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Protective Effects of Dietary Co-Administration of Probiotic *Lactobacillus casei* on CP-Induced Reproductive Dysfunction in Adult Male Kunming Mice

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Abstract: Cyclophosphamide (CP), an anti-cancer alkylating agent is known to be a male reproductive tract toxin. The aim of this study was to investigate the possible protective effects of *Lactobacillus casei* as a probiotic product and potent antioxidant on CP-induced testicular and spermatozoal toxicity associated with oxidative stress in male mice. Seventy two healthy adult male Kunming mice were divided into eight groups of nine mice each. The blank group (P1) and the CP Model group (P2) were fed the basal pellet diet without any probiotics. The mice in the *Lactobacillus casei*+CP groups P3, P4, P5 and P6 were fed the basal pellet diet with 10^5, 10^6, 10^7, 10^8 CFU g^{-1} (diet) of *Lactobacillus casei*. The P7 and P8 group mice administered with *Lactobacillus casei* but not CP as a control group. For the 1st 5 days, mice in the P2, P3, P4, P5 and P6 groups were injected intraperitoneally with CP. All treatments were maintained for 8 weeks. At the end of the treatment period, body weight and the weight of the reproductive organs was determined. Histological and biochemical analyses of sperm and testicular tissue and serum sex hormone levels were performed. Administration of CP resulted in significant decreases in epididymal sperm concentration and motility and significant increases in the number of abnormal sperm and Malondialdehyde (MDA) and Catalase (CAT) levels. However, *Lactobacillus casei* co-treatment prevented the development of CP-induced sperm and testicular damage. Compared with the CP groups, the *Lactobacillus casei*-treated groups showed increases in testicular and epididymal weights, Superoxide Dismutase (SOD) and sperm concentrations, percentages of morphological normality and serum testosterone levels. No significant differences were observed in the number of abnormal sperm in the epididymides and histomorphology of the testes between the P4, P5, P6, P7 and P8 groups and blank group. *Lactobacillus casei* has a significant protective effect on CP-induced toxicity in the male mice reproductive system.

Key words: Cyclophosphamide, probiotic, *Lactobacillus casei*, antioxidant, reproductive dysfunction

INTRODUCTION

Probiotics which are defined as live microbial food supplements that benefit human health (McFarland, 2000; Salminen, 2001; Nikfar *et al*., 2010, 2008; Rahimi *et al*., 2008a, b; Elahi *et al*., 2008) have been rated as safe by the United States Food and Drug Administration (FDA) and the European Food Safety Authority (Ceragioli *et al*., 2009). Viable lactic acid bacteria have long been assumed to be a probiotic product suitable for human use and have been approved for oral administration as a live microbial formulation for bacterioprophylaxis and bacteriotherapy (Hoa *et al*., 2000). A broad spectrum of literature supports the use of oral administration of lactic acid bacteria for both the prevention and treatment of a wide variety of gastrointestinal disorders (Hong *et al*., 2005; Williams *et al*., 2009). This bacterial constituent of probiotic foods has several scientifically established and/or clinically proved health effects such as improvement of the intestinal microbial balance by antimicrobial activity, maintenance of the numbers of healthy bacteria in the intestines, reduction and prevention of diarrheas of different origin, prevention of food allergy, enhancement of immune potency, alleviation of lactose intolerance symptoms and anti-tumorigenic activity (McFarland, 2000; Salminen, 2001;

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Andersson *et al*., 2001). Moreover, many studies indicate that certain lactic acid bacteria possess antioxidative activity (Peuhkuri *et al*., 1996; Kullisaar *et al*., 2002, 2003; Songisepp *et al*., 2004; Zhang *et al*., 2009; Spyropoulos *et al*., 2011; Kaizu *et al*., 1993; Martarelli *et al*., 2011). For example, *Lactobacillus casei* decreases the risk of Reactive Oxygen Species (ROS) accumulation in a host organism indicating potential utility in probiotic food supplements to reduce oxidative stress (Songisepp *et al*., 2004).

Recently, there has been increasing interest in understanding the roles and mechanisms of probiotic products as inhibitors of oxidative stress which has attracted considerable attention as antioxidants (Peuhkuri *et al*., 1996; Kullisaar *et al*., 2002, 2003; Songisepp *et al*., 2004; Zhang *et al*., 2009; Spyropoulos *et al*., 2011; Kaizu *et al*., 1993; Martarelli *et al*., 2011). Investigation of various defense strategies has shown that Superoxide Dismutase (SOD) is upregulated following probiotic supplementation. These data provide a possible mechanism for the benefits of a supplemented formula for decreasing the severity of Necrotizing Enterocolitis (NEC) by preserving the antioxidant systems (D'Souza *et al*., 2010).

