (in the dark); 5 µl propidium iodide (PI) was then added, mixed and incubated for another 5 min. For each replicate sample, two slides were prepared and appropriately 200 spermatozoa were counted per slide. The green or red fluorescent sperma-

tozoa was counted under a fluorescence microscope (Axio, Carl Zeiss, Goettingen, Germany) fitted with a 488 nm excitation filter and the percentage of membrane-intact (green fluorescent) spermatozoa was calculated.

ROS level

To detect H_2O_2 (ROS level), 2, 7-dichlorodihydrofluorescein diacetate (H_2DCFDA DCF; Molecular Probes, Inc., Eugene, Oregon, USA) was used (17). The mean fluorescence intensity (MFI) of DCF was measured to evaluate intracellular mean H_2O_2 per viable sperm population. Data were expressed as the percentage of viable sperm with H_2O_2 (high DCF fluorescence).

Apoptosis index

To evaluate apoptosis (phosphatidylserine translocation: PS translocation), Annexin V-FITC apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA) was used as described by Rahman *et al*. (40). Briefly, frozen-thawed spermatozoa were centrifuged at 300 x g for 5 min. Following disregarding the supernatant, sperm pellet was washed twice using 1X Dulbecco's phosphate buffered saline (DPBS; 14190, Gibco, Grand Island, NY, USA). Thereafter, the pellet was resuspended (1×10^6 sperm/ml) in 1X Annexin binding buffer (10 mM HEPES/NaOH[pH 7.4], 140 mM NaCl and 2.5 mM $CaCl_2$) at room temperature. Annexin V-FITC (5 μl) and 5 μl PI or nothing were added to sperm suspension (100 μl). The tubes with samples were gently mixed and incubated at room temperature for 15 min in the dark. After incubation, 1 x Annexin binding buffer (400 μl) was added to each tube. Flow cytometric analysis was conducted within 1 h. The different labelling patterns in the Annexin (AN)/PI analysis were classified as follows: viable (AN−/PI−); viable but phosphatidyl serine (PS) translocated (AN+/PI−); nonviable and PS translocated (AN+/PI+); and nonviable and late necrotic sperm (AN−/PI+). The apoptosis index was calculated as the ratio between AN+/PI− spermatozoa and total viable (PI−) spermatozoa.

Flow cytometry

All fluorescence signals of labeled spermatozoa were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser. This technique was used in the assays for determining ROS level and apoptosis index.

RNA isolation

The procedure of RNA isolation and cDNA synthesis was performed according to Rahman *et al.* (40). RNA samples were obtained from frozen-thawed spermatozoa of 0.1% G-rutin, 5 mM GSH, 10 mM CATA and 50 mM β-ME treatment groups, respectively. Total RNA was extracted using RiboEx kit (GeneAll®, Biotechnology Co., LTD, Seoul, South Korea) according to the manufacturer's protocol.

Gene expression

Quantitative real-time PCR (qPCR) was carried out to assess transcript abundance using oligonucleotide primers sequences (Table 2). Primers were designed and tested for specificity using the Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time, accessed 19 April 2016, 40). For each selected gene, optimal amplification conditions (with RNA adjusted for an equal concentration) were selected to achieve a high efficiency among all selected genes. Relative quantification was performed in triplicate using the SYBR Green qPCR Master Mix TaKaRa One Step SYBR® PrimeScript™ RT-PCR kit II (Takara Bio USA, Inc., Mountain View, CA, USA). The reaction was carried out using a Real-Time PCR System (7500 Fast System, Applied Biosystems®) according to the manufacturer's instructions. The PCRs were performed using the following program; stage 1, reverse transcription: 5 min at 42ºC and then 10 sec at 95ºC; stage 2, PCR reaction: 40 cycles of 5 sec at 95ºC and 34 sec at 56ºC; and stage 3, dissociation. The data were analyzed using the Livak and Schmittgen equation, where fold change = $2^{-\Delta\Delta CT}$ (26). GAPDH was used as internal control (40).

Experimental design

Experiment 1: The effect of G-rutin supplementation in freezing extender on sperm progressive motility, ROS and apoptosis in dog sperm cryopreservation

To assess the effect of G-rutin supplementation, dog spermatozoa were frozen in freezing extender supplemented with 0.001, 0.01, and 0.1% G-rutin. Sperm progressive motility, ROS level and apoptosis following thawing were evaluated as described above.

