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Antioxidative activity and protective effect of probiotics against high-fat diet-induced sperm damage in rats

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In this study, antioxidant capability and protective effect of probiotics on reproductive damage induced by diet oxidative stress were investigated. Thirty male Sprague-Dawley rats were randomly divided into three groups with 10 rats in each group. The control group consumed a normal standard diet (5% fat, w/w). The other two treatment groups were fed with a high-fat diet (20% fat, w/w), and a high-fat diet supplemented with 2% probiotics (w/w), respectively. At the end of the experimental period, that is, after 6 weeks, rats were killed. Activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), contents of nitric oxide (NO) free radical and malondialdehyde (MDA) in serum and sperm suspension were examined. Sperm parameters including sperm concentration, viability, motility and DNA integrity were analyzed. The results showed that high-fat diet could induce oxidative stress, shown as significant increases in lipid peroxidation, NO free radical, significant decrease in activities of SOD, GSH-Px, significant reduction in sperm concentration, viability and motility, and damage in sperm DNA ($P < 0.05$), compared with the control group. These alterations were significantly reversed in the probiotics-supplemented group and had no significant difference in antioxidant capability, lipid peroxidation and sperm parameters compared with the control group. The percentage of sperm with DNA damage was significantly lower than the high-fat diet group and still higher than the control group, which means that probiotics could attenuate sperm damage to some extent. The present results indicated that dietary probiotics had antioxidant activity and the protective effect against sperm damage induced by high-fat diet to some extent.

Keywords: probiotics, antioxidant, oxidative stress, sperm

Implications

The nutritional benefits of probiotics in animals have been well documented, but studies on the effect of probiotics on sperm damage are lacking. The results of this study show that feeding probiotics could improve the antioxidant capacity and protect sperm against high-fat diet-induced oxidative damage in rats. This is the first report regarding the protective effects of probiotics against sperm injury. The present observations suggest a protective effect of probiotics on sperm injury, which could be of interest for improving animal reproduction performance using probiotics supplementation.

Introduction

With the fast development of intensive livestock breeding, high-yielding farm animals are very prone to the unfavorable

consequences of oxidative stress and appear to be more vulnerable to infection, have shortened longevity potential and reproductive performance (D'Silva and Stevenson, 2011). Oxidative stress in a living organism arises when the production of reactive oxygen species (ROS) or/and reactive nitrogen species surpasses the scavenging capacity of antioxidant systems (Sies, 1991). Excessive ROS can damage macromolecules including lipids, proteins, polysaccharides and DNA, lead to cell injury and have detrimental effects in the form of gastrointestinal tract ulcers, reduction in immune functions, cessation of growth and reproduction and death from failure of adaptive mechanisms (Dantzer and Mormede, 1983).

Reproductive consequences of ROS damage include disruption in the function of spermatozoa. It is assumed that the ROS could affect the fertility of males, and some reports have supported this viewpoint (Gagnon *et al.*, 1991). The ROS have both advantageous and pernicious influences on sperm and thus influence the fertilization process in male animals. Antioxidant status may be one determinant of reproductive function in poultry and livestock. The effects

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of antioxidants on reproductive function may be more pronounced because of the increased metabolic rates associated with cellular hyperthermia. Diets high in fats, which increase fat-mediated oxidative stress and ROS levels in a variety of tissues and decrease antioxidant capacity, have long been recognized (Sreekumar *et al.*, 2002; Feillet-Coudray, 2009). Decreasing the oxidative damage could be feasible by increasing the antioxidant level in the body. Overwhelming evidence from laboratory and farm animals has supported a protective role of antioxidants on reproductive function (Tarin *et al.*, 1998).

With the cognition of side effects and ban of antibiotic growth promoters in animal feeding, poisonless and harmless probiotics feed additive has risen as a viable alternative means to antibiotics to improve animal health and protect against infectious challenges. Probiotics feed supplementation may benefit the animal host directly, by preventing the infection and combating the causative agent of the intestinal disorder, or indirectly by balancing the disrupted equilibrium of the enteric flora and augmenting the host's immune responses. Recently, antioxidant properties of probiotics, such as lactic acid bacteria (Mikelsaar *et al.*, 2008) and yeast (Tovar-Ramírez *et al.*, 2010), have received more attention. However, there are few reports concerning the effect of dietary probiotics on the oxidative damage of antioxidant system and reproductive performance in animals and fewer reports concerning the involvement of probiotics in the antioxidant system as feed additives.

