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Outcomes of food deprivation and exogenous melatonin on oxidative stress markers and apoptosis; an experimental study

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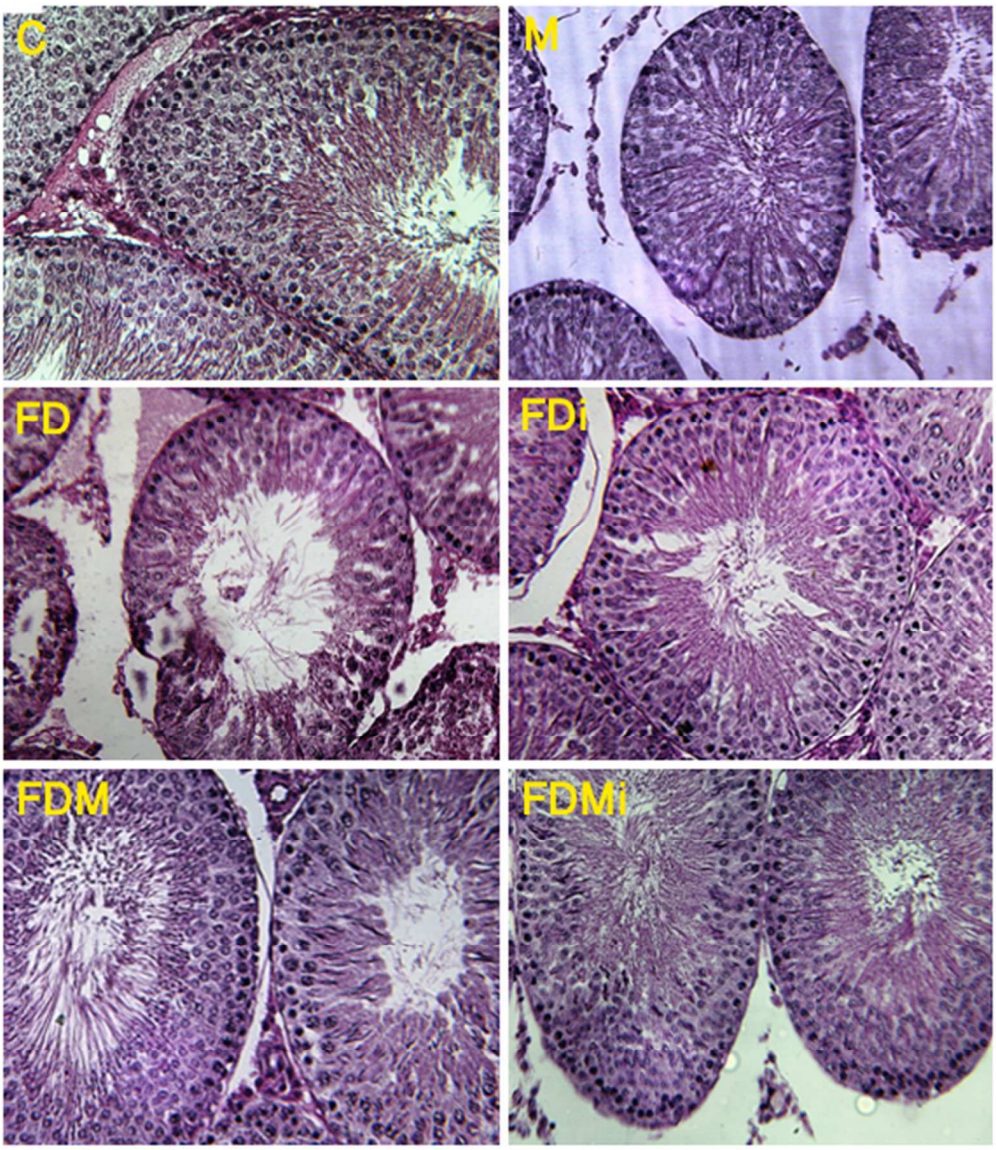

H&E staining, Magnification: 400, Groups;C:Control, M:Melatonin, FD: Food deprivation, FDi: Food deprivation isolated, FDM: Food deprivation and melatonin, FDMi: Food deprivation isolated with melatonin.

