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EXPRESSION AND ACTIVITY OF ANTIOXIDANT ENZYMES IN THE LIVER OF MALE AND FEMALE FRUCTOSE-FED RATS

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ЕКСПРЕСИЈА И АКТИВНОСТ ЕНЗИМА АНТИОКСИДАТИВНЕ ЗАШТИТЕ У ЈЕТРИ МУЖЈАКА И ЖЕНКИ ПАЦОВА НАКОН ИСХРАНЕ ОБОГАЋЕНЕ ФРУКТОЗОМ

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Expression and activity of antioxidant enzymes in the liver of male and female fructose-fed rats

Abstract

Fructose overconsumption has been related to metabolic syndrome and its sequels. Oxidative stress has been proposed as a mechanism underlying adverse metabolic effects of fructose. The aim of this study was to learn whether fructose-rich diet induces hepatic oxidative stress, thus contributing to aggravation of metabolic disturbances in a gender-dependent manner.

Toward that aim, we exposed male and female rats to moderate and high fructose diet over a period from weaning to adulthood, and subsequently evaluated: daily food, liquid and energy intake; physiological and biochemical parameters; expression and/or activity of hepatic antioxidant enzymes, and markers of lipid peroxidation and protein damage.

A decrease in food intake; an increase in liquid intake, energy intake and triglyceridemia; and no changes in body mass, insulinemia and the level of hepatic triglycerides were observed in all fructose-fed rats as compared to controls, irrespectively of the gender and the diet regime. Females on moderate and males on high fructose diet displayed increased adiposity. Plasma levels of NEFA were increased only in males on moderate diet. The activity and level of hepatic antioxidant enzymes, and markers of lipid peroxidation and protein damage were not altered in rats of both genders in response to both diets, the only exception being mitochondrial SOD2 function in males.

In conclusion, moderate fructose diet led to gender-specific metabolic disturbances in young rats, resulting from activation of adipose tissue lipolysis in males and lipogenesis in females. Fructose overconsumption did not provoke hepatic oxidative stress in the rats of any gender. Nevertheless, a possible mediatory role of mitochondrial SOD2 in development of insulin resistance needs further investigation.

Key words: Antioxidant enzymes, fructose-fed rat, liver, oxidative stress, male rat, female rat.

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Експресија и активност ензима антиоксидативне заштите у јетри мужјака и женки пацова након исхране обогаћене фруктозом

Сажетак

Прекомерно конзумирање фруктозе повезује се са метаболичким синдромом и његовим последицама, а оксидативни стрес је један од претпостављених механизама на којима се заснивају штетни ефекти фруктозе. Циљ ове студије био је да се испита да ли исхрана богата фруктозом индукује оксидативни стрес у јетри и на тај начин доприноси погоршању метаболичких поремећаја на полно специфичан начин.

У том циљу, изложили смо мужјаке и женке пацова умерено- и високо-фруктозној исхрани током периода од одвајања од мајке до полне зрелости и потом измерили: дневни унос хране, течности и енергије; физиолошке и биохемијске параметре; експресију и активност антиоксидативних ензима, маркера липидне пероксидације и оштећења протеина у јетри.

Код свих пацова храњених фруктозом, без обзира на пол и режим исхране, запажено је смањење уноса хране; повећање уноса течности и енергије, и триглицеридемије; као и непромењена телесна маса, инсулинемија и ниво триглицерида у јетри. Женке на умереној и мужјаци на високо-фруктозној исхрани имали су повећан индекс адипозности. Ниво масних киселина у плазми био је повећан само код мужјака на умереној дијети. Активност и ниво антиоксидативних ензима, као и маркера липидне пероксидације и протеинских оштећења нису били промењени у јетри пацова оба пола храњених фруктозом. Једини изузетак био је митохондријски ензим СОД2 код мужјака.

У закључку, умерено-фруктозна исхрана довела је до полно специфичних метаболичких поремећаја код младих пацова, што је резултат активације липолизе у масном ткиву мужјака и липогенезе у масном ткиву женки. Прекомерни унос фруктозе исхраном није изазвао оксидативни стрес у јетри пацова оба пола. Ипак, потребно је даље испитати могућу медијаторну улогу митохондријске СОД2 у развоју инсулинске резистенције.

Кључне речи: Антиоксидативни ензими, пацов храњен фруктозом, јетра, оксидативни стрес, мужјак пацова, женка пацова.

Научна област: Биологија

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Abbreviations

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CAT	Catalase
ECF	Enhanced chemifluoroscence
GLUT	Glucose transporter
GPx	Glutathion peroxidase
GR	Glutathion reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
Hsp	Heat shock protein
NEFA	Nonesterified fatty acids
PBS	Phosphate-buffered saline
PVDF	Polivinilidene difluoride
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD1	Cytoplasmic copper-zinc superoxide dismutase
SOD2	Mitochondrial manganese superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
UCP	Uncoupling proteins

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1. INTRODUCTION

1 INTRODUCTION

1.1 Fructose

1.1.1 Chemical and physical properties of fructose

Fructose, or fruit sugar is a monosaccharide discovered by French chemist Augustin-Pierre Dubrunfaut in 1847 (Hewitt, 1940). In the past it was named levulose, after its levorotatory property of rotating plane polarized light to the left (in contrast to glucose which is dextrorotatory). Fructose and glucose molecules have the same molecular formula but differ structurally, as fructose has a keto-group on the second carbon while glucose has an aldehyde group on the first carbon (Figure 1.1).

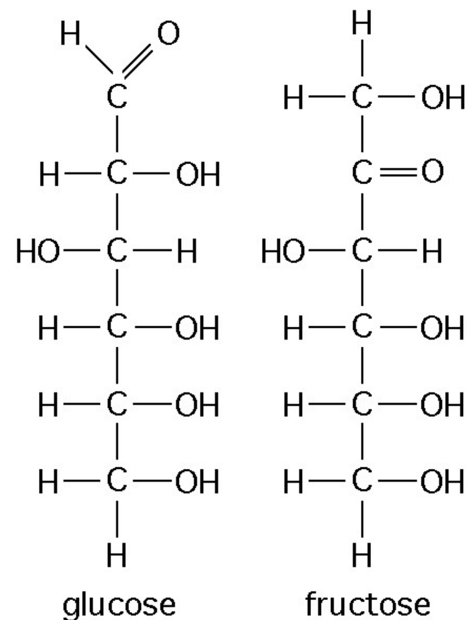

Figure 1.1 Structural formulae of glucose and fructose

Table 1.1 Physical properties of fructose

Molecular Formula	$C_6H_{12}O_6$
Molar mass	180.156 Da
Density	1.694 g/cm^3
Melting point	103 °C
Water Solubility	3750 g/L (20 ºC)

Fructose is ketohexose, a reducing sugar that exists in at least five tautomers in solution (Figure 1.2). At tautomeric equilibrium (20 °C in H_2O or D_2O) the distribution of the β-pyranose, β-furanose, α-furanose, α-pyranose and the *keto* tautomers was found to be approximately 69%, 22%, 6%, 2.5% and 0.50%, respectively (Shallenberger, 1978; Barclay *et al.*, 2012).

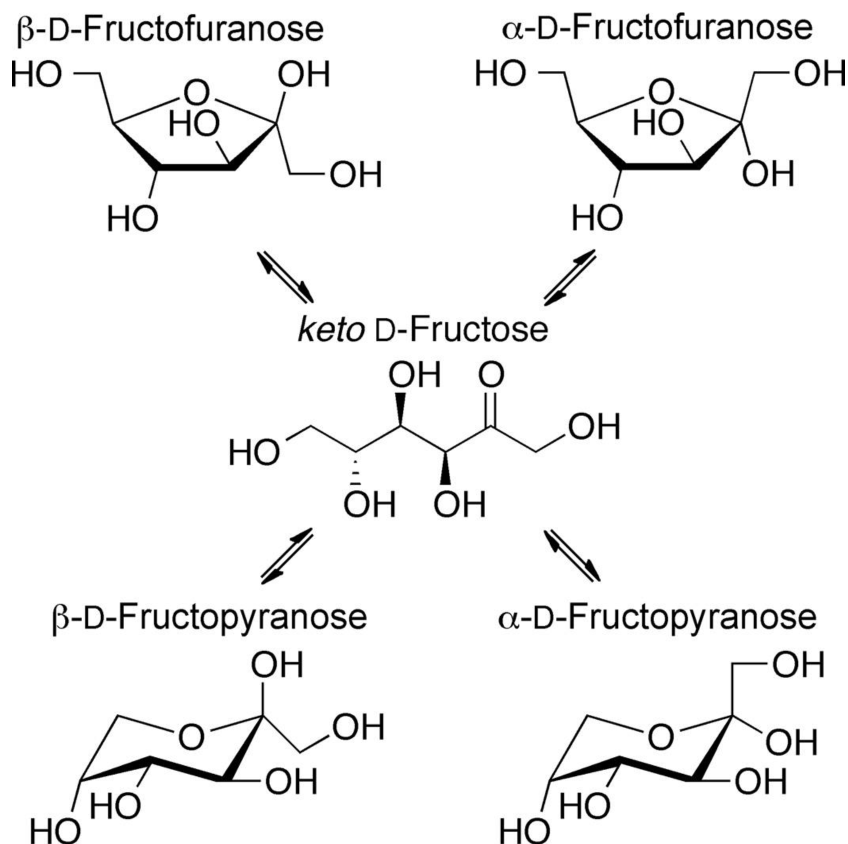

Figure 1.2 Tautomeric forms of D-fructose in solution.
(*reproduced from Shallenberger, 1978*)

Pure, dry fructose is a very sweet, white, odourless, crystalline solid. Fructose has higher solubility as compared to other sugars, which makes it difficult to crystallize from an aqueous solution (Table 1.1). Because of its greater solubility, fructose-containing sugar mixes, such as candies, are softer than those containing other sugars. Fructose has a greater effect on freezing point depression than disaccharides or oligosaccharides, which may be undesirable in soft-serve or hard-frozen dairy desserts.

