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Communication

Frequent inadequate supply of micronutrients in fast food induces oxidative stress and inflammation in testicular tissues of weanling rats

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Abstract

Fast food is high in energy density and low in essential micronutrient density, especially zinc (Zn), of which antioxidant processes are dependent. We have tested the hypothesis that frequent fast food consumption could induce oxidative damage associated with inflammation in weanling male rats in relevance to Zn deprivation, which could adversely affect testis function. Zn and iron (in plasma and testicular tissue), plasma antioxidant vitamins (A, E, and C), as well as testicular superoxide dismutase (SOD) and reduced glutathione (GSH), lipid peroxidation indexes (thiobarbituric acid reactive substances (TBARS) and lipoprotein oxidation susceptibility (LOS)), and inflammatory markers (plasma C-reactive protein (CRP) and testicular tumour necrosis factor-alpha (TNF-α)) were determined. Serum testosterone and histological examination of the testis were performed also. We found a severe decrease in antioxidant vitamins and Zn, with concomitant iron accumulation. Zinc deficiency correlated positively with SOD, GSH, antioxidant vitamins and testosterone, and negatively with TBARS, LOS, CRP and TNF-α, demonstrating a state of oxidative stress and inflammation. We concluded that micronutrient deficiency, especially Zn, enhanced oxidative stress and inflammation in testicular tissue leading to underdevelopment of testis and decreased testosterone levels.

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Introduction

'Fast food' represents the main dietary pattern for a large proportion of the population, especially children, because it is quick and appeals to all age groups (French 2003). Fast food provides more than one-third of the day's energy, total and saturated fat, it is high in energy density and poor in micronutrients (Ebbeling et al 2002). The lower intake of vitamins and essential elements results in a decrease of micronutrient density with increased frequency of fast food consumption (Bowman & Vinyard 2004). This can provide the basis of chronic oxidative stress as well as inflammation (Dandona et al 2007). Zinc is one of the most important essential elements for normal functioning of the male reproductive system; testis and prostate have a higher concentration of zinc than any other organ of the body (reviewed by Bedwal & Bahuguna (1994)). It is an effective antioxidant and anti-inflammatory agent. Zinc deficiency is associated with male hypogonadism (reviewed by Prasad (2008)), atrophy of seminiferous tubules and decreased testosterone levels (Ueda et al 1991). It has more pronounced effects on testis during the prepubertal state than in later life (Leathern 1970).

We have studied the effect of a fast food diet on testicular tissue in young weanling rats through estimation of testicular tissue content of zinc and antioxidant vitamins. We evaluated the extent of the association between such micronutrient imbalance and oxidative stress and inflammation. Finally, we have examined the possible detrimental effects such a biochemical pattern could have on the histology of testicular tissue with regards to its function and testosterone level.

Materials and Methods

Experimental animals

Twenty male Wistar rats (50 ± 10 g, 3-weeks-old) were supplied by the Egyptian Organization for Biological Products and Vaccines. 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 two experimental groups. Ten rats were placed on a typical fast food hamburger diet, prepared in our laboratory. The diet was composed of 16.5% protein, 16.7% total fats, 6.12% total saturated fatty acid, 8.77% total unsaturated fatty acid, 0.81% total trans fatty acid, 37.2% carbohydrates, 12.5% water, 0.8% minerals and vitamins mixture according to USDA National Nutrient Database for Standard Reference, Release 20 (USDA SR20). Ten rats (controls) were fed a normal chow diet containing 20% protein, 10% total unsaturated fatty acids, 25% carbohydrate, 38.5% sugar, 3% fibre, 1% vitamin mix and 2.5% mineral mix (L'Abbé & Fischer 1984) for 16 weeks. At the end of the study, blood samples were collected for plasma and serum separation. These samples were stored at −80°C for further analysis, while some of the testes were directed for microscopical examination and the others were stored at −80°C until biochemical assays were performed.