Cyclophosphamide (CP) is extensively used for the treatment of various cancers as well as an immunosuppressant in organ transplantation (Perini *et al*., 2007; Uber *et al*., 2007). However, despite its wide spectrum of clinical uses, CP as an anti-cancer alkylating agent is known to be a male reproductive tract toxin (Rezvanfar *et al*., 2008). Studies on rats and mice have confirmed the potential of CP to cause testicular weight loss, transitory oligospermia, reduced DNA and protein synthesis in spermatogonia and spermatids and histological alterations in the testes and epididymides (Meistrich *et al*., 1995; Kaur *et al*., 1997). The precise mechanism by which CP causes testicular and other organ toxicity is not fully understood. However, numerous studies have shown that exposure to CP disrupts the redox balance of tissues, suggesting that biochemical and physiological disturbances result from oxidative stress (Selvakumar *et al*., 2005a, b). Free radicals are normally generated in subcellular compartments of the testis, particularly mitochondria which are subsequently scavenged by the antioxidant defense systems of the corresponding cellular compartments (Agarwal *et al*., 2008). However, this balance is easily disturbed by chemicals such as CP which disrupt the pro-oxidant/antioxidant balance, leading to cellular dysfunction (Howell and Shalet, 2005). Furthermore, the mitochondrial membrane of spermatozoa is more susceptible to lipid peroxidation because this compartment is rich in polyunsaturated fatty acids and has been shown to contain low amounts of antioxidants (Agarwal *et al*., 2008; Aitken and McLaughlin, 2007).

Earlier studies have shown that CP is a potential toxin that may influence male reproductive health and *Lactobacillus casei* is known to increase antioxidant activity in lipid peroxidation which may protect the reproductive system from CP toxicity (Rezvanfar *et al*., 2008). *Lactobacillus casei* has long been considered a probiotic product and antioxidant and has wide clinical use. Therefore, this suggests the benefit of using probiotics in the protection of the reproductive system along with protection of intestinal gut flora. This study was designed to investigate the protective effect of *Lactobacillus casei* against CP-induced changes in the characteristics of epididymal spermatozoa and testicular tissue associated with CP-induced oxidative stress in mice.

MATERIALS AND METHODS

Chemicals and bacterial strains: CP was purchased from Jiang Su Heng Rui Medicine Co., Ltd., China. All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). *Lactobacillus casei* strain 6002 was obtained from the China Center of Industrial Culture Collection (CICC).

Animal selection and experimental design: Seventy two healthy adult male Kunming mice (aged 5 weeks) were obtained from the Experimental Animal Center of Chongqing Medical University (China) and were housed under standard laboratory conditions (temperature, 24±3°C; humidity, 40-60%; 12 h light-dark cycle). A commercial pellet diet (Xinxiwang Food Co., Chongqing, China) and fresh drinking water were available *ad libitum*. All the animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Chongqing (Certificate number: scxk-YU-2007-0001). The body weight of the animals was measured and occurrence of toxic signs was examined on a weekly basis. The Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) were followed throughout the experimental schedule.

After 10 days quarantine period, mice were weighed and divided into eight groups of nine animals based on body weight randomization as follows: the blank group (P1) was fed the basal pellet diet and injected intraperitoneally (ip) with 0.8% NaCl for the 1st 5 days (daily dose of 50 mL kg^{-1} body weight); the CP toxic model group (CP Model group, P2) was fed the basal pellet diet and administered 0.8% NaCl by gavage as a

placebo; the treated groups P3, P4, P5, P6, P7 and P8 were fed the basal pellet diet and administered 10^5, 10^6, 10^7, 10^8, 10^7 and 10^8 CFU g^{-1} (diet) of *Lactobacillus casei* 6002 by gavage, respectively (Zhai *et al*., 2010). For the 1st 5 days, the P2, P3, P4, P5 and P6 groups were injected (ip) with CP (daily dose of 50 mg kg^{-1} body weight) as described by L. The P7, P8 group mice administered with *Lactobacillus casei* but not CP as a control group. The spermatogenic cycle including spermatocytogenesis, meiosis and spermiogenesis lasts 48-52 days in mice (Bennett and Vickery, 1970). Therefore, the treatment period in this study was set at 56 days.