Fructose is estimated to be approximately 1.2-1.8 times sweeter than glucose, although the perception of sweetness depends on a variety of factors, such as concentration, pH, temperature and individual taste buds. The sweetness of fructose is perceived earlier than that of sucrose or glucose, and the taste sensation reaches a peak, which is higher and diminishes more quickly than that of sucrose. Fructose can also enhance other flavours (Hanover & White, 1993).

1.1.2 Fructose in our daily food

Fructose was always a part of human diet. Free fructose, together with free glucose, is present in fruits and honey, and in smaller amounts in vegetables (Table 1.2). Fructose polymers - fructans can be found in some vegetables and wheat. The level of fructose consumption remained low until the 19th century, when sugar became widely available at a low cost due to colonial trade. The main part of today's dietary fructose intake comes from sucrose, a disaccharide composed of one molecule of glucose linked to a molecule of fructose through an alpha 1-4 glycoside bond. In addition to natural sources, fructose may be found in commercially produced high fructose corn syrup. The production process of high fructose corn syrup was developed by Marshall and Kooi in 1957 (Marshall & Kooi, 1957). The industrial production process was refined by Dr. Takasaki from Japan up to 1970, and since that time

the syrup was rapidly introduced in many processed foods and soft drinks (Bray *et al.*, 2004).

Table 1.2 Sugar content of selected common plant foods (g/100g). (*reproduced from http://fnic.nal.usda.gov/databases*)

Food Item	Total Carbohydrate	Total Sugars	Free Fructose	Free Glucose	Sucrose	Fructose/ Glucose Ratio	Sucrose as a % of Total Sugars
Fruits							
Apple	13.8	10.4	5.9	2.4	2.1	2.0	19.9
Apricot	11.1	9.2	0.9	2.4	5.9	0.7	63.5
Banana	22.8	12.2	4.9	5.0	2.4	1.0	20.0
Fig, dried	63.9	47.9	22.9	24.8	0.07	0.93	0.001
Grapes	18.1	15.5	8.1	7.2	0.2	1.1	1.0
Peach	9.5	8.4	1.5	2.0	4.8	0.9	56.7
Pear	15.5	9.8	6.2	2.8	0.8	2.1	8.0
Pineapple	13.1	9.9	2.1	1.7	6.0	1.1	60.8
Plum	11.4	9.9	3.1	5.1	1.6	0.66	16.2
Vegetables							
Beet, Red	9.6	6.8	0.1	0.1	6.5	1.0	96.2
Carrot	9.6	4.7	0.6	0.6	3.6	1.0	70.0
Corn, Sweet	19.0	6.2	1.9	3.4	0.9	0.61	15.0
Red Pepper, Sweet	6.0	4.2	2.3	1.9	0.0	1.2	0.0
Onion, Sweet	7.6	5.0	2.0	2.3	0.7	0.9	14.3
Sweet Potato	20.1	4.2	0.7	1.0	2.5	0.9	60.3
Sugar Cane		13 – 18	0.2 – 1.0	0.2 – 1.0	11 - 16	1.0	100
Sugar Beet		17 – 18	0.1 – 0.5	0.1 – 0.5	16 - 17	1.0	100

Until recently, fructose has not been present in large amounts in the human diet; however, since the introduction of high-fructose corn syrup in 1970s its daily intake has largely increased. For example, before 1900 Americans consumed approximately 15 g of fructose per day (4 % of total cal), mainly through consumption of fruits and vegetables. By 1940s, fructose intake had increased to 24 g per day (5 % of total cal); by 1977, it was 37 g per day (7 % of total cal); and by 1994, 55 g per day (10 % of total cal). Between 2005 and 2010 approximately 13% of adults' total caloric intake came from added sugars (Ervin & Ogden, 2013). Among adults, one-third of calories from added sugars came from beverages, while in children and adolescents, 40% of calories from added sugars came from beverages (Ervin *et al.*, 2012). Interestingly, the most recent studies show that estimated dietary sugars intake is either stable or decreasing (Wittekind & Walton, 2014).

Fructose exhibits numerous useful physical and functional attributes, which can be use in food and beverage industry, such as sweetness, flavour enhancement, colour and flavour development, freezing-point depression, and osmotic stability (Hanover & White, 1993). Therefore, fructose is usually added to foods and drinks in order to enhance palatability and taste, as well as for browning of some foods, such as baked goods. It is extensively used in breakfast cereals, baked goods, condiments, and prepared desserts sweetened with sucrose or high-fructose corn syrup.

To date, high-fructose corn syrup represents approximately 40% of all added sweeteners used in production of soft drinks and fruit juices. The usage of high-fructose corn syrup in food industry was encouraged due to its functional advantages over sucrose such as greater sweetness and palatability, better solubility, better preservative features, liquid form enabling easier handling, etc. In addition, the preference for high-fructose corn syrup over glucose or sucrose in commercial food production can also be attributed to low cost and high production efficiency (Hanover & White, 1993).

Since fructose metabolism is not dependent on insulin secretion, at least not in the initial steps, and because fructose ingestion causes only a limited rise in glycemia, fructose was initially proposed as a natural substitute of sucrose for diabetic patients. However, the data collected in the past decades, implied that increase in fructose consumption correlates with the rising prevalence of metabolic disorders, which prompted the research toward understanding the metabolic fate of fructose and the mechanisms underlying its possible harmful effects.

1.1.3 Fructose metabolism

Both glucose and fructose are hexoses, but due to structural difference they do not share the same metabolic fate and differ in digestion, absorption and metabolism. Namely, in the intestine, glucose is absorbed by sodium-glucose cotransporter, while fructose absorption occurs further down in the duodenum and jejunum, and is facilitated by a non-sodium-dependent process. After absorption, glucose and fructose enter the portal circulation and either enter the liver, or pass into the general circulation. In the liver, fructose undergoes a specific metabolism which differs markedly from that of glucose. Namely, hepatic fructolysis, unlike glycolysis, is not regulated by insulin or inhibited by high concentrations of ATP or citrate.

Glucose enters the cells by insulin dependent GLUT4 transporter. Inside the cell, glucose is phosphorylated by glucokinase to glucose-6-phosphate, from which the intracellular metabolism of glucose begins. Hepatic glucose metabolism is limited by the capacity of the liver to store glucose as glycogen and by the inhibition of glycolysis and further glucose uptake resulting from allosteric inhibition of phosphofructokinase by citrate and ATP.

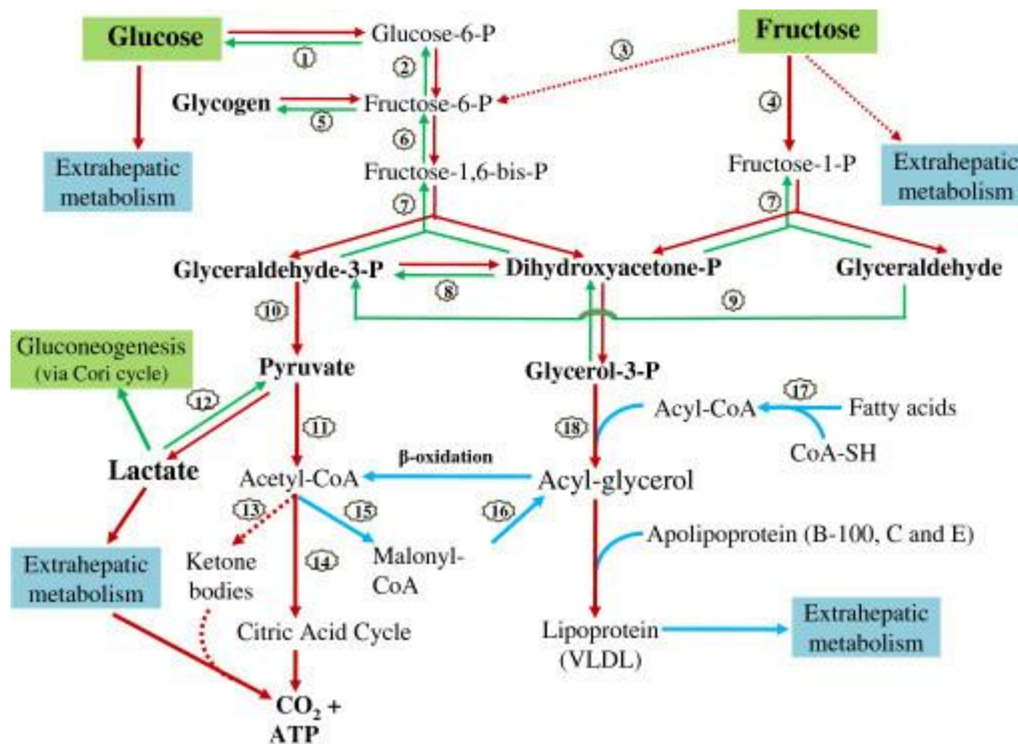

Figure 1.3. Major metabolic pathways and flux of dietary glucose and fructose. P = phosphate. For enzymes numbered in circles: 1 = hexokinase/glucokinase or glucose-6-phosphatase, 2 = phosphoglucose isomerase, 3 = hexokinase, 4 = fructokinase, 5 = glycogen synthase or phosphorylase, 6 = phosphofructokinase, 7 = aldolase, 8 = triose phosphate isomerase, 9 = triose kinase, 10 = several enzymes including pyruvate kinase, 11 = pyruvate dehydrogenase complex, 12 = lactate dehydrogenase, 13 = ketothiolase and other 3 enzymes, 14 = enzyme group related to citric acid cycle, 15 = acetyl CoA carboxylase, 16 = multienzyme complexes, 17 = acyl CoA synthase, 18 = glycerol-phosphate acyl transferase and triacylglycerol synthase complex. The dashed-lines and arrows represent minor pathways or will not occur under a healthy condition or ordinary sugar consumption. The compound names in **bold** would be major metabolic intermediates or end products of glucose or fructose metabolism. (*reproduced from (Sun & Empie, 2012)*.

