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Alterations in mitochondrial function and spermatozoal motility in goat spermatozoa following incubation with a human lysozyme plasmid

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ABSTRACT

Spermatozoa mediated gene transfer has become a promising technology to generate transgenic animals with disease resistance. However, exogenous DNA invasion may cause changes in spermatozoon natural defense system which result in spermatozoon dysfunction. The objective of this study was to investigate the changes of mitochondrial function and motility in goat spermatozoa after pre-incubation and incubation with and without the human lysozyme plasmid pFLAG-hLY. The results demonstrated that human lysozme plasmid pFLAG-hLY could bind to the surface of the spermatozoon membrane at 186,000 copies/spermatozoa and incorporate to spermatozoon nucleus at 78 copies/spermatozoa after incubation. However, the treated spermatozoon samples showed a significant lower motility (29.7 ± 2.2% vs. pre-incubation control 48.0 ± 1.4% and incubation control 54.5 ± 1.5%, $P < 0.05$), and percentage of rapid progressive motile spermatozoa (16.4 ± 2.4% vs. pre-incubation control 31.4 ± 0.6% and incubation control 37.0 ± 0.5%, $P < 0.01$). Meanwhile, the incubation with plasmids caused significant reduction of mitochondrial membrane potential (31.44 ± 2.17% vs. pre-incubation control 51.79 ± 2.08% and incubation control 58.81 ± 1.76%, $P < 0.05$). In addition, dichlorofluorescin relative fluorescence intensity (32.81 ± 2.41%) and malondialdehyde levels (2.18 ± 0.21 nM/10^8 spermatozoa), which represents mitochondrial function, showed a significant increase after incubation ($P < 0.05$). The cytochrome *c* release from the mitochondrial inner membrane space, and enzymatic activities of caspase-3 (0.086 ± 0.024) and caspase-9 (0.083 ± 0.019) ($P < 0.05$) also increased, in which resulted in spermatozoon dysfunction. In conclusion, this study confirmed that goat spermatozoa could capture human lysozyme plasmid pFLAG-hLY, but the incubation with the plasmids resulted in a decrease of spermatozoa motility and partial rupture of mitochondrial membrane, and further prompted the expression of cytochrome *c*, and generation of oxidative stress in vitro and finally led to spermatozoon dysfunction.

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1. Introduction

Transgenic animals have a great potential to increase animal disease resistance and improve livestock breeds by introducing specific new genetic material into animal cells or organism. Goat is a potentially appropriate species for the production of large amounts of recom-

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binant proteins from transgenic organisms (Houdebine, 2000, 2002). For example a recent study (Li et al., 2009) has indicated that human lysozyme is an important recombinant protein for innate immunity and its antibacterial activity is many times greater than that of other animal species milk lysozyme. Thus, we consider that, by means of transgenic technology, the human lysozyme gene may be used to reduce the high incidence of mastitis infection.

Currently, transgenic animals may be conventionally produced through various developed transgenic technologies, such as pronuclear microinjection (Jon and Frank, 1983), nucleus transfer (Karatzas et al., 1999), sperm-mediated (Lavitrano et al., 1992) and embryonic stem cells (Richard et al., 1984). One alternative method is sperm-mediated gene transfer (SMGT), which uses spermatozoon as a natural vector to introduce new genes into animals. In contrast with other established methods, this method has the advantages of simplicity and cost-effectiveness (Smith, 2002; Garcia-Vazquez et al., 2009). Currently, this gene transfer technique can be widely applied in many species (Garcia-Vazquez et al., 2009) and has been efficient in successfully producing large transgenic offspring including pigs (Aitken et al., 1998) and cattle (Shemesh et al., 2000; Donovan et al., 2005; Wall et al., 2005). So far, it has been found that spermatozoa of many animal species are able to uptake spontaneously exogenous DNA of mouse (Lavitrano et al., 1992), rabbit (Fan and Watanabe, 2003), ram and buck (Castro et al., 1992) and bull (Schellander et al., 1995). However, it has been reported that spermatozoon interactions with exogenous DNA can trigger endogenous nuclease(s) which cleave exogenous and genomic DNA (Spadafora, 1998), and induced a significant decrease in spermatozoa motility (Schellander et al., 1995). Also, nuclease activation may indicate the presence of a significant increase in spermatozoon genomic DNA damage after the binding of exogenous DNA (Kang et al., 2008). Alternatively, endonuclease activation may result in the disruption of spermatozoon DNA repair mechanisms (Anzar and Buhr, 2006) and increased susceptibility to oxidative stress and the presence of apoptotic-like process.

In last decade, many experiments in SMGT have attempted to identify the effect of different treatments including incubation time, temperature, spermatozoon selection and optimization of DNA uptake on spermatozoa motility (Lavitrano et al., 2003; Wu et al., 2009). However, there has been no examination of mitochondrial function alterations of spermatozoa after spermatozoa with exogenous DNA. The objective of the present study was to investigate the effect of spermatozoon incubation with plasma pFLAG-hLY (human lysozyme plasmid expressing the FLAG-tagged) on spermatozoa motility and mitochondrial functions.