Sample collection and preparation: Mice were sacrificed under light ether anesthesia at the end of week 8. The testes and epididymides were removed, cleared of adhering connective tissue and weighed. The right testicle was fixed in 10% neutral formalin solution for histochemical examination. The left testicle was stored at -20°C for subsequent biochemical analyses. Blood samples were collected and centrifuged at 3000 g for 10 min to obtain plasma and stored at -80°C for subsequent biochemical analysis of Luteinizing Hormone (LH) and testosterone.

Sperm preparation and analyses: The epididymis was minced using scissors in a Petri dish containing 5 mL 5% glucose and placed in a rocker for 15 min at 37°C to release sperm from the epididymal tissue.

The epididymal tissue was isolated for analyses by filtration (3 μm). The epididymal sperm concentration was determined using a hemocytometer according to a modification of the method described by Yokoi *et al*. (2003). Sperm suspensions (5 μL) were diluted into 95 μL phosphate-buffered saline (PBS, pH 7.4) solution containing 5% glucose. Approximately 10 μL of the diluted sperm suspension was transferred to each counting chamber of the hemocytometer and was allowed to stand for 5 min. The spermatozoa in chambers were counted under a light microscope (x200 magnification).

Percentages of viability and morphological abnormalities were determined after vital eosin staining, (0.5% eosin was prepared by diluting Y eosin in PBS: 0.01 M phosphate buffer, 0.027 M KCl, 0.137 M NaCl, pH 7.4). Non-stained cells were considered viable and expressed as a percentage of the total sperm cell count. Cell counting was performed by placing a semen drop mixed with a drop of eosin between slide and cover slip followed by examination under a bright field light microscope (x200 magnification). This microscopy preparation was also used to evaluate sperm morphology (Orgebin-Crist, 1969). In all cases, 200 sperm cells were counted. Subsequently, semen samples from all groups were diluted (1:50, v/v) with warmed PBS and sperm motility was evaluated under a phase-contrast microscope (x200 magnification) maintained at 37°C. Motility (progressive and *in situ*) was expressed as a percentage of motile sperm (200 cells).

Biochemical analyses: Testis samples were used for the measurement of Malondialdehyde (MDA), reduced Glutathione (GSH), Catalase (CAT) and SOD activities. Weighed testicular tissues were homogenized using a Teflon-coated glass homogenizer in a buffer containing 1.15% KCl to obtain a dilution 1:10 (w/v) of whole homogenates. MDA was determined in whole homogenates. Homogenates were centrifuged at 18,000 g (4°C) for 15 min in cold physiological saline on ice to determine GSH concentrations, SOD activity and CAT activity (Yilmaz *et al*., 2006).

Determination of MDA levels: Testicular tissue lipid peroxidation levels were determined as Thiobarbituric Acid-Reactive Substances (TBARS) (Ohkawa *et al*., 1979). Generation of MDA was used as an index of lipid peroxidation. In the TBARS test reaction, MDA or MDA-like substances and TBARS react resulting in the production of a pink pigment with a maximum absorption at 532 nm. Briefly, a volume of test sample and two volumes of the stock reagent (15%, w/v) Trichloroacetic Acid (TCA) in 0.25 M HCl and thiobarbituric acid (0.375%, w/v) in 0.25 M hydrochloric acid) was mixed in a centrifuge tube. The solution was heated in boiling water for 15 min and centrifuged at 1500 g for 10 min to the remove the sediment. The supernatant absorbance was read at 532 nm in a spectrophotometer (2R/UV-visible; Shimadzu, Tokyo, Japan) against a blank containing all reagents except the test sample. MDA content is expressed as μM.

Determination of GSH levels: Decreased GSH content of testicular tissue was determined at 412 nm by the method described by Sedlak and Lawrence (Sedlak and Lindasy, 1968; Lawrence and Burk, 1976). Briefly, samples were precipitated with 50% Trichloracetic Acid (TCA) and then centrifuged at 1000 g for 5 min. Supernatant (0.5 mL) was mixed with 2.0 mL Tris-EDTA buffer (0.2 M, pH 8.9) and 0.1 mL 0.01 M 5, 5-dithio-bis- 2-nitrobenzoic acid and maintained at room temperature for 5 min before the absorbance was read at 412 nm. Concentrations of GSH are expressed as μM.