In contrast to glucose, fructose is rapidly and almost completely taken up from the portal vein by the liver, in order to be converted into glucose, glycogen, lactate and fat, or to be oxidized within hepatocytes. Fructose enters the cells predominantly through GLUT5 and/or GLUT2 transporters irrespective of energy needs, or circulating glucose levels. Once inside the cell, fructose carbons, like glucose, are utilized through glycolysis, gluconeogenesis, glycogenolysis, tricarboxylic acid cycle, lactate production (Cori cycle), pentose phosphate shunt and lipid synthesis pathways, to provide substrates for glycogen, amino acids, carbohydrates, fat, ATP, etc (Figure 1.3). However, fructose and glucose enter metabolic pathways differently. Fructose is phosphorylated by fructokinase to form fructose-1-phosphate, which can directly enter glycolysis. This unique characteristic of fructose metabolism enables bypassing tightly regulated glycolytic checkpoints, especially phosphofructokinase. Fructose-1-phosphate can be cleaved by aldolase to form trioses that are the backbone for phospholipid and triacylglycerol synthesis. Fructokinase and aldolase B are not regulated by insulin and the energy status of the cell, therefore the majority of fructose is rapidly converted into triose-phosphate. As a consequence, a transient ATP depletion occurs in hepatocytes, which can be followed by formation of AMP and its degradation to uric acid. Also formed triose-phosphates can be converted into lactate or glucose and released into the circulation; or directed to glycogen synthesis. Finally, fructose can be converted to acetyl coenzyme A and citrate to provide carbon for *de novo* lipogenesis (Havel, 2005; Tappy, 2012).

1.1.4 Fructose and metabolic disorders

Since the introduction of high-fructose corn syrup in 1970s, daily consumption of this sugar has largely increased. Emerging body of evidence suggests that high-fructose diet promotes profound metabolic alterations in the liver and adipose tissue (Havel, 2005; Dekker *et al.*, 2010). Epidemiological evidence suggest that increased intake of added sugars and/or sugar-sweetened beverages is associated with dislypidemia, insulin resistance, fatty liver, visceral adiposity, type 2 diabetes, cardiovascular diseases, metabolic syndrome, chronic kidney diseases, hyperuricemia and gout (Bomback *et al.*, 2010; Hostmark, 2010; Perez-Pozo *et al.*, 2010; Nomura & Yamanouchi, 2012; Stanhope *et al.*, 2013; Bruun *et al.*, 2015; Malik & Hu, 2015; Stanhope *et al.*, 2015; Baena *et al.*, 2016). Enhanced hepatic *de novo* lipogenesis, oxidative stress, inflammation and hyperuricemia have been proposed as underlying mechanisms responsible for adverse metabolic effects of fructose (Johnson *et al.*, 2013; Bruun *et al.*, 2015; Keane *et al.*, 2015).

1.1.5 Gender differences in prevalence of metabolic disorders

Previous studies reported gender differences in susceptibility and progression of metabolic disturbances (Vistisen *et al.*, 2014; Rochlani *et al.*, 2015). Although women seem to have higher risk of developing metabolic syndrome, less severe metabolic disturbances and/or later onset of adverse phenotypes were observed in females as compared to males. For instance, epidemiological studies indicate that prevalence of insulin resistance-related disorders is higher in men compared to women (Vistisen *et al.*, 2014). Also, men display a higher cardiovascular risk due to differences in prevalence of individual components of metabolic syndrome (Geer & Shen, 2009; Rochlani *et al.*, 2015). Studies examining differences in lipid profile patterns between men and women have shown that men tend to have more pathogenic lipid fraction pattern than women, which leads to an increased risk of cardiovascular diseases

(Johnson *et al.*, 2004; Rochlani *et al.*, 2015). However, the prevalence of obesity is greater in women than in men, and it is accelerated after menopause (Meyer *et al.*, 2011; Garawi *et al.*, 2014). A fall in estrogen levels following menopause has been associated with increased visceral obesity, impaired glucose metabolism and increased risk of cardiovascular diseases, implying that postmenopausal women share the same risk category as men for development of metabolic diseases (Wang *et al.*, 2012).

Although the underlying mechanisms responsible for fructose-mediated metabolic disturbances are not quite clear, previous studies have revealed a link between nutritional excess and oxidative stress, suggesting that redox disbalance may participate in development and progression of metabolic diseases (Nomura & Yamanouchi, 2012). Interestingly, gender-related dimorphism in oxidative capacity and activities of antioxidant enzymes points to a higher protection against oxidative damages in females (Vina *et al.*, 2005a; Pajovic & Saicic, 2008; Giergiel *et al.*, 2012). Namely, males were shown to be more prone to insulin resistance-related disorders as compared to females, and the later onset and less severe metabolic phenotypes in females have been related to the better antioxidative capacity of females (Busserolles *et al.*, 2002; Borras *et al.*, 2003; Baba *et al.*, 2005).

1.2 Oxidative stress and antioxidant defence system

1.2.1 Reactive oxygen species

Free radicals were discovered by Moses Gomberg more than a century ago (*https://www.acs.org/content/acs/en/education/whatischemistry/landmarks/freeradicals.html*). The scientific community began recognizing the importance of free radicals in 1929, when Friedrich Paneth and Wilhelm Hofeditz produced the methyl free radical (Commoner *et al.*, 1954). Yet, due to their short life time and extremely high chemical activity their presence in biological systems remained undiscovered till the early 20th century. However, immediately upon the discovery of the presence of free radicals in biological systems they were linked to diverse human pathologies (Gerschman *et al.*, 1954), and aging processes (Harman, 1956), which launched the field of free radical research in living organisms. In the 1970s, Sies and Chance evaluated catalase function *in vivo*, and provided information regarding the steady-state hydrogen peroxide levels in perfused rat liver (Sies & Chance, 1970). In the 1980s, it became clear that the generation and elimination of free radicals in living organisms is well-balanced, while imbalances between their generation and elimination underlie various pathophysiological states. Subsequently, their role in numerous biochemical processes, including intracellular messaging, cellular differentiation, growth arrestment, apoptosis, immunity and defence against microorganisms has been revealed (Valko *et al.*, 2007; Forman *et al.*, 2010; Forman, 2016). Since their discovery the gathered knowledge on the involvement of free radicals in living processes has increased enormously.

Radicals derived from oxygen are considered the most important class of radical species generated in living systems (Valko *et al.*, 2007). Reactive oxygen species (ROS) are a highly reactive, short-lived free-radical and non-radical derivatives of oxygen metabolism, produced in all biological systems in response to extracellular and intracellular stimuli.

Mitochondria are a major source of cellular ROS generation, where ROS formation results from imperfectly coupled electron transport (Murphy, 2009). The primary ROS produced in aerobic organisms is superoxide anion radical ($O_2^{\bullet -}$), derived by the addition of one electron to oxygen molecule. Hydrogen peroxide (H_2O_2) although not a free radical in itself, is a biologically important oxidant because of its ability to generate extremely reactive hydroxyl radical ($HO^{\bullet}$). Namely H_2O_2 is a powerful oxidizing agent, which in the presence of reduced transition metals such as Cu^+ or Fe^{2+}, can be converted to the highly reactive $HO^{\bullet}$ through Fenton or Haber–Weiss reactions. $HO^{\bullet}$ unspecifically reacts with all kinds of biomolecules at a diffusion-limited rate. Due to their unstable electron configurations ROS can react with cellular macromolecules. ROS are capable of initiating chain reactions, resulting in protein, lipid and nucleic acid damages (Aruoma *et al.*, 1991; Imlay, 2003; Valko *et al.*, 2007).

1.2.2 Oxidative stress

Oxidative stress is a condition that occurs when the balance between the formation and the removal of ROS is disturbed, thereby resulting in the accumulation of oxidized and damaged biomolecules. It is a consequence of either increased generation of free radicals or impaired antioxidant defense (Valko *et al.*, 2007; Lushchak, 2014). The excess ROS can damage cellular lipids, proteins, or DNA, thus decreasing their biological activity, which can lead to alterations in cell signalling, metabolic dysregulations and other cellular functions. Oxidative stress was implicated in the pathogenesis of various disease states, including diabetes, obesity, atherosclerosis, cancer, neurodegenerative disorders, hypertension, cardiovascular diseases, and heart failure (Valko *et al.*, 2007; Grattagliano *et al.*, 2008; Reuter *et al.*, 2010; Rains & Jain, 2011; Miljkovic & Spasojevic, 2013; Balmus *et al.*, 2016). The potential role of oxidative stress in initiation and progression of metabolic disorders is rapidly evolving.

However, the delicate balance between beneficial and harmful effects of ROS represents an important aspect of living organisms. Namely, low levels of ROS as signaling molecules are necessary for proper cell functioning and biological effects of these highly reactive species are controlled by a wide spectrum of antioxidant mechanisms (Valko *et al.*, 2007).

1.2.3 Antioxidant defence system

The main physiological mechanism, by which cells regulate ROS concentration within non-toxic homeostatic levels, thereby enabling appropriate signaling, as well as the protection of macromolecules from oxidative damage, includes a complex set of enzymes and non-enzymatic low-molecular weight endogenous and dietary antioxidant compounds (Figure 1.4). Cells maintain levels of antioxidants, often defined as their antioxidant potential, through dietary intake and/or *de novo* synthesis.

