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Food deprivation and social inequality may lead to oxidative damage: a study on the preventive role of melatonin in the male rat reproductive system

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Abstract. Spermatogenic cells are susceptible to oxidative stress and apoptosis. Food deprivation (FD) has been reported as a stressor that could increase reactive oxygen species. In the present study, FD-induced oxidative stress and apoptosis, as well as the protective effects of melatonin, were evaluated in the testes. Wistar rats in the control group were fed a standard diet, whereas a sham group was administered saline as the melatonin vehicle. A third group received daily injections of melatonin (5 mg kg^{-1} bodyweight). These rats were further divided into four groups of rats that were either subjected to FD, FD + isolation, FD + melatonin injection and FD + melatonin injection + isolation. Testicular tissues were evaluated for malondialdehyde (MDA) and reduced glutathione (GSH) concentrations, as well as and DNA damage. FD increased MDA and reduced GSH concentrations, whereas melatonin treatment improved these parameters. Immunohistochemistry for capsase-3 and terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling revealed that the number of apoptotic cells was increased in rats subjected to FD alone. Melatonin treatment offset the number of apoptotic cells following FD. The results provide evidence that FD can increase oxidative stress, leading to activation of apoptosis, and that melatonin has the ability to protect the testes against oxidative damage induced by FD.

Additional keywords: apoptosis, testis.

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Introduction

Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage (Agarwal *et al.* 2006).

Oxidative stress is induced by a wide range of environmental factors, including food deprivation (Blokhina *et al.* 2003; Morales *et al.* 2004; Santos *et al.* 2009; Moradi *et al.* 2012). In recent years, levels of human infertility have increased worldwide by almost 50% since 1955 (Sarvari *et al.* 2010). An estimated 6% of adult men are thought to be infertile (Carrell and Peterson 2010). Studies have shown that infertility in men increases significantly with increasing environmental stress (Sarvari *et al.* 2010). Some studies have shown that oxidative stress can damage spermatozoa, and this seems to have a significant role as one of the major factors leading to infertility (Benoff *et al.* 2004).

Starvation and food deprivation have been reported as oxidative stressors that could increase levels of superoxide dismutase (SOD), catalase and malondialdehyde (MDA), and they are also being considered responsible for changing tissue content of reduced glutathione (GSH; Bhardwaj *et al.* 1998; Domenicali *et al.* 2001; Morales *et al.* 2004; Al-Majed 2011).

Similarly, Santos *et al.* (2009) reported that food deprivation increases oxidative stress parameters, such as lipid peroxidation and hydrogen peroxide, and decreases antioxidant reagents such as GSH, SOD and vitamin E. Free radicals and oxidative stress are known to be important factors in causing apoptosis.

Apoptosis is programmed cell death seen in multicellular organisms whereby specific cells are killed and removed for the benefit of the organism. Oxidants such as hydrogen peroxide can trigger apoptosis. Intracellular ROS generation may be critical for the induction of apoptosis by these agents (Clutton 1997; Ferlini *et al.* 1999; Blokhina *et al.* 2003).

In a previous study, we showed that food deprivation decreases the quality of the semen (Nasiraei-Moghadam *et al.* 2014). We observed a reduction in the number and motility of spermatozoa, as well as a significant increase in the number of spermatozoa with abnormal morphology following food deprivation in male Wistar rats. This reduction in the number of spermatozoa was also observed following examination of the testis tissue and cell generator layers. In addition, food deprivation reduced the number of Sertoli and Leydig cells, which, in turn, affects sperm proliferation (Nasiraei-Moghadam *et al.* 2014). Considering these findings, we hypothesised that oxidative stress and apoptotic events may explain the events in testis tissue of starved rats. The results reported herein indicate that dietary restriction induces oxidative stress and apoptosis in the testis tissue of rats. Finally, we show that administration of melatonin, a potent free radical scavenger, may improve the severe effects of food deprivation on testicular tissue.

Materials and methods

Reagents

Adult male Wistar rats, weighing 200–220 g, were supplied by the Neuroscience Research Center, Shahid Beheshti University of Medical Sciences (Tehran, Iran). Melatonin, normal goat serum (NGS), diaminobenzidine (DAB), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA) and eosin were obtained from Sigma-Aldrich (St Louis, MO, USA). Melatonin powder (m5250) was dissolved in saline and alcohol (95%/5%, v/v). Xylene, ethanol, Bouin's solution, phosphate-buffered saline (PBS) and haematoxylin were purchased from Merck (Germany). Anti-capsase-3 rabbit polyclonal antibody (1 : 100 dilution; 9661S) was purchased from Cell Signaling Technology (Beverly, MA, USA). Proteinase K and the In Situ Cell Death detection kit POD (catalogue no. 11684817910) were obtained from Roche (Germany). Streptavidin biotin secondary antibodies (anti-rabbit IgG and anti-mouse IgG) were obtained from Abcam (Germany).