Determination of catalase activity: Testicular tissue catalase activity assay was measured by hydrogen

peroxide decomposition at 240 nm, according to the method described by Aebi (1984). Catalase activity is expressed as kU g^{-1} protein.

Determination of SOD activity: Testicular SOD activity was determined by xanthine and xanthine oxidase generation of superoxide radicals and Nitro Tetrazolium (NBT) reaction (Folhe and Otting, 1984). Briefly, each sample was diluted 1:10 with phosphate buffer (50 mM, pH 7.5). The assay solution containing sodium carbonate buffer (50 mM, pH 10), 0.1 mM xanthine, 0.025 mM NBT, 0.1 mM EDTA, xanthine oxidase (0.1 U mL^{-1} in 2 M ammonium sulfate) and sample were mixed in a cuvette. A unit of SOD activity is defined as the amount of enzyme required to inhibit 50% NBT reaction. SOD activity (and the degree of inhibition) was measured at 560 nm and is expressed as U mL^{-1} (Turk *et al*., 2010).

Estimation of serum sex hormone (LH and testosterone) concentrations: Serum sex hormone (LH and testosterone) concentrations were measured using Radioimmunoassay kits (Wuhan USCN Sciences Co., Ltd., China) according to the instructions provided by the manufacturer. Concentrations of LH are reported as IU/L. Concentrations of testosterone are reported as ng/dL.

Histopathological analysis: For histopathological examination, the right testis from each mouse was pierced with a needle, fixed in 10% neutral buffered formalin and processed according to standard procedures described by Rouquie *et al.* (2009). Specimens embedded in paraffin were sectioned (5-8 μm) and stained with Hematoxylin and Eosin staining (H&E) prior to examination by light microscopy (x100-400 magnification). A total of 200 cross-sections of seminiferous tubules from each sample were examined for evidence of damage as indicated by empty and atrophic tubules.

Statistical analysis: All data are presented as the arithmetic mean±SEM. Data were analyzed using SPSS Version 10.0 (SPSS Inc., Chicago, IL, USA). One-way Analysis of Variance (ANOVA) and post-hoc Tukey's HSD tests were used to evaluate differences between groups. The $p<0.01$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Effects on body, testicular and epididymal weights: No morbidity or mortality was observed in any of the experimental groups during the study period. The body weights and the weights of testes or epididymides were significantly higher in the blank group (P1) and *Lactobacillus casei*-treated groups (P3, P4, P5, P6, P7 and P8) compared with the CP Model groups (P2) (Table 1). There were no significant differences between the *Lactobacillus casei*-treated groups (P4, P5 and P6) and the blank group (P1). But the body weights and the weights of testes or epididymides were significantly higher in the P7 and P8 group compared with the blank group (P1) (Table 1).

Epididymal sperm concentration, sperm motility, viability and abnormal sperm: Sperm motility, viability and concentration in the epididymides of mice treated with *Lactobacillus casei* and those in the blank group (P1) were significantly higher than in the CP Model group (P2) ($p<0.01$). The percentage of abnormal sperm in the epididymides of *Lactobacillus casei*-treated groups (P3, P4, P5, P6, P7 and P8) and those in the blank group (P1) was significantly lower than in the CP Model group (P2) ($p<0.01$). No differences were observed in the percentage of motility and abnormal sperm between the experimental mice treated with *Lactobacillus casei* (P4, P 5, P6, P7 and P8) and the mice in the blank group (P1) ($p>0.05$) (Table 2).

MDA, GSH, SOD and CAT levels in the testis: Testicular MDA levels were significantly lower in the blank group (P1) and in mice treated with *Lactobacillus casei* groups (P3, P4, P5, P6, P7 and P8) compared with the CP Model group (P2) ($p<0.01$). There was no difference between the *Lactobacillus casei*-treated groups (P3, P4, P5 and P6) ($p>0.05$). Testicular MDA levels were significantly lower

Table 1: Effect on body, testis and epididymal weight of mice of treatment with or without concurrent *Lactobacillus casei* treatment