The primary antioxidant enzymes include, but are not limited to: cytoplasmic copper-zinc superoxide dismutase (SOD1) and mitochondrial manganese superoxide dismutase (SOD2), which rapidly and specifically reduce superoxide anion radicals to hydrogen peroxide (Figure 1.4). Hydrogen peroxide is further decomposed to water by catalase (CAT) and glutathione peroxidase (GPx). Cytosolic GPx detoxifies hydrogen peroxide in the presence of reduced glutathione (GSH), which is thus converted to oxidized glutathione (GSSG) and subsequently recycled by glutathione reductase (GR) (Figure 1.4). The non-enzymatic compounds such as GSH, vitamins A, E, C, beta-carotene, uric acid, bilirubin, etc play an essential role in maintaining redox balance by trapping free radicals and preventing chain reactions (Figure 1.4). Finally, numerous proteins including heath shock proteins Hsp70 and Hsp90, assist in reparation of oxidatively damaged cellular biomolecules.

1.2.4 *Antioxidant enzymes*

Superoxide dismutase (SOD) (EC 1.15.1.1) is an antioxidant enzyme that catalyses the dismutation of $O_2^{\bullet -}$ to O_2 and the less reactive ROS H_2O_2, with extremely high reaction rates (Fridovich, 1995). Humans express three types of SODs: mitochondrial Mn SOD, cytosolic Cu/Zn SOD and extracellular SOD (Mates *et al.*, 1999). The dismutation of $O_2^{\bullet -}$ is achieved by successive oxidation and reduction of the transition metal ion at its active site in a Ping Pong type mechanism (Meier *et al.*, 1998). The respiratory chain in mitochondria is a major source of oxygen radicals. Mn-SOD (SOD2) is a mitochondrial homotetrameric enzyme which contains one manganese ion per subunit. Cu/Zn-SOD is believed to play a major role in the first line of antioxidant defense. Cu/Zn-SOD (SOD1) is homodimer. Each subunit contains a metal cluster, containing copper and zinc ions bridged by a histamine residue in the active site.

Catalase (EC 1.11.1.6) is a homotetrameric enzyme that reacts with H_2O_2 to form water and molecular oxygen, or facilitates the reduction of organic hydroperoxides using hydrogen donors (methanol, ethanol, formic acid, or phenols). The enzyme consists of four identical tetrahedrally arranged subunits. Each subunit contains a single ferriprotoporphyrin group. Catalase has one of the highest turnover rates for all enzymes (Mates *et al.*, 1999)

Glutathione peroxidase (GPx) (EC 1.11.1.19) is a selenium-containing tetrameric enzyme that reduces H_2O_2, lipoperoxides and other organic hydroperoxides to their corresponding hydroxylated compounds using reduced glutathione (GSH) as a hydrogen donor. Each of the four identical subunits contains a single selenocysteine (Sec) residue (Chaudiere & Ferrari-Iliou, 1999). Mammals express five GPx isoenzymes that differ in intracellular localization and substrate specificity. The distribution and the level of isoenzymes is regulated in a tissue specific manner.

Glutathione reductase (GR) (EC 1.8.1.7) is disulfide oxidoreductase that catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form (GSH). GR is homodimer which utilizes an FAD prosthetic group and NADPH to reduce GSSG using a Ping-Pong mechanism. The enzyme has three substrates (NADPH, H^+ and GSSG) and two products (GSH and GSH). It is crucial for maintaining a reducing intracellular milieu - high GSH and low GSSG levels (Couto *et al.*, 2016).

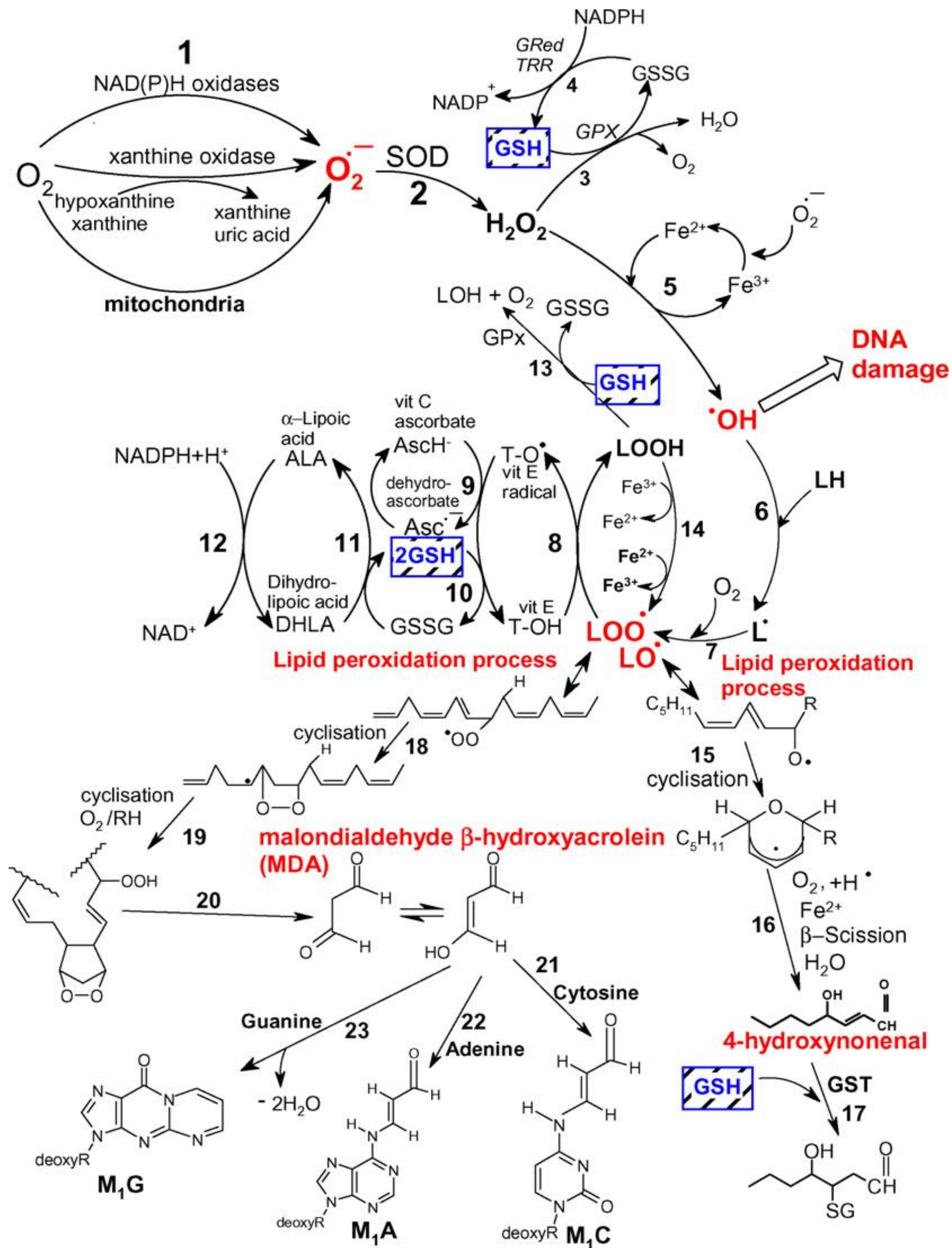

Figure 1.4. Pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C, lipoic acid) in the management of oxidative stress. Reaction 1: The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain.

Reaction 2: Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide. Reaction 3: Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor. Reaction 4: The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred) which uses NADPH as the electron donor. Reaction 5: Some transition metals (e.g. Fe^{2+}, Cu^{+} and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction). Reaction 6: The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centred lipid radical (L•). Reaction 7: The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxyl radical (LOO•). If the resulting lipid peroxyl radical LOO• is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 18–23 and 15–17). Reaction 8: The lipid peroxyl radical (LOO•) is reduced within the membrane by the reduced form of Vitamin E (T-OH) resulting in the formation of a lipid hydroperoxide and a radical of Vitamin E (T-O•). Reaction 9: The regeneration of Vitamin E by Vitamin C: the Vitamin E radical (T-O•) is reduced back to Vitamin E (T-OH) by ascorbic acid (the physiological form of ascorbate is ascorbatemonoanion, AscH−) leaving behind the ascorbyl radical (Asc•−). Reaction 10: The regeneration of Vitamin E by GSH: the oxidised Vitamin E radical (T-O•) is reduced by GSH. Reaction 11: The oxidised glutathione (GSSG) and the ascorbyl radical (Asc•−) are reduced back to GSH and ascorbatemonoanion, AscH−, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to α-lipoic acid (ALA). Reaction 12: The regeneration of DHLA from ALA using NADPH. Reaction 13: Lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor. *Lipid peroxidation process*: Reaction 14: Lipid hydroperoxides can react fast with Fe^{2+} to form lipid alkoxyl radicals (LO•), or much slower with Fe^{3+} to form lipid peroxyl radicals (LOO•). Reaction 15: Lipid alkoxyl radical (LO•) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide. Reaction 16: Six-membered ring hydroperoxide udergoes further reactions (involving β-scission) to from 4-hydroxy-nonenal. Reaction 17: 4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST, glutathione *S*-transferase). Reaction 18: A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical. Reaction 19: This radical can then either be reduced to form a hydroperoxide (reaction not shown) or it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide. Reaction 20: Formed compound is an intermediate product for the production of malondialdehyde. Reactions 21, 22, 23: Malondialdehyde can react with DNA bases Cytosine, Adenine, and Guanine to form adducts M_1C, M_1A and M_1G, respectively. (*reproduced from Valko et al, 2007*).

1.2.5 Oxidative stress and metabolic syndrome

Oxidative stress is a condition that can occur when pro-oxidant challenge overwhelms the antioxidant defense. Recent studies support the concept that increased oxidative stress may play an important role in initiation and progression of metabolic syndrome related manifestations (Ceriello & Motz, 2004; Hopps *et al.*, 2010). Namely, the observation that oxidative stress was present in various metabolic disorders including insulin resistance, obesity, atherosclerosis, type 2 diabetes, has implied that it might be common unifying mechanism underlying development of metabolic dysfunction (Ceriello & Motz, 2004; Furukawa *et al.*, 2004; Grattagliano *et al.*, 2008; Henriksen *et al.*, 2011; Yubero-Serrano *et al.*, 2013; Manna & Jain, 2015).

