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Reproductive disorders in male rats induced by high-fructose consumption from juvenile age to puberty

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There is compelling evidence that a hypercaloric, high-fructose diet can cause metabolic syndrome (MetS) and a whole range of other metabolic changes. In the context of androgen deficiency, MetS in boys merits special attention, but the effects of fructose-rich diet in youth on future male reproductive function are still poorly evidenced. The aim of this study was to address this issue and analyse the effects of high-fructose intake starting from weaning to puberty (postnatal day 23 up to 83) on the reproductive function of male rats. For this purpose juvenile male Wistar rats were divided in two groups: control and the group receiving 10 % fructose solution instead of drinking water. Reproductive function was evaluated in terms of fertility, sperm count, testes/epididymis morphology, and serum sex hormones. The fructose-treated group showed a decrease in testosterone and twofold increase in luteinising and follicle-stimulating hormone levels in the serum. This was accompanied with lower testis/epididymis weights, sperm count, and changed testis/epididymis morphology. Their fertility remained unchanged, but the fertility of females mating with these males diminished. In addition, pre-implantation and post-implantation embryonic death rate rose in these females. Our results have confirmed that high fructose consumption from early age until puberty can impair the reproductive function of male rats, and call for further animal and epidemiological investigation.

KEY WORDS: epididymis; fertility; FSH; LH; male reproductive function; metabolic syndrome; MetS; sperm count; testes; testosterone

Today, there is no doubt that the roots of metabolic disorders lie in childhood and adolescence (1, 2). The high prevalence of obesity, especially in developed countries, leads to a significant increase in the number of children and adolescents diagnosed with metabolic syndrome (MetS) (3). MetS has also been associated with high fructose corn syrup consumption by both children and adults (4) and is one of the greatest healthcare challenges worldwide (5).

There is also compelling evidence that diets high in fructose have implications beyond excess caloric consumption (6). High-fructose diet has been shown to induce a whole range of metabolic changes not only in animal models but also in humans. Bettaieb et al. (7) reported typical pathogenic features of MetS (insulin resistance, obesity, dyslipidaemia, and hypertension) in experimental animals. In humans, hypercaloric fructose intake has been associated with increased plasma uric acid as a byproduct of uncontrolled fructose metabolism and a potential cause of MetS (8). Other reports (9, 10) implicate advanced glycation end products because of their direct protein modifications and indirect effects on inflammation and oxidative stress with subsequent impairment of several tissues and organ functions. According to DiNicolantonio et al. (11), fructose-induced inflammation activates cortisol and causes visceral adiposity. Other authors propose other mechanisms and effects, such as high plasma triacylglycerol and intrahepatic fat deposition (12), ATP depletion and dysfunctional energy metabolism (13), and ectopic fat accumulation, particularly in the liver (14). In addition, recent reports of significantly higher total sugar and fructose intakes seem to coincide with the current epidemics of obesity and related metabolic disorders (15–17).

A critical period for the development of MetS is adolescence (from the onset of puberty to its completion), as manifested by increased insulin resistance both in healthy adolescents and those with diabetes mellitus (18, 19). At the same time, MetS is three times more common in teen boys than girls (20) and deserves special attention.

MetS and obesity in childhood are believed to result in poor hormonal or metabolic "programming" that persists throughout life (21), including androgen deficiency (reduced testosterone levels) and hypogonadism, whose frequency and severity seems to reflect obesity and carbohydrate metabolism disorders (22). However, this association is far from clear, and the evidence of long-term effects of MetS in adolescence on male reproductive function in adulthood is still scarce.

Taking into account all of the reports referred to above and a recent animal study (23) showing the adverse effects

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of high fructose corn syrup intake on the antioxidant state of testes and sperm counts in 12–16 weeks old rats, we focused on the effects of fructose-rich diet from the weaning age on future male reproductive function, including fertility, sperm count, germ cell morphology, and serum hormones [testosterone (TS), luteinizing hormone (LH), follicle-stimulating hormone (FSH)].