2. Materials and methods

2.1. Reagents and media

Except for otherwise stated, all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) with the highest available purity. The basic dilutions and culture media were cell culture grade routinely used in our laboratory.

2.2. Preparation and measurement of goat spermatozoa

2.2.1. Semen collection and processing

Five clinically healthy and fertile Saanen bucks at 2–3 years old were selected from China's first animal cloning base in Yangling, Shaanxi Province. A total of 160 ejaculates (32 from each animal) were obtained, frozen and thawed (Hidalgo et al., 2007). Aliquots of each spermatozoon sample were assessed subjectively for the following variables: volume, concentration, morphology and motility.

The samples of frozen–thawed semen in this study were selected at $300 \times g$ with 40:80% PureSperm density gradient (40/80, Nidacon International, Goteborg, Sweden) for 20 min at room temperature (Ricci et al., 2009). After the swim-up technique, motile spermatozoa were suspended in Tyrode's bicarbonate buffer-HEPES (TALP-HEPES, 114 mM NaCl, 25 mM $NaHCO_3$, 2.10 mM $CaCl_2.2H_2O$, 3.20 mM KCl, 0.34 mM $NaH_2PO_4{\cdot}H_2O$, 1.50 mM $MgSO_4{\cdot}7H_2O$, 10.07 mM HEPES and 50 μg/ml gentamycine) described in detail (Nedambale et al., 2006) with 5 mg/ml BSA fraction V. The viable spermatozoa were suspended for further experiments.

2.2.2. Detection of spermatozoa motility

Upon incubation, spermatozoa motility (percentage) was assessed immediately under a light Olympus BX 51 microscope (Tokyo, Japan) at a 37 °C warming plate. The percentage of rapid progressive motile spermatozoa (RPMS) was estimated under a phase-contrast microscope at 200× magnification by two independent observers.

2.3. The pFLAG-hLY plasmid and incubation with spermatozoa

The pFLAG-hLY plasmid was constructed in our Laboratory and was linearized by digesting with HindIII and purified from solution using a plasmid purification kit from Promega Corporation (Madison, WI, USA). The plasmid DNA was stored at 0.5 μg/ml in Tris–EDTA solution.

Following the preliminary protocol, the selected samples (1×10^6/ml) were incubated with or without linearized pFLAG-hLY (0.05 μg/ml) in 1 ml of modified TALP-Fert medium (114 mM NaCl, 25 mM $NaHCO_3$, 2.10 mM $CaCl_2.2H_2O$, 3.20 mM KCl, 0.34 mM NaH_2PO_4·H2O, 1.50 mM $MgSO_4{\cdot}7H_2O$, 0.222 mg/ml sodium pyruvate, 20 μM penicillinamine, 10 μM hypotaurin, 2 μM epinephrine, 6 mg/ml fatty acid free BSA, 50 μg/ml gentamycine and 1 μg/ml heparin) for 30 min at 37 °C and 5% CO_2 using 8 μl of FuGene 6 transfection Reagent (Roche, Germany). In the pre-incubation control group, the selected samples were suspended only with TALP-Fert medium.

2.4. Real-time PCR quantification in spermatozoa incubated with pFLAG-hLY

2.4.1. DNA isolation from whole spermatozoa

Spermatozoon genomic DNA extraction was performed using a QIAamp DNA mini kit (QIAGEN, Germany) accord-

ing to the manufacturer's instruction (Hoelker et al., 2007). Briefly, after the spermatozoon sample incubated with pFLAG-hLY was centrifugated and washed in TALP-HEPES buffer, 200 μl sample was added with 20 μl of QIAGEN protease (stock solution) and 4 μl of RNase A (stock solution), and lysed in buffer AL (stock solution) for 30 min at 56 °C. Each sample was pulse-vortexed for 15 sec and centrifuged at 12,000 × *g* for 5 min. The DNA was further purified with DNA fragment purification kit from TaKaRa (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China). DNA quality was examined by a 1.0% (w/v) agarose gel electrophoresis and DNA amount was quantified by measuring absorbance at 260 nm using a computerized Microplate Reader 550 from the Bio-Rad Company (Hercules, CA, USA). The recovered DNA was stored in Tris-EDTA buffer (pH 8.0) at −20 °C and used as template for quantitative real-time PCR.