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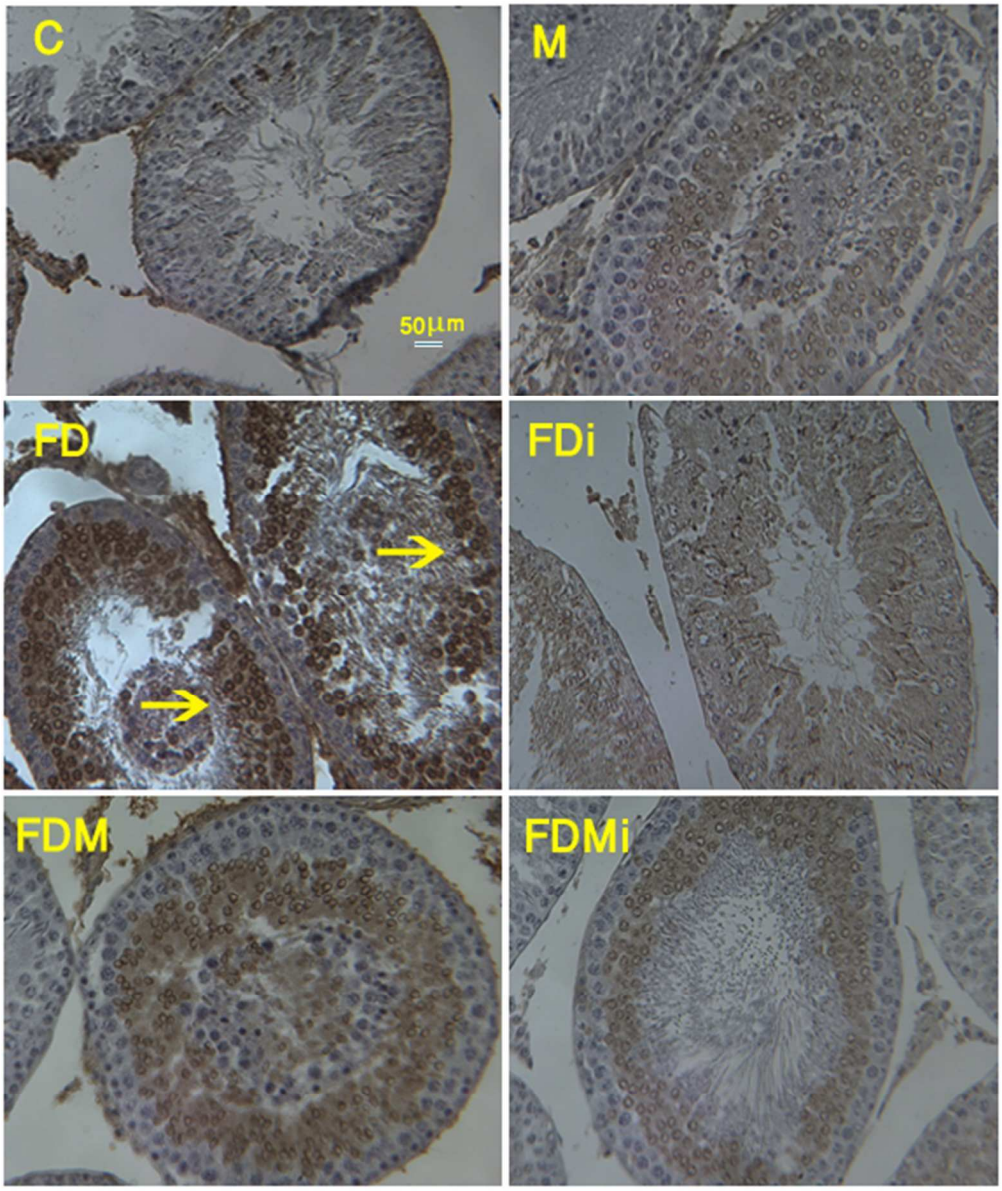