MATERIALS AND METHODS

Study design

For the study we used male Wistar rats aged 23 postnatal days and weighing 50–70 g at entry. They were kept under controlled conditions (temperature 22–24 °C, relative humidity 40–70 %, and 12 h light/dark cycle) and fed on a standard pellet feed (Research Limited Liability Company "F.U.D", Tetiiv, Ukraine).

High-fructose intake protocol was reproduced from Bettaieb et al. (12). Animals from different litters were divided in two groups of 42.The control group was receiving drinking water in addition to standard feed, whereas the high-fructose (HF) group was receiving a 10 % fructose solution (100 g/L) instead of drinking water. The physiological outcomes associated with supraphysiological concentrations of fructose cannot be used to extrapolate the effects to human health. We have therefore opted for a lower fructose concentration that reflects those found in sugar-sweetened beverages (~10 % w/v) (24).

The solutions were prepared by mixing crystalline fructose (Shandong Xiwang Sugar Industry Co., Ltd., Binzhou, China) with drinking water on a daily basis and given to the HF group ad libitum for 60 days.

On the morning of day 61, the rats were anaesthetised with mild ether (80 µL/L) for about 5 min and then decapitated. Testes and epididymis were removed for immediate histological examination.

The procedure followed the 1986 UK Animals (Scientific Procedures) Act (25) and the EU Directive 2010/63/EU (26) and was approved by the Institute's Animal Care and Use Committee (approval number 01/07/15).

Fertility evaluation

On day 47 of the experiment, the males from both groups were separated from each other and paired with healthy, unexposed females at the 1:1 ratio for 14 nights (three oestrous cycles), during which the HF group males were receiving water instead of fructose solution. On the first day of pregnancy, the females were placed in individual cages, and the males returned to their initial cages, in which the HF group continued receiving the 10 % fructose solution. The first day of pregnancy was established in the morning by vaginal cytology (sperm detection in the vagina) according to the generally accepted guidelines for fertility studies in laboratory rats (27). Most males impregnated females within the first five days of cohabitation (i.e. at the first oestrus), but some demonstrated infertility. This was taken into account when we evaluated male fertilising capacity using the following formula:

Male fertilising capacity (%) = number of pregnant females/number of females paired with individual male x 100 [1]

Embryonal/foetal loss determination

Pregnancy was confirmed by laparotomy at necropsy after having sacrificed female rats under mild ether anaesthesia by cervical dislocation on day 20 of pregnancy.

We immediately counted the number of *corpora lutea* in the ovaries, implantation sites, and live and dead foetuses in each uterine horn and calculated the percentages embryonic/foetal loss before and after implantation according to standard procedure (27) using the following formulas:.

- Preimplantational loss (%) = (number of *corpora lutea* – number of implantation sites) / number of corpora lutea x 100 [2]
- Postimplantational loss (%) = number of lost foetuses / number of implantation sites x 100 [3]

Serum testosterone, LH, and FSH

Immediately before the male rats were sacrificed, we took their blood from the femoral vein. Serum was separated and samples kept frozen at -70 °C. Testosterone (T), luteinising hormone (LH), and follicle-stimulating hormone (FSH) levels were measured using testosterone, LH, and FSH ELISA kits (DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's instructions.

Spermatogenesis parameter evaluation

For all morphological and morphometric parameters we used the right testicle and epididymis. The tissues were fixed in Bouin's solution (BS) for 6–8 h. Picric acid from BS was first removed by washing the fixed tissues in 70 % ethanol and then by transferring the specimen to a saturated lithium carbonate solution in 70 % ethanol, which was changed at least three times before the tissue was rid of the yellow colour of BS. The tissues were then dehydrated with graded ethyl alcohol and embedded in paraffin. Tissue sections (6 µm) were stained with haematoxylin and eosin and histologically examined under a light microscope Olympus BX41 (Tokyo, Japan).

Testicle spermatogenesis was evaluated based on the estimated number of cell layers, types of cells, and the presence of late spermatids in the seminiferous tubules. The evaluation grades (corresponding to spermatogenesis stages) were as follows: 1 – only spermatogonia present; 2 – spermatogonia and spermatocytes present; 3 – spermatogonia, spermatocytes, and round (early) spermatids present with <5 late spermatids per tubule; and 4 –

spermatogonia, spermatocytes, and round spermatids present with <25 late spermatids per tubule (26). Spermatogenesis index was calculated as the ratio of the sum of grades to the number of examined seminiferous tubules (200 per testis of each animal).