2.4.2. DNA isolation from spermatozoa nuclei

For spermatozoa nuclei isolation, the sample was incubated with pFLAG-hLY and centrifuged and washed twice with 150 mM NaCl at 600 × *g* for 3 min, and finally was resuspended at 1×10^8/ml in the nuclei isolation buffer (pH 9.0, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM PMSF and 1 μg/ml pepstatin A). The spermatozoon suspension was collected by centrifugating at 600 × *g* for 3 min and washed three times with the isolation buffer (fresh DTT 25.4 mM, final concentration) and incubated for 30 min on ice. Afterwards, the sample was added with CTAB buffer (10% CTAB, 10 mM DTT) and was incubated again for 60 min on ice. The obtained spermatozoon nuclei were immediately centrifugated at 10,000 × *g* for 5 min and washed twice with the nuclei isolation buffer as template for quantitative real-time PCR.

2.4.3. Real-time PCR quantification

Quantitative analyses of the human lysozyme (hLY) gene was carried out by comparing the copy number of plasmid with the copy number of samples as previously described (Hoelker et al., 2007). The concentration of purified DNA samples was quantified fluorometrically using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, USA) and a VersaFluor fluorometer (Bio-Rad). The same amount of DNA was used in each Real-Time PCR. SYBR Green as a specific dye was used for quantitative Real-Time PCR reaction, in triplicate, in an ABI Prism 7500 Sequence Detection System. In the quantification of bind and internalization of plasmid pFLAG-hLY by goat spermatozoa, primer sets were designed using Primer 5.0 and criteria used during primer design were that primers have predicted Tm = 60.0 °C and that they generate to amplify a 130-bp partial gene fragment and primers bind to the conserved regions of human lysozyme gene (accession no.: NM000239). The primers containing the FLAG epitope were designed as follows: forward primer 5′-CAAGGATGACGACGATAAGATGAAG-3′ and reverse primer 5′-GCCATCCATTCCCAATCTTTTC-3′. The PCR amplifications were carried out in a 20 μl reaction volume containing 10.0 μl of SYBR® Green Master Mix (PE Biosystems), 6.8 μl of double-distilled water, 0.6 μl of 10 μM forward, 0.6 μl of 10 μM reverse primers and 2.0 μl of the purified DNA solution (10–20 ng). The thermal profile for SYBR RT-PCR was 50 °C 2 min, 95 °C 10 min followed by 50 cycles of 95 °C 10 s and 60 °C 1 min. The CT value was used to determine the copy numbers of human lysozyme gene in the unknown spermatozoon samples incubated with pFLAG-hLY based on the standard curve. The real-time standard curves were derived from a serial of linearized plasmid dilutions containing between 10^9 and 10^1 target gene copies calculated directly from the concentration of extracted standard plasmids. The copy numbers of standard plasmids and human lysozyme genes of the unknown sample from the pFLAG-hLY groups were determined using a standard curve. All reactions were repeated at least 3 times independently to ensure the reproducibility of the results

2.5. Mitochondrial membrane potentials ($\Delta\Psi m$)

The $\Delta\Psi$m changes of treated spermatozoa were evaluated using a FACSCalibur flow cytometer (BDBioscience, San Jose, CA) and a JC-1 (lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) Mitochondrial Membrane Potential Detection Kit (Beyotime Institute of Biotechnology, Haimen, China). Briefly, the spermatozoa sample (2×10^6/ml) was stained with 28 μl of JC-1 (stock solution) and 5 μl of PI (stock solution) in 100 μl (final volume) of TALP solution. After incubation at 37 °C in the dark for 30 min, the samples were collected and placed on ice using the method previously described (Pant et al., 2008). High membrane potential was associated with emission at 590 nm (red), and low membrane potential at 530 nm (green) when spermatozoa were excited at 488 nm. $\Delta\Psi$m was determined by a ratio of fluorescence intensity at 590 nm to that at 530 nm. Data were processed by using the CellQuest program (BDBioscience, San Jose, CA). Spermatozoa were treated with 10 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which is a protonophore which can cause dissipation of $\Delta\Psi$m, were used as a negative control.

2.6. Assay of reactive oxygen species (ROS) generation

The levels of intracellular reactive oxygen species were estimated using the membrane-permeable fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA) and a FACSCalibur flow cytometer. The treated spermatozoa (1×10^6/ml) before the end of each experiment and, in three groups, were incubated in 1 ml of mPBS containing DCFH-DA dye (10 μM) at 37 °C for 15 min. Propidium iodide (PI) was then added to DCFH-DA-loaded cell suspensions at a final concentration of 10 μg/ml for approximately 1 min before measurement. The DCHF-DA probe passively diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped inside cells. The ROS produced by spermatozoa oxidizes DCHF to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Then, spermatozoa fluorescence was measured and analyzed by flow cytometry. When DCF was oxidized by ROS, they emit green fluorescence at 510–540 nm after excitation at 488 nm with an argon ion laser. The intracellular ROS levels were expressed as DCF relative fluorescence intensity per 10^6 cells from independent experiments.