Groups	Testis weight (mg)	Epididymis weight (mg)	Body weight (g)	Testis weight (mg g^{-1} body wt.)	Epididymis weight (mg g^{-1} body wt.)
P1	185.36±2.59*	46.24±1.50*	37.66±1.18*	4.96±0.17*	1.24±0.06*
P2	92.71±1.11$^{\#}$	23.59±0.82$^{\#}$	28.60±1.14$^{\#}$	3.28±0.13$^{\#}$	0.84±0.05$^{\#}$
P3	128.58±2.88$^{\#*}$	29.22±1.27$^{\#}$	30.76±0.60$^{\#}$	4.20±0.14*	0.95±0.05$^{\#}$
P4	144.70±2.03$^{\#*}$	31.82±1.27$^{\#*}$	33.18±0.91*	4.39±0.14*	0.97±0.05$^{\#}$
P5	151.42±4.16$^{\#*}$	37.47±0.85$^{\#*}$	34.39±0.78*	4.43±0.18*	1.09±0.03*
P6	167.40±5.99$^{\#*}$	38.53±0.47$^{\#*}$	34.51±1.01*	4.89±0.24*	1.12±0.03*
P7	205.69±2.26$^{\#*}$	46.51±1.64*	39.89±0.92$^{\#*}$	5.17±0.11*	1.17±0.05*
P8	206.17±2.94$^{\#*}$	47.42±1.41*	39.97±1.08$^{\#*}$	5.18±0.13*	1.20±0.06*

Data shown as mean values±SEM ($^{*}p<0.01$; $^{\#}p<0.01$) are marked with * and $^{\#}$. $^{\#}p<0.01$ compared with the blank group. $^{*}p<0.01$ compared with the CP Model group

Table 2: Epididymal sperm analysis

Groups	Motility (%)	Sperm concentration ($\times 10^6$) (million/g tissue)	Abnormal sperm rate (%)	Sperm viability (%)
P1	82.79±1.66*	66.36±2.87*	11.11±0.95*	90.56±1.68*
P2	40.94±1.91#	32.69±3.74#	19.0±0.630#	53.56±1.94#
P3	69.44±3.49#*	53.24±4.32*	14.33±1.14*	77.22±1.66#*
P4	82.67±2.26*	65.86±2.93*	10.22±0.85*	90.22±1.24*
P5	81.74±2.24*	65.54±2.46*	10.11±0.48*	90.33±1.58*
P6	81.23±3.13*	65.64±2.36*	9.89±0.51*	91.78±1.39*
P7	83.03±1.13*	66.62±2.21*	9.89±0.61*	90.67±0.76*
P8	83.21±1.55*	66.65±2.10*	9.78±0.82*	90.78±1.08*

Data shown as mean values±SEM (*p<0.01; #p<0.01) are marked with * and #. #p<0.01 compared with the blank group. *p<0.01 compared with the CP Model group

Table 3: Biochemical analyse of testes

Groups	MDA (μM)	CAT (kU g^{-1} protein)	GSH (μM)	SOD (U mL^{-1})
P1	83.20±2.300*	8.24±0.13*	7.66±0.43*	1.92±0.13
P2	151.41±2.960#	7.51±0.19#	5.34±0.34#	1.41±0.13#
P3	64.70±1.410#*	8.14±0.18*	8.03±0.50*	1.98±0.12
P4	61.23±0.500#*	8.28±0.14*	8.01±0.40*	2.18±0.13*
P5	60.91±0.400#*	8.16±0.10*	8.2±0.420*	2.13±0.19*
P6	60.39± 0.18#*	8.1±0.070*	8.1±0.430*	2.17±0.18*
P7	79.77±2.330#*	8.22±0.11*	8.46±0.20#*	2.36±0.12#*
P8	78.96±1.980#*	8.24±0.06#*	8.49±0.18#*	2.38±0.10#*

Data shown as mean values±SEM (*p<0.01; #p<0.01) are marked with * and #. #p<0.01 compared with the blank group. *p<0.01 compared with the CP Model group; MDA = Malondialdehyde; CAT = Catalase; GSH = Reduced Glutathione; SOD = Superoxide Dismutase

in the P7 and P8 group compared with the blank group (P1). Testicular SOD and Catalase (CAT) levels in mice fed diets supplemented with *Lactobacillus casei* were significantly higher than in the CP Model group (P4, P5, P6, P7 and P8) ($p<0.01$). Testicular GSH levels in the CP Model group were no difference between the *Lactobacillus casei*-treated groups (P3, P4, P5 and P6). No significant changes were observed in the testicular GSH, SOD and CAT levels of male mice treated with different doses of *Lactobacillus casei* (P3, P4, P5 and P6) and those in the blank group ($p>0.05$). Testicular SOD levels in mice of P7 and P8 were significantly higher than in the blank group (Table 3).