Animals

The experimental protocol was performed in accordance with the Declaration of Helsinki for experiments involving humans and EU Directive 2010/63/EU for animal experiments. Forty-two male Wistar rats were maintained under standard laboratory conditions under a 12-h light–dark cycle and *ad libitum* access to food (for control (C) and melatonin (M) groups) and water (for all of groups) throughout the experiment. In the present study, we evaluated the effects of food deprivation, with and without melatonin treatment and isolation of rats, compared with *ad libitum* access to food and water. Rats were randomly assigned to one of seven groups. Rats in the C group C were untreated and were maintained in the animal room during the study, with free access to food (~22 g per day). The sham control group (S) was injected with saline as the melatonin vehicle. The M group was treated with daily injections of melatonin (5 mg kg^{-1}, i.p.). The S and M groups had free access to food. The remaining four groups of rats were subjected to food deprivation alone (FD), food deprivation with isolation (FDi), food deprivation and melatonin injection (FDM) or food deprivation and melatonin injection with isolation (FDMi). Under the food deprivation conditions, rats were given one-third of the normal daily food ration (7.5 g per day). 'Isolation' referred to conditions under which the rats (six rats in one cage) could not see or smell other rats' food in different cages. Rats were weighed twice, once at the beginning and once at the end of the experiment (14 days). At the end of the experimental period, rats were anaesthetised with CO_2 and killed, followed by the removal, washing and weighing of the testes. The dimensions of the testes were then measured using callipers.

Histopathological procedures

Haematoxylin and eosin staining

Right testis tissues were fixed in Bouin's solution for 20 h and prepared for histopathological evaluation. After processing and embedding in paraffin, tissues were sectioned on a rotary microtome (530 577; LEITZ, Germany) at 5 μm before being stained with haematoxylin and eosin according to standard staining protocols. Tissues were evaluated histopathologically under a light microscope (Listed 7GA9; Labo America Inc.).

Terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling

Apoptotic cells were detected using a commercially available *In Situ* Cell Death detection kit (Roche) according to the manufacturer's instructions. Briefly, paraffin sections were cut (5 μm) and mounted on poly-L-lysine-coated slides (Sigma, St Louis, MO, USA). Sections were deparaffinised using xylene and hydrated by processing through a graded series of alcohol. At room temperature, 3% H_2O_2 was used for inactivation of endogenous peroxidase for 15 min. Then, sections were digested with 20 μg mL^{-1} proteinase K for 45 min in a dark and humidified chamber at 37°C. The sections were incubated with terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) reaction mixture (terminal deoxynucleotidyl transferase [TdT], which catalyses polymerisation of labelled nucleotides to free 3′-OH DNA ends in a template-independent manner) for 60 min at 37°C. The slides were then incubated with converter POD for 1 h at 37°C. At each step, sections were washed three times using PBS as the washing buffer. Slides were developed with 0.1% DAB and stained for 15 min at 37°C. Then, samples were washed three times in distilled water, counterstained with haematoxylin for 1 min, dehydrated and mounted. Testis tissues treated with DNase were used as positive controls. As a negative control, the TUNEL reaction mixture was substituted with Label Solution (nucleotide mixture in reaction buffer). For assessment of apoptosis, the percentage of seminiferous tubules with apoptotic cells was determined by scoring 100 randomly selected tubules per section on at least four sections from four different rats. The number of apoptotic cells per tubule was assessed in three sections. In each section, two microscopic fields (×400 magnification) were randomly selected.

Immunohistochemistry

Testis sections (5 mm) that fixed in the Bouin's solution and embedded in paraffin were deparaffinised, rehydrated and then subjected to antigen retrieval in a microwave. Slides were placed in a plastic jar containing 200 mL of 0.1 M citrate buffer, pH 6.0,

under boiling point conditions (300 W microwave irradiation) for 10 min. Endogenous peroxidase was inactivated by 3% H_2O_2 in methanol for 20 min. The sections were subsequently washed three times in PBS (pH 7.4) and then incubated with 10% NGS (blocking buffer) for 60 min and incubated at 4°C overnight with the cleaved-caspase-3-antibody. Sections were again washed in PBS and subsequently incubated for at least 30 min with the streptavidin (A) and biotin (B) components of the ABC staining kit (Abcam). Both components (A and B) were ready to use. Slides were washed again in PBS; bound antibody was visualised after addition of a 1 mg mL^{-1} solution of DAB in PBS. The slides were subsequently counterstained with Mayer's haematoxylin. Control sections, in which the primary antibody was replaced by NGS, were similarly processed. Neonate rat thymus tissue treated with dexamethasone (0.1 M) was used as a positive control.