Experiment 2: Comparative effects of G-rutin, β-ME, GSH and CATA supplementation in freezing extender on sperm progressive motility, viability and gene expression in dog sperm cryopreservation

To evaluate the comparative effect of 0.1% G-rutin, 10

Table 2. Overview of primer sequences used to characterize gene expression in dog spermatozoa

Gene	Primer sequence	Position	Fragment length (bp)	Accession no.
GAPDH	F: TGACGACATCAAGAAGGTAGTGA R: GTCATTGAGGGCAATGCCAG R: CGCTCTCGAAGGAAGTCCAG	821-843 971-952 495-476	151	NM_001003142.2
Bcl-2	F: CCCCTCATCCAAGAATGCAA R: TCCCGGTTATCGTACCCTGT	300-319 470-451	171	NM_001002949.1
SMCP	F: GTCTGGAGCCAAAACCTGAA R: GCCCCACTAAGTGATGGGTA	355-374 539-520	185	NM_001031636.1

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, Bcl-2: B-cell lymphoma 2 (anti-apoptotic), SMCP: sperm mitochondrial-associated cysteine-rich protein.

mM β-ME, 5 μM GSH and 50 μM CATA supplementation in freezing extender, dog spermatozoa were frozen in freezing extender supplemented with 0.1% G-rutin, 10 mM β-ME, 5 μM GSH or 50 μM CATA. After thawing, sperm progressive motility, viability and gene expression were assessed as described above.

Statistical analyses

Five replicates of pooled semen were conducted for each experiment. Data expressed as percentages were subjected to an arcsine transformation before analysis. All data are presented as mean ± SE. Data were analysed using one-way ANOVA followed by Duncan's multiple range test and all calculations were performed in the Statistical Analysis System ver. 8x (SAS, Cary, NC, USA). P values < 0.05 were considered to indicate significant differences.

Results

The effect of G-rutin supplementation in freezing extender on sperm progressive motility, ROS and apoptosis in dog sperm cryopreservation

G-rutin supplementation in dog sperm freezing extender significantly affected post-thaw sperm motility compared to control (Table 3). The percentage of progressively motile spermatozoa in all G-rutin groups was higher ($P < 0.05$) than control. In addition, ROS level was significantly decreased ($P < 0.05$) after adding of 0.1% and 0.01% G-rutin groups compared to the control and 0.001% G-rutin groups (Table 3, Fig 1). However, apoptosis was not significantly different among groups (Table 3, Fig 2).

Table 3. Effect of G-rutin on progressive motility, ROS level and apoptosis of dog spermatozoa following freezing and thawing

G-rutin concentration (%)	Progressive motility (%)	ROS levels (%)	Apoptosis index
Control	43.0 ± 2.0^c	20.3 ± 2.9^a	14.5 ± 2.1
0.1	61.0 ± 1.7^a	10.5 ± 1.8^b	19.1 ± 2.8
0.01	56.0 ± 1.8^b	10.7 ± 0.3^b	17.0 ± 2.0
0.001	51.0 ± 2.4^b	19.9 ± 2.3^a	16.9 ± 1.4

Control: no G-rutin in freezing extender, ROS: reactive oxygen species. Values are expressed as the mean ± SE. [a,b,c]Different superscripts indicate significant differences within columns ($P < 0.05$).

Comparative effects of G-rutin, β-ME, GSH and CATA supplementation in freezing extender on sperm progressive motility, viability and gene expression in dog sperm cryopreservation

Bcl-2 (anti-apoptotic) expression of G-rutin group was higher ($P < 0.05$) than β-ME and CATA groups (Fig 3A)