Determination of lipogram pattern in plasma and testicular tissues

Plasma total cholesterol (TC), triacylglycerols (TAG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were estimated enzymatically, using Spinreact diagnostics kits (Girona, Spain). Total lipids were extracted from testicular tissues using the method of Folch et al (1957). Testicular tissue (0.5 g) was homogenized using 10 mL chloroform:methanol mixture (2:1). After homogenization, the sample was evaporated. The sediment was suspended in 10 mL of the same homogenate solvent and then mixed with 2 mL distilled water containing 0.017% $MgCl_2$. After vortexing the mixture was separated into two layers. The lower layer was taken for analysis of TC and TAG content using Spinreact diagnostics kits (Girona, Spain).

Determination of oxidative stress markers

Determination of thiobarbituric acid reactive substance (TBARS) in testes homogenates

For estimation of the different oxidative stress parameters and the antioxidant enzymes, a sample of testis (0.25 g) was ice-cooled, homogenized in 2.5 mL phosphate buffer (pH 7.4), and then centrifuged at 3000 *g* for 15 min at 4°C. The supernatant was collected and lipid peroxidative products were quantified by measuring the formation of TBARS as described by Preuss et al (1998), using 1,1 3,3-tetramethoxypropane as the standard. The data were expressed as malondialdehyde equivalents (nm MDA (g tissue)$^{-1}$).

Plasma lipoprotein oxidation susceptibility (LOS)

LOS was measured according to the principle of Dujovne et al (1994). Briefly, very low-density lipoprotein cholesterol (VLDL-C) and LDL-C were precipitated from a 500-μL plasma sample by 100 μL dextran sulfate/magnesium chloride. The pellet was dissolved in 4% saline solution. A volume of the redissolved precipitate containing 100 μg non-HDL-C was mixed with 4% sodium chloride to give 500 μL total solution. Copper solution (0.5 μM $CuCl_2$) was added and incubated at 37°C for 5 h in a shaking water bath. The solution was assayed for TBARS as an index for oxidation. The MDA (nmol) present in the samples was estimated from a standard curve prepared using 1,1,3,3 tetraethoxypropane.

Determination of antioxidant parameters

Determination of plasma antioxidant vitamins

Vitamin C in plasma was extracted as follows: plasma protein (100 μL) was precipitated with 400 μL 60% methanol and 1 mM EDTA (60% methanol/EDTA), incubated for 10 min at 4°C and centrifuged at 12000 *g* for 8 min. The clear phase was evaporated to dryness under nitrogen. The dried extracts were dissolved in 100 μL methanol before injecting into the HPLC system. Vitamin E and β-carotene in plasma were extracted as follows: plasma was deproteinized with ethanol, extracted with chloroform and then centrifuged for 5 min. The organic layer was extracted and evaporated to dryness under nitrogen. The dried extracts were dissolved in 100 μL methanol before injection in the HPLC system.

Determination of antioxidant enzymes in testes homogenates

Superoxide dismutase (SOD) activity was determined spectrophotometrically. The rate of inhibition of pyrogallol autooxidation is directly proportional to the activity of SOD in the testicular tissue. Reduced glutathione contents were measured following its reaction with 5,5-dithiobis-(2-nitrobenzoic acid) in phosphate buffer, pH 8.

Determination of inflammatory markers

Serum acute phase protein (C-reactive protein; CRP) and testicular tumour necrosis factor-α (TNF-α) concentration were evaluated using ELISA kits purchased from DiaMed Eurogen (Belgium) and Biosource (Camarillo, CA, USA). A 100-mg testis sample was homogenized in 0.1 M phosphate buffer (pH 7.2), containing 0.05% (w/v) sodium azide at 4°C. The testis homogenate was centrifuged at 2000 *g* for 10 min and the supernatant fraction was used for determination of TNF-α.

Determination of zinc and iron

Plasma and testes samples were wet-washed with 16 M nitric acid, evaporated and then diluted with 0.1 M nitric acid. Trace element concentrations were determined by flame atomic absorption spectrophotometer (Unicam Solaar 969).

Determination of serum testosterone

Serum testosterone was detected by the ElectroChemiLuminescence ImmunoAssay (ECLIA) (Roche Diagnostics, Indianapolis, USA).

