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Full Length Research Paper

Pattern of fatty acids as modulator for dietary iron overload and its influences on testicular function of experimental rats

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An iron enriched diet was adopted by different health authorities as an effective tool to combat iron deficiency anemia. However, dietary fat may alter absorption and utilization of iron either in human or animal models. Being a potent pro-oxidant, iron can lead to generation of reactive oxygen species, inducing oxidative damage and inflammations in different tissues. Present study aimed mainly to illustrate any alterations in testicular function of experimental rats kept on dietary iron. Long intake for 10 weeks may affect the testicular function, supplementation of dietary fat of various pattern may alter iron effect. We found a significant increase in serum iron, ferritin, nitrogen oxide (NO), TNF-α, testicular hydroxyproline, thiobarbituric acid reactive substances (TBARS) along with decrease in testosterone level in the group that received dietary iron only (control group). Iron overload induced adhesion of seminiferous tubules, disorganization of germinal epithelium, multiple collagen fibers and iron deposition were also observed. While co-supplementation of palm oil with dietary iron intake significantly decreased testosterone level, olive oil intake induced significant increase. That effect was associated with moderate alleviation of fibrosis and mild regeneration of testicular tissues. We concluded that the iron overloaded diet enhances oxidative stress and inflammation leading to decreased spermatogenesis and testosterone secretion (testicular function). Therefore, supplementation of dietary fats can modulate iron effect.

Key words: Dietary iron, lipid peroxidation indices, olive-palm oils, testicular function.

INTRODUCTION

Previous studies illustrated that dietary fat (type and amount) might affect iron absorption and utilization in animal models (Droke and Lukaski, 1996; Pabon and Lonnerdal, 2001). For example, saturated fatty acids like stearic acid can increase iron absorption and utilization as well as the liver iron content in iron deficient rats (Johnson et al., 1987; Fields and Lewis, 1999). However the reverse was true for oil rich in unsaturated fatty acids like safflower oil. In spite of inducing no increase in liver iron content, safflower oil promoted the development of iron deficiency state (Rao et al., 1983; Fields and Lewis, 1999).

In humans, a diet rich in polyunsaturated fatty acids like linoleic acid can reduce iron retention and balance as compared to highly saturated fatty acids (Lukaski et al., 2001) and in turn affectting mineral status (Milin et al., 2001). According to some researchers, olive oil may exert certain influence on iron status and utilization which may be related to certain alterations in iron absorption or fatty acid composition of cellular membranes (Shotton and

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Droke, 2004).

In this study, we studied the effect of biscuits enriched with iron (BEI) on testicular tissues in experimental rats through estimation of certain inflammatory marker, serum iron, ferritin, testosterone, nitric oxide (NO), and testicular iron, reduced glutathione (GSH), lipid peroxidation, "thiobarbituric acid reactive substances" (TBARS), and hydroxyproline (Hyp) contents. We have evaluated also the potential effect of an oil rich in saturated fatty acids (palm oil) and another rich in unsaturated fatty acid (olive oil) on these markers using the previously mentioned markers in addition to histological pattern in testicular tissues. The level of dietary iron used was BEI containing 0.3% w/w ferrous sulphate, a dietary iron level adopted by the Egyptian Ministry of Education and used in Egyptian schools and institutes to combat anemia.

MATERIALS AND METHODS

Experimental animals

Sixty male Wistar rats (180 ± 20 g, 12 weeks old) were supplied by the Egyptian Organization for Biological Products and Vaccines. The rats were subjected to controlled temperature (25 ± 2°C) and illumination (12 h light/dark) and allowed free access to a normal rat chow diet and water. This protocol was approved by the Animal Care and Use Committee of the Biochemistry department, Faculty of Pharmacy, Zagazig University.