Biochemical investigations

Determination of testicular MDA levels

The MDA concentration, as an index of thiobarbituric acid-reactive substances (TBARS), was measured as described by Nasri *et al.* (2011) and Roghani and Baluchnejadmojarad (2010). Briefly, dissected testes were cleaned of extra tissues, blotted dry and weighed before being made into an approximate 5% tissue homogenate in ice-cold 0.9% NaCl solution. The homogenate was centrifuged (1000*g*, 4°C, 10 min) and the supernatant collected. To measure MDA concentrations in the supernatant, 1.0 mL of 20% TCA and 1.0 mL of 1% TBARS reagent were added to 100 µL supernatant, mixed and incubated at 100°C for 80 min. After cooling on ice, samples were centrifuged at 1000*g* for 20 min at room temperature and absorbance read at 532 nm. The TBARS results are expressed as MDA equivalents using tetraethoxypropane as the standard.

Determination of testicular GSH levels

GSH levels were estimated according to the method of Sedlak and Lindsay (1968), which is based on the reaction between thiol groups and 1,2-dithiobis nitrobenzoic acid (DNTB) as substrate to produce a compound that absorbs light at 412 nm. The amount of GSH was determined from a standard curve obtained simultaneously under the same conditions using various concentrations of GSH (standard curve is 5–25 µM; maximum absorbance (1) is 25; our data is in the median of the curve).

Data and statistical analysis

All values are given as the mean ± s.d. The significance of differences was determined using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered significant.

Results

Rat weight

All rats retained a relatively good health status throughout the experiment. Bodyweight and testis weight are given in Table 1. The percentage of bodyweight gain in the control group was 66%, whereas decreases in bodyweight of 26%, 7%, 8%, 1% and 11% were observed in the FD, FDi, FDM, FDMi and M groups, respectively. There were no treatment-related changes in the absolute and relative weights of the testes in the treatment groups compared with the control group. Because there was no significant difference between the C and S groups, the results of the S group were ignored.

Apoptosis detection by TUNEL and immunohistochemistry

The TUNEL assay and immunohistochemistry (IHC) of capsase-3 were used to evaluate apoptotic cells in the testis of different groups. In this study, the number of TUNEL-positive germ cells per tubule cross-section increased in the FD compared with C group. TUNEL-positive spermatogonia, spermatocytes and spermatids were the main germ cells undergoing apoptosis. Fig. 1 shows results of TUNEL analysis in the C, FD, FDi, FDM, FDMi and M groups. The TUNEL assay is an *in situ* detection method by which apoptotic cells can be identified on the basis of their darkly stained nuclei. There was a low incidence of apoptosis in the control rats. The number of total apoptotic cells (spermatogonia, spermatocytes, and spermatids) in the C, FD, FDi, FDM, FDMi and M groups was 14.8 ± 4.6, 32.7 ± 3.2, 21.3 ± 3.7, 21.4 ± 3.6, 16.1 ± 2.4 and 12.3 ± 4.2, respectively (Fig. 2).

These results were confirmed by IHC, where the number of capsase-3-positive cells was detected in 100 randomly selected tubules per rat in all groups. Fig. 3 shows results of the capsase-3 assay in the C, FD, FDi, FDM, FDMi, and M groups. Consistently, the results indicate that FD-induced apoptosis in testis tissues. Apoptotic cells were mainly detected in inner regions. We counted the number of capsase-3-positive cells per testis and the rate of positive cells (number of caspase-3 positive cells to all of the cells) in the C, FD, FDi, FDM, FDMi and M groups was 27.8 ± 3.4, 46.7 ± 4.8, 31.4 ± 3.7, 29.5 ± 5.1, 25.4 ± 1.9 and 26.3 ± 3.3, respectively (Fig. 4).