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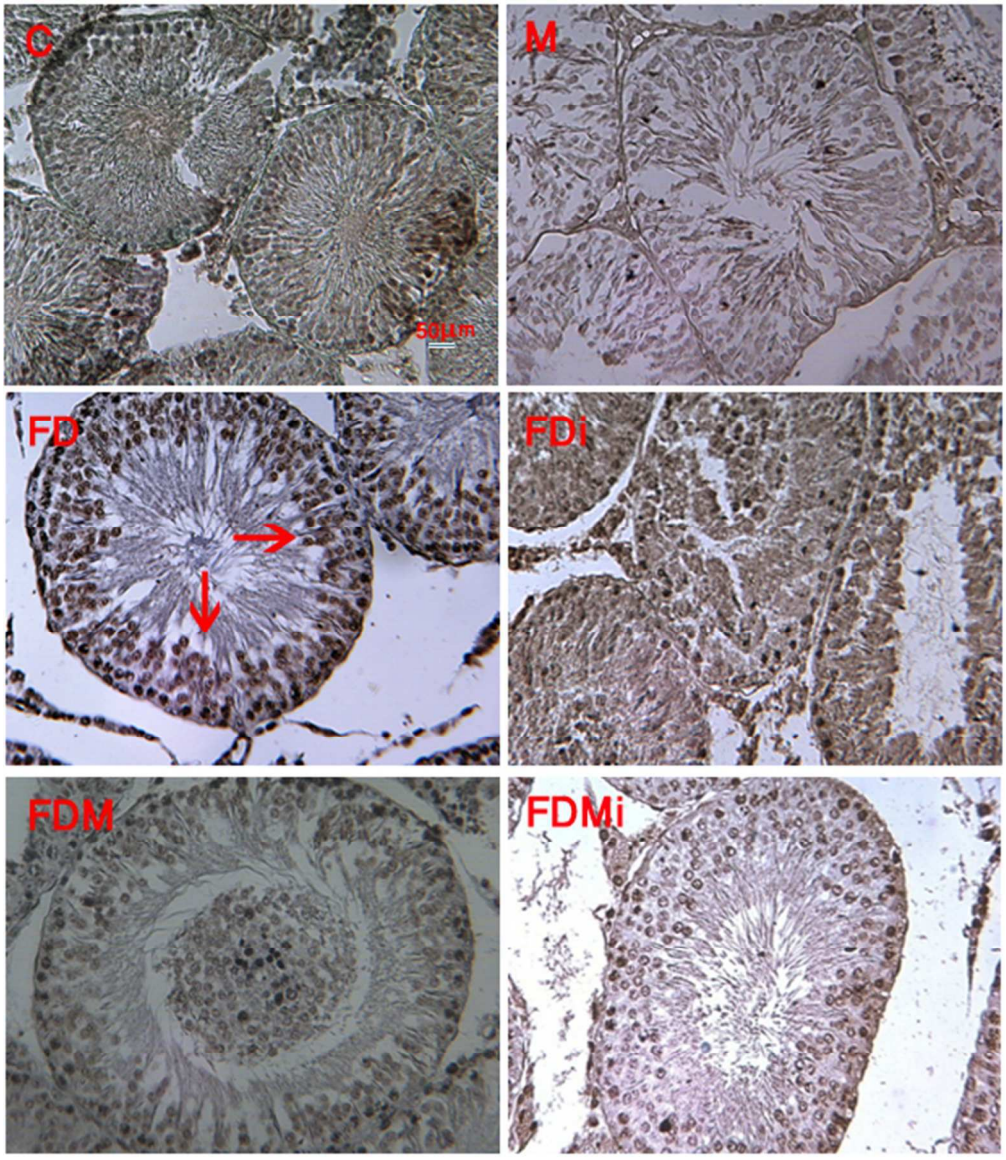

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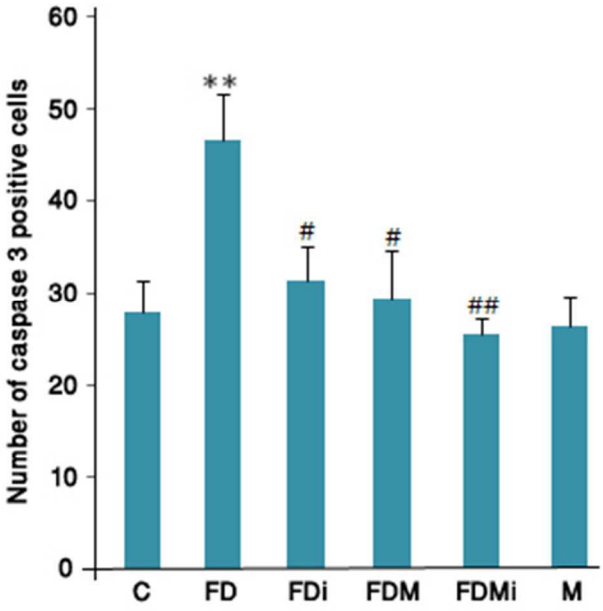

The number of caspase 3 positive cells
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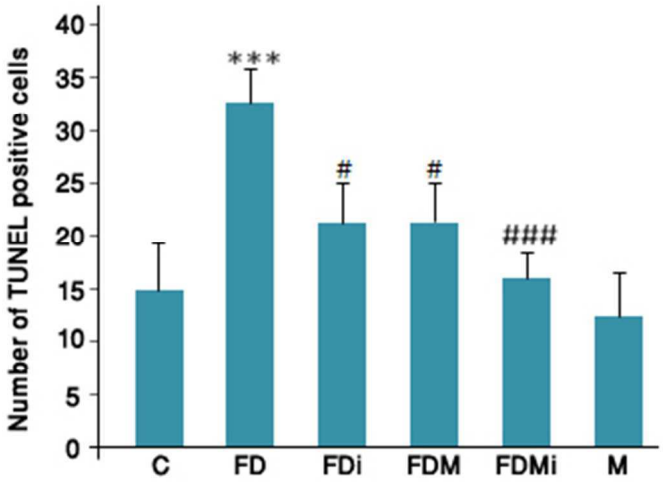