Histological study

Small pieces of testes organ were sampled and fixed in 10% neutral buffer formalin and in Bowan's fixative, dehydrated and embedded in paraffin blocks. Sections 5-μm thick were stained with haematoxylin and eosin (H&E) under $\times$400 magnification.

Statistical analysis

All the data were expressed as mean $\pm$ s.d. Statistical analyses were performed using unpaired *t*-test. Pearson correlation was used to study any association between variables. *P* values <0.05 were considered statistically significant.

Results

Effect of frequent fast food consumption on lipid profile

Frequent fast food consumption increased significantly the plasma TAG level up to 41.01% and the testicular tissue content of TAG up to 84.45% at $P<0.0001$. Although there were no significant changes of total cholesterol in plasma or testicular tissue, plasma HDL-C concentration was decreased (31.4 ± 4.11 vs 40.3 ± 5.3, $P<0.001$) in association with plasma LDL-C concentration increase (62.1 ± 5.8 vs 50.2 ± 6.3, $P<0.0001$) in comparison with the normal control group (Table 1).

Effect of frequent fast food consumption on plasma antioxidant vitamins and lipid peroxidation markers

The chromatograms depicted in Figure 1A and B show the significant decrease in serum lipid soluble vitamin A (94.8 ± 10.1 vs 283.3 ± 47.0, $P<0.0001$) and vitamin E (79.0 ± 13.9 vs 247.7 ± 39.2, $P<0.0001$). Figure 1C and D show the decrease in water soluble vitamin C (304 ± 80.6 vs 755.3 ± 120.7, $P<0.0001$). Significant correlation between tissue zinc and vitamins A ($r=0.717$, $P<0.0001$), E ($r=0.920$, $P<0.0001$) and C ($r=0.685$, $P<0.0001$) were recorded. Regarding testicular enzymatic antioxidants, SOD and GSH activity were significantly reduced by 39.59% and 57.08% and correlated ($r=0.65$ and 0.895) with low levels of testicular zinc at $P<0.0001$, respectively. As a consequence of this, testicular TBARS reached 2.61-fold while plasma LOS was elevated up to 2.06-fold at $P<0.0001$ (Table 2). Reduced zinc levels negatively correlated with testicular TBARS ($r=-0.911$, $P<0.0001$) and plasma LOS ($r=-0.925$, $P<0.0001$).

Table 1 Effect of frequent fast food consumption on lipogram pattern and weight gain in weanling male rats

Parameter	Rats fed normal diet	Rats fed fast food diet
Plasma TC ($mg\,dL^{-1}$)	99.8 ± 11.4	103.2 ± 15.5*
Testicular TC ($mg\,g^{-1}$)	29.4 ± 3.7	31.4 ± 2.8
Plasma TAG ($mg\,dL^{-1}$)	82 ± 10.7	115.7 ± 12.6*
Testicular TAG ($mg\,g^{-1}$)	90.7 ± 9.1	167.3 ± 18.57*
Plasma HDL ($mg\,dL^{-1}$)	40.3 ± 5.3	31.4 ± 4.11**
Plasma LDL ($mg\,dL^{-1}$)	50.2 ± 6.3	62.1 ± 5.8*
Weight gain	196 ± 14.6	211 ± 23.15

All values are expressed as mean $\pm$ s.d., n = 10. *$P<0.0001$ and **$P<0.001$ as compared with rats fed a normal diet.

Effect of frequent fast food consumption on inflammatory markers and testosterone level

CRP and TNF-α demonstrated significant decreases (3.5 ± 0.57 vs 1.28 ± 0.32, and 91.9 ± 15.8 vs 10 ± 2.1, $P<0.000$, respectively) in comparison with the normal group (Figure 2A and B). Zn deficiency correlated significantly with CRP ($r=-0.721$, $P<0.0001$) and with TNF-α ($r=-0.766$, $P<0.0001$). Serum testosterone was sharply decreased (1.16 ± 0.24 vs 2.43 ± 0.4, $P<0.0001$) in comparison with the normal rats (Figure 2C). Testosterone correlated positively with serum Zn ($r=0.872$, $P<0.0001$).