2.7. Determination of lipid peroxidation

The level of malondialdehyde (MDA) production, a measure of lipid peroxidation, was measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, the spermatozoon suspensions (approximately 5×10^8/ml) and the reaction mixture containing 0.5 ml thiobarbituric acid reagent (1:1, v:v, mixture of 0.67% thiobarbituric acid and acetic acid) were mixed, shaken, and heated at 95 °C for 40 min. After cooling, the tubes were centrifuged at $4000 \times g$ for 10 min at 4 °C and the supernatants were removed for the detection of lipid peroxidation. The absorbance of the clear supernatant was determined at 532 nm using a computerized microplate reader 550. The MDA content was expressed as nM/10^8 spermatozoa.

2.8. Cytochrome *c* release

Cytochrome *c* levels in both mitochondrial and cytosolic fractions were analyzed by western blotting (Hanzel and Verstraeten, 2009). The spermatozoa (5×10^7/ml) were harvested before the end of each experiment by centrifugation at $600 \times g$ for 10 min at 4 °C,then washed twice with TALP buffer. The spermatozoon pellets were then lysed by incubation at 56 °C overnight with gentle agitation in Tris buffer (pH 8.3) containing 0.45% NP-40, 0.45% Tween-20, 400 μg/ml proteinase K, 1 mM PMSF, 40 mM DTT, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 μg/ml aprotinin and 40 μg/ml bestatine. After centrifugation twice at $600 \times g$ for 15 min at 4 °C, the supernatants were again centrifuged twice at $7000 \times g$ for 20 min at 4 °C to obtain sediments considered as the mitochondria fraction. The mitochondrialis fractions were suspended in 10 mM Tris–HCl (pH 8.0) buffer containing 0.3 mM sucrose, 0.05% (v/v) Igepal, 5 mM $CaCl_2$, 10 μM Aprotinin, 10 μM Pepstatin, 10 μM Leupeptin and 1 mM PMSF. Cytosolic fractions were obtained by centrifugation at $100,000 \times g$ for 1 h at 4 °C. After centrifugation, the protein content of both fractions was measured using a Bradford protein assay kit (KeyGEN Biotech. Co. Ltd., Inc., Nanjing, China). Briefly, equal amounts of protein were subjected to SDS–PAGE, followed by transfer to PVDF membranes (Millipore, USA). Membranes were then blocked in 5% powdered non-fat milk in a Tris/Tween solution (20 mM Tris–HCl, pH 7.6, 0.1% Tween 20 in saline) for 2 h. The location and change in mitochondrial cytochrome *c* level probed with specific anti-cytochrome *c* antibodies (diluted at 1:500) from Cell Signaling (Abcam, USA). Following incubation with appropriate secondary anti-rabbit antibodies at a 1:5,000 dilution, immunoreactive protein bands were visualized by an enhanced chemiluminescence detection kit (Invitrogen, USA) and were recorded on X-ray film (Kodak, China). As control, the blots were reprobed with anti-β-actin antibody (KeyGEN Biotech. Co. Ltd., Nanjing, China) at 1:1500 dilutions. Finally, the membranes were exposed for varying periods ranging from 10 s to 5 min. The band signal intensities were quantified with a Bio-Rad GS-800 Calibrated Densitometer and Bio-Rad personal computer analysis software (Bio-Rad, USA).

2.9. Caspase-3 and -9 activities

Activities of caspase-3 and -9 of spermatozoa samples were determined at an absorbance (OD, optical density) at F405 nm using a caspase-3 and -9 Colorimetric Protease Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). 5×10^6/ml spermatozoon samples were washed with cold PBS by centrifugation at $600 \times g$ at 4 °C for 5 min, and then resuspended in lysis buffer in a HEPES buffer (pH 7.4) of 50 mM containing 0.1% Igepal, 1 mM DTT, 0.1 mM EDTA, and 150 mM NaCl, and were incubated on ice for 15 min. The lysate was centrifuged at $16,000 \times g$ at 4 °C for 15 min. Proteins content was measured in the supernatant using the Bradford method (Zor and Seliger, 1996) with bovine serum albumin as a standard, and the protein was adjusted to 2 μg/μl. Subsequently 80 μl reaction buffer, 10 μl of protein solution and 10 μl 2 mM caspase-3 substrate (acetyl-Asp-Glu-Val-Asp p-nitroanilide, Ac-DEVD-pNA) or caspase-9 substrate (Ac-Leu-Glu-His-Asp-paranitroaniline, Ac-LEHD-pNA) were mixed sequentiallyand the samples were incubated at 37 °C for 4 h. The cleavage and release of paranitroanilide (pNA) was qualified by determining the absorbance with a computerized microplate reader 550 (Bio-Rad, USA) at 405 nm using spectrophotometer, expressed as OD_{405} (test). A total of 90 μl of reaction buffer and 10 μl 2 mM of the corresponding substrate buffer were used for pre-incubationsand defined as OD_{405} (pre-incubation). The activities of caspase 3 and -9 were expressed as OD_{405} (test)/OD_{405} (pre-incubation). Assays were performed in duplicate, and at least three independent tests were performed.