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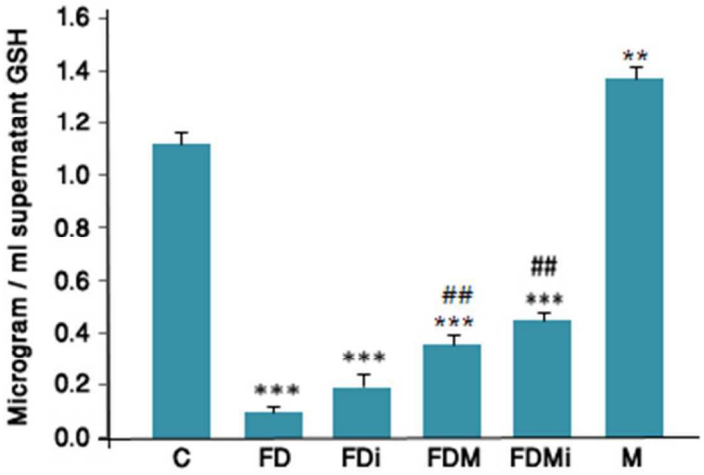

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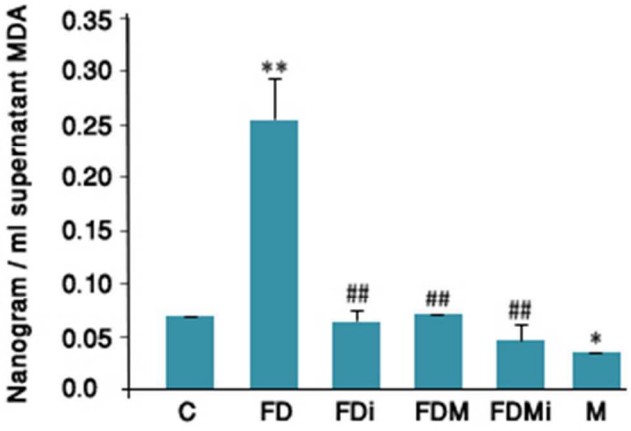

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Outcomes of food deprivation and exogenous melatonin on oxidative stress markers and apoptosis; an experimental study

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Email address: shiva_nasiraei@yahoo.com; Tel: +98 21 22248958; Fax: +98 21 22432047Abbreviations:

FD: Food deprivation

FDM: Food deprivation with melatonin

FDi: Food deprivation with isolation

FDMi: Food deprivation with melatonin and isolation

Inequality: FDi - FD = animals inequality.

Inequality: Different results between FD and FDi groups revile inequality sense.

Running title: Food deprivation and exogenous melatonin on oxidative stress and apoptosis

Abstract:

Spermatogenic cells constitute one of the tissues that are susceptible to oxidative stress and apoptosis. Food deprivation has been reported as a stressor that could increase reactive oxygen species (ROS). In this study, food deprivation-induced oxidative stress and apoptosis, as well as protective effects of melatonin were evaluated in testes.

Wistar rats in the control group were given a standard diet while a sham group was administered saline as a melatonin vehicle. A third group received daily injections of melatonin. Other four groups included food deprivation (FD), food deprivation with isolation (FDi), food deprivation with melatonin injection (FDM), and food deprivation with melatonin injection and isolation (FDMi). The testicular tissues were evaluated for MDA and GSH concentrations and DNA damage.

Food deprivation increased MDA and reduced the GSH concentration while the melatonin treatment improved these observations.

Immunohistochemistry of Capsase-3 and TUNEL assay revealed that the numbers of apoptotic cells were increased in the FD group. Melatonin treatment could offset the number of apoptotic cells following food deprivation.

Results confirm evidence that food deprivation could increase oxidative stress leading to the activation of the apoptosis, and melatonin has the ability to protect testes against the oxidative damages promoted by food deprivation.

Keywords: Apoptosis, Food deprivation, Melatonin, Oxidative stress, Testis.