Experimental design

One week after acclimatization, rats were randomly divided into six experimental groups. Ten rats were kept on BEI (0.3% w/w ferrous sulphate) for 10 weeks, expressed as iron overloaded group while the second group of 10 rats received iron free biscuits and served as a normal control group. The biscuits were composed of flour, starch, cane sugar, hydrogenated vegetable oil, eggs, flavours and vanillin. The third group of 10 rats were given palm oil; the fourth group of 10 rats received olive oil; the fifth group received 15 percent palm oil premixed with BEI; and the sixth and final group received 15 percent olive oil premixed with BEI for 10 weeks. Palm oil was selected since it is rich in saturated fatty acids (plam oil) while olive oil represented an oil rich in monounsaturated fatty acids (oleic acid). At the end of the study, blood samples were collected for serum separation and kept at -80°C until biochemical assays were performed. Rats were killed by decapitation; testicular tissues were removed, rinsed with cold normal saline, divided into parts and dried with filter paper. First part was quickly frozen in liquid nitrogen (-170°C) then stored at -20°C for determination of biochemical parameters. The other part was kept in 10% formalin-saline at 4°C for 1 week, subsequently dehydrated with a series of ethanol solution from 75 to 100% before embedding in paraffin. Cross sections (5 µm thick) were stained with hematoxylin and eosin (H & E) for microscopical examination and Perl´s Prussian blue stain to localize deposited iron and Mallory trichrome stain to illustrate collagen fibers and any fibrotic changes.

Analytical procedures

Serum

Serum iron was determined by colorimetrics using commercial kit (Spinreact, S.A., Spain) (Burits and Ashwood, 1999), NO was measured as nitrite (Moshage et al., 1995), TNF-α was evaluated using ELISA kit purchased from Biosource Int (Ca., USA) (Chen et al.,1998).

Testicular tissues

Determination of TBARS in testes homogenate: 0.5 G tissue was homogenized in 5 ml phosphate buffer (pH = 7.2), centrifuged at 3000g for 15 min at 4°C. The supernatant was collected and lipid peroxidative products were determined (Buege and Aust, 1978) using 1,1,3,3 tetramethoxy propane as a standard. The data were expressed as malondialdehyde equivalents (nmol MDA/g tissue).

Determination of GSH: Glutathione (GSH) was determined spectrophotometrically using Ellman's reagent according to modified method (Ahmed et al., 1991). 0.1 G of tissue was homogenized in 1 ml phosphate buffer (pH = 8) at 4°C. 0.5 ml of homogenate was mixed with 0.5 ml 10% TCA in 5 mM ethylenediaminetetraacetic acid (EDTA) sodium, mixed well and centrifuged at 2,000 g for 5 min. Supernatant was used for determination of reduced GSH.

Determination of iron content: The iron content was determined by flame atomic absorption spectrophotometer. Briefly, 0.1 g tissue was incubated with a mixture of 2 ml conc. nitric acid and 2 ml perchloric acid at room temperature for 24 h for digestion. After incubation, the mixture was filtered, diluted and absorption was measured at 248 nm (Basset et al., 1986).

Determination of hydroxyproline (Hyp): This was determined spectrophotometrically by Ehrlich reagent (Fujita et al., 2003). 0.01 g tissues was pulverized with 500 µl of 6 N HCl, incubated overnight at 120°C. 5 µl of the acid hydrolysate was mixed with 5 µl of citrate acetate buffer and 100 µl chloramines T in ELISA plate and incubated for 20 min at room temperature before addition of Ehrlich solution.

Statistical analysis

All values were expressed as mean ± standard deviation "SD". Analysis was performed using statistical package for social sciences (SPSS) program for windows version 10 (SPSS, Chicago, USA) "student t-test", the analysis of variance "one way ANOVA" was used for the comparison between groups. Pearson correlation was used to study any association between variables. P values < 0.05 were considered statistically significant.

RESULTS

Table 1 shows that rats fed with the iron enriched diet demonstrated significant increase in serum iron, ferritin, TNF-α ($P < 0.001$) and NO ($P < 0.01$), while testosterone was found to be significantly decreased ($P < 0.001$) as compared to normal control iron-free group. Administration of palm oil significantly decreased TNF-α, NO ($P < 0.001$), testosterone ($P < 0.01$) in serum. However olive oil administration significantly increased testosterone level in serum ($P < 0.05$). Table 2 showed that rats receiving palm oil premixed with iron enriched diet demonstrated significant decrease in serum testosterone,

Table 1. Effect of palm oil, olive oil intake and iron enriched diet on serum biochemical parameters