We also observed cell exfoliation (shedding of the epithelial elements), epithelium desquamation (detachment) from the tubule basal membrane, and the presence of cell-free regions (vacuoles) as indicators of disrupted spermatogenesis.

Sperm count in *cauda epididymis* was estimated as described by Chitra et al. (28) using Goryaev's counting chamber and a light microscope (200x).

Statistical analysis

The obtained numerical data were recorded and processed with STATISTICA v. 10 (StatSoft, Inc., Tulsa, OK, US). The normality of distribution of continuous variables was tested with one-sample Kolmogorov-Smirnov test. Continuous variables with normal distribution are presented as mean (±standard deviation [SD]), geometric mean, and 95 % confidence intervals and compared with independent samples Student's *t*-test. Variables without normal distribution are reported as median (and interquartile range [IQR]) and compared with the Mann-Whitney U test. The frequencies of categorical variables were compared with either Pearson's chi-squared or Fisher's exact test, as appropriate. Differences were considered statistically significant at $p<0.05$.

RESULTS

Serum sex hormone levels and gonad findings

The HF group showed a significant drop in serum testosterone levels (28 % compared to control), accompanied by a two-fold increase in LH and FSH levels (Table 1).

It also showed significantly lower absolute weight and volume of the testicles, lower absolute weight of epididymis and 26 % lower sperm count than controls (Table 2).

Histopathological findings

High fructose intake was accompanied by destructive changes in the seminiferous epithelium. Table 3 shows a moderate yet statistically significant drop in spermatogenesis index in comparison with the control group. The spermatogenic cell population was also affected through inhibited mitotic activity and a 17 % drop in the number of spermatogonia in seminiferous tubules compared to control.

Table 1 Comparison of serum sex hormone levels between the high-fructose-treated and control group

Parameters	Groups		*P* value
	Control	High-fructose	
T (nmol/L)	14.96±3.10 14.69 (12.09–17.82)	9.34 (10.25; 12.25)	0.024*
LH (ng/mL)	0.301±0.231) 0.174 (0.124–0.478)	0.691±0.189 0.622 (0.505–0.797)	0.003*
FSH (ng/mL)	0.10±0.06 0.077 (0.044–0.157)	0.218 (0.171; 0.235)	0.021*

* significantly different; T – testosterone; LH – luteinising hormone; FSH – follicle-stimulating hormone. Normally distributed data are presented as means ± standard deviations and geometric mean (95th confidence intervals); Data not distributed normally are reported as medians and interquartile ranges (IQR)

Table 2 Comparison of gonad weight, volume, and sperm counts between the high-fructose-treated and control group

Parameters	Groups		*P* value
	Control	High-fructose	
Testes absolute weight (g)	3.49±0.24 3.48 (3.31–3.68)	3.21±0.19 3.20 (3.06–3.36)	0.014*
Testes relative weight (% of body weight)	1.17±0.08) 1.16 (1.10–1.23)	1.28±0.17 1.27 (1.15–1.41)	0.09
Testes volume (cm^3)	3.81±0.39 3.79 (3.53–4.10)	3.37±0.45 3.34 (3.05–3.69)	0.029*
Epididymis absolute weight (g)	0.96 (0.95; 0.98)	0.88±0.11 0.88 (0.81–0.95)	0.047*
Epididymis relative weight (% of body weight)	0.33±0.04 0.32 (0.30–0.35)	0.35±0.02 0.35 (0.32–0.38)	0.217
Sperm count (million/mL)	70.26±6.96 68.43 (53.23–87.28)	52.57±3.19 52.01 (46.31–58.82)	0.046*

* significantly different. Normally distributed data are presented as means ± standard deviations and geometric mean (95th confidence intervals); Data not distributed normally are reported as medians and interquartile ranges (IQR)

In addition, the number of cells in the 12th stage of spermatogenesis (characterised by meiosis of the primary spermatocytes) was 1.4 times lower than in the control group.

We also observed degenerative changes in the testes of the HF group, such as epithelium exfoliation into the lumen of seminiferous tubules (Table 3).