In a previous study, we showed that probiotics have antioxidant properties and positively modulate free radical metabolism in rats, increasing the activities of antioxidant enzymes and decreasing the content of malondialdehyde (MDA) and nitric oxide (NO; Chen *et al.*, 2010). It is proposed that dietary supplementation of probiotics with antimicrobial and antioxidant properties may be a promising strengthening agent for increasing pathogen resistance in animals. Reproduction is likely to fail long before life is endangered by deficiency of any required nutrient, including dietary antioxidants (Miller and Brzezinska-Slebodzinska, 1993). To date, no study has been found reporting on the effects of antioxidative probiotics on reproductive performance and oxidative status in animals, and only preliminary reports are available. The aims of this study using rats as the model were to examine the effects of oxidative stress on the quality of sperm, and to evaluate the influence of probiotics supplementation on animal growth, antioxidant ability and repair function of sperm oxidative damage induced by diet stress *in vivo*.

Material and methods

Animals

Thirty male s.d. rats (300 ± 30 g, SIPPR/BK Experimental Animal Ltd, Shanghai, China) were used and housed in individual cages in a room at 22°C to 24°C , with 60% relative humidity and 12-h dark-light cycles (light on from 0800 to 2000 h). Animal experiments were carried out in

accordance with the Guidelines for Animal Experimentation of Shanghai Jiao Tong University (Shanghai, China) and the protocol was approved by the Animal Ethics Committee of this institution.

Experimental design

After 1 week of adaptation to a standard diet, rats were randomly assigned to one of three groups with 10 rats in each group. Group I (Control) received only a standard diet containing 5% fat, 20% CP, 5% crude fiber, 8% ash, 1.2% calcium, 0.8 phosphorus, 50% nitrogen-free extract and 10% moisture. Group II received a high-fat diet comprising the standard diet supplemented with 20% (w/w) pure sunflower seed oil. The final fat content of this high-fat diet was 20%. Group III was fed the high-fat diet in conjunction with 2% (w/w) probiotics, which is a commercial product produced by Chuangbo Modern Natural Agricultures Group (Shanghai, China) for animals and is mainly based on *Lactobacillus* spp., *Bacillus* spp., beer yeast and photosynthetic bacteria culture, and the bacterial counts normally reach 5.5×10^9 cfu/g. Rats were allowed free access to their respective diets and tap water *ad libitum* for 6 weeks. At the end of the experimental periods, after an overnight fast the rats were anesthetized with ethyl ether, weighed and killed. Serum was obtained from blood samples after centrifugation ($500 \times g$ for 10 min at 4°C) and stored at -20°C until analysis. The testis and epididymis were dissected and weighed to calculate relative organ weight expressed as (organ weight/BW) $\times 100\%$.

Preparation of sperm suspensions

Sperm collection and preparation of suspension was carried out according to the procedure described by Suresh *et al.*, (2010). In brief, the caudal portion of the right epididymides of rats was placed in 30-mm dishes containing 2 ml physiological saline and minced with a fine scissors to allow spermatozoa to swim out for 10 to 15 min at 37°C . After incubation, the epididymal tissue–fluid mixture was filtered using a strainer in order to separate the supernatant from tissue particles. The sperm supernatant fluid was used to analysis of antioxidant properties, lipid peroxidation, sperm parameters and DNA integrity.

Determination of enzymatic antioxidant activities in serum and sperm

The activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in serum and sperm were assayed using colorimetric methods with a spectrophotometer (Thermo Spectronic, Cambridge, England). The activity of SOD was measured by the xanthine oxidase method, which monitored the inhibition of reduction of nitro blue tetrazolium by the sample. The activity of GSH-Px was detected with 5,5'-dithiobis-nitrobenzoic acid, and the change of absorbance at 412 nm was monitored using a spectrophotometer. The test kits for measuring the activity of SOD (A001-1) and GSH-Px (A005) were purchased from Nanjing Jiancheng Institute

of Bioengineering (Nanjing, Jiangsu, China) according to the instructions of the manufacturer. The SOD and GSH-Px activity in sperm suspension was expressed as U/g protein. The protein concentration was measured by the method of Lowry *et al.* (1951).