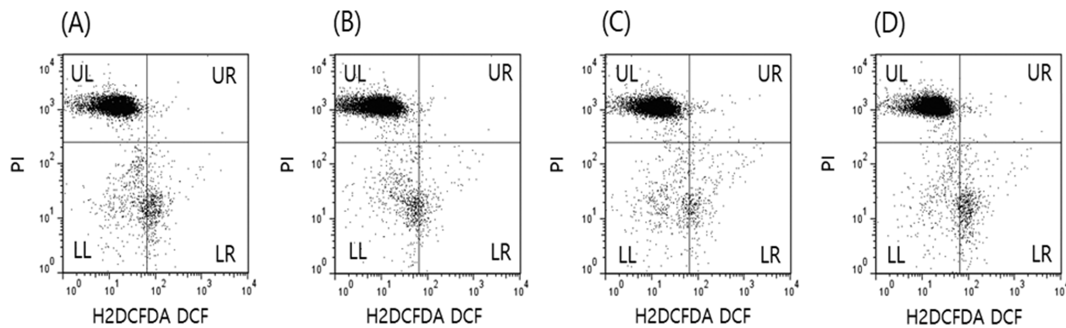

Fig 1. Effects of G-rutin supplementation in freezing extender on dog sperm reactive oxygen species (ROS) levels after freezing and thawing. (A) control, (B) 0.1% G-rutin, (C) 0.01% G-rutin, (D) 0.001% G-rutin. The 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCFDA DCF) assay was assessed for ROS detection. The upper panel show dot plot cytograms measuring the sperm population based on the level of intracellular H_2O_2 level, LL: viable sperm with a low intracellular H_2O_2 level, LR: viable sperm with a high intracellular H_2O_2 level, UL: dead sperm with a low intracellular H_2O_2 level. UR: dead sperm with a high intracellular H_2O_2 level.

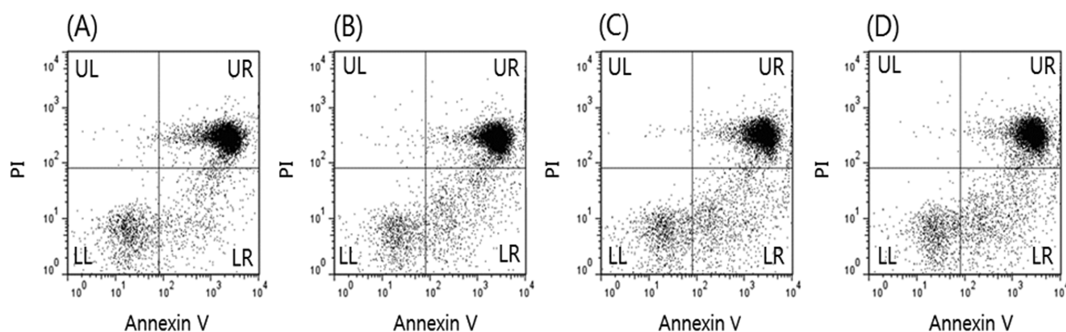

Fig 2. Effects of G-rutin supplementation in freezing extender on dog sperm phosphatidylserine (PS) translocation after freezing and thawing. (A) control, (B) 0.1% G-rutin, (C) 0.01% G-rutin, (D) 0.001% G-rutin. Annexin V-FITC-PI fluorescent staining was assessed for PS translocation. LL: viable spermatozoa with no signs of PS translocation, LR: viable spermatozoa showing PS translocation. UL: dead spermatozoa with no signs of PS translocation, UR: dead spermatozoa showing PS translocation.

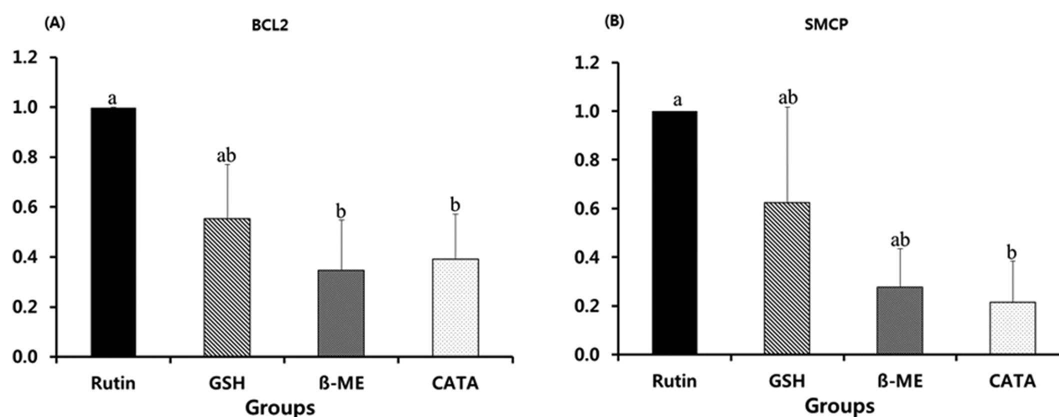

Fig 3. Gene expression of Bcl-2 (A) and SMCP (B) following dog sperm cryopreservation using 0.1% G-rutin, 5 μM GSH, 10 mM β-ME and 50 μM CATA in freezing extender. BCL-2: B-cell lymphoma 2 (anti-apoptotic), SMCP: sperm mitochondrial-associated cysteine-rich protein. Real-time qPCR was used to determine the gene expression profiles. The fold changes were calculated by the ΔΔCT method following normalization against a reference gene (GAPDH). Values are expressed as the mean ± SE. Bars with different superscript are significantly different ($P < 0.05$).