Previous studies have shown an increase in oxidative stress biomarkers in obese adults and children (Faienza *et al.*, 2012; Kotani & Yamada, 2012; Gonzalez-Muniesa *et al.*, 2013). In children, oxidative stress and adipokine levels worsen throughout the continuum of obesity and especially in the presence of the components of metabolic syndrome (Kelly *et al.*, 2006). Fujita et al (2006) have demonstrated a strong association between systemic oxidative stress, visceral fat accumulation and metabolic syndrome. Biomarkers of oxidative stress were found to be elevated in individuals with metabolic syndrome as compared to individuals with no metabolic dysfunction (Armutcu *et al.*, 2008; Rao *et al.*, 2010; Demir *et al.*, 2014; Sabir *et al.*, 2016). Van Gulinder et al, implied that increased oxidative and inflammatory stress may contribute to the greater risk of coronary heart disease and cerebrovascular disease in obese adults with metabolic syndrome (Van Guilder *et al.*, 2006). Oxidative stress and obesity have also been related to insulin resistance and type 2 diabetes (Urakawa *et al.*, 2003; Katsuki *et al.*, 2004; Das *et al.*, 2016). Boden et al have shown that acute excessive caloric intake can induce oxidative stress and a consequent oxidation and carbonylation of numerous proteins, including

GLUT4, which might be related to development of insulin resistance in healthy men (Boden *et al.*, 2015).

Previous studies on rodents have shown that fructose-rich diet can induce most features of metabolic syndrome, including hypertension, insulin resistance, abdominal obesity, hepatic steatosis, endothelial dysfunction and inflammation (Dekker *et al.*, 2010; Tappy *et al.*, 2010). Thus, fructose-fed rats appeared to be a commonly used animal model for studying diet-induced metabolic disturbances (Tran *et al.*, 2009).

The induction of some features of metabolic syndrome by chronic fructose feeding, and even by a single dose of fructose (Moreno & Hong, 2013), was associated with oxidative stress and the disruption of antioxidant mechanisms (Francini *et al.*, 2010), suggesting a causative role of oxidative stress (Grattagliano *et al.*, 2008; Rains & Jain, 2011). However, a large discrepancy in the course and the intensity of fructose-induced alterations in antioxidant enzyme functioning can be found. On the other hand, protective effects of fructose and its phosphorylated forms after short-term application were also demonstrated in oxidative stress-related conditions, and several studies have reported their antioxidative and cytoprotective effects (Frenzel *et al.*, 2002; Spasojevic *et al.*, 2009a; Semchyshyn & Lozinska, 2012; Semchyshyn, 2013). In general, it appears that negative effects of fructose emerge mostly after long-term exposure, while its acute application seems to protect cells and can be beneficial under some pathophysiological conditions (Semchyshyn, 2013). In addition, the question whether fructose-induced oxidative stress represents an early event in the pathogenesis of metabolic diseases or a consequence of metabolic disturbances remains opened.

2. AIM

2 AIM

Increased fructose consumption coincides with the rising incidence of obesity, metabolic syndrome and type 2 diabetes. The underlying mechanisms responsible for fructose-mediated metabolic disturbances are not quite clear, and previous studies revealed the link between nutritional excess and oxidative stress, suggesting that redox disbalance may participate in the initiation and/or progression of metabolic disorders, often in a gender-specific manner. However, the relationship between fructose consumption and oxidative stress has appeared to be complex, since both, prooxidant and antioxidant effects of fructose were reported. Moreover, the ability of antioxidant defence system to react efficiently under conditions of disturbed homeostasis is largely dependent on age, and the effects of fructose rich-diet on young population, which is at increased risk of developing metabolic disorders in the adulthood, have not been fully elucidated.

The general aim of this study is to investigate whether fructose-rich diet applied, over a period from weaning to adulthood, induces hepatic oxidative stress, thus contributing to the induction and/or aggravation of metabolic disturbances in later adulthood.

The specific aims are:

- To examine the effects of long-term moderate fructose-enriched diet (10% fructose in drinking water) or high-fructose diet (60% fructose in drinking water) on physiological and biochemical parameters in rats subjected to dietary regime immediately after weaning;

- To determine whether long-term moderate fructose-enriched diet (10% fructose in drinking water) affects expression and activity of antioxidant enzymes in the liver of female and male rats;

- To investigate the effects of high-fructose diet (60% fructose in drinking water) on expression and activity of antioxidant enzymes in the liver of male rats which are more prone to development of insulin-resistance related disorders as compared to females.

3. MATERIAL AND METHODS

3 MATERIAL AND METHODS

3.1 Animals and treatment

To examine the effects of moderate fructose-rich diet, young male and female Wistar rats (21 days old) were randomly divided in 2 experimental groups (9 animals per group): control group (C) fed with commercial standard chow and drinking water, and fructose group (F10) fed with the same chow and 10% (w/v) fructose solution instead of drinking water. Both experimental groups had *ad libitum* access to food and drinking fluid during 9 weeks. The choice of fructose concentration was based on the data that 10% fructose solution closely resembles the intake of sweet solutions characteristic for Western diet (Ventura *et al.*, 2011).

To examine the effects of high-fructose diet, male Wistar rats aged 21 days at the beginning of the treatment, were randomly divided in 2 experimental groups according to diet regime applied during 9 weeks (n = 9 animals per group). Control group (C) had standard commercial laboratory chow and drinking water available ad libitum, while fructose group (F60) had the same chow and both 60 % fructose solution and drinking water available *ad libitum*. Additional drinking water was provided for fructose group in order to prevent kidney hypertrophy, glomerular hypertension and cortical vasoconstriction, as possible consequences of disturbed water balance (Sanchez-Lozada *et al.*, 2007).

All animals were kept under standard conditions, 22°C with a 12 h light/dark cycle. The detailed composition of the laboratory chow obtained from Veterinary Institute, Subotica, Serbia, is presented at Table 3.1. During the 9 week treatment, food and liquid intake was measured daily. Energy intake was calculated as sum of calories ingested as food and liquid. Body mass was measured at the beginning and at the end of the treatment, while the livers and visceral adipose tissue were weighted immediately after sacrifice.

The procedures were complied with the EEC Directive (86/609/EEC) on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

Table 3.1. Composition of the laboratory chow (Veterinary Institute, Subotica, Serbia).

Chemical composition of the standard diet	
Metabolizable energy, not less than	11000 kJ/kg
Protein, not less than	20%
Moisture, not more than	13 %
Ash, not more than	10 %
Cellulose, not more than	8 %
Calcium, not less than	1 %
Lysine, not less than	0.90 %
Methionine + Cystine, not less than	0.75 %
Phosphorus, not less than	0.50 %
Sodium	0.15-0.25 %
Vitamin A, not less than	10 000 IU/kg
Vitamin D3, not less than	1600 IU/kg
Vitamin E, not less than	25 mg/kg
Vitamin B12, not less than	0.02 mg/kg
Zinc, not less than	100 mg/kg
Iron, not less than	100 mg/kg
Manganese, not less than	30 mg/kg
Copper, not less than	20 mg/kg
Iodine, not less than	0.5 mg/kg
Selenium, not less than	0.1 mg/kg
Antioxidant, not less than	100 mg/kg

3.2 Blood plasma preparation and tissue collection

After overnight fasting, animals were sacrificed by rapid decapitation with a guillotine (Harvard-Apparatus, USA). Livers were perfused with cold 0.9% NaCl, quickly excised and stored in liquid nitrogen until use. Visceral adipose tissue was carefully isolated and weighed. Adiposity index (%) was calculated as [(adipose tissue mass/body mass) x100].

Trunk blood was rapidly collected into EDTA containing tube and agitated slowly. Blood plasma was isolated by centrifugation at 1600xg for 10 min at room temperature and stored at -20ºC for subsequent processing.

3.3 Determination of biochemical parameters

Glucose and triglycerides concentrations were determined in the blood immediately after sacrifice using MultiCare strips (Biochemical Systems International, Italia).

Plasma insulin level was determined by radioimmunoassay, using RIA kit for insulin (INEP, Zemun, Serbia). Radioactivity was counted in Rackbeta liquid scintillation counter (LKB). Assay sensitivity was 0.6 mIU/l and an intraassay coefficient of variation was 5.24 %.

Level of nonesterified fatty acids (NEFA) was determined in the blood plasma using a modified version of Duncombe's (1964) method. Serial dilutions of palmitic acid were used for standard curve construction.

Liver triglycerides were isolated from 100 mg of liver tissue by modified Folch method (1957) and analysed by modified colorimetric method by Fletcher (1968).

3.4 Determination of antioxidant enzymes activity

For the preparation of whole cell extracts, livers were homogenized in 10 vol. (w/v) of buffer (50 mM Tris, 0.25 M sucrose, 1 mM EDTA, pH 7.4), sonicated (3 x 10 s at 10 MHz on ice) prior to 60 min centrifugation at 105000 x g. Final supernatants were used as whole cell extracts.

Total SOD activity was determined in the whole cell extracts by the adrenaline method (Misra & Fridovich, 1972). SOD units were defined as the amount of the enzyme necessary to decrease the rate of adrenalin autooxidation by 50%, at pH 10.2. For determination of SOD2 activity, the assay was performed after preincubation with 8 mM KCN. The SOD1 activity was calculated as the difference between total SOD and SOD2 activities.

CAT activity was determined according to Claiborne´s method (1985). One unit of CAT activity was defined as the amount of enzyme necessary to decompose 1 mmol H_2O_2 per minute at 25°C and pH 7.0.