Estimation of free radical and lipid peroxidation in serum and sperm

The content of NO free radical and lipid peroxidation production, MDA in serum and sperm was estimated. The NO content was expressed by the content of NO metabolites (nitrite and nitrate). Nitrate was reduced first to nitrite by the action of nitrate reductase, then the reaction was initiated by the addition of the Griess reagent, and absorbance of the mixture at 550 nm was determined. The MDA content was analyzed with 2-thiobarbituric acid, monitoring the change of absorbance at 532 nm with the spectrophotometer. The test kits for measuring the contents of MDA and NO were purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, China) according to the instructions of the manufacturer. The content of MDA and NO in sperm suspension was expressed as nmol/g protein and μ mol/g protein, respectively.

Measurements of sperm number, viability and motility

The sperm supernatant fluid was diluted 1:100 with a solution containing 5 g sodium bicarbonate, 1 ml formalin (35%) and 25 mg eosin per 100 ml ultra pure water for analysis of sperm number, viability and motility.

The sperm number of diluted sperm suspension was counted with a haemocytometer (Hawksley and Sons Ltd, England, UK). An aliquot of 10 μ l of dilution was added to counting chamber. After standing for 5 min, the sperm number was counted under the 400 \times magnification of a light microscope (Olympus Optical Co. Ltd., Tokyo, Japan). The total sperm counts ($\times 10^6$ /ml) were calculated.

The determination of sperm viability and motility of diluted sperm suspension was carried out. An aliquot of 20 μ l of diluted sperm suspension was mixed with an equal volume of 0.05% eosin-Y and nigrosin in a slide. The mixture was incubated at room temperature for 2 min and analyzed under a light microscope, at 400 \times magnification. Live sperms were white (unstained) and dead sperms were stained. 100 sperms per rat was counted and viability percentages (%) was calculated. Sperm motility and percentage of rapid progressive motile sperm (grade a, percentage of the sperms move forward rapidly) were categorized

and assessed according to the World Health Organization standards (2010).

Assessment of sperm nuclear DNA integrity (comet assay)

Comet assay, also called a single-cell gel electrophoresis (SCGE), determining a combination of single-strand breaks, double-strand breaks and alkaline labile sites was performed to investigate the effect of probiotics on sperm DNA integrity using a commercial kit (Trevigen, Gaithersburg, MD, USA). As directed in the instructions of the kit manufacturer, all sperm suspensions were washed twice with cold 1 \times PBS (phosphate-buffered saline) by centrifugation (10 min, 300 \times g, 4°C) and then resuspended in 1 ml of 1 \times PBS (1 $\times 10^5$ sperm/ml of PBS). The sperm suspension was suspended in low-melting Comet LMAgarose (1% w/v, Trevigen, MD, USA) at a ratio of 1:10 (v/v) at 37°C. Of this preparation, 75 μ l was pipetted to the surface of a pre-coated comet slide (Trevigen) and covered with a coverslip (24 mm \times 60 mm). The slide sandwiches were placed horizontally at 4°C in the dark for 10 min to allow the agarose to solidify. Then the coverslides were gently removed and slides were submerged in a pre-chilled lysis solution (Trevigen) at 4°C for 60 min. Following cell lysis, the slides were immersed in the chilled alkaline solution (pH > 13, NaOH, 0.6 g; EDTA (ethylenediaminetetraacetic acid), 200 mmol) for 10 min in the dark at 0°C. The slides were placed in a horizontal electrophoresis unit in 1 \times TBE (Tris-borate EDTA) buffer and Electrophoresis was conducted at 25 V, 250 mA for 15 min. When electrophoresis was complete, the slides were washed twice with PBS, immersed in an ethanol solution (70%, v/v) for 5 min and air-dried. The slides were stained with DNA fluorochrome SYBR Green (Trevigen), and a coverslip was applied before image analysis. Cells were visualized under a fluorescence microscope (Olympus Co., Tokyo, Japan) at 200 \times magnification. At least 50 randomly selected images were analyzed from each sample with the comet assay software project (CASP 1.2.2, University of Wroclaw, Poland). In our study, DNA damage was evaluated by the percentage of DNA in the tail (% tail DNA), tail length (μ m), tail moment and olive tail moment and obtained by the software automatically. Tail moment is calculated as the product of the tail length (μ m) and the fraction of DNA in the tail (% tail DNA). Olive tail moment represents the product of the percentage of total DNA in the tail (% tail DNA) and the distance between the center of the mass of head and tail regions (olive tail moment = (tail mean – head mean) \times percentage of DNA in the tail).