Effect of frequent fast food consumption on Zn and iron

Zn levels decreased significantly in fast food fed rats in testicular tissue and in plasma (32.57% and 65.72% at $P<0.0001$, respectively). Simultaneously, iron accumulated up to 23.45% ($P<0.0001$) in testis tissues and in plasma by 57.74% ($P<0.0001$) in comparison with normal rats (Table 3).

Examination using light microscopy

Histological examination of the testis of normal control rats showed normal spermatogenesis, with seminiferous tubules comprising a complex stratified epithelium containing many layers of different generations of spermatogenic cells and supporting cells (Sertoli cells), as well as a tubular lumen and normal interstitium (Figure 3A). The testicular tissue of weanling rats fed fast food for 16 weeks was dramatically affected, with degenerative changes of the germinal epithelium. The lumen in the seminiferous tubules was absent and spermatogenesis had become extremely disorganized, whereas elongated spermatids were absent or severely reduced in number. Furthermore, spermatogenic cells appeared with dark small pyknotic nuclei, and there was severe germ cell sloughing of several seminiferous tubules. Figure 3B (H&E $\times$400) shows the area of vacuolation in the interstitium in addition to mononuclear cell infiltration.

Discussion

Oxidative stress is a major causal factor in altered steroidogenesis, spermatogenesis, and perhaps male infertility (Reddy et al 2006). Frequent fast food consumption resulted in a

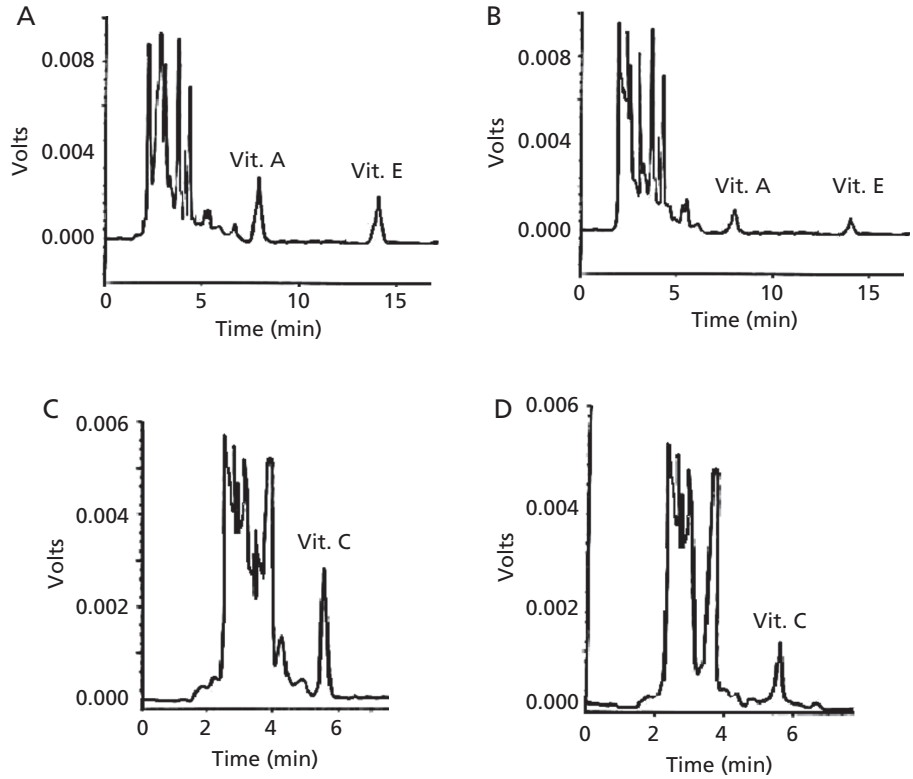

Figure 1 Chromatograms to show the effect of frequent fast food consumption on plasma antioxidant vitamins. A, Vitamins A and E in rats fed a normal diet; B, vitamins A and E in rats fed fast food; C, vitamin C in rats fed a normal diet; D, vitamin C in fast food fed rats.