Testicular MDA levels

MDA levels were used as an index of lipid peroxidation in testis homogenates from rats in each of the different groups. MDA concentrations increased significantly in the FD compared with C group ($P < 0.01$). There was a significant decrease in MDA levels in the FDM and FDi groups compared with the FD group

Table 1. Bodyweight and testes weight of rats after 14 days treatment in the control (C), melatonin-treated (M), food-deprived (FD), food-deprived + isolated (FDi), food-deprived + melatonin-treated (FDM) and food-deprived + melatonin-treated + isolated (FDMi) groups

Data are the mean ± s.d. of six rats in each group. *$P < 0.05$ compared with the control group; †$P < 0.05$ compared with the FD group

	Control	FD	FDi	FDM	FDMi	M
Final bodyweight (g)	270 ± 35	190 ± 13*	176 ± 7*	179 ± 28*	205 ± 13*†	249 ± 9
Testis weight (g)	1.46 ± 0.23	1.27 ± 0.22	1.26 ± 0.17	1.35 ± 0.28	1.40 ± 0.29	1.41 ± 0.31

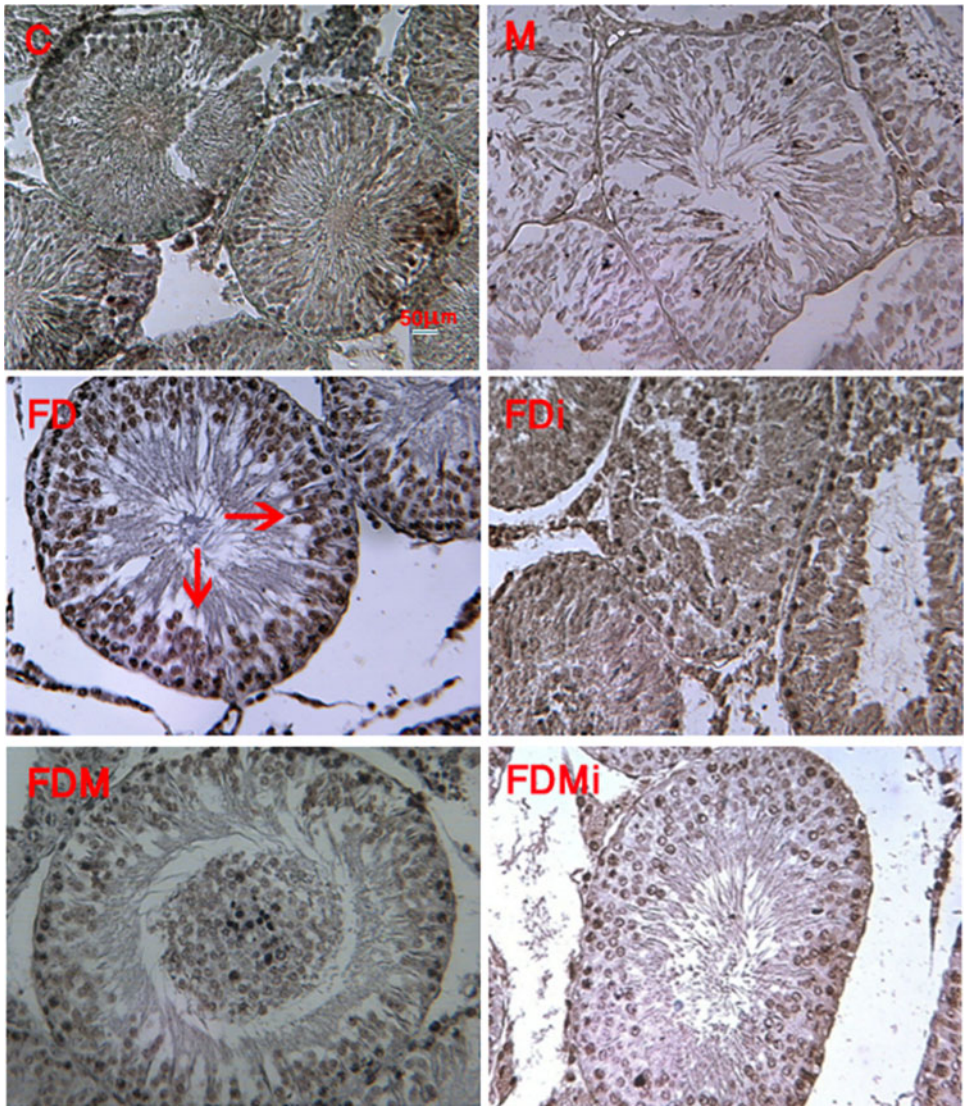

Fig. 1. Cross-sections (5 µm) of the testis in the control (C), melatonin-treated (M), food-deprived (FD), food-deprived + isolated (FDi), food-deprived + melatonin-treated (FDM) and food-deprived + melatonin-treated + isolated (FDMi) groups showing representative results of the terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) assay (original magnification ×400). Arrows indicate TUNEL positive nucleus.