Table 1 Effects of oxidative stress induced by high-fat diet and probiotics on BW and reproductive organ weight relative to BW of rats

Item	Control	High-fat diet	High-fat diet + 2% probiotics
Initial BW (g)	381 \pm 15	382 \pm 16	379 \pm 15
Final BW (g)	487 \pm 26	514 \pm 24	496 \pm 23
Relative testis weight (% BW)	0.69 \pm 0.02 ^a	0.66 \pm 0.03 ^b	0.70 \pm 0.03 ^a
Relative epididymis weight (% BW)	0.24 \pm 0.00 ^a	0.22 \pm 0.00 ^b	0.24 \pm 0.01 ^a

Means within a row without a common superscript alphabets differ ($P < 0.05$).

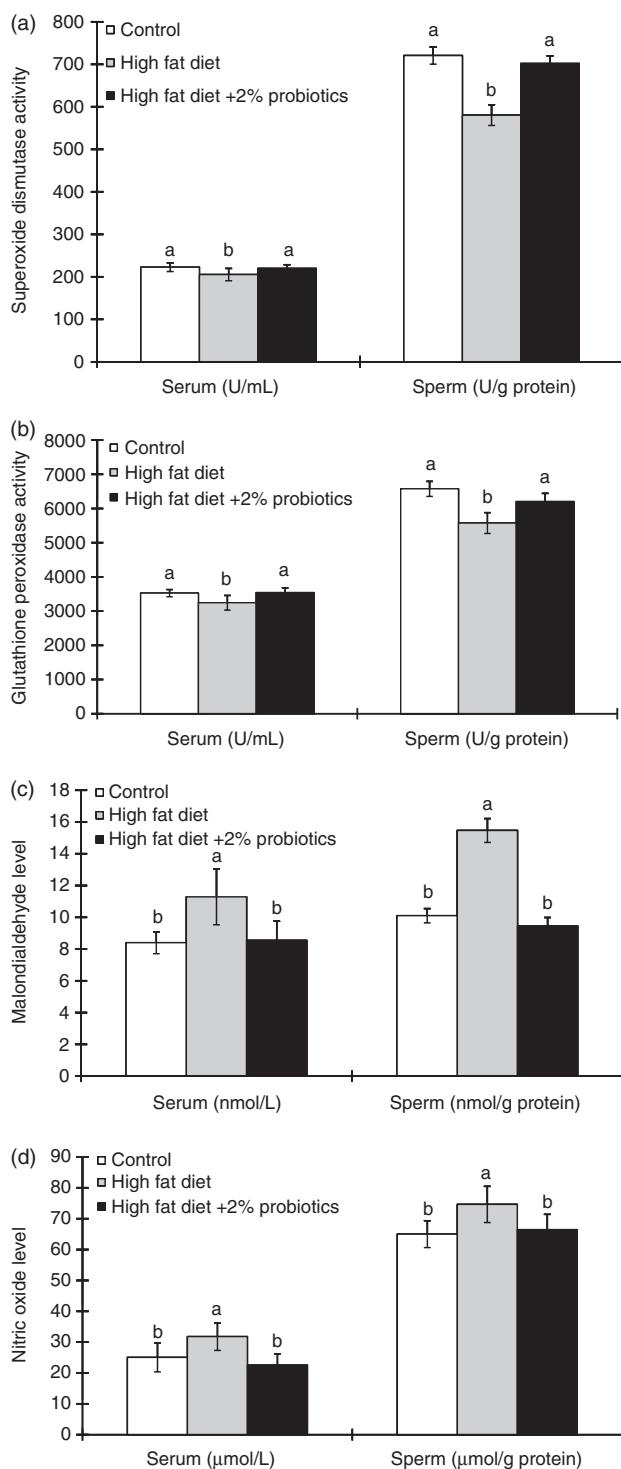


Figure 1 Effect of oxidative stress induced by high-fat diet and probiotics on the activity of superoxide dismutase and glutathione peroxidase, level of malondialdehyde and nitric oxide in serum and sperm. The mean differences between the bars bearing values with different superscript letters are statistically significant ($P < 0.05$).

Statistical analysis

All the results were expressed as mean \pm s.d. of 10 rats in each group. Statistical significance of difference in means was analyzed by a one-way analysis of variance, followed by least significant difference *post hoc* test. A difference of $P < 0.05$

was considered statistically significant. Analysis was carried out with SPSS 11.5 (SPSS Inc., Chicago, Illinois, USA).