Table 4. Comparative dog sperm parameters following freezing in G-rutin and other antioxidants supplemented in freezing extender

Groups	Progressive motility (%)	Viability (%)
G-rutin	50.0 ± 1.5	50.0 ± 1.2
GSH	56.0 ± 1.8	51.2 ± 1.9
β-ME	52.0 ± 3.7	49.8 ± 5.5
CATA	49.0 ± 2.4	50.3 ± 3.2

G-rutin: 0.1% alpha-glucosyl rutin. GSH: 5 μM glutathione. β-ME: 10 mM beta-mercaptoethanol. CATA: 50 μM catalase. Values are expressed as the mean ± SE.

while the expressions of SMCP (related to motility) in G-rutin group were higher ($P < 0.05$) than in CATA group (Fig 3B). However, the sperm progressive motility and viability were not significantly different among G-rutin, GSH, β-ME and CATA groups (Table 4).

Discussion

Spermatozoa are highly sensitive to ROS damage due to the high polyunsaturated fatty acid content in their cell membranes (6,27). Increased accumulation of ROS is responsible for the damage of mammalian spermatozoa and subsequent dysfunction associated with reduced sperm quality (4-6) especially during cryopreservation process (35,37). Therefore, antioxidants are needed to minimize the negative effect of oxygen-induced damage and lipid peroxidation (7,9,10,12).

Therefore, we treated dog spermatozoa with different concentrations of G-rutin, which is polyphenolic flavonoid compounds found mainly in plants and recently has been used to prevent number of diseases in human due to its antioxidants activity (22,48,50). In the present study, ROS levels of G-rutin groups except 0.001% groups compared to control were significantly reduced. These results indicate that supplementation of G-rutin in the range of 0.1-0.01% can reduce the ROS generation with increase of sperm motility during dog sperm cryopreservation. On the other hand, 1 mM rutin compared to 0.1 mM was effective on ram and red deer spermatozoa during the incubation at 37°C after freezing and thawing (28,29). However, rutin showed an inhibitory effect on motility even though it efficiently removes free radicals and protects DNA and membranes from oxidation (28). In this study, all G-rutin groups increased the sperm progressive motility compared to the control with the highest motility in 0.1% G-rutin group. The discordance between these studies could be due to the different addition time of rutin, following thawing or prior to freezing. That is, supplementation of rutin in freezing extender before freezing can be more effective in improving sperm motility than supplementation in thawing extender.

In the second experiment, we compared G-rutin with other antioxidants generally used in sperm freezing. G-rutin group showed the highest value of SMCP gene expression compared to other antioxidant groups. SMCP is cysteine-and proline-rich protein in the mitochondrial capsule and enhances sperm motility (19). However, the progressive motility in G-rutin group was not different to other antioxidant groups. In the present study, gene expression was evaluated using all alive and dead spermatozoa while the assay for progressive motility was based on live spermatozoa with fast moving pattern. Therefore, we suggest that the potential ability for total motility including slow and moderate moving patterns can be demonstrated using SMCP gene expression rather than progressive motility.

In this study, expression of apoptosis related genes, Bcl-2 was compared in 5 μM GSH, 10 mM CATA and 50 μM β-ME groups. The mRNA expression level of Bcl-2 gene in spermatozoa cryopreserved in 0.1% G-rutin group was significantly higher than β-ME and CATA groups. The increase of expression of anti-apoptotic Bcl-2 gene supports the positive effect of G-rutin supplementation on ROS in experiment 1. This result indicates the significant ability of G-rutin (0.1%) compared to other antioxidants to protect dog spermatozoa during cryopreservation against ROS damage and consequent apoptosis. This could be due to the potent anti-oxidative and anti-apoptotic efficiency of G-rutin compared to the other antioxidants. Ahmed and Zaki (1) reported that

rutin positively impacted on intracellular GSH level related to an anti-oxidative role in cells (1). On other hand, the sperm membranes are the most susceptible and the first part of sperm damaged by the cold shock during cryopreservation due to its high contents of polyunsaturated fatty acids (8). In this study, supplementation of all antioxidants including G-rutin has no significant difference in sperm viability with showing the similar values.

In conclusion, this study provided that the effective concentration range of G-rutin supplemented in dog sperm freezing extender is 0.1-0.01% confirmed through increased progressive motility and decreased ROS level. G-rutin showed a potential antioxidative effect with higher expression level of anti-apoptotic Bcl-2 and SMCP gene expression.

Therefore, G-rutin can be used for the cryopreservation of dog spermatozoa. The effect of G-rutin extender on *in vivo* fertility still has to be evaluated.

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