Parameters	Normal control group	Iron overloaded group	Palm oil group	Olive oil group
Iron (mg/dl)	264.6 ± 44	518 ± 90***	242.3 ± 64	207.5 ± 73
Ferritin (ng/ml)	4.83 ± 0.7	10.5 ± 1.6***	4.8 ± 1	5.2 ± 0.5
Testosterone (ng/dl)	123.6 ± 24.9	43.9 ± 9.3***	67 ± 14**	165.7 ± 24*
NO (µmol/l)	42.98 ± 3.6	55.7 ± 5**	26.1 ± 3.4***	32 ± 3.6***
TNF-α (pg/ml)	24.2 ± 4.6	66.3 ± 6.3***	21.6 ± 4.7**	23.5 ± 4.6**

*significantly different from normal at p < 0.05. **significantly different from normal at p < 0.01. ***significantly different from normal at p < 0.001, (n =10).

Table 2. Effect of iron enriched diet either in combination with palm oil or olive oil on serum biochemical parameters

Parameters	Iron overloaded group	Palm oil + iron group	Olive oil + iron group
Iron (mg/dl)	518 ± 90	609.7 ± 95	444.4 ± 66
Ferritin (ng/ml)	10.5 ± 1.6	11.6 ± 2.2	12.5 ± 1.66
Testosterone (ng/dl)	43.9 ± 9.3	28.4 ± 6.7**	80 ± 13**
NO (µmol/l)	55.7 ± 5	45.6 ± 3**	45.3 ± 3.6**
TNF-α (pg/ml)	66.3 ± 6.3	36.3 ± 7**	32.5 ± 5.5*

*significantly different from control iron group at p < 0.05. **significantly different from control iron group at p < 0.01, (n =10).

Table 3. Effect of palm oil, olive oil intake and iron enriched diet on certain testicular parameters

Parameters	Normal control group	Iron overloaded group	Palm oil group	Olive oil group
TBARS (nmol/g tissue)	669.2 ± 49.6	1439 ± 70.6 ***	515.6 ± 47.9***	466.4± 50***
GSH (nmol/g protein)	38 ± 1.9	18.6 ± 2.5**	41.7 ± 2.2**	42.7 ± 2.5**
Hyp (µg/g tissue)	131 ± 7	213.6 ± 4.3***	131.1 ± 7.4	130.6 ± 5.9
iron (µg/g tissue)	122.7 ± 7.8	265.2 ± 11***	125.7 ± 11.1	119.3 ± 7.6

*significantly different from normal at p < 0.05. **significantly different from normal at p < 0.01.*** significantly different from normal at p < 0.001, (n =10).

Table 4. Effect of iron enriched diet either in combination with palm oil or olive oil on certain testicular parameters

Parameters	Iron overloaded group	Palm oil + iron group	Olive oil + iron group
TBARS (nmol/g tissue)	1439 ± 70.6	1240 ± 81.2***	1115.2 ± 91.9***
GSH (nmol/g protein)	18.6 ± 2.5	28.1 ± 3.2*	30.2 ± 2.2**
Hyp (µg/g tissue)	213.6 ± 4.3	207.1 ± 3**	206.1 ± 2.9**
iron (µg/g tissue)	265.2 ± 11	271 ± 10.1	263.5 ± 11.8

*significantly different from control iron group at p < 0.05. **significantly different from control iron group at p < 0.01. ***significantly different from control iron group at p < 0.001, (n =10).

NO, TNF-α ($P < 0.01$). Administration of olive oil premixed with iron enriched diet resulted in significant decrease of NO ($P < 0.01$) and TNF-α in serum ($P < 0.05$), while testosterone level demonstrated significant increase in serum ($P < 0.05$) as compared to iron overloaded group.

Table 3 showed that testicular tissues of rats fed iron enriched diet demonstrated significant increase in TBARS, Hyp, iron ($P < 0.001$) together with marked decrease in GSH content ($P < 0.01$) as compared to normal control group. Administration of palm oil resulted in significant decrease of TBARS ($P < 0.001$) along with increased GSH ($P < 0.01$). Olive oil administration induced nearly similar changes in palm oil group compared to iron overloaded group. Table 4 illustrated that testicular tissues of rats fed iron enriched diet premixed with palm

Table 5. Correlation coefficient between different serum and testicular biochemical parameters

Parameters	Serum iron	Testicular iron content
Serum TNF-α	0.57*	
Serum ferritin	0.8*	
Serum testosterone	-0.72*	
Testicular TBARS		0.95*
Testicular Hyp		0.98*
Testicular GSH		-0.86*

*significantly different at p < 0.05.