2.10. Data analysis and statistics

Analysis of variance was applied to determine possible effects of incubation with pFLAG-hLY on mitochondrial function, oxidative damage and sperm characteristics. Data were analysed for main effects of the pre-incubation control, the incubation control and pFLAG-hLY groups using one-way analysis of variance (ANOVA) of SPSS for Windows 17.0 Software (SPSS, Inc. Chicago, IL, USA). The independent tests were repeated three times in each group. All the values are presented as mean ± standard error of the mean (SEM). Error bars represent the 95% confidence interval of the mean percentage. A probability (p) value of <0.05 or <0.01 was considered statistically significant (two-tailed).

3. Results

3.1. The ability of goat spermatozoa binding human lysozyme gene

To quantify the copy number of plasmid pFLAG-hLY binding to and internalizating into spermatozoon, a serials of dilutions of extracted genomic DNA or nucleus were assayed by primers (forward primer sequence, 5′-CAAGGATGACGACGATAAGATGAAG-3′; Reverse primer sequence, 5′-GCCATCCATTCCCAATCTTTTC-3′), the resultant CT value against the amount of plasmid DNA was

Table 1
Change of goat sperm motility and RPMS after incubation with pFLAG-hLY for 30 min.

Parameters	Pre-incubation control	Incubation control	pFLAG-hLY
Sperm motility (%)	48.0 ± 1.4^a	54.5 ± 1.5^a	29.7 ± 2.2^b
RPMS (%)	31.4 ± 0.6^a	37.0 ± 0.5^a	16.4 ± 2.4^c

a, b and a, c represent significant differences among groups (a, b: $P < 0.05$, a, c: $P < 0.01$). Results come from triplicate experiment average and standard deviation.

calculated and finally a standard curves was drawn. A linear correlation coefficient between CT values and log template DNA concentration was determined as $R2 = 0.996 \pm 0.001$. After 30-min incubation, human lysozyme gene evidently bound on the surface of goat spermatozoa about 186,000 copies/spermatozoon in pFLAG-hLY. Also, this gene effectively incorporated into spermatozoon nucleus at 78 copies/spermatozoon. These observations indicated that goat spermatozoa had the ability to captured exogenous DNA spontaneously.

3.2. Spermatozoa motility

After treatment with pFLAG-hLY, goat spermatozoon motility and RPMS showed a significant decrease (Table 1). However, there was no significant difference between incubation control and pre-incubation control, although their motility of both groups showed reduction tendency. The results indicate that incubation with plasmid pFLAG-hLY impaired spermatozoon motility and RPMS.

3.3. Measurement of spermatozoa mitochondrial membrane potentials ($\Delta\Psi m$)

Decreased $\Delta\Psi$m is a sensitive indicator of mitochondrial damage, which was assessed by measuring cellular retention of the fluorescent probe JC-1. The potential across the inner mitochondrial membrane was shown in Fig. 1. In the control group, 34.8% pre-incubation and 30.3% incubation spermatozoa showed normal mean red fluorescent intensity, while this proportion decreased significantly ($P < 0.05$) to 23.74 in the pFLAG-hLY group. The mean green fluorescent intensity in spermatozoa was significantly higher than the control ($74.06 \pm 3.96\%$ vs. pre-incubation control $55.16 \pm 3.16\%$ and incubation control $63.74 \pm 3.55\%$, $P < 0.05$). Further, spermatozoa in plasmid pFLAG-hLY group had a significantly lower ratio of red/green fluorescence intensities of JC-1 (Table 2). These results indicate that incubation with plasmid pFLAG-hLY caused a marked decrease in the mitochondrial membrane potential (MMP) of goat spermatozoa.

3.4. Determination of spermatozoa MDA and ROS levels

The generation of ROS and MDA were induced by 30 min incubation with plasmid pFLAG-hLY. The changes of ROS level in treated spermatozoa were determined by DCF relative fluorescence intensity, as measured by flow cytometry using DCFH-DA as an indicator. There were significant differences in DCF relative fluorescence intensity between the pFLAG-hLY group ($32.81 \pm 2.41\%$) and the pre-incubation control ($15.26 \pm 1.48\%$) and incubation control groups ($16.78 \pm 2.05\%$) (Table 2). ROS production was greater in the pFLAG-hLY group than the other two control groups ($P < 0.05$). Similarly, there was a significantly higher level of MDA in the pFLAG-hLY group (2.18 ± 0.21 nM/10^8 spermatozoa) than in the pre-incubation control (1.43 ± 0.17 nM/10^8 spermatozoa) and incubation control groups (1.54 ± 0.11 nM/10^8 spermatozoa) ($P < 0.05$). In addition, the MDA level moderately or markedly increased throughout the incubation period, though the MDA level in the incubation group was not different from control group (Table 2).