Figure 1 (A through D) shows the photomicrographs of rat testes. In the control group, seminiferous tubules and layers of seminiferous epithelium had normal structure. In the HF group we found both normal tubules and those undergoing pathological changes. In the former, all layers of the seminiferous epithelium were represented, and dystrophic changes in the germ cells were absent. In the latter, we observed dissolution of the seminiferous epithelium, indicated by wide gaps between neighbouring cells and enlarged intercellular spaces, and a drop in the number of spermatogonia and spermatocytes (Figure 1D). Some tubules had dystrophic spermatocytes without clear boundaries and nuclear membranes. Some primary spermatocytes also showed margination of large chromatin granules. These structures were strongly stained with haematoxylin, which could indicate apoptotic cell death.

The lumens of some tubules showed exfoliated germ cells, including dystrophic spermatids, primary spermatocytes, and a small number of spermatozoids.

Figure 2 (A through D) shows the photomicrographs of rat epididymis. The control rats had normal spermatozoon density and intact basement membrane. Epididymis tubules of HF rats did not differ significantly from control. Some, however, did show dystrophic epithelial cells. In control animals, the epididymis tubules were covered by a two-layered pseudostratified high epithelium with round nuclei, but the tubules of HF rats showed hyperplasia of epithelial clear cells accompanied by increased debris in the lumen. Their epithelial cells also showed vacuolation of the cytoplasm and pyknotic nuclei. We also observed luminal sperm stasis.

Male rat fertility and embryonic/foetal development of offspring

The fertilising capacity of male rats receiving high-fructose did not differ from control (Table 4).

Nevertheless, the fertility rate of females not exposed to high-fructose treatment and mated with these males had a tendency to decrease (Table 5).

High-fructose treatment significantly increased preimplantational loss of embryos/foetuses (Table 6). The level of postimplantational embryonic and foetal death was also higher.

Total embryonic/foetal loss in the HF group was 12 % higher than in controls.

DISCUSSION

Between juvenile age and puberty, male rats and mice fully develop the hypothalamic-pituitary-gonadal (HPG) axis and reproductive capacity (29) and critically rely on the androgen-oestrogen balance for normal pubertal development (30). Spermatogenesis and steroidogenesis are not fully established until puberty, which makes the male genital system more susceptible to external adverse effects early in life (31). Our findings clearly show that high-fructose intake caused primary hypogonadism in rats. This was confirmed by lower serum testosterone counts and higher LH and FSH levels compared to controls, as described elsewhere (32).

A decrease in the relative and absolute testis weight in the HF group indicates reproductive impairment. Reduced testis weight generally reflects germ cell loss and decreased tubule fluid production (33), but in our experiment it may

Table 3 Spermatogenesis epithelium findings

Parameters	Groups		*P* value
	Control	High-fructose	
Spermatogenesis index (stages of spermatogenesis total / number of examined tubules)	3.44±0.21 3.43 (3.42–3.45)	3.34±0.05 3.34 (3.31–3.38)	<0.001*
Number of spermatogonia (per tubular cross section)	71.83±5.43 71.64 (68.38–75.28)	59.80±4.24 59.64 (56.99–62.61)	<0.001*
Cells at the 12th stage of spermatogenesis (%)	4.00 (3.0; 4.5)	2.83±1.21 2.53 (2.03–3.64)	0.008*
Exfoliation of epithelium (%)	1.0 (1.0; 2.0)	2.5 (1.5; 3.0)	1.000

* significantly different. Normally distributed data are presented as means ± standard deviations and geometric mean (95th confidence intervals); Data not distributed normally are reported as medians and interquartile ranges (IQR)

Table 4 Fertilising capacity of male rats

Groups	Number of mated females	Number of pregnant females	Fertilising capacity
Control	42	38	91 %
High-fructose group	42	41	98 %
P value	-	-	0.809