($P < 0.01$). Furthermore, there was a significant decrease in MDA levels in the M group compared with the C group ($P < 0.05$; Fig. 5). The mean (± s.d.) MDA levels in testes in the C, FD, FDi, FDM, FDMi and M groups were 0.06 ± 0.00, 0.25 ± 0.03, 0.06 ± 0.00, 0.07 ± 0.00, 0.04 ± 0.01 and 0.03 ± 0.00 ng mL^{-1}, respectively (Fig. 5). Although decreased tissue MDA levels were detected in the FDi and FDM groups ($P < 0.05$), there was no synergistic effect of melatonin and isolation on testis MDA levels in the FDMi group.

Testicular GSH levels

Testicular GSH levels in all groups are shown in Fig. 6. GSH concentrations decreased significantly in the FD compared with C group ($P < 0.001$). In the FDM group, there was a significant ($P < 0.01$) increase in GSH levels compared with the FD group. Moreover, there was a significant ($P < 0.01$) increase in GSH levels in the M versus C group (Fig. 6). GSH concentrations in the supernatant from the C, FD, FDi, FDM, FDMi and M groups were 1.12 ± 0.04, 0.09 ± 0.02, 0.19 ± 0.05, 0.35 ± 0.03, 0.45 ± 0.03 and 1.37 ± 0.04 µg mL^{-1}, respectively (Fig. 6). Although tissue GSH levels increased in the FDi group, the difference was not significant different compared with the FD group ($P > 0.05$).

Discussion

The negative impact of food deprivation on health is gaining increasing interest, and the relationship between socioeconomic

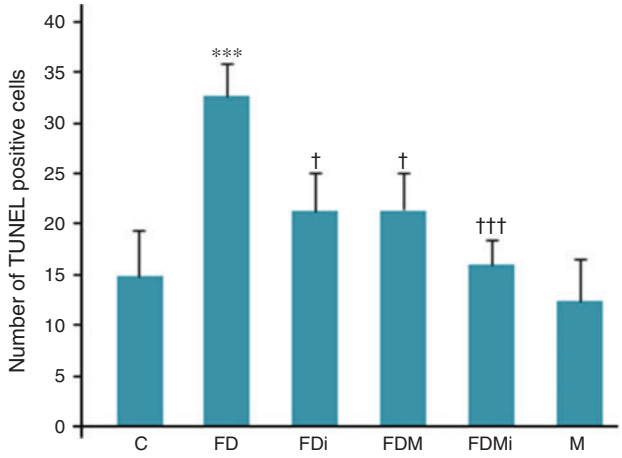

Fig. 2. Number of terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL)-positive spermatogenic cells per unit area (~500 μm^2) in the control (C), melatonin-treated (M), food-deprived (FD), food-deprived + isolated (FDi), food-deprived + melatonin-treated (FDM) and food-deprived + melatonin-treated + isolated (FDMi) groups. Data are the mean ± s.d. of six rats in each group. ***$P < 0.001$ compared with the control group; $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ compared with the FD group.

status and various diseases is well documented in the literature (Heidary *et al.* 2012, 2013).

Several studies have investigated the effects of food deprivation on enhancement of oxidative stress. Santos *et al.* (2009) and Domenicali *et al.* (2001) reported that food deprivation promotes oxidative imbalance in the rat brain and liver, respectively. Pascual *et al.* (2003) reported that dietary restriction of *Sparus aurata* for 46 days resulted in increased hepatic MDA levels, suggesting increased oxidative stress in the liver. The results in the present study are consistent with these findings, because food deprivation increased oxidative stress in the testis tissue of male rats. MDA is a known index of lipid peroxidation and has been widely used as a marker of oxidative damage to membranes and therefore of oxidative stress (Buege and Aust 1978; Lepage *et al.* 1991). In the present study, we investigated MDA levels in the testis tissue to confirm that fasting conditions lead to oxidative stress in rat testes. We observed significant increases in MDA levels in rat testes after 14 days of food deprivation: MDA levels in the FD group were threefold greater than in the control group.

In order to evaluate the effect of limited food availability as a source of oxidative stress, we also investigated the ratio of GSH in rat testis. GSH (L-γ-glutamyl-L-cysteinylglycine) is an important sulfydryl compound in mammalian cells with different functions that coordinates body defence systems against oxidative stress. GSH effectively scavenges free radicals and has an important role in the elimination of superoxide anions and hydrogen peroxide through oxidation and reduction. The role of GSH in protecting cells against the harmful effects of free radicals is now well established (Grosshans and Calvin 1985). In the present study, GSH levels in the testes decreased in the FD group, suggesting a state of oxidative stress in the testes following food deprivation. The results of the present study demonstrate that food restriction causes marked depletion of GSH levels (eightfold) in the testicles of Wistar rats. These results confirm that food deprivation can induce oxidative stress.