3.5. Determination of mitochondrial cytochrome c expression

The average level of cytochrome *c* release was determined by band densitometry analysis of western blotting (Fig. 2) from three separate experiments. This experiment demonstrated that the fact of spermatozoa taken up pFLAG-hLY significantly induced cytochrome *c* release (11.5 kDa) from the mitochondria into the cytoplasm in the three groups (Fig. 2(a) and (b)). The cytochrome *c* level increased in the cytosolic compartment (204.10 ± 27.67 vs. pre-incubation control 114.90 ± 27.61 and incubation control 142.70 ± 30.91), and this level decreased proportionally in the mitochondria (606.20 ± 29.69 vs. pre-incubation control 707.40 ± 27.18 and incubation control 663.10 ± 32.67). The intensity of cytochrome *c* bands in spermatozoa pellets and supernatants were quantified (Fig. 2(c) and (d)). Comparing with the other two control groups, the levels of cytochrome *c* release were significantly lower in the pellets fractions (mitochondrial) of the

Table 2
Change of MMP, ROS, MDA and Caspase-3 and -9 after goat spermatozoon incubation with pFLAG-hLY.

Parameters	Pre-incubation control	Incubation control	pFLAG-hLY
$\Delta\Psi m$ (I_{590}/I_{530}, %)	51.79 ± 2.08^a	58.81 ± 1.76^a	31.44 ± 2.17^b
DCF relative fluorescence (%)	15.26 ± 1.48^a	16.78 ± 2.05^a	32.81 ± 2.41^b
MDA (nM/10^8 spermatozoa)	1.43 ± 0.17^a	1.54 ± 0.11^a	2.18 ± 0.21^b
Caspase-9 (OD_{405})	0.061 ± 0.025^a	0.062 ± 0.019^a	0.083 ± 0.019^b
Caspase-3 (OD_{405})	0.035 ± 0.021^a	0.035 ± 0.025^a	0.086 ± 0.024^b

a, b represent significant differences among groups ($P < 0.05$). Results represent triplicate experiment average and standard deviation.