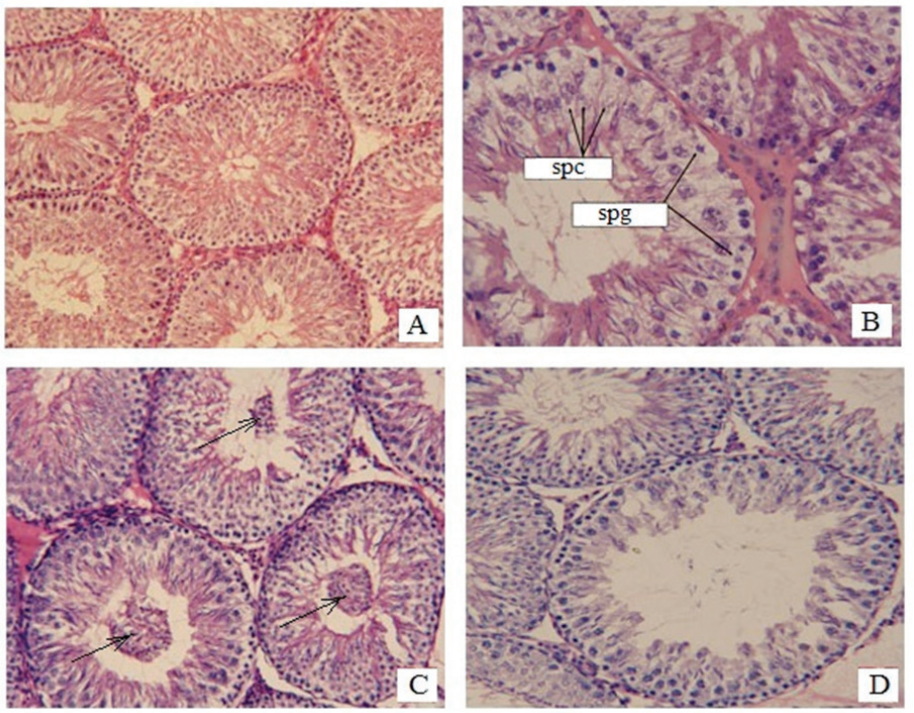

Figure 1 Photomicrographs of rat testes stained with haematoxylin and eosin; (A) normal structure of seminiferous tubules in control rats (200x magnification); (B) focal dystrophic changes in spermatogonia (spg) and primary spermatocytes (spc) in HF rats (400x magnification); (C) spermatogenic epithelium exfoliation into the lumens of seminiferous tubules in HF rats (200x magnification); (D) decrease in the number of spermatogenic epithelium layers and expansion of the seminiferous tubules in HF rats (400x magnification)

have also been related to delayed puberty onset (34). Similarly, epididymis weight was also lower in the HF group. Taking into account our results on serum testosterone decrease, changes in testicular weight and volume and in epididymis weight can be attributed to altered testicular endocrine function. In the testes, increased or decreased testosterone secretion usually provokes morphological changes.

Our morphometric findings and testosterone, LH and FSH levels are in good accordance with the lower sperm count and destructive changes in the spermatogenic epithelium in the HF group of rats. High fructose intake led to lower spermatogenesis compared to control males. This is strong evidence of germ cell death, which is also evidenced by the presence of pyknotic nuclei and lower pachytene spermatocyte count at stage 12. The death is

Table 5 Fertility rate of unexposed females mated with HF males

Groups	Parameters	
	Total number of foetuses	Number of foetuses per female
Control	319	9.5 (7.5; 10.5)
High-fructose group	300	9 (6; 9)
P value	-	0.092

Data are reported as medians and interquartile ranges (IQR)

Table 6 Effects of paternal high-fructose treatment on embryogenesis/foetogenesis in unexposed females on day 20 of gestation

Parameters	Groups		*P* value
	Control	High-fructose	
Number of pregnant females (N)	38	41	-
Total number of corpora lutea (N)	362	394	-
Number of corpora lutea per female (N)	10.5 (8.5; 11)	10 (9; 11)	1.000
Preimplantational loss (N /%)	25 / 10	60 / 15	0.012*
Preimplantational loss per female (N)	0 (0; 1)	1 (0; 2)	0.028*
Postimplantational loss (N /%)	6 / 2	19 / 6	0.013*
Postimplantational loss per female (N)	0 (0; 0)	0 (0; 1)	0.041*
Total embryonal/foetal death (%)	11.88	23.86	<0.001*