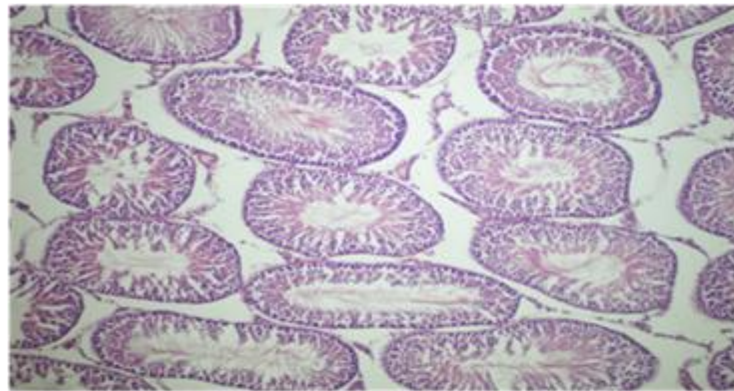

Plate 1. A photomicrograph of adult male albino rat testis (control group) showing seminiferous tubules separated by interstitial tissue (double arrow) (H&E ×100).

oil demonstrated significant decrease in TBARS ($P < 0.001$), Hyp ($P < 0.01$) while GSH showed significant increase ($P < 0.05$), while premixing of olive oil with the dietary iron have induced nearly similar effects compared to iron overloaded group. Correlations between studied biochemical parameters are illustrated in Table 5.

Histopathological study results

The iron enriched diet induced adhesion of some testicular seminiferous tubules associated with disorganized germinal epithelium, extensive areas of exudates, sloughed cells in the lumen, few sperms, multiple vacuoles within interstitial tissues and wide spaces separating spermatogenic cells (Plate 2) as compared to control group (Plate 1). Iron deposition in interstitial tissues (Plate 6) was also evident in addition to massive collagen deposition (Plate 10) in comparison with control group (Plates 5 and 9). However, tissue pattern in rats fed with olive oil in combination with iron demonstrated few exudates, narrow interstitial space, some seminiferous tubules had empty lumen, while others had many sperms (Plate 3), iron precipitation (Plate 7) and mild fibrosis (Plate 11) was also observed. Dietary intake of palm oil in combination with iron resulted in disorganized, compressed germinal epithelium of tubules, wide interstitial space, fewer sperms (Plate 4), iron deposition (Plate 8) and moderate fibrosis (Plate 12).

DISCUSSION

Present study demonstrates that rats fed BEI exhibit significant increase in serum iron, ferritin (2 fold) as compared to the normal group, in agreement with earlier reported studies (Silvana et al., 2003; Elmegeed et al., 2005; Zhao et al., 2005). Certain evidences suggested that dietary iron overload can specifically activate target genes in the liver (L ferritin and procollagen), further studies supported such suggestion (Pietrangelo et al., 1990; Valerio and petersen, 2000). Testicular iron content also showed significant increase in accordance with reported studies (Lucesoli and Fraga, 1999; Lucesoli et al., 1999). The testis represents a secondary target for iron accumulation (Galleano and Puntarulo, 1997). It can also produce its own transferrin and participate in an iron shuttle system to fulfill its requirements of iron for spermatogenesis (Sylvester and Griswold, 1993).