Results

BW and reproductive organ weight coefficient

The consumption of water and feed during the period of experiment was similar among the groups (data not shown). The mean final BW at the end of experiment was generally higher in treatment groups, compared with controls, but did not reach statistical significance as shown in Table 1. The testis and epididymis weight coefficient (expressed as relative to BW) of rats fed with high-fat diet was significantly lowered compared with control rats. Supplementation with probiotics along with the high-fat diet had a similar reproductive organ weight coefficient with those of the control rats.

Oxidative stress induced by high-fat diet

The activities of SOD and GSH-Px and the content of MDA and NO in serum and sperm were determined to evaluate the antioxidant ability, lipid peroxidation and free radical metabolism in rats, and results are shown in Figure 1. In this study, feeding of the high-fat diet for 6 weeks resulted in the development of oxidative stress in experimental rats. Activities of SOD and GSH-Px in serum and sperm were significantly lowered in rats fed high-fat diet compared with control rats. The content of MDA, the end products of lipid peroxidation and NO free radical were significantly increased in rats fed with high-fat diet compared with the control group, indicative of aggravating oxidative stress and subsequent lipid peroxidation in rats fed the high-fat diet.

Antioxidant ability of probiotics

As shown in Figure 1, supplementation with probiotics along with the high-fat diet restored the activities of SOD and GSH-Px and decreased the content of MDA and NO in the serum and sperm of rats to near those of the control rats ($P > 0.05$), and had a significant difference compared with those in rats fed the high-fat diet.

Sperm parameter analysis

To ascertain whether oxidative stress induced by high-fat diet influences the reproductive performance of male rats and protective effect of probiotics, sperm characteristic analysis was conducted. Sperm concentration, viability and motility were significantly decreased in rats fed the high-fat diet; these alterations were significantly reversed in group III fed the high-fat diet supplemented with probiotics when compared with the control group. No significant changes were observed in the progressive motility study in all the experimental groups (Table 2).

Sperm DNA damage

To evaluate the protective effect of antioxidants on sperm DNA integrity, the DNA migration of the sperm was assessed by the comet assay. As shown in Table 3, the high-fat diet could provoke more DNA damage for rats, and was shown to

Table 2 Effects of oxidative stress induced by high-fat diet and probiotics on sperm parameters in rats

Parameters	Control	High-fat diet	High-fat diet + 2% probiotics
Concentration ($\times 10^6/\text{ml}$)	$30.0 \pm 2.6^{\text{a}}$	$23.8 \pm 4.1^{\text{b}}$	$29.0 \pm 5.5^{\text{a}}$
Viability (%)	$89.3 \pm 3.5^{\text{a}}$	$78.2 \pm 2.4^{\text{b}}$	$86.2 \pm 3.7^{\text{a}}$
Motility (%)	$87.8 \pm 4.2^{\text{a}}$	$78.4 \pm 5.4^{\text{b}}$	$86.8 \pm 6.5^{\text{a}}$
Progressive motility ($\times 10^6/\text{ml}$)	7.6 ± 1.9	6.4 ± 2.1	8.0 ± 1.3

Means within a row without a common superscript alphabets differ ($P < 0.05$).

Table 3 Effects of oxidative stress induced by high-fat diet and probiotics on extent of sperm DNA damage in rats: a comet assay

Item	Control	High-fat diet	High-fat diet + 2% probiotics
Tail length (μm)	$3.94 \pm 1.34^{\text{c}}$	$12.56 \pm 4.76^{\text{a}}$	$8.13 \pm 2.75^{\text{b}}$
Tail DNA percent (%)	$0.33 \pm 0.15^{\text{c}}$	$4.0 \pm 0.98^{\text{a}}$	$3.12 \pm 0.06^{\text{b}}$
Tail moment	$0.03 \pm 0.01^{\text{c}}$	$0.75 \pm 0.20^{\text{a}}$	$0.31 \pm 0.13^{\text{b}}$
Olive tail moment	$0.11 \pm 0.07^{\text{c}}$	$1.52 \pm 0.87^{\text{a}}$	$0.60 \pm 0.02^{\text{b}}$

Means within a row without a common superscript alphabets differ ($P < 0.05$).

significantly increase tail length, percentage of DNA in tail, tail moment and olive tail moment. Supplementation of probiotics could protect DNA against oxidative damage induced by high-fat diet to some extent, and tail length, percentage of DNA in tail, tail moment and olive tail moment were significantly decreased. However, the percentage of sperm DNA damage was higher than that in the control group ($P < 0.05$).