Serum sex hormones (LH and testosterone) levels: Levels of the serum sex hormone, testosterone in the experimental mice treated with *Lactobacillus casei* (P3, P4, P5, P6, P7 and P8) and those in the blank group (P1) were significantly higher than in the CP Model group (P2) ($p<0.01$). LH levels were no difference in mice treated with *Lactobacillus casei* (P3, P4, P5 and P6) groups but there were significantly higher in P7 and P8 groups than in the CP Model group (P2) ($p<0.01$). Concentrations of testosterone were significantly lower in the P2 and P3 compared with the P1 group ($p<0.01$). There was no difference in the concentrations of testosterone in the P4, P5, P6, P7 and P8 groups ($p>0.05$) (Table 4).

Table 4: Estimation of serum sex hormones (LH, testosterone) concentrations

Groups	LH (IU L^{-1})	Testosterone (ng dL^{-1})
P1	64.72±2.57	714.12±11.77*
P2	61.16±2.54	583.53±12.04#
P3	62.48±2.42	659.6±17.560#*
P4	62.46±1.55	717.88±10.01*
P5	62.72±3.24	720.84±9.950*
P6	62.27±2.58	721.01±9.630*
P7	64.88±2.02*	722.14±8.270*
P8	65.42±1.99*	724.01±14.58*

LH = Luteinizing Hormone; data shown as mean values±SEM (*p<0.01; #p<0.01) are marked with * and #. #p<0.01 compared with the blank group. *p<0.01 compared with the CP Model group

Histology of the testis: Histological examination showed that the testicular structure in the P1, P4, P5, P6, P7 and P8 groups was as follows: seminiferous tubule with recognizable initial germ cells, spermatogonia and spermatocytes, evident lumen, minimal damage to the seminiferous tubules with many tubules showing undamaged spermatogenesis (Fig. 1. P1, P4, P5, P6, P7 and P8). In the CP Model group (P2), the structural changes appeared in the form of epithelial sloughing, cellular detachment and degeneration, abnormal germ cells and seminiferous tubular atrophy (Fig. 1. P2) compared with the normal cellular content of the blank group and control group P7 and P8 (Fig. 1. P1, P7 and P8) which was also reflected in the decrease in testicular weights (Table 1). Although, seminiferous tubules were found to retain some peripheral integrity, the number of spermatogenic cell layers ranged from one to three (Fig. 1. P2). Few degenerating germ cells were observed in the atrophic seminiferous tubules of the P3 group (Fig. 1. P3).

The average percentage of damaged seminiferous tubules in the blank group (P1) and control group (P7 and P8) were 2.5±0.1-2.5±0.2%. The average percentage of damaged seminiferous tubules in the toxic model group (P2) was significantly higher, 73.2±3.5%. In contrast, histological sections of seminiferous tubules in the testis from *Lactobacillus casei*-treated animals (P3, P4, P5 and P6) showed significantly fewer damaged tubules (5.2±0.8, 2.9±0.6, 2.9±0.5 and 2.8±0.5, respectively) (Table 5, Fig. 2).

Lactobacillus casei has long been used as a probiotic in humans and animals although, its mechanism of action is not yet fully understood (Hong *et al*., 2005). Viable lactic acid bacteria have been associated with well-balanced intestinal microflora, antimicrobial activity, stimulation of the immune system and antioxidative properties. Several studies have been conducted to evaluate the effects of probiotic use in livestock farming where it has been reported to contribute to enhanced weight gain, improved feed conversion ratio, reduced incidence of liquid feces and post-weaning diarrhea and lower mortality rates (Aly *et al*., 2008; Alexopoulos *et al*., 2004; La Ragione and Woodward, 2003). In this study, adult male Kunming mice were fed diets containing 10^5, 10^6, 10^7 and 10^8 CFU g^{-1} *Lactobacillus casei*.