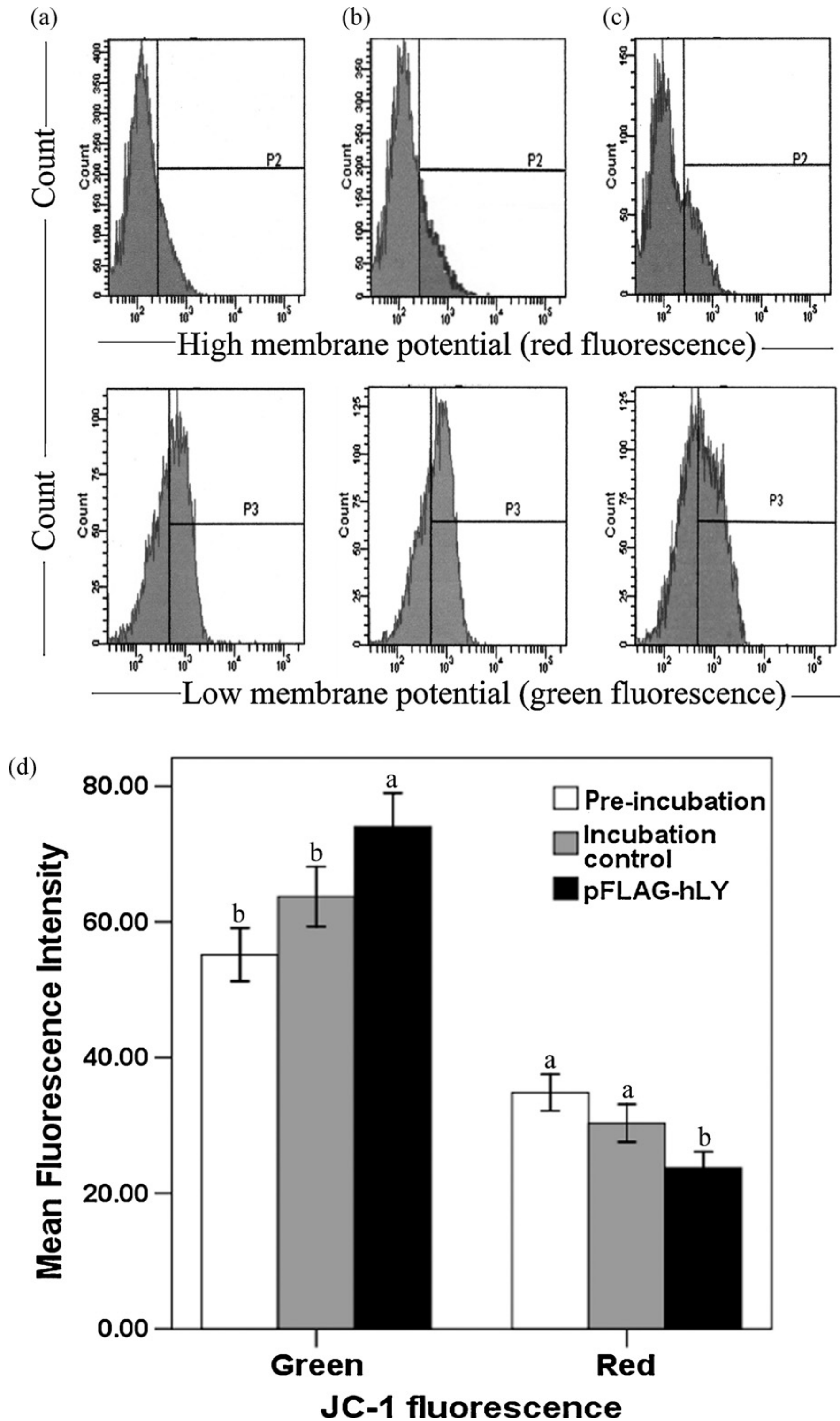

Fig. 1. Flow cytometry study of the mitochondrial membrane potential. (1) A typical picture of flow cytometric analysis of the mitochondrial membrane potential from three groups: the pre-incubation control, the incubation control and pFLAG-hLY. $\Delta\Psi$m was evaluated by flow cytometric analysis of JC-1-stained spermatozoa in figure (a), (b) and (c). Green fluorescence represents the monomeric form of JC-1 with low $\Delta\Psi$m. Red fluorescence represents the mitochondrial aggregate form of JC-1 with high $\Delta\Psi$m. (2) The percentage of red fluorescence intensity and green fluorescence intensity from three groups in figure (d). Different letter a and b represent significant differences among three groups ($P < 0.05$).

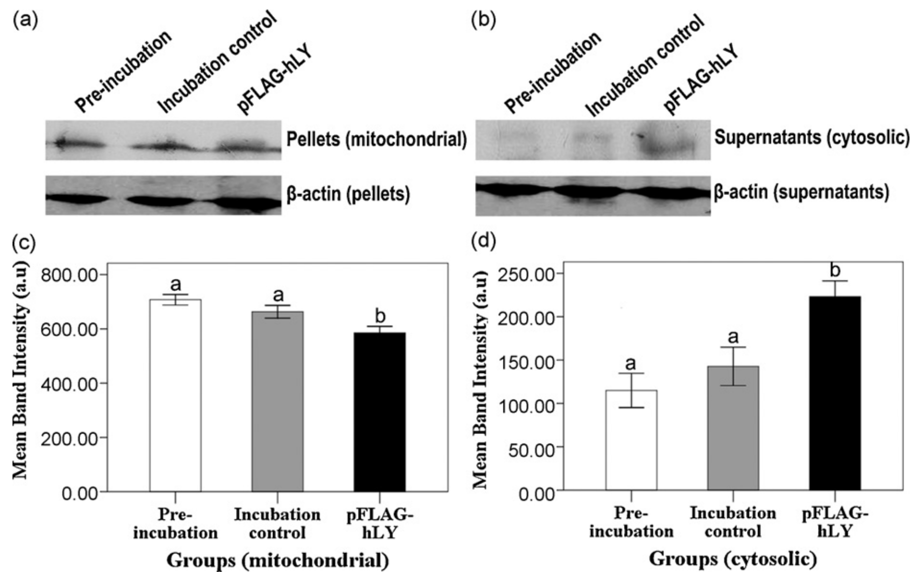

Fig. 2. Western blotting analysis of cytochrome *c* in spermatozoon pellets and supernatants. (1) A representative western blotting of cytochrome *c* expression in spermatozoon pellets (a) and supernatants (b) of three groups; β-actin antibody as loading control and cytochrome *c* was probed using anti-cytochrome *c* antibody. (2) One representative statistical analysis of two similar western blotting from mitochondrial fractions (c) and cytosolic fractions (d) using densitometric analysis software. The densitometric data in cytochrome *c* release was expressed in arbitrary units (a.u.) corresponding bands in spermatozoon pellets from three groups. Different letter a and b represent significant differences among three groups ($P < 0.05$).

pFLAG-hLY group ($P < 0.05$) and there were significantly higher in the supernatants fractions (cytosolic) ($P < 0.05$).

3.6. Colorimetric analysis for the activities of caspase-3 and -9

The enzymatic activities of caspase-3 and -9 were activated in different groups. Caspase-9 is known to be an important trigger in mitochondria and its expression results in spermatozoon dysfunction. Caspase-3 plays a crucial role in mitochondrial oxidative damage. Our study confirmed that 30 min incubation with plasmid pFLAG-hLY induced an increase of caspase-3 and -9 activities (Table 2). The relative levels of caspase-3 and -9 were determined by absorbance at 405 nm. The results showed that the levels of caspase-3 (0.086 ± 0.024) and -9 (0.083 ± 0.019) were higher in treatment group than the controls ($P < 0.05$). No significant differences were observed in enzyme activities between the pre-incubation and incubation control groups.