There are studies indicating that oxidative stress is one of the most important factors inducing germ cell apoptosis in the testis (Rao and Shaha 2000; Yang *et al.* 2001). Because the testis is very sensitive to oxidative stress (Carrell and Peterson 2010), it has high amounts of antioxidants, including GSH (Rao and Shaha 2000; Yang *et al.* 2001) that play important roles in spermatogenesis and protecting the testes against oxidative damage (Fraga *et al.* 1991). Accumulation of lipid peroxidation products, such as MDA, following the oxidative stress can result in impairment of cell function (Troyer-Caudle 1993; Adams *et al.* 1995; Sucu *et al.* 2002) and apoptosis (Clutton 1997). Our data illustrate that food deprivation increases MDA levels and decreases GSH levels in the testicular cells of Wistar rats. Therefore, we concluded that high concentrations of MDA, as well as low amounts of GSH, may induce apoptosis in the testis. We then examined apoptotic cells in the testes of rats subjected to food deprivation. TUNEL and IHC assays of capsase-3 confirmed that food restriction induced apoptosis in germ cells (Figs 2, 4). In another study, we found reductions in the number of spermatozoa, Sertoli and Leydig cells following starvation in Wistar rats (Nasiraei-Moghadam *et al.* 2014). We suggest that these findings may result from apoptosis in the tissue.

The protective effect of melatonin against oxidative stress in rat testis tissue after 2 weeks starvation was also examined. Melatonin is an important hormone of the pineal gland that has key roles in defence against oxidative stress. Melatonin is a scavenger of ROS and acts as an effective antioxidant in addition to glutathione (Hardeland *et al.* 1993; Pieri *et al.* 1994; Reiter *et al.* 1997, 2002; Narayana *et al.* 2002; Okutan *et al.* 2004; El-Sokkary *et al.* 2005). Other studies have shown that melatonin increases the activity of antioxidant defence systems and demonstrated direct effects of melatonin on animal male reproductive systems (Yu *et al.* 1994; Cagnacci and Volpe 1996).

In the present study, we observed that MDA levels in the testis decreased significantly in the FDM compared with FD group, almost reaching levels seen in the control group. This suggests that melatonin probably prevents lipid peroxidation and the negative effects of oxidative stress after food deprivation, as evidenced by biochemical markers. Wakatsuki *et al.* (1999) also reported that melatonin inhibits the peroxidation of membrane lipids. In addition, results from the present study demonstrate that melatonin administration decreases oxidative stress, as evidenced by increased GSH levels in the testis of rats in the FDM group. These protective effects were also supported by studies into apoptotic cells in testis tissue after melatonin treatment. Melatonin suppressed the food deprivation-induced apoptosis of testicular germ cells (Fig. 2). The results of the TUNEL assay indicated that the number of TUNEL-positive or apoptotic cells in the FDM group was significantly lower than in the FD group. This was also confirmed by IHC evaluating capsase-3-positive cells. Taking all these findings of into consideration, it can be suggested that melatonin may be used clinically as an effective agent in male reproductive problems caused by oxidative damage.

In addition, it was demonstrated in the present study that the isolation situation was efficient in preventing the side effects of