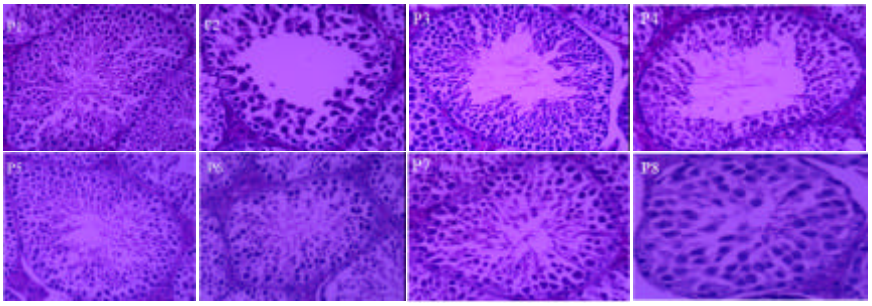

Fig. 1: The effect of *Lactobacillus casei* co-treatment on the testicular damage induced by injections of Cyclophosphamide (CP). Cross-sections of CP-treated mice were characterized by empty and atrophic seminiferous tubules (P2) compared with the normal cellular content of the blank group and controls (P1, P7 and P8). Seminiferous tubules exhibiting cellular degeneration, atrophy, thinning and detached spermatogenic cells. Cross-sections of seminiferous tubules exhibiting degenerating germ cells in the epithelium (P2). In the *Lactobacillus casei*-treated and blank groups, the damage to the seminiferous tubules was considerably less severe with many tubules showing undamaged spermatogenesis (P3, P4, P5, P6, P7 and P8)

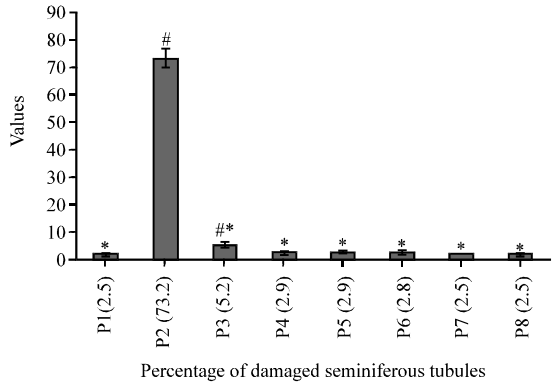

Fig. 2: Average percentage of damaged tubules in the testes of mice. CP-induced a significant increase in the percentage of damaged seminiferous tubules. Significant protection was conferred by *Lactobacillus casei* against the CP-induced testicular damage to the tubules. #p<0.01 compared with the blank group. *p <0.01 compared with the CP Model group

Table 5: Average percent of damaged seminiferous tubules

Groups	Percentage
P1	2.5±0.2*
P2	73.2±3.5#
P3	5.2±0.8#*
P4	2.9±0.6*
P5	2.9±0.5*
P6	2.8±0.5*
P7	2.5±0.1*
P8	2.5±0.2*

Data shown as mean values±SEM (*p< 0.01; #p<0.01) are marked with * and #. #p<0.01 compared with the blank group. *p<0.01 compared with the CP Model group

Compared with the CP Model group and blank group (P1), the *Lactobacillus casei*-treated group showed improved growth performance. Weight gain was higher in the mice fed the diets supplemented with 10^7 and 10^8 CFU g^{-1} of *Lactobacillus casei* compared with the mice in other *Lactobacillus casei*-treated groups, this effect did reach the level of statistical significance.

Earlier studies have shown that lactic acid bacteria have the ability to scavenge ROS and increase plasma antioxidant levels or protect plasma lipids from oxidation to different degrees (Martarelli *et al*., 2011; Lin and Yen, 1999; Lin and Chang, 2000). Furthermore, lactic acid bacteria were shown to prolong resistance of the lipoprotein fraction to oxidation, lower levels of peroxidized lipoproteins, oxidized LDL, 8-isoprostanes and glutathione redox ratio and enhance total antioxidative activity (Kullisaar *et al*., 2003). Proposed mechanisms of antioxidative activity include metal ion chelating ability, scavenging of ROS, enzyme inhibition and reduced activity of intracellular cell-free extracts of lactic acid bacteria (Lin and Yen, 1999).

Cytotoxic chemotherapy is associated with significant gonadal damage. Alkylating agents such as CP are the most common agents involved in the pathogenesis of this injury. In this study, it was observed that intraperitoneal administration of a daily dose (50 mg kg^{-1} body weight) of CP to male mice during the 1st 5 days of the 56 days experimental period significantly decreased body weight and the reproductive system was seriously affected. Similar results were obtained by Rezvanfar (2008). The effects on the male mouse reproductive system were characterized by both mild germ cell necrosis in the seminiferous tubule and a significant decrease in the concentration of the serum sex hormone, testosterone, a concomitant decrease in SOD and CAT levels and increased MDA levels, an apparent histopathological

change in either the seminiferous tubules or spermatogenic cell layers and a significant decrease in either testicular weight or sperm count and motility.