4. Discussion

The sperm-mediated gene transfer, that uses spermatozoon as a natural vector to introduce new genes into animals, has been considered as a direct, fast and low cost method. However, the effect of this technology is often questionable because of its low efficiency and contradictory reports. Some reports indicated that exogenous DNA invasion may cause change of spermatozoon natural defense system, which led to spermatozoon dysfunction (Kang et al., 2008). Here, our study further analyzed the reason of low efficiency using sperm-mediated vector for transgenic animals by observing sperm mitochondrial function.

The present study showed that after incubation of goat spermatozoa with human lysozyme gene pFLAG-hLY, the plasmid pFLAG-hLY could be bound to the surface of spermatozoon membrane and internalized into spermatozoon nucleus. However, after binding the exogenous pFLAG-hLY plasmid DNA, goat spermatozoa displayed an abnormal motility. At the same time, percentage of rapid progressive motile spermatozoa also changed. This finding was in agreement with previous other animal reports on boars (Gandolfi et al., 1996) and bull (Schellander et al., 1995; Anzar and Buhr, 2006) spermatozoa.

Spermatozoon motility is closely associated with the functional status of spermatozoa mitochondria (Ly et al., 2003) and spermatozoa plasma membrane (Ponzio et al., 2007). Spermatozoa might undergo marked changes in their intracellular and extracellular environment during incubation with exogenous DNA. The most ordinary appearance is reactive oxygen species (ROS) generation. The ROS was responsible for the loss of spermatozoon motility (Bilodeau et al., 2002). Motility is ATP-dependent (Nascimento et al., 2008) and ROS causes a rapid decrease in intracellular ATP concentration (Delamirande and Gagnon, 1992). During incubation of the spermatozoa with pFLAG-hLY, extracellular depletion of ATP in the presence of ROS has a detrimental effect on motility. Intracellular ATP could not neutralize the toxic effect of high ROS formation due to the absent of iron and other transition metal, which led to further decline of spermatozoa motility.

The result of the present study indicated that goat spermatozoa incubation with pFLAG-hLY caused significant decrease in spermatozoon mitochondrial membrane potential ($\Delta\Psi$m). It was also possible that incubation with plasmid pFLAG-hLY had changed culture environment and increased the ROS production, which might interfere spermatozoon mitochondrial function (Guthrie and Welch,

2006). We observed here that significant high level of LPO in treatment group was detected by MDA production (Table 2). This observation reflected that lipid peroxidation (LPO) was an important contributor to mitochondrial dysfunction (Sen et al., 2006) and there was a high positive correlation between percentage of abnormal spermatozoa and membrane LPO (Zheng and Zhang, 1997). Therefore, the potential anomalies of mitochondrial membrane and lipid peroxidation resulted in alterations of mitochondrial function following incubation of plasmid pFLAG-hLY.

Cytochrome *c* release from the mitochondrial inner membrane space has an important role in the induction of oxidative damage. It occurs via mitochondrial permeability transition pore-induced mitochondrial dysfunction (Orrenius et al., 2007). Opening of the permeabilization transition pore complex is thought to mediate release of cytochrome *c* from the mitochondria (Ansari et al., 2006). Mitochondrial permeability transitionresults indisruption of the outer membrane, release of cytochrome *c*, apoptosis inducing factor (AIF) and the activation of caspases (Alksne et al., 2000; Petersen et al., 2000; Gravance et al., 2001; Cordero et al., 2009). Our study showed that the incubation with plasmid pFLAG-hLY caused release of cytochrome *c* via the mitochondrial signaling pathway (Fig. 2). Western blotting analysis revealed that the amount of cytochrome *c* remaining in the mitochondrial pellets displayed significantly reduction. In similar experiments, cytochrome *c* release in the cytosol were markedly increased (Fig. 2). Release of cytochrome *c* is coupled with the increase of activation of caspases-3 and -9 (Table 2). These results strongly supported the fact that the change in mitochondrial membrane potential companied by a partial release of cytochrome *c* from the insoluble fraction to the cytosol. Cytochrome *c* released can interact with the Apaf-1 protein and the inactive form of caspase-9, which leads to activation of caspase-3 (Gravance et al., 2001; Cordero et al., 2009). Therefore, the incubation with plasmid pFLAG-hLY altered spermatozoon mitochondria and induced the expression of cytochrome *c*, the activation of caspase-3 and -9, which led to cytotoxic effect and even spermatozoon death.

In summary, the present study demonstrated that goat spermatozoa could capture human lysozyme plasmid pFLAG-hLY, but the incubation with plasmid pFLAG-hLY resulted in decrease of spermatozoa motility and partial rupture of the spermatozoa mitochondrial membrane, and further promoted the expression of cytochrome *c* and generation of oxidative stress in vitro and finally led to spermatozoon dysfunction. Therefore, a new method needs to be considered to protect DNA-loading spermatozoa from alterations of mitochondrial function and motility during sperm-mediated gene transfer.

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