Present results demonstrated the pro-oxidant properties of iron in testicular tissues, in turn generation of reactive oxygen species (ROS) to be implicated latter in oxidative

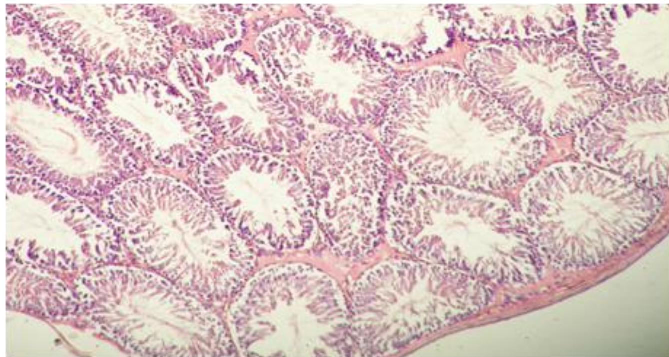

Plate 2. A photomicrograph of adult male albino rat testis of iron overload group showing adhesion of seminiferous tubules. Some tubules revealed disorganized germinal epithelium. Extensive area of exudates can be seen (H&E × 100).

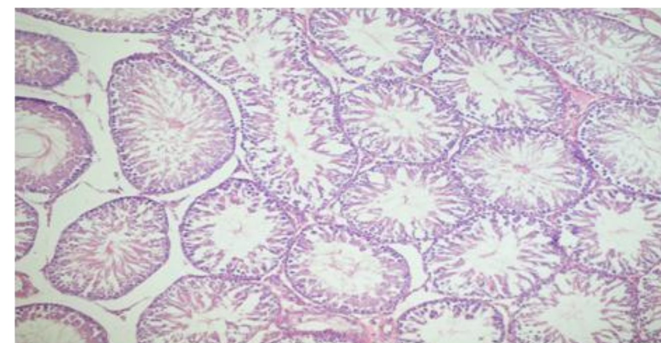

Plate 3. A photomicrograph of adult male albino rat testis of iron overload group treated with olive oil showing a few of exudates and narrow interstitial space. Some seminiferous tubules have empty lumen, while others have many sperms (s) (H&E × 100).

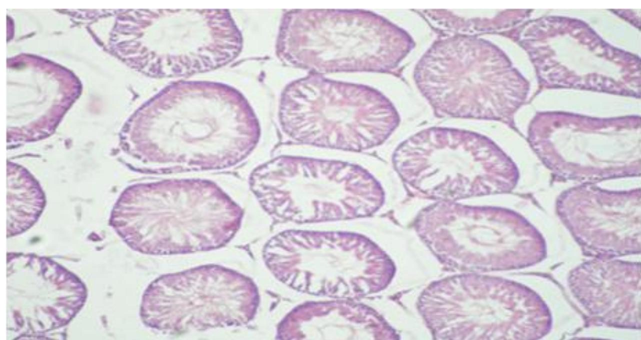

Plate 4. A photomicrograph of adult male albino rat testis of iron overload group treated with palm oil showing some tubules with disorganized germinal epithelium and wide interstitial space few sperms can be seen (H&E × 100).

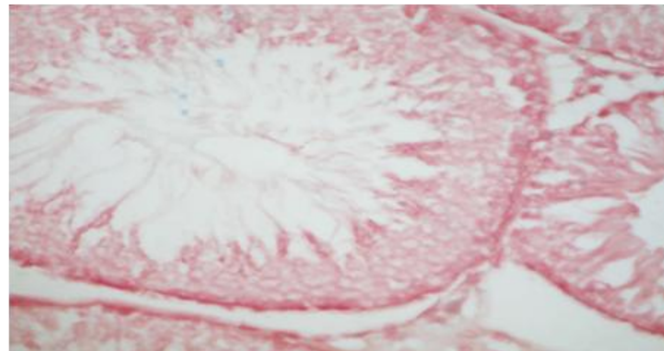

Plate 5. A photomicrograph of of control group showing negative Perl´s Prussian blue stain (Perl´s Prussian blue × 400).

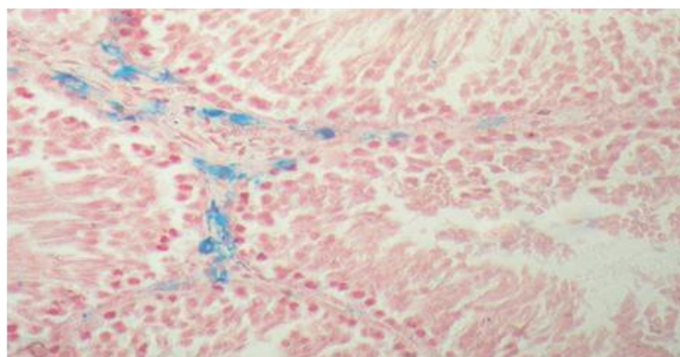

Plate 6. A photomicrograph of adult male albino rat testis (iron overload group) showing positive Perl´s prussian blue stain in interstitial tissue (Perl´s Prussian blue × 400).