1. 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, as one of human society's problems infertility has increased by almost 50% since 1955 worldwide (Sarvari et al., 2010). An estimated 6% of adult males are thought to be infertile (Carrell et al., 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 could 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 the levels of superoxide dismutase (SOD), catalase, and malondialdehyde (MDA); and they are also being considered responsible for changing tissue glutathione (GSH) content (Morales et al., 2004, Abdulhakeem and Al-Majed, 2011, Domenicali et al., 2000, Bhardwaj et al., 1998).

Similarly, Santos et al. (2009) reported that FD 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 induction of apoptosis by these agents (Clutton 1997, Blokhina et al., 2003, Ferlini et al., 1999).

In our previous study, we showed that FD decreases the quality of semen liquid. We observed a reduction in number and motility of sperm as well as a significant increase in the number of sperm with abnormal morphology following FD in male Wistar rats. This reduction was also observed in examination of the tissue and cell generator layers. In addition, FD reduced the number of Sertoli and Leydig cells, which in turn affects sperm proliferation (Nasiraei-Moghadam et al., 2012). Considering these findings, we hypothesized 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, might improve the severe effects of FD on testicular tissue.

2. Materials and Methods:

2.1. 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 (USA). Melatonin powder (m5250) was resolved in saline and alcohol (V/V 95–5%). Xylene, ethanol, Bouin's solution, PBS (phosphate buffered saline), and hematoxylin were all purchased from Merck (Germany). Anti-Capsase-3 rabbit polyclonal antibody (dilution 1:100) was purchased from Cell Signaling Technology (9661S). Proteinase K, In Situ Cell Death detection kit, POD were obtained from Roche (Cat. No. 11684817910, Germany). Streptavidin biotin secondary antibodies (anti-rabbit IgG and anti-mouse IgG) were obtained from Abcam (Germany).

2.2. Animals:

The experimental protocol was in accordance with the Declaration of Helsinki for experiments involving humans; EU Directive 2010/63/EU for animal experiments. Forty-two male Wistar rats were kept under standard laboratory conditions with a 12-h light/dark cycle and ad libitum access to food and water throughout the experiments. In this study, the effects of ad libitum access to food and water and melatonin treatment were evaluated with and without a FD and isolation situation. Animals were randomly assigned to seven groups. Animals in the control group (C) remained intact and were kept in the animal room during the study. They received normal food without any limitation, approximately 22 g a day. The sham control group (S) received saline as a melatonin vehicle. The third group (M) received daily intraperitoneal melatonin (5 mg/kg body weight). The remaining four groups including food deprivation (FD), food deprivation with isolation (FDi), food deprivation with melatonin injection (FDM), and food deprivation with melatonin injection and isolation (FDMi) underwent a food deprivation condition and received one-third of the normal daily food, 7.5 g/day. Isolation was the condition under which animals (6 animals in 1 cage) could not see or smell other animals' food. The rats were weighed twice, at the beginning and at the end of the experiment. Animals were anesthetized with CO_2 and

killed, followed by the removal, washing, and weighing of the testes. The dimensions of the testes were then measured by caliper.

2.3. Histopathological procedure

2.3.1 .H&E staining:

The right testis tissues were fixed in the Bouin's solution for 20 hours and prepared for histopathological evaluation. After processing and embedding in paraffin, they were sectioned by rotary microtome (LEITZ, 530 577, Germany) with 5-µm thickness and stained with hematoxylin and eosin, according to the standard staining protocol. Histopathological evaluation of the tissues was performed by light microscopy (Labo America Inc., Listed 7GA9).

2.3.2. The TUNEL assay:

Apoptotic cells were detected using an in situ cell death detecting kit according to the manufacturer's instructions. Briefly, paraffin sections were cut (5 µm) and mounted on poly-L-lysine coated slides (Sigma, US). Sections were deparaffinized using xylene and hydrated by processing with a series of graded alcohols. At room temperature, 3% H_2O_2 was used for inactivation of endogenous peroxidase for 15 minutes. Then the sections were digested with 20 µg/ml proteinase K for 45 minutes in a dark and humidified chamber at 37 ºC. The sections were incubated with TUNEL reaction mixture (terminal deoxynucleotidyl transferase [TdT], which catalyzes polymerization of labeled nucleotides to free 3'-OH DNA ends in a template-independent manner) for 60 min at 37 ºC. The slides were then incubated with converter-POD for 1 h at 37 ºC. In each step, washing was performed three times using PBS as the washing buffer. The slides were developed with 0.1% diaminobenzidine (DAB), and stained for 15 minutes at 37 ºC. Afterwards, the specimens were washed three times in distilled water, counterstained by hematoxylin for 1 minute, dehydrated, and mounted. Testis tissues treated with DNase were used as positive controls. In the negative control, 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 animals. The number of apoptotic cells per tubule was assessed on three sections. In each section, two microscopic fields (400X) were randomly selected.