The activity of GPx was determined by the GSH reduction of t-butyl hydroperoxide, using a modification of the assay described by Paglia and Valenine (1967). One unit of GPx activity was defined as the amount of enzyme needed to oxidaze 1 mmol NADPH per minute at 25°C and pH 7.0.

GR activity was determined by the method of Glatzle (1974). One unit of GR activity is defined as the amount of enzyme needed to oxidaze 1 nmol NADPH per minute at 25°C and pH 7.4.

All enzyme activities are expressed as arbitrary units per mg of protein (AU/mg). Protein concentration was determined by the method of Spector (1978) using bovine serum albumin as a standard.

3.5 Assessment of TBARS

For the measurement of lipid peroxidation products, livers were homogenized in 10 vol. (w/v) of Tris buffer pH 7.4 without sucrose, sonicated and centrifuged at 6 000 x g. The degree of lipid peroxidation was assessed by estimating the thiobarbituric acid reactive substances (TBARS) (Rehncrona *et al.*, 1980). The absorbance was measured at 530 nm. TBARS concentration was calculated using the molar extinction coefficient of malondialdehyde ($\varepsilon530=15\ 600\ M^{-1}\ cm^{-1}$), and expressed in nmol per mg of protein.

3.6 SDS-PAGE and immunoblotting

Proteins were resolved according to Laemmly (1970) on 12% SDS-polyacrylamide gels using Mini-Protean Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA). Western transfer of proteins from acrylamide gels to PVDF membranes (Amersham Pharmacia Biotech, UK) was performed in 25 mM Tris buffer, pH 8.3 containing 192 mM glycine and 20% (v/v) methanol, at 135 mA overnight in Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked by Phosphate-buffered saline (PBS) comprised of 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 2.7 mM KCl, 0.14 M NaCl, at pH 7.2 which contained 1% non-fat dry milk. After blocking, the membranes were incubated with primary antibody. The list of antibodies used for immunodetection of target proteins is presented in Table 3.2.

Table 3.2. Characteristics of primary antibodies used in Western blot detection.

Target protein	Molecular weight (kDa)	Source	ID number Manufacturer	Dilution
SOD1	18	Rabbit	Ab13498 Abcam	1:5000
SOD2	27	Rabbit	Ab13533 Abcam	1:5000
CAT	60	Rabbit	Ab16731 Abcam	1:2000
GPx	22	Rabbit	Ab22604 Abcam	1:5000
GR	58	Rabbit	Ab16801 Abcam	1:2000
Hsp70	73	Mouse	SPA-820 Stressgen	1:1000
Hsp90	90	Mouse	SPA-830 Stressgen	1:1000
β-actin	43	Mouse	AC-15 Sigma-Aldrich	1:5000

After washing with PBS containing 0.1% Tween 20 the membranes were incubated with appropriate alkaline phosphatase-conjugated secondary antibody (1:20000) under the same conditions, and subsequently washed with PBS containing 0.1% Tween 20. The immunoreactive proteins were visualized on STORM (Amersham Biosciences Limited, UK) using an enzyme-amplified chemifluoroscence (ECF) method (Amersham Biosciences Limited, UK). Quantitative analysis of immunoreactive bands was done by ImageQuant software (GE Healthcare). Probing for each protein was followed by stripping with 0.2 M NaOH and blocking. β-actin was used as equal load control.

3.7 Statistical analyses

Each assay was performed in triplicate per sample. The morphological and biochemical parameters are given as means ± SD. The enzyme activities and Western blot data are presented as means ± SEM. Between-group differences were assessed by Student's unpaired *t*-test. A probability level of $P<0.05$ was considered statistically significant. Statistical analyses were performed by using GraphPad Prism v5 Software (GraphPad Software, Inc. La Jolla, CA,USA).

4. RESULTS

4 RESULTS

4.1 The effects of moderate fructose-rich diet (10% fructose in drinking water) on physiological parameters and hepatic antioxidant enzymes function in female rats

4.1.1 Physiological and biochemical parameters of fructose-fed rats and rats on standard diet

Daily food and liquid intake was measured during 9 weeks of application of the diet. A decrease in chow intake and increase in liquid intake were observed in fructose-fed female rats, as compared to controls (Table 4.1). Despite increased energy intake observed in fructose-fed rats, body mass remained unaltered. However, high-fructose diet led to an increase in visceral adipose tissue mass and adiposity index as compared to controls. Also, the diet regime led to an increase in the liver mass, but liver to body mass ratio remained unaltered (Table 4.1).

Table 4.1. Food, liquid and energy intake, and physiological parameters in female rats subjected to moderate fructose-rich diet.

	Control	10% Fructose
Food intake (*g/day/animal*)	17.41 ± 2.99	12.59 ± 0.67 **
Liquid intake (*mL/day/animal*)	33.02 ± 6.58	56.19 ± 20.41 *
Total energy intake (*kJ/day/animal*)	191.51 ± 32.89	236.06 ± 30.21 *
Body mass (*g*)	259.00 ± 28.66	271.23 ± 25.44
Mass of liver (*g*)	7.93 ± 0.97	9.37 ± 1.69 *
Liver-to-body mass ratio (*x100*)	3.07 ± 0.33	3.41 ± 0.89
Mass of visceral adipose tissue (*g*)	3.20 ± 1.32	5.06 ± 2.48 *
Adiposity index (%)	1.24 ± 0.52	1.82 ± 0.75 *

The data are presented as means ± SD (n = 9 animals per group). Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test. A value of P<0.05 was considered statistically significant. Asterisks indicate significant differences. *P<0.05, **P<0.01.

Fructose-rich diet led to a decrease in fasting blood glucose level while plasma insulin level remained unaltered after the treatment. At the same time, the diet has induced triglyceridemia while plasma NEFA level remained unchanged. The diet had no effect on the level of hepatic triglycerides (Table 4.2).

Table 4.2. Biochemical and hormonal parameters in female rats subjected to moderate fructose-rich diet

	Control	10% Fructose
Glucose (mmol/l)	5.38 ± 0.65	4.58 ± 0.84 *
Insulin (mIU/l)	6.60 ± 2.50	8.87 ± 3.97
NEFA (mmol/l)	0.84 ± 0.17	0.83 ± 0.18
Triglycerides (mmol/l)	1.39 ± 0.29	1.87 ± 0.38**
Triglycerides in the liver (mmol/l)	1.45 ± 0.35	1.53 ± 0.22

Blood glucose and triglyceride levels were determined at the end of the application of 9-week fructose-rich diet, after overnight fasting of animals. Insulin and NEFA levels were measured in isolated plasma samples. Liver triglycerides were isolated from fresh liver tissue. The data are presented as means ± SD (n = 9 animals per group). Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test. A value of $P<0.05$ was considered statistically significant. Asterisks indicate significant differences. *$P<0.05$, **$P<0.01$.

4.1.2 Activity and expression of antioxidant enzymes in the liver of female rats exposed to moderate fructose-rich diet

To explore possible pro-oxidative effects of long-term moderate-fructose diet we determined the activity and protein level of antioxidant enzymes SOD1, SOD2, CAT, GPx, and GR in the livers of control and fructose-fed rats. The activities of SOD1, SOD2, CAT, GPx and GR were determined spectrophotometricaly, and their protein levels were examined by Western blotting. As shown on Figure 4.1, both the activity and expression of SOD1 remained unaltered after the treatment.

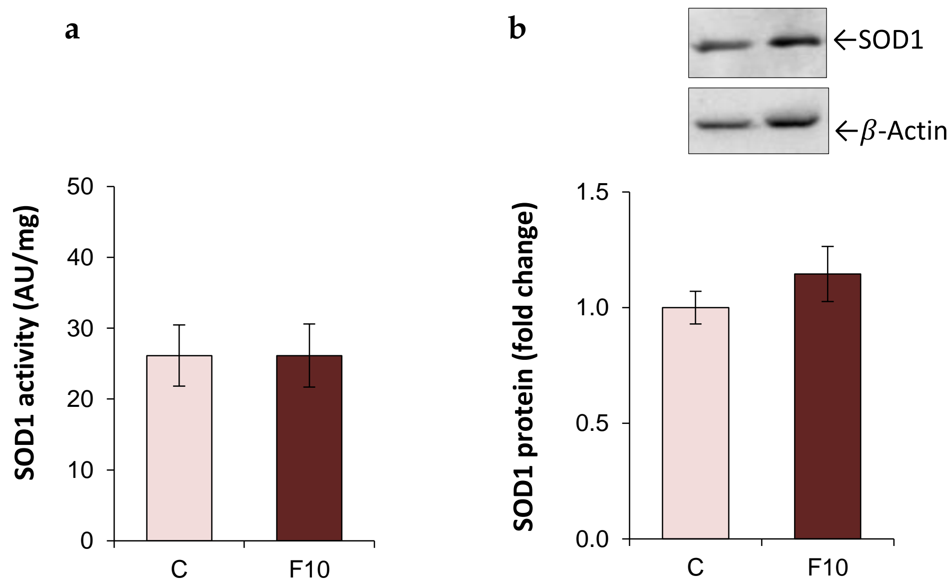

Figure 4.1. Activity and expression of SOD1 in the liver of female rats exposed to moderate fructose-rich diet over a period from weaning to adulthood. (**a**) SOD1 activity in hepatic whole cell extracts of control (C) and fructose-fed rats (F10) was determined spectrophotometrically, and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Hepatic whole cell extracts (50 µg protein) were subjected to SDS-PAGE and Western blotting. *β*-Actin was used as loading control. Representative blots and relative quantification of SOD1 level in control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

Mitochondria are the major source of ROS in the cells. When the activity and the expression of mitochondrial SOD2 were examined, it was found that both parameters remained unaltered after long-term moderate fructose-rich diet (Figure 4.2).