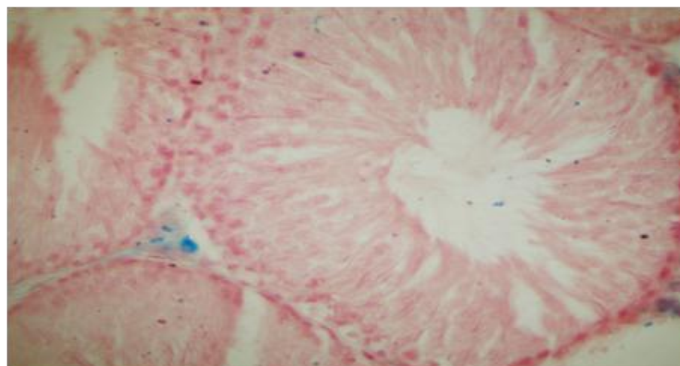

Plate 7. A photomicrograph of adult male albino rat testis of iron overload group treated with olive oil showing positive Perl´s prussian blue stain in interstitial tissue (Perl´s Prussian blue × 400).

damage of cellular components (Jagetia et al., 2004; Harandi et al., 2005). Iron is essential also for normal collagen synthesis acting as cofactor for prolyl-hydroxylase as reported before in various models of iron overload state (Poli and Parola, 1997). Consequently, intake of dietary iron significantly increased testicular hydroxyproline content, in agreement with the ealier reported study (Zhang et al., 2006). Previous reports recorded that iron overload induces moderate fibrosis in testicular interstitium and Leydig cells (Lucesoli et al.,

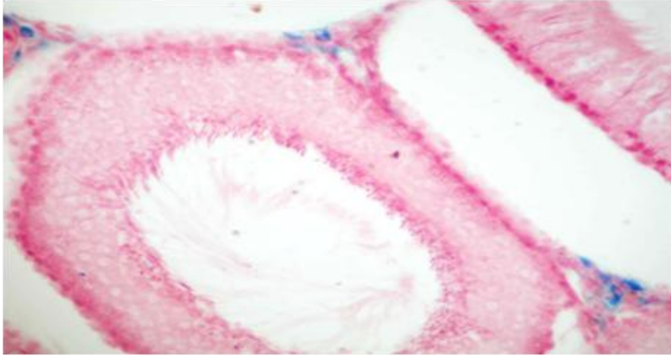

Plate 8. A photomicrograph of adult male albino rat testis of iron overload group treated with palm oil showing positive Perl´s prussian blue stain in interstitial tissue (Perl´s Prussian blue × 400).

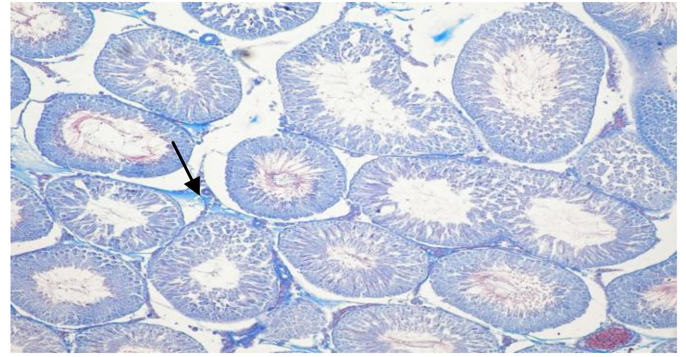

Plate 11. A photomicrograph of adult male albino rat testis of iron overload group treated with olive oil showing mild fibrosis (arrow) (Mallory trichrome × 100).