* significantly different. Data are reported as medians and interquartile ranges (IQR)

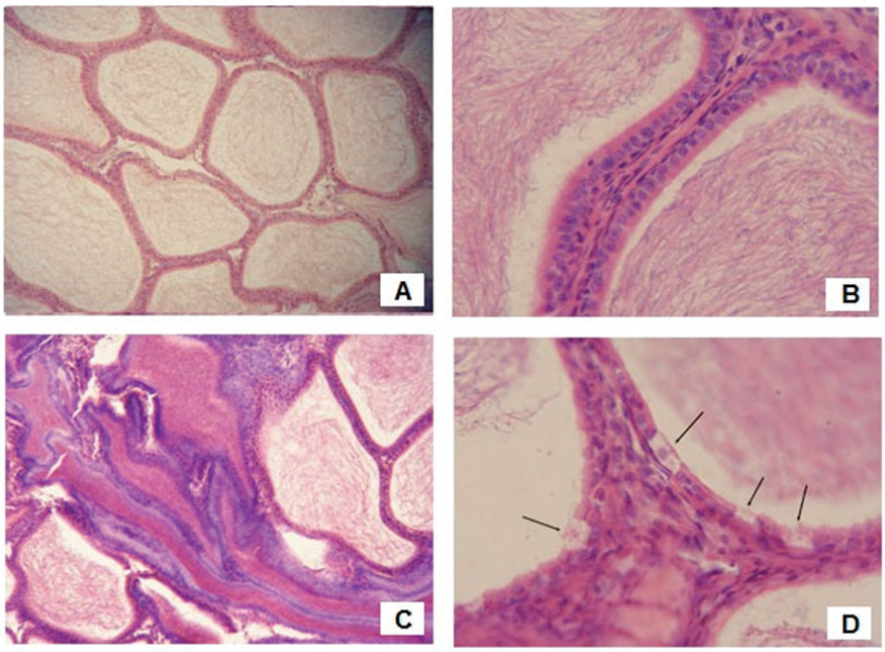

Figure 2 Photomicrographs of rats epididymis stained with haematoxylin and eosin; (A and B) normal epididymis tubule structure in control rats (100x and 400x magnification, respectively); (C) focal luminal sperm stasis in HF rats (100x magnification); (D) epithelial clear cell hyperplasia in HF rats (400x magnification)

usually apoptotic, and dying spermatocytes generally develop cytoplasmic eosinophilia and nuclear pyknosis, while round spermatids show chromatin margination (33). This is the pattern we observed when evaluating histopathological changes in the testes of rats with high fructose intake.

The germ cell depletion in HF rats can also be due to epithelium exfoliation into the lumens of seminiferous tubules. It evidences the loss of adhesion between Sertoli and germ cells and abnormal transport of these cells to the rete testis and epididymis (33). Collectively, the morphological changes in HF rat testes can be associated with lower intratesticular testosterone levels, which in healthy animals are about 50 times higher than serum testosterone levels (33). Our morphological findings in HF rats are in good accordance with the recent data published by Meydanli et al. (35).

In the group of rats with high fructose intake, we have also detected hyperplasia/hypertrophy of clear cells in the cauda epididymis (including cells with signs of degeneration) accompanied by increased debris in the lumens. The presence of debris, in turn, points to activated spermophagy (36).

Some efferent ducts of the HF rats showed sperm stasis. It could be a result of increased fluid resorption or blockage in blind-ending ducts. Considering our results, however, this was probably due to lower sperm production by the testes (36). Sperm stasis can also occur because of disrupted fluid dynamic and smooth muscle contraction in the epididymis and vas deferens (36).