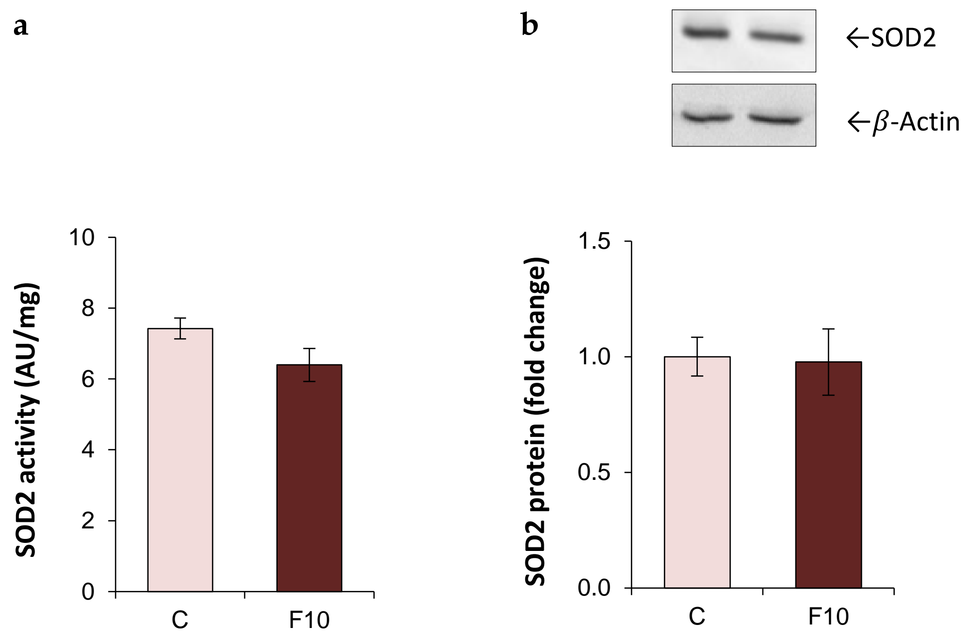

Figure 4.2. Activity and expression of SOD2 in the liver of female rats exposed to moderate fructose-rich diet over a period from weaning to adulthood. (**a**) SOD2 activity in hepatic whole cell extracts of control (C) and fructose-fed rats (F10) was determined spectrophotometrically, and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of SOD2 level in hepatic whole cell extracts (50 µg protein) of control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. *β*-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

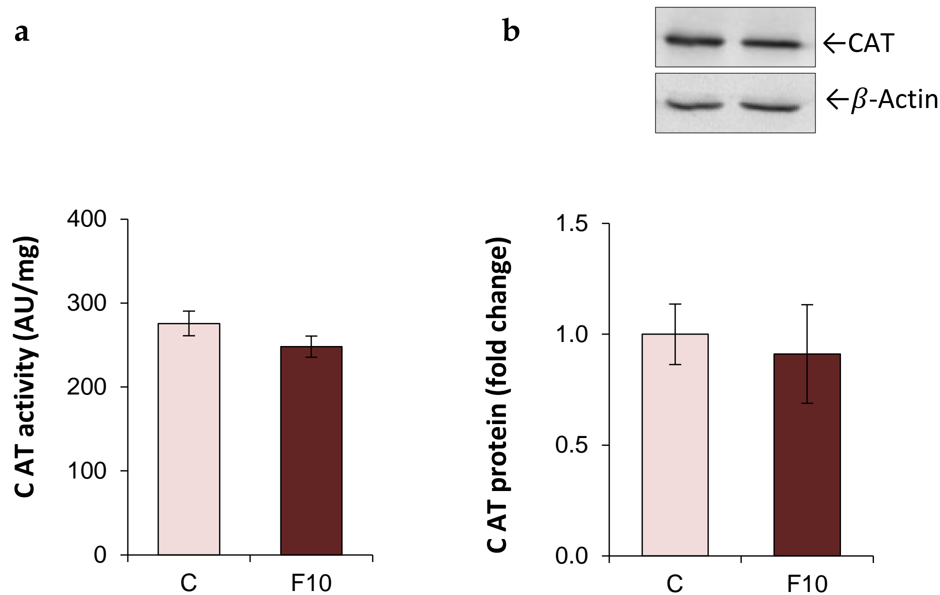

Figure 4.3. Activity and expression of CAT in the liver of female rats exposed to moderate-fructose diet over a period from weaning to adulthood. (**a**) CAT activity was determined spectrophotometrically and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of CAT level in hepatic whole cell extracts (50 µg protein) of control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. *β*-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

Similarly, no significant differences in the activity and expression of CAT, GPx and GR between fructose-fed rats and rats on standard diet were observed (Figure 4.3 and 4.4).

For the measurement of lipid peroxidation products the level of TBARS was determined. Unchanged levels of TBARS suggest that the moderate fructose-rich diet did not affect the level of lipid peroxidation in the liver (Figure 4.5).

The level of Hsp70 and Hsp90 as markers of accumulation of damaged proteins was determined by semi-quantitative Western blot. Unaltered levels of Hsp70 and Hsp90 after the applied diet regime imply that the levels of damaged proteins in the liver were not increased (Figure 4.5).

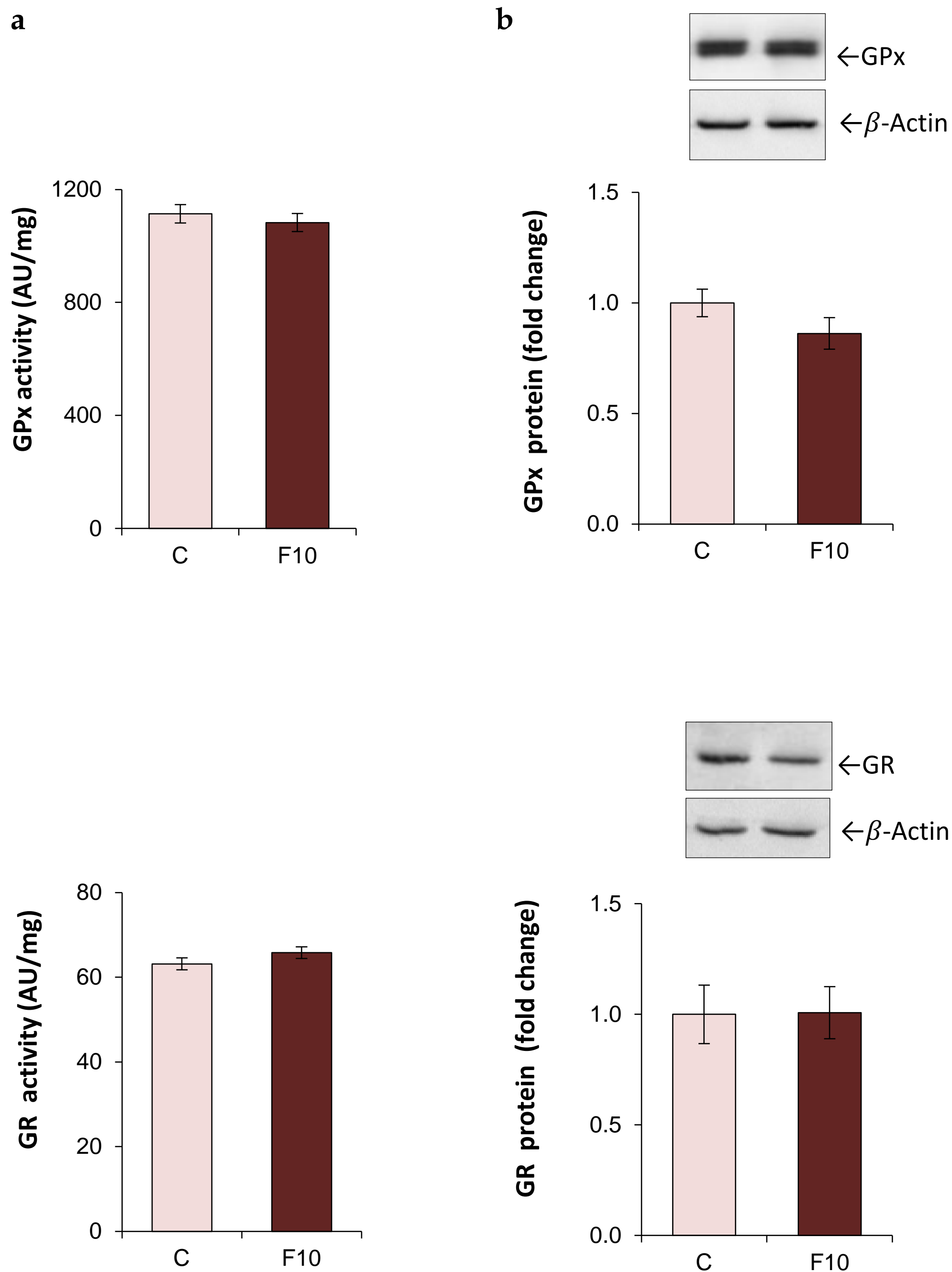

Figure 4.4. Activity and expression of GPx and GR in the liver of female rats exposed to moderate-fructose diet over a period from weaning to adulthood. (**a**) Enzyme activities were determined spectrophotometrically and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of GPx and GR level in hepatic whole cell extracts (50 µg protein) of control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. *β*-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