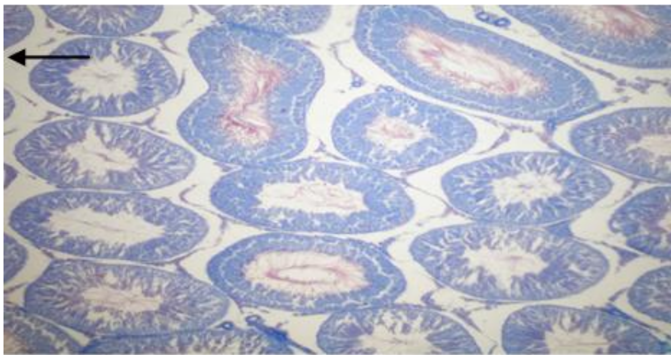

Plate 9. A photomicrograph of adult male albino rat testis (control group) showing distinctive boundary (arrows) formed of collagen fibers (Mallory trichrome × 100).

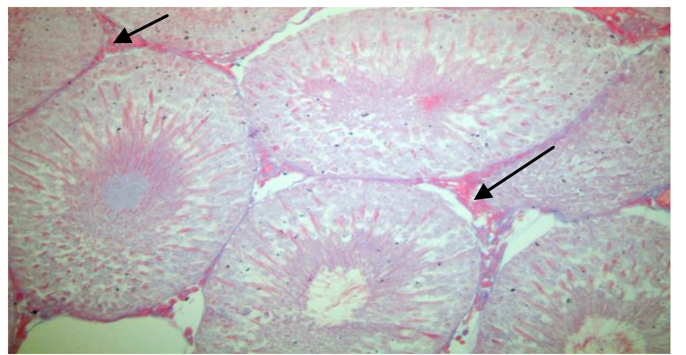

Plate 12. A photomicrograph of adult male albino rat testis of iron overload group treated with palm oil showing moderate fibrosis (arrows) (Mallory trichrome × 100).

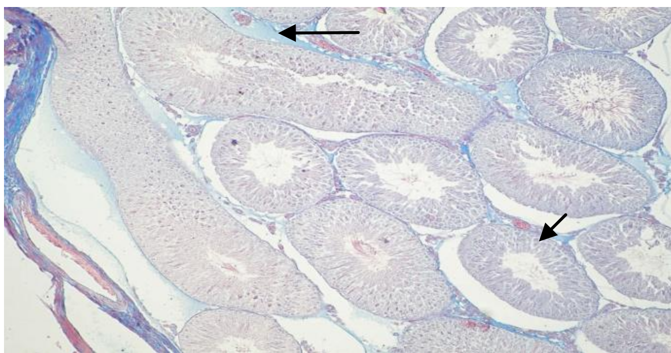

Plate 10. A photomicrograph of adult male albino rat testis (iron overload group) showing multiple collagen fibers (arrows) (Mallory trichrome × 100).

1999). TNF-α showed also significant increase in control iron group, in agreement with others (Elmegeed et al., 2005).

Generally iron can exacerbate various types of liver injury where NF-kB-driven genes are implicated. Accordingly, ferrous serves as a direct agonist to activate NF-kB, and TNF-α promoter activity and finally TNF-α protein. This was observed in cultured Kupffer cells in a redox status-dependent manner. These finding may offer a molecular basis for iron-mediated accentuation of TNF-α dependent liver injury (Hongyun et al., 2002).

In a recent study, it has been proposed that chronic iron overload enhanced proliferation and increased TNF-α mRNA in iron loaded livers. Iron therefore may act as a direct hepatic mitogen (Brown et al., 2006). Iron group also demonstrated significant increase in NO in agreement with reported studies (Elmegeed et al., 2005). Complex relationship between iron and NO was reported before (Galleano et al., 2004), indicating that iron overload can enhance iron uptake by liver Kupffer cells and NO increase via inducible nitric oxide synthase (iNOS). NO expression is also controlled by the redox-sensitive transcription factor NF-$_k$B (Kleinert et al., 2004). This led others to conclude that NO response is mediated by CIO and represents a molecular mechanism affording protection against iron toxicity (Cornejo et al., 2007).