Changes in spermatogenesis and normal sperm production following long-term fructose consumption seem to occur through various mechanisms. One is a decrease in testosterone, observed in our HF rats, and testosterone plays a key role in insulin regulation (37). It is known that insulin resistance disturbs normal sperm morphology and function (38). Another mechanism is dyslipidaemia, which affects normal sperm development and function on molecular level and increases the levels of reactive oxygen species (ROS), inducing thus intracellular oxidation and inflammation on the metabolic level (37, 39, 40). Several studies have reported significant correlations between changes in semen and high levels of ROS, indicating that oxidative stress adversely affects male fertility (41). Spermatozoa seem to be highly susceptible to oxidative stress because of their inadequate cell repair system, insufficient antioxidant defences, and high content of polyunsaturated fatty acids in their plasma membrane (42). If we accept that prolonged consumption of fructose leads to the development of MetS, it is also necessary to take into account the deregulation of glucose homeostasis, which is extremely important for the control of the testicular microenvironment and may be one of the causes of disturbed spermatogenesis (43, 44).

Surprisingly, in spite of the changes in spermatogenesis, cellular content of the epididymis, and lower sperm count, we observed no decline in the fertilising capacity of the HF group. However, unexposed females mating with the HF males still demonstrated lower fertility rate than females mating with controls. They had a higher preimplantational embryonic/foetal loss, which usually points to lethal mutations in the germ cells of males (45). Considering our assumption that prolonged high fructose intake can increase ROS generation in germ cells, it is important to note that ROS adversely affects sperm nuclear DNA, including DNA

fragmentation, chromatin cross-linking, base-pair modifications, and chromosomal microdeletions (46). In turn, sperm DNA damage is known to be associated with lower embryo quality, implantation rates, and, possibly, early onset of some childhood diseases (46).

CONCLUSIONS

Our findings confirm that high fructose intake starting at juvenile age can impair the reproductive function of male rats. High fructose intake leads to hypogonadism (confirmed by lower serum testosterone and higher LH and FSH levels), smaller sexual organ weight, disturbed spermatogenesis, changed cellular content in the epididymis, and lower sperm count. In addition, embryonic loss in females mating with males exposed to high fructose intake suggests lethal mutations in male germ cells. Our findings call for further investigation of the links between high fructose consumption and male reproductive disorders in animal models and epidemiology.

Conflict of interests

None to declare.

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Poremećaji reprodukcijske funkcije u mužjaka štakora uzrokovani unosom tekućine bogate fruktozom od 23 dana starosti do puberteta

Postoje snažni dokazi da hiperkalorična prehrana bogata fruktozom može uzrokovati metabolički sindrom (MetS) i cijeli niz drugih promjena u metabolizmu. U smislu androgene deficijencije, MetS u dječaka izaziva posebnu pažnju, ali nema mnogo spoznaja o učincima prehrane bogate fruktozom u ranoj mladosti na buduću reprodukcijsku funkciju u muškaraca. Stoga je cilj ovoga istraživanja bio analizirati učinke unosa tekućine bogate fruktozom u mladih mužjaka štakora od trenutka kad su prestali sisati (23 dana starosti) do puberteta (83 dana starosti) na njihovu reprodukcijsku funkciju. U tu su svrhu muški Wistar štakori podijeljeni u dvije skupine: kontrolnu i onu koja je primala 10 %-tnu otopinu fruktoze umjesto vode za piće. Parametri procjene reprodukcijske funkcije obuhvatili su plodnost, broj spermija, morfologiju testisa (sjemenika) i epididimisa (pasjemenika) te razine spolnih hormona u serumu. U skupini koja je primala fruktozu zamijećeno je smanjenje razine testosterona i dvostruko povećanje razina luteinizirajućega i folikulostimulirajućega hormona u serumu u odnosu na kontrolnu skupinu. Te su promjene popraćene padom težine testisa i epididimisa, broja spermija te promjenama u morfologiji testisa i epididimisa. Plodnost im se nije promijenila, ali je zato plodnost ženki koje su se parile s mužjacima izloženima fruktozi bila smanjena. Osim toga, u tih se ženki povećala smrtnost embrija prije i nakon implantacije u odnosu na ženke koje su se parile s kontrolnim mužjacima. Naši rezultati potvrđuju pretpostavku da konzumacija hrane i pića bogatih fruktozom od rane dobi do puberteta može oštetiti reprodukcijsku funkciju u štakora. Stoga je potrebno provesti daljnja istraživanja u životinja te epidemiološka istraživanja u ljudi.

KLJUČNE RIJEČI: epididimis; FSH; LH; metabolički sindrom; MetS; plodnost; spermiji; testis; testosteron