In the group co-treated with 10^6, 10^7 and 10^8 CFU g^{-1} (diets) of *Lactobacillus casei*, it was observed that the testis and epididymis weights were both reduced with significantly less damage to the seminiferous tubules compared with the CP Model group. The testis weights of the CP+*Lactobacillus casei*-treated animals were significantly higher than in the CP group. The testicular protection effects of *Lactobacillus casei* not only decreased the dramatic effects of CP on cell number but also prevented the slow recovery of sperm from the epididymis.

Two mechanisms of CP toxicity in the reproductive system have been reported as a result of numerous studies. It is proposed that CP mediates direct DNA a damaging effect on testicular germ cells or indirectly via oxidative stress (Turk *et al*., 2010; Ceribasi *et al*., 2010). CP disrupts the redox balance of the tissues and biochemical and physiological disturbances may result from oxidative stress (Selvakumar *et al*., 2005a, b; Ahmadi *et al*., 2008). Evidence now suggests that ROS-mediated damage to sperm is a significant factor contributing to pathology in 30-80% of cases (Agarwal *et al*., 2006). ROS including oxygen ions, free radicals and peroxides, cause infertility by two principal mechanisms. Firstly, ROS are known to damage the sperm membrane which in turn reduces sperm motility and the ability to fuse with the spermatocyte. Secondly, ROS directly damages sperm DNA, compromising the paternal genomic contribution to the embryo. Despite the common association between compromised sperm quality and oxidative damage, men are rarely screened for oxidative stress or treated for this condition. Direct treatment of oxidative stress and increased application of existing drugs such as *Lactobacillus casei* may allow for natural conception, thereby conserving scarce medical resources.

Production cell of testicular have an antioxidant mechanisms to combat the effects of ROS. The most important mechanism involves production of oxygen and two molecules of oxygen from H_2O_2 in reactions catalyzed by enzymes such as SOD, GSH and CAT (Selvakumar *et al*., 2005a, b; Ilbey *et al*., 2009). In this study, treatment with *Lactobacillus casei* significantly increased SOD and CAT activity compared with the CP Model group.

Many strains of lactic acid bacteria have been consumed worldwide for decades (Ceragioli *et al*., 2009). As a probiotic, lactic acid bacteria are known to have several beneficial properties including antioxidant activity (Martarelli *et al*., 2011; Lin and Yen, 1999; Lin and Chang, 2000; Hong *et al*., 2009). To the knowledge, this is the first comprehensive assessment of the protective effect of dietary co-administration of probiotic *Lactobacillus casei* on CP-induced damage in the male reproductive system. In this study, the groups treated with 10^6, 10^7 and 10^8 CFU g^{-1} (diets) of *Lactobacillus casei* showed increased development of the testes and epididymides, increased SOD and CAT activity in the testis, increased serum testosterone levels and decreased MDA levels compared to the CP Model group. There were no significant differences in the percentage of abnormal sperm in the epididymides and the histology of the testes in the *Lactobacillus casei*-treated groups compared with the blank group. This study demonstrated that long-term administration of *Lactobacillus casei* significantly increased the protections against CP-induced toxicity in the male mouse reproductive system. Little is known about the mechanism underlying the protective effect of *Lactobacillus casei* although it can be speculated that *Lactobacillus casei* acts as an antioxidant (Aly *et al*., 2008; Ciabattini *et al*., 2004; Huang *et al*., 2008; Luiz *et al*., 2008) to counteract the effects of CP through a mechanism similar to other antioxidants such as Satureja Khuzestanica Essential Oil (SKEO), lycopene and ellagic acid, prostasomes, vitamin C and E (Rezvanfar *et al*., 2008; Turk *et al*., 2010; Saez *et al*., 1998; Rolf *et al*., 1999).

CONCLUSION

This study shows that the results of this study suggest that *Lactobacillus casei* exerts regulatory antioxidant effects on CP-induced toxicity in the testes and sperm and can therefore be used to reduce sperm damage in CP-treated cancer patients and in autoimmune disease patients following post-transplant combination treatment with CP. Further experimental studies such as TUNEL or Caspase 3 and the analysis of sperm chromatin are required to elucidate the molecular mechanism of the protective effect of probiotic *Lactobacillus casei* and to observe the changes in fecal bacterial flora in CP-induced toxicity following treatment with *Lactobacillus casei*.

NOMENCLATURE

CFU = Colony-Forming Units
CP = Cyclophosphamide
MDA = Malondialdehyde
PBS = Phosphate Buffered Saline
SOD = Superoxide Dismutase
CAT = Catalase
GSH = Reduced Glutathion
LH = Luteinizing Hormone

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