Serum testosterone showed significant decrease in iron group as compared to normal ($P < 0.01$), mostly attributed to many causes. Sexual dysfunction due to

hypogonadotrophic hypogonadism represents a well recognized disturbances among male patients suffering from β-thalassaemia. This was attributed formerly to damage induced by hemosiderin deposition in pituitary gland in consequent to iron overload (Seracchioli et al., 1994). Hypogonadism was also demonstrated in hemochromatosis patients, attributed mostly to iron-induced cellular damage to the gonadotrophs. Accordingly cytotoxic effect of iron regarding gonadotrophs may take place as soon as iron overload threshold is reached (Sparacia et al., 2000).

Gupta et al. (2004) pointed out the possible involvement of oxidative stress in the suppression of steroidogenesis via substantial reduction in the mRNA of steroid acute regulatory protein as well as activities of testicular Δ^5-3β and 17- β hydroxysteroid dehydrogenases via strong affinity of divalent heavy metal for the thiol groups of these proteins and enzymes. TNF-α may be implicated also as a mediator of many diseases related with hypothalamic-pituitary-testicular (HPT) function. Van der Poll et al. (1993) demonstrated that TNF-α induced an early and transient increase in serum luteinizing hormone (LH) levels followed by transient decrease in serum testosterone levels, keeping follicle-stimulating hormone (FSH) unchanged.

Administration of olive oil or palm oil either individually or in combination with dietary iron induced no change in serum iron and ferritin levels, in agreement with previous studies (Perez-Grandos et al., 2000; Mesembe et al., 2004). Both oils however induced significant decrease in serum TNF-α level. Hydroxytyrosol (HT), a phenolic compound from virgin olive oil, was reported to block NF-kB activation (Carluccio et al., 2003; Maiuri et al., 2005) that is, release of TNF-α and IL-1β. This could modulate immune response selectively (Reimund et al., 2004). Others illustrated also that rats fed palm oil rich diet demonstrated reduced expression of Cox-2 and TNF-α (Nanji et al., 1997; Nanji et al., 2001), mostly attributed to decreased lipid peroxidation rate, TNF-α and Cox-2 levels.

Karsten et al. (1994) reported that palmitic acid can enhance the release of interferon-gamma (IFN-γ) which decreases TNF-α production. They suggested that saturated fatty acids may exhibit more potent effect than unsaturated one regarding cytokine production. Accordingly modulation of free fatty acid (FFA) ratios may be an effective means for fine tuning of the immune system. Administration of olive oil or palm oil or their combination with iron significantly reduced serum NO level. This outcome may be through blocking NF-kB activation by the phenolic constituents of olive oil (HT) (Maiuri et al., 2005) or to be mediated by oxidized low density lipoprotein (ox-LDL) by palmitic acid in palm oil (Moers and Schrezenmeir, 1997).

Serum testosterone level demonstrated significant increase in olive oil group, the reverse was true for palm oil. Lu et al. (2003) concluded that palmitic and stearic acids induced apoptosis in testicular Leydig cells through ceramide production and arachidonic acid can partly prevent such apoptotic effect. Gromadzka-Ostrowska et al. (2002) indicated that diet rich in MUFA content can stimulate testicular function in rats through stimulating 17β-HSD activity, the most important key-enzyme in the testosterone synthesis pathway in male rat gonads and androgen secretion. However, saturated fatty acids may exert inhibitory effect leading finally to opposite results.

Administration of both oils significantly reduced testicular hydroxyproline content, in agreement with reported study (Fernandez et al., 1997). Experimental studies indicated that olive oil administration significantly decreased collagen ratio and connective tissue in CCl_4 induced fibrosis and supported hepatocyte recovery regarding its ultrastructural and morphometric values (Szende et al., 1994; Fernandez et al., 2005). Palmitic acid in palm oil potentially induced hepatic stellate cells deactivation and significantly decreased collagen type I expression reducing fibrosis (Abergel et al., 2006).

Taken together, the results reported in this study suggested that chronic dietary iron overload induces testicular tissue damage, as illustrated by increased oxidative stress parameters, Hyp, TNF-α, depletion of antioxidants and decreased testosterone level. We found also that olive oil demonstrated a protective effect on testicular tissues. This was illustrated by decreased oxidative stress parameters, TNF-α, Hyp and increased testosterone level. Like olive oil, palm oil demonstrated also a protective effect on testis. Unlike olive oil, it significantly reduced serum testosterone level which makes supplementation of olive oil to be highly recommended.

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