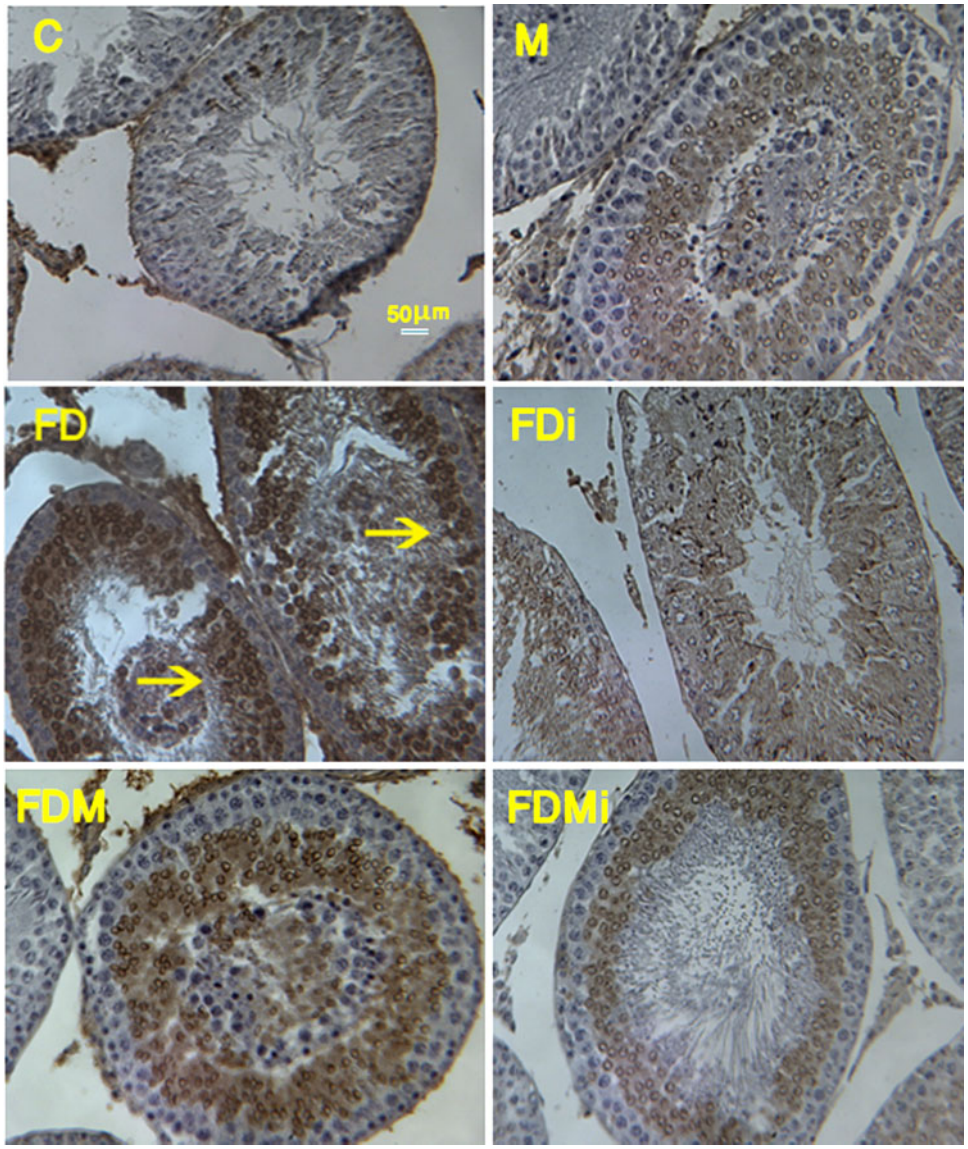

Fig. 3. Cross-sections (5 µm) of the testis in the control (C), melatonin-treated (M), food-deprived (FD), food-deprived + isolated (FDi), food-deprived + melatonin-treated (FDM) and food-deprived + melatonin-treated + isolated (FDMi) groups showing representative results of immunohistochemistry for caspase-3 (original magnification ×400). Arrows indicate brown granules representing cytcaspase3.

food deprivation. The protection offered by isolation was similar to that afforded by melatonin. Some studies have reported that the isolation situation improves the detrimental effects of food deprivation (Mojarab *et al.* 2010; Moradi *et al.* 2012). In the present study, isolation decreased MDA levels ($P < 0.05$) in the testes of rats in the FDi group compared with those in the FD groups. In the case of GSH, isolated increased GSH levels in the testis of FDi rats, whereas starvation significantly decreased GSH levels (Fig. 6). Increased testicular GSH levels, along with reduced MDA levels, in FDi rats are probably the consequence of inhibition of oxidative stress resulting from food deprivation. Moreover, our data showed a reduction in the number of TUNEL- and caspase-3-positive cells in the FDi compared with FD group, indicating a decrease in apoptosis following isolation. The decrease in oxidative stress and apoptosis in the FDi group favours the hypothesis that the isolation condition during a starvation period may attenuate the damaging effects of the food deprivation. These results may suggest that a sense of deprivation could induce oxidative stress in the testis tissue of male Wistar rats.