Discussion

The present study explored the effect of high-fat diet on antioxidant capacity and reproductive cell DNA damage and the protective effect of probiotics on oxidative damage in rats. We found that high-fat diet could provoke oxidative stress and sperm damage in rats, and probiotics could repair oxidative damage and improve sperm quality to some extent.

High-fat diet inducing oxidative stress has been shown in this study and was in agreement with those reported in other studies (Cui *et al.*, 2009; Feillet-Coudray, 2009). Antioxidants can dispose, scavenge and suppress the formation of free radicals or oppose ROS chain actions in the body and play an important role in animal health. Our results showed that probiotics supplement recovered antioxidant capacity and decreased lipid peroxidation, suggesting that probiotics had antioxidant ability and could restore oxidative damage induced by high-fat diet to some extent. The main antioxidant components produced by the probiotics are assessing in our lab recently.

It is well known that sperm cell membranes are rich in polyunsaturated fatty acids and are very susceptible to free radical attack. Lipid peroxidation of sperm cell membrane is one type of cell damage induced by free radicals, which causes an increase in membrane permeability, an interruption in respiratory chain and ATP production and a decrease in phosphorylation of axonemal proteins (Flaherty *et al.*, 2006). Sperm quality in rats fed the high-fat diet significantly

declined may be because of the decreased protective function of antioxidant enzyme in the body (Figure 1) and the increased free radicals. Excessive free radicals could decrease sperm number and result in poor viability and motility of sperm (Table 2), even infertility. Sperm motility decreased by free radicals was presumably due to a rapid loss of intracellular ATP, which altered axoneme structure and caused tail abnormality and decrease in sperm motility (Syntin and Robaire, 2001). Free radicals not only damage sperm lipid membrane, but also affect sperm cell DNA integrity. It is well known that oxidative damage of DNA occurs in all aerobic cells (may including sperm), which are rich in mitochondria, because ROS produced during oxidative respiratory in the mitochondria. Sperm DNA under oxidative stress leads to more than 20 different types of base damage, producing oxidized and ring-fragmented nitrogen bases (Slupphaug *et al.*, 2003). In the present study, decrease in the activity of SOD and GSH-Px in sperm suspension in rats fed the high-fat diet indicates either reduced synthesis or elevated degradation or inactivation of the enzyme and excessive ROS production. Injury of cell DNA induced by excessive ROS led to the production of some peroxidation products such as 8-oxo-7,8-dihydroxyguanosine, which causes fragmentation and has a mutagenic effect (Menezo *et al.*, 2007). High levels of sperm DNA damage have been reported to decrease male fertility and antioxidants could be a treatment to alleviate male infertility associated with sperm DNA damage from the clinical point of view (Greco *et al.*, 2005).

Antioxidant supplementation can theoretically protect and prevent peroxidative damage. Probiotics are live microbial preparations that have beneficial effects on host health, growth performance, disease resistance ability and intestinal health (Gaggia *et al.*, 2010; Le Bon *et al.*, 2010). To our knowledge, however, few studies were related to reproductive system of animals. Kistanova (2005) showed that probiotic (BioPro-1) can be used for improvement of the reproductive performances of rams during the breeding

season. Su *et al.* (2009) reported that the sperm motility rate, acrosome integrity rate and GSH-Px active in stock boars supplemented with selenium-enriched probiotics were better than that in control stock boars fed a basal diet. In the present study, sperm quality was significantly increased in rats fed the high-fat diet supplemented with probiotics compared with that in rats fed the high-fat diet, which demonstrated that incorporation of probiotics in feed positively influenced the reproductive performance of rats in terms of higher sperm viability and motility, and lower sperm DNA fragmentation. Our study was the first report on the effect of incorporated probiotics on repair of reproductive damage induced by oxidative stress. It is hypothesized that the repair effect on sperm damage of probiotics might be a result of the increase in antioxidant system and the decrease in sperm cell apoptosis in the testis, which would need more avenues to approve in future.

In conclusion, the results of the present investigation confirmed that probiotics have antioxidant properties in response to oxidative stress and have the potential to restore the quality of sperm damage induced by diet stress in rats to some extent. The mechanism and subsequent application of probiotics for improving reproductive performance or fertility in animals need further study.

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