2.3.3. Immunohistochemistry:

Testis sections (5-mm thickness) that were fixed in the Bouin's solution and embedded in paraffin were deparaffinized, rehydrated, and then antigen retrieval was performed in a microwave. Slides were placed in a plastic jar containing 200 ml 0.1 M citrate buffer, pH 6.0 under boiling point condition (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% normal goat serum (blocking buffer) for 60 min and incubated at 4 ºC overnight with the Capsase-3 antibody. Sections were again washed in PBS and subsequently incubated for at least 30 min with the components streptavidin (A) and biotin (B) of the ABC staining kit. Both components (A and B) were ready to use. Slides were washed again in PBS; bound antibody was visualized after addition of a 1 mg/ml solution of DAB in PBS. The slides were subsequently counterstained with Mayer's hematoxylin. Control sections, in which the primary antibody was replaced by normal goat serum, were similarly processed. Neonate rat thymus tissue treated with dexamethasone (0.1M) was used as positive controls.

2.4. Biochemical Investigation:

2.4.1. Determination of testicular MDA levels:

The MDA concentration (thiobarbituric acid reactive substances, TBARS) was measured as Nasri et al. (2011) and Roghani et al., 2009 described previously. Briefly, after removing the testis and its cleansing of extra tissues, it was blotted dry and weighed, then made into about 5% tissue homogenate in ice-cold 0.9% NaCl solution. A supernatant was obtained from tissue homogenate by centrifugation (1000 × g, 4 °C, 10 min). To measure MDA concentration in the supernatant, 1.0 ml of 20% trichloroacetic acid and 1.0 ml of 1% TBARS reagent were added to 100 µl of the supernatant, then mixed and incubated at 100 °C for 80 min. After cooling on ice, samples were centrifuged at 1000 × g for 20 min and absorbance of the supernatant was read at 532 nm. TBARS results were expressed as MDA equivalents using tetraethoxypropane as the standard.

2.4.2. Determination of testicular glutathione levels:

GSH levels were estimated by Sedlak and Lindsay's method (1968), which is based on the reaction between thiol groups and 1,2-dithio-bis nitro benzoic acid (DTNB) as substrate to produce a compound that absorbs light at 412 nm. The amount of GSH was determined from a standard curve simultaneously obtained under the same conditions with various concentrations of GSH.

2.5. Data and statistical analysis:

All values were given as means ± SD. Statistical analysis was determined using the one-way ANOVA followed by Tukey post hoc test. A statistical p value less than 0.05 was considered significant.

3. Results:

3.1. Animals' weight:

All the rats retained relatively good health status during the experiment. Data on their body and testis weights are presented in Table 1. The percentage of bodyweight gain in the control group was 66%, while 26%, 7%, 8%, 1%, and 11% decreases were observed for the FD, FDi, FDM, FDMi, and M-treated animals, respectively. There were no treatment-related changes in the absolute and relative weights of the testes in the treated groups, as compared with the control group. Since there was no statistically significant difference between the control and the sham groups, the results of the sham group were ignored.

(Table 1)

3.2. Apoptosis; TUNEL and IHC:

We used the TUNEL assay and immunohistochemistry (IHC) of Capsase-3 to evaluate apoptotic cells in testis tissues of different groups. In this study, the number of TUNEL positive germ cells per tubule cross-section increased following FD, as compared to the control group. The TUNEL positive spermatogonia, spermatocytes, and spermatids were the main germ cells undergoing apoptosis. Fig. 3 shows the histograms of TUNEL assay analyzed by light microscopy in the control, FD, FDi, FDM, FDMi, and M groups, respectively. The TUNEL assay is an in situ detection method, by which the apoptotic cells can be identified by their darkly stained nuclei. We observed a low incidence of apoptosis in the control rats. The number of total apoptotic cells (spermatogonia, spermatocytes, and spermatids) 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 in the control, FD, FDi, FDM, FDMi, and M groups, respectively (Fig. 5).

Our results were confirmed by IHC, where the numbers of Capsase-3-positive cells were detected in 100 randomly selected tubules per animal in all investigated groups. Fig. 2 shows the histograms of Capsase-3 assay in the control, FD, FDi, FDM, FDMi, and M groups, respectively. Consistently, our results showed 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 compared the values between different groups. The rate of positive cells 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 in the control, FD, FDi, FDM, FDMi, and M groups, respectively (Fig. 4).