Conclusion

The findings of the present study demonstrate that food deprivation increases the number of apoptotic spermatogenic cells and oxidative markers in the testes. According to differences in

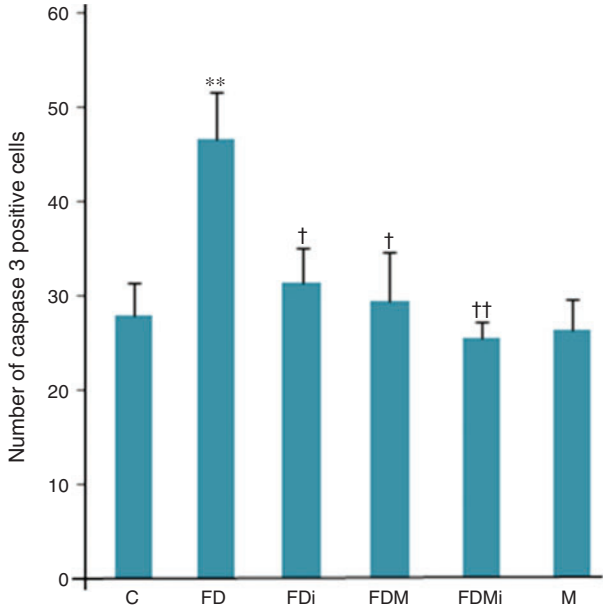

Fig. 4. Number of caspase-3-positive spermatogenic cells per unit area (~500 μm^2) in the control (C), melatonin-treated (M), food-deprived (FD), food-deprived + isolated (FDi), food-deprived + melatonin-treated (FDM) and food-deprived + melatonin-treated + isolated (FDMi) groups. Data are the mean ± s.d. of six rats in each group. *$P < 0.05$ compared with the control group; $^{\dagger}P < 0.05$ compared with the FD group.

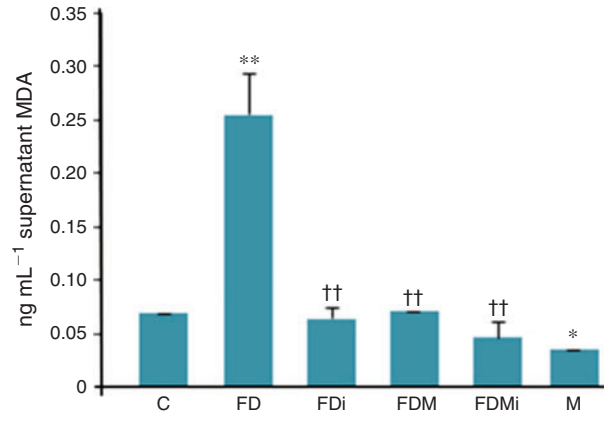

Fig. 5. Malondialdehyde (MDA) concentrations in testes homogenates in the control (C), melatonin-treated (M), food-deprived (FD), food-deprived + isolated (FDi), food-deprived + melatonin-treated (FDM) and food-deprived + melatonin-treated + isolated (FDMi) groups. Data are the mean ± s.d. of six rats in each group. *$P < 0.05$, **$P < 0.01$ compared with the control group; $^{\dagger\dagger}P < 0.01$ compared with the FD group.

spermatogenic cells between the FDi and FD groups, it is suggested that the isolation situation could limit the oxidative and apoptotic effects of food deprivation.

The results of the present study further show that melatonin treatment during food deprivation has a protective effect against the oxidative effects of food deprivation. In addition, melatonin reduces the apoptotic effect of food deprivation on spermatogenic cells.

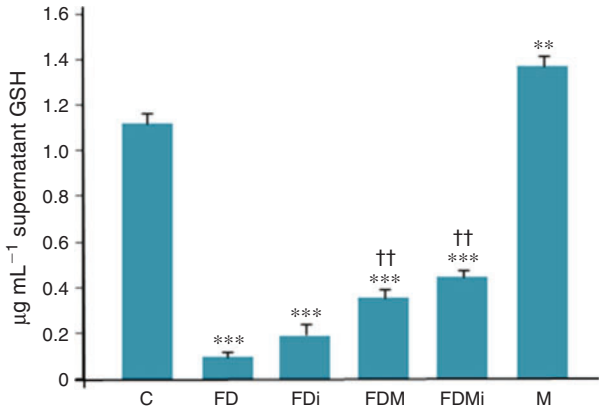

Fig. 6. Reduced glutathione content (GSH) in testes homogenates in the control (C), melatonin-treated (M), food-deprived (FD), food-deprived + isolated (FDi), food-deprived + melatonin-treated (FDM) and food-deprived + melatonin-treated + isolated (FDMi) groups. Data are the mean ± s.d. of six rats in each group. **$P < 0.01$, ***$P < 0.001$ compared with the control group; $^{\dagger\dagger}P < 0.01$ compared with the FD group.

The next step would be to investigate the pathways through which these factors affect apoptotic spermatogenic cells of the testes.

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