Table 2 Effect of frequent fast food consumption on enzymatic antioxidants and lipid peroxidation markers in weanling male rats

Parameter	Rats fed normal diet	Rats fed fast food diet
SOD (U (mg protein)$^{-1}$)	64.4 ± 9	38.9 ± 7.8*
GSH (nmol (mg protein)$^{-1}$)	75.5 ± 10.6	32.4 ± 6.9*
TBARS (nmol MDA mg^{-1})	67.9 ± 12.1	177.3 ± 31.2*
LOS (nmol MDA (mg non-HDL)$^{-1}$)	50.7 ± 9.8	104.9 ± 14.9*

All values are expressed as mean ± s.d., n = 10. *$P < 0.0001$ as compared with rats fed a normal diet.

significant decrease of Zn content in plasma and testicular tissue. This decrease was positively correlated with the antioxidant enzymes and negatively with lipid peroxidative products to reveal a state of oxidative stress. The zinc-specific effect was localized within the testis. Zn can antagonize redox-active transition metal catalysts, such as copper and iron, in the Fenton pathway, thereby inhibiting the production of reactive oxygen species (ROS) (Powell 1994). Actually, we found obvious increases in iron with concomitant decrease in the Zn content in the testicular tissue and plasma. Iron accumulation in testis has been reported to be associated with oxidative damage as a potential source of ROS (Oteiza et al 1995). The latter can interact with polyunsaturated fatty acids, principally located in cellular membranes and lipoproteins, to initiate lipid peroxidation as revealed by the increment in TBARS and LOS. Furthermore, zinc functions as an antioxidant in CuZn-SOD, and can stimulate GSH synthesis through reduction of the GSH redox potential (Ha et al 2006). Zn deficiency impairs antioxidant enzymes and enhances ROS endogenously (Chen et al 2007) and particularly in testicular tissue (Nair et al 2005). Changes in expression of antioxidant enzymes can increase testicular TBARS (Reddy et al 2006) and LOS (Guo et al 2001). Moreover, antioxidant vitamins A, E and C decreased significantly and in correlation with Zn deficiency to contribute to the observed decline in testosterone secretion observed by Cao et al (2004). They react with free radicals, notably peroxyl radicals, and with singlet molecular oxygen. They also promote the production of scavenger antioxidant enzymes and inhibit the lipid peroxidation indexes; testicular TBARS (Jialal et al 1991; Ernster et al 1992; El-Missiry 1999) in addition to LOS (Jialal et al 1991). Ascorbic acid protects enzymatic antioxidants and the loss of zinc from testis (El-Missiry 1999). Despite the fact that fast food meals are already poor in vitamins (Paeratakul et al 2003), vitamins are definitely affected by Zn levels, possibly affecting absorption (Noh & Koo 2003), metabolism (Sundaresan et al 1977) and transport of vitamin A (Christian & West 1998). A low level of Zn also impairs the absorption of vitamin E (Kim et al 1998). Redox state regulates the inflammatory signals in a ROS-dependent mechanism (Haddad 2002). Zn deficiency is inversely correlated with serum CRP (Ghayour-Mobarhan et al 2005) and TNF-α, which are known to generate ROS and alter the expression of the antioxidative enzymes. This is one