3.3. Testicular MDA level:

MDA level was used as an index of lipid peroxidation in testis homogenates of the animals in the control and experimental groups. Our results showed that MDA concentration increased significantly in the group with food deprivation (FD group; $p < 0.01$) compared to the control. Animals treated with melatonin or isolation following FD treatment (FDM and FDi groups) showed a significant decrease ($p < 0.01$) when compared with the FD group. Furthermore, the group treated with melatonin alone (M group) showed a significant decrease ($p < 0.05$) in the MDA level compared with the control group (Fig. 6). The mean ± SD values of MDA levels of testes in the control, 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, respectively (Fig. 6). Although decreased tissue MDA levels were detected in the FDi and FDM groups ($p < 0.05$), we did not observe a cumulative effect of melatonin and isolation on testis MDA levels in the FDMi group.

3.4. Testicular glutathione level:

The results of rat testicular GSH levels in all groups are shown in Fig. 7. Our results showed that GSH concentration decreased significantly in the FD group ($p < 0.001$) compared to the control group. Animals treated with melatonin following FD treatment (FDM group) showed a significant increase ($p < 0.01$) when compared with the FD group. Moreover, the group treated with melatonin alone (M group) showed a significant increase ($p < 0.01$) in the GSH levels relative to the control group (Fig. 7). GSH concentrations of the supernatant 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 in the control, FD, FDi, FDM, FDMi, and M groups, respectively (Fig. 7). Although tissue GSH level increased in the FDi group, the difference was not statistically significant compared to the FD group ($p > 0.05$).

4. Discussion:

The negative impact of food deprivation on health is gaining increasing interest while relationship between socioeconomic status and various diseases have been well presented in the literature. Heidary et al (2012)(2013).

There are several studies concerning the effects of FD on enhancement of oxidative stress. Santos et al. (2009) and Domenicali et al. (2000) reported that FD promotes oxidative imbalance in rat brain and liver, respectively. Pascual et al. (2003) reported that dietary restriction of Sparus aurata for 46 days results in an increase of liver MDA levels, suggesting increased oxidative stress in the liver. The results obtained in the current study are consistent with these findings, as FD increased oxidative stress in testis tissue of male rats. MDA has been known as an index of lipid peroxidation and has been widely used as a marker of oxidative damage to membranes and therefore of oxidative stress (Lepage et al., 1991, Buege et al., 1978). In the current study, we studied the levels of MDA 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 FD. It was shown that the MDA levels increased three times compared to the control group.

In order to evaluate the effect of limited food availability as a source of oxidative stress, we also studied the ratio of reduced glutathione (GSH) in rat testis. GSH (L-g-glutamyl-L-cysteinylglycine) is an important sulfydryl compound in mammalian cells with different functions and that coordinates body defense systems against oxidative stress. Reduced glutathione effectively scavenges free radicals and has an important role in 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 et al. 1985). In the present investigation, glutathione levels of testes decreased in the FD group, suggesting a state of oxidative stress in the testes following FD. The results of the present study demonstrate that food restriction caused marked depletion of GSH levels (eightfold) in the testicles of Wistar rats. These results confirm that FD could induce oxidative stress.

There are studies indicating that oxidative stress is one of the most important factors inducing germ cell apoptosis in the testis (Pieri and Shaha. 2000, Yang et al., 2001). Because the testis is very sensitive to oxidative stress (Carrell DT, Peterson, 2010), it has high amounts of antioxidants including GSH (Pieri 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 functions (Adams et al. 1995, Troyer-Caudle 1993, Sucu et al. 2002) and apoptosis (Glutton, 1997). Our data illustrate that FD would increase MDA levels and decrease GSH levels in the testicular cells of Wistar rats. Therefore, we concluded that high concentrations of MDA, as well as low amounts of GSH, might induce apoptosis in the testis. We then examined the existence of apoptotic cells in the testes of rats that experienced limitation in food

availability. TUNEL and IHC assays of Capsase-3 confirmed that food restriction would induce apoptosis in germ cells (Fig. 4 and 5). In our previous work (submitted for publication), we showed reduction in the number of sperm, and Sertoli and Leydig cells, following starvation. We suggest that these findings might result from apoptosis in the tissue.