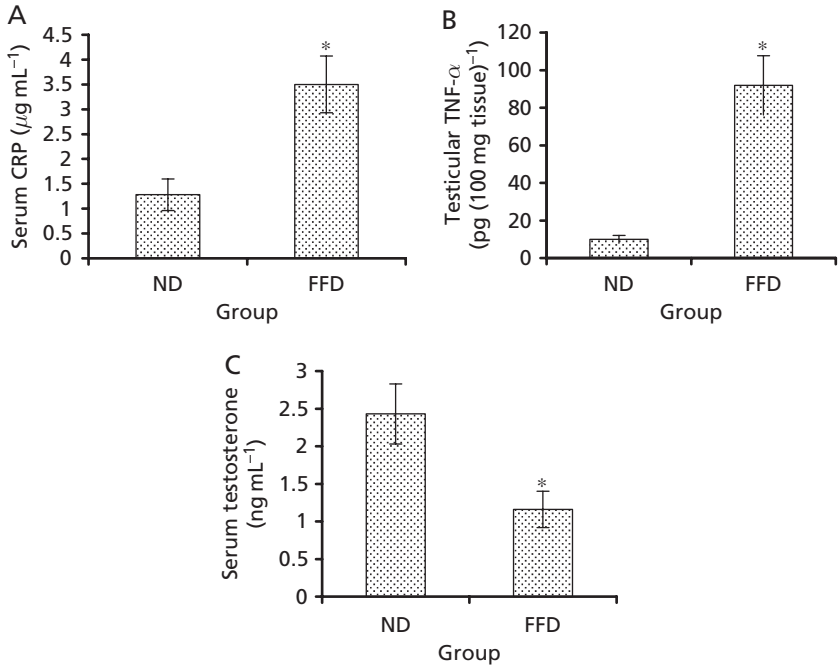

Figure 2 Effect of frequent fast food consumption on (a) serum CRP (μg mL^{-1}), (b) testicular TNF-α (pg/100 mg tissue), and (c) serum testosterone (ng mL^{-1}) in weanling rats. All values were expressed as mean ± s.d., n = 10. *$P < 0.0001$ as compared with normal rats. ND, normal diet; FFD, fast food diet.

Table 3 Effect of frequent fast food consumption on zinc and iron in plasma and testis of weanling rats

Element	Rats fed normal diet		Rats fed fast food diet	
	Plasma (μmol L^{-1})	Testis (nmol (g wet tissue)$^{-1}$)	Plasma (μmol L^{-1})	Testis (nmol (g wet tissue)$^{-1}$)
Zinc	24.8 ± 1.6	223.82 ± 4.25	8.5 ± 0.8*	150.9 ± 11.9*
Iron	33.2 ± 4.7	318.5 ± 3.7	52.37 ± 3.3*	393.2 ± 14.4*

All values are expressed as mean ± s.d., n = 10. *$P < 0.0001$ as compared with rats fed a normal diet.

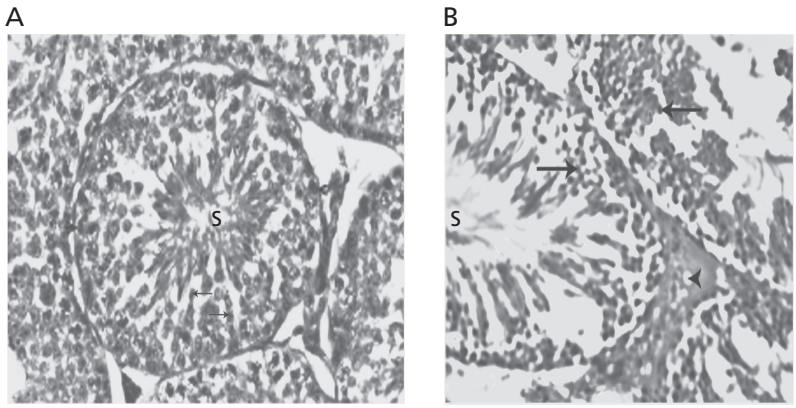

Figure 3 Photomicrographs to show sections of testis of weanling rats after 16 weeks consumption of either a normal diet (A) or a fast food diet (B). A. Normal histology of seminiferous tubules, where seminiferous cords consisted of different types of spermatogenic cells (arrow), Sertoli cells (arrow head), sperms in the centre of tubules (S), and normal interstitium. B. Severe degenerative changes of the seminiferous cords. Focal area showed a decrease in spermatogenesis. Most of the spermatogenic cells appeared with dark small pyknotic nuclei (arrow). Severe germ cell sloughing and sperms were absent or reduced in number (S). Enlarged and congested interstitium with vacuolation and monomolecular cellular infiltrate.

additional mechanism by which zinc may be functioning as an antioxidant. TNF-α severely affects seminiferous tubules and consequently causes the decrease in testosterone levels (reviewed by Prasad (2008)).

It is well established that fast food is deficient in micronutrients, and when weanling male rats were fed a fast food diet for a long time it caused a severe decrease in Zn levels in plasma and testicular tissue. This decrease consequently led to damage of the seminiferous tubules with concomitant reduction of testosterone level. This may have been due to oxidative damage of testis lipids and regulation of pro-inflammatory signals.

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