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

In our study, we observed that the level of testis MDA significantly decreased in the FDM group, and reached near the control group. This suggests that melatonin probably prevents lipid peroxidation and the negative effects of oxidative stress after FD, as evidenced by biochemical markers. Wakatsuki et al. (1999) also reported that melatonin inhibits the peroxidation of membrane lipids. Further data also demonstrate that melatonin administration decreases oxidative stress as shown by increased levels of testis GSH in the FDM group. These protective effects were also supported by evaluation of apoptotic cells in testis tissue after melatonin treatment. We observed that melatonin suppressed the fasting induction of testicular germ cell apoptosis (Fig. 5). The results of TUNEL assay indicated that the number of TUNEL positive or apoptotic cells in the FDM group significantly decreased compared to the FD group. This was also confirmed by the IHC method through evaluation of Capsase-3-positive cells. Considering the findings of the present study, it can be suggested that melatonin may be used clinically as an effective agent in male reproductive problems caused by oxidative damage.

Additionally, it has been revealed that the isolation situation was also efficient in preventing side effects of FD. The protection offered by the isolation situation was similar to melatonin. Some studies showed that the isolation situation could improve FD effects (Moradi et al., 2010, Mojarab et al., 2010). Isolation decreased MDA levels ($p < .05$) in testes of the FDi group compared with FD rats. A similar result was obtained for the GSH biomarker whereby GSH levels increased in the testis of FDi rats. Starvation significantly decreased GSH levels (Fig. 7). Increased testicular levels of GSH along with reduction of MDA levels in FDi rats are probably the result of the inhibition of oxidative stress derived from the food

deprivation. Moreover, our data showed reduced numbers of TUNEL and Caspase-3 positive cells in the FDi group compared to the FD group that indicated decreasing of the apoptosis phenomena following isolation. The decrease in oxidative stress and apoptosis in the FDi group favors 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 testis tissue of male Wistar rats.

5. Conclusion

Our findings demonstrated that FD increases the number of apoptotic spermatogenic cells and oxidative markers in the testes.

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

The results of this study further showed that melatonin treatment, together with FD, has a protective role against the oxidative effects of FD. It also reduces the apoptotic effect of FD on spermatogenic cells.

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

6. Acknowledgements:

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Tables and figures legends:

Table 1: Body weight (g) and relative organ weight (g) of rats in FD, FDi, FDM, FDMi, and M groups after 14 days.

The data are expressed as mean ± SD (Standard deviation) for six rats in each group. Significant difference ($p < 0.05$) between the control group and other groups is shown by (*), and significant difference ($p < 0.05$) between the FD group and the FDi, FDM, and FDM groups is shown by (#)

Fig. 1: Cross-sections of the testis, 5 µm thickness, H&E staining, magnification x400, C: Control, FD: Food deprivation, FDM: Food deprivation with melatonin, FDi: Food deprivation with isolation, FDMi: Food deprivation with melatonin and isolation, and M: melatonin groups, magnification x400.

Fig. 2: Cross-sections of the testis, 5 µm thickness, Immunohistochemistry of Caspase-3, magnification x400, C: Control, FD: Food deprivation, FDM: Food deprivation with melatonin, FDi: Food deprivation with isolation, FDMi: Food deprivation with melatonin and isolation, and M: melatonin groups, Scale bar means 50 µm, magnification x400.

Fig. 3: Cross-sections of the testis, 5 µm thickness, TUNEL assay, magnification x400, C: Control, FD: Food deprivation, FDM: Food deprivation with melatonin, FDi: Food deprivation with isolation, FDMi: Food deprivation with melatonin and isolation, and M: melatonin groups, Scale bar means 50 µm, magnification x400.

Fig. 4: The number of Caspase-3 positive spermatogenic cells in unit area about 500µm^2, magnification x400. The data are expressed as mean ± SD (Standard deviation) for six rats in each group. Significant difference ($p < 0.05$) between the control group and other groups is shown by (*), and significant difference ($p < 0.05$) between the FD group and the FDi, FDM, and FDM groups is shown by (#).

Fig. 5: The number of TUNEL positive spermatogenic cells in unit area about 500µm^2, magnification x400. The data are expressed as mean ± SD (Standard deviation) for six rats in each group. Significant difference ($p < 0.05$) between the control group and other groups is shown by (*), and significant difference ($p < 0.05$) between the FD group and the FDi, FDM, and FDM groups is shown by (#).

Fig. 6: Nanogram/ml supernatant MDA., The data are expressed as mean ± SD (Standard deviation) for six rats in each group. Significant difference ($p < 0.05$) between the control group and other groups is shown by (*), and significant difference ($p < 0.05$) between the FD group and the FDi, FDM, and FDM groups is shown by (#).

Fig. 7: Microgram/ml supernatant GSH. The data are expressed as mean ± SD (Standard deviation) for six rats in each group. Significant difference ($p < 0.05$) between the control group and other groups is shown by (*), and significant difference ($p < 0.05$) between the FD group and the FDi, FDM, and FDM groups is shown by (#).

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