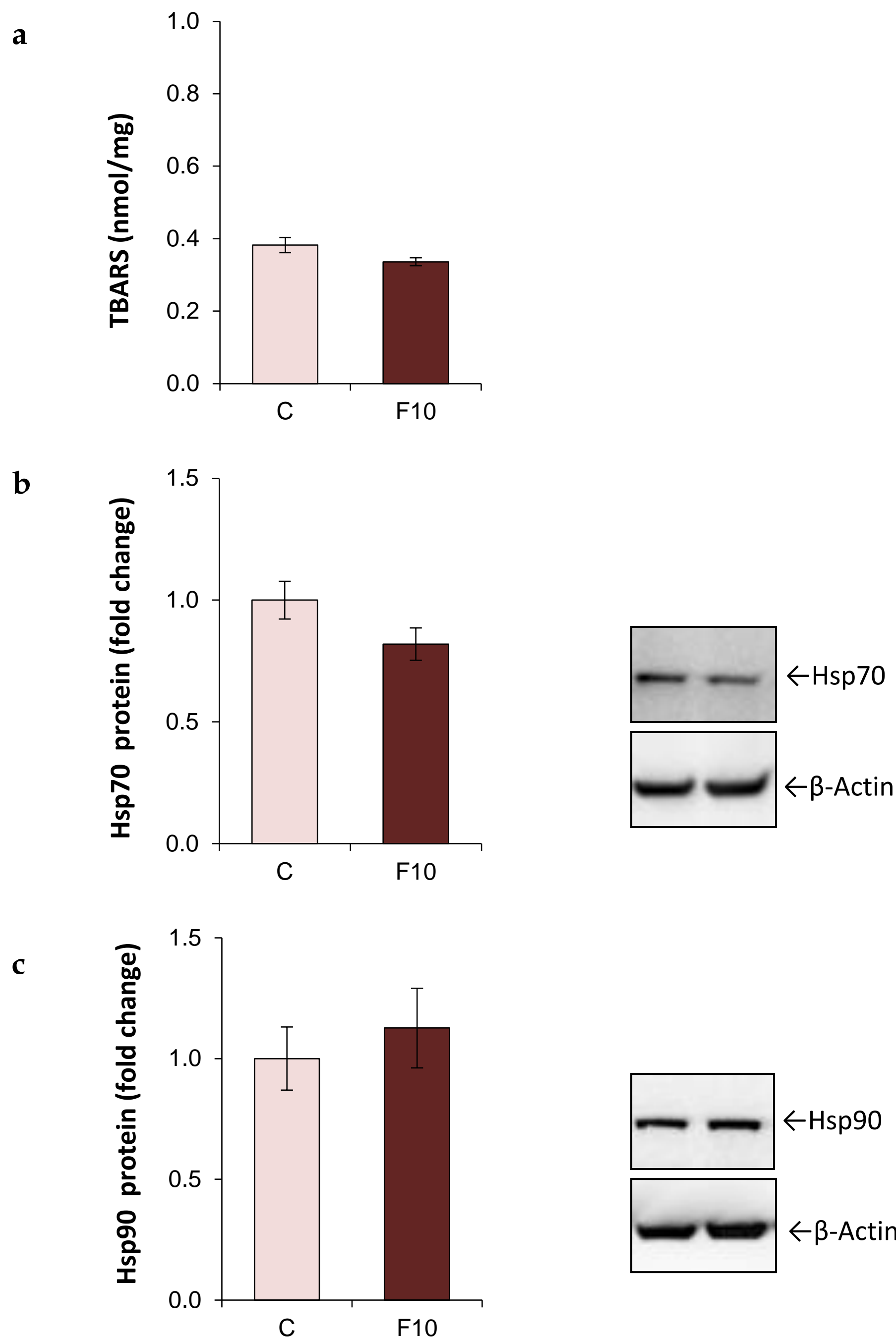

Figure 4.5. Levels of TBARS, Hsp70 and Hsp90 in the liver of female rats subjected to moderate fructose-rich diet in a period from weaning to adulthood. (a) The level of TBARS was determined spectrophotometrically and expressed as nmol per mg of protein. Relative protein levels of Hsp70 **(b)** and Hsp90 **(c)** were determined by Western blotting. Representative Western blots of Hsp70 and Hsp90 in in the hepatic whole cell extracts of control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. *β*-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

4.2 The effects of moderate fructose-rich diet (10% fructose in drinking water) on physiological parameters and hepatic antioxidant enzymes function in male rats

4.2.1 Physiological and biochemical parameters of fructose-fed rats and rats on standard diet

As shown in Table 4.3, moderate fructose-rich diet led to a decrease in chow intake and increase in liquid intake and total energy intake in fructose-fed male rats, as compared to controls. Despite increased energy intake observed in fructose-fed rats, body mass remained unaltered. Also, visceral adipose tissue mass and adiposity index, as well as liver mass and liver to body ratio remained unaltered after the 9-week fructose-rich diet (Table 4.3).

Table 4.3. Food, liquid and energy intake, and physiological parameters in male rats subjected to moderate fructose-rich diet.

	Control	10% Fructose
Food intake (*g/day/animal*)	20.57 ± 2.63	14.20 ± 2.01 *
Liquid intake (*mL/day/animal*)	49.33 ± 5.39	74.10 ± 11.36 *
Total energy intake (*kJ/day/animal*)	226.23 ± 28.90	283.65 ± 24.51 *
Body mass (*g*)	338.89 ± 40.45	311.25 ± 37.58
Mass of liver (*g*)	12.48 ± 1.82	10.77 ± 1.46
Liver-to-body mass ratio (*x100*)	3.67 ± 0.25	3.48 ± 0.41
Mass of visceral adipose tissue (*g*)	2.59 ± 1.14	3.16 ± 1.80
Adiposity index (%)	0.75 ± 0.28	1.01 ± 0.48

The data are presented as means ± SD (n = 9 animals per group). Comparisons between fructose-fed and control rats were made by unpaired Student's t-test. A value of $P<0.05$ was considered statistically significant. Asterisks indicate significant differences. *$P<0.05$.

The blood glucose concentration and plasma insulin level in male rats exposed to moderate fructose-rich diet did not differ between the groups (Table 4.4). An increase in blood triglycerides and plasma NEFA level was observed in male rats subjected to moderate fructose-rich diet as compared to controls. The level of hepatic triglycerides remained unaltered after the treatment (Table 4.4).

Table 4.4. Biochemical and hormonal parameters in male rats subjected to moderate fructose-rich diet

	Control	10% Fructose
Glucose (mmol/l)	5.20 ± 0.42	4.84 ± 0.62
Insulin (mIU/l)	12.68 ± 6.33	7.51 ± 4.94
NEFA (mmol/l)	0.61 ± 0.08	0.70 ± 0.08*
Triglycerides (mmol/l)	1.63 ± 0.30	2.07 ± 0.27*
Triglycerides in the liver (mmol/l)	2.04 ± 0.31	1.63 ± 0.38

Blood glucose and triglyceride levels were determined at the end of the application of 9-week fructose-rich diet, after overnight fasting of animals. Insulin and NEFA levels were measured in isolated plasma samples. Liver triglycerides were isolated from fresh liver tissue. The data are presented as means ± SD (n = 9 animals per group). Comparisons between fructose-fed and control rats were made by unpaired Student's t-test. A value of P<0.05 was considered statistically significant. Asterisks indicate significant differences. *P<0.05.

4.2.2 Activity and expression of antioxidant enzymes in the liver of male rats exposed to moderate-fructose rich diet

To further examine possible gender differences in prooxidative effects of long term fructose consumption we determined the activity of antioxidant enzymes SOD1, SOD2, CAT, GPx, and GR in the livers of male rats subjected to moderate fructose-enriched diet in the period from weaning to adulthood. There were no significant differences in the activity and the expression of SOD1 between fructose-fed and control rats (Figure 4.6).

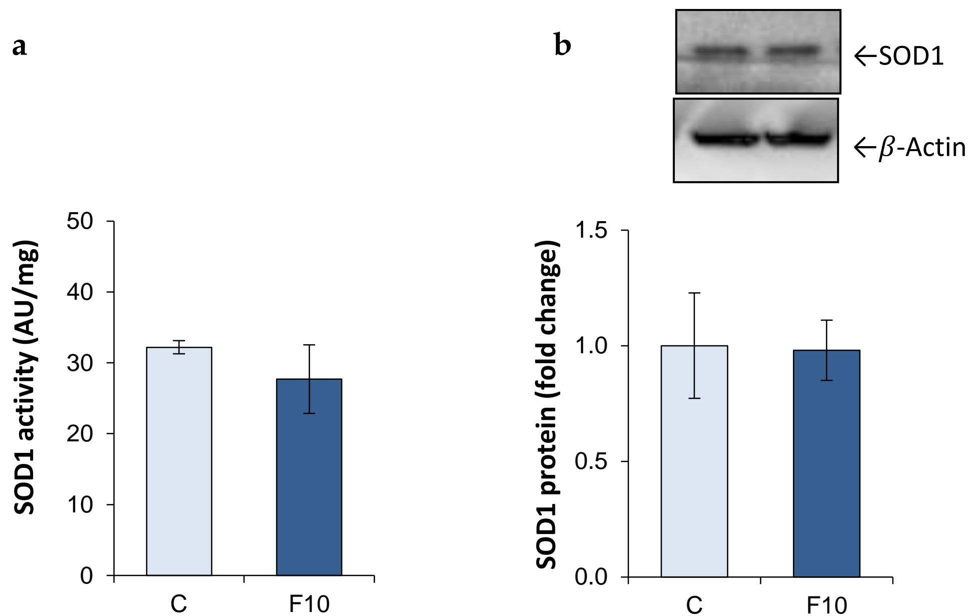

Figure 4.6. Activity and expression of SOD1 in the liver of male rats exposed to moderate-fructose diet over a period from weaning to adulthood. (**a**) SOD1 activity in hepatic whole cell extracts of control (C) and fructose-fed rats (F10) was determined spectrophotometrically, and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of SOD1 level in hepatic whole cell extracts (50 µg protein) of control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. β-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

On the other hand, moderate fructose-rich diet has affected SOD2 function. The expression of SOD2 was significantly elevated by 50% in fructose-fed group, as compared to control, while the activity of SOD2 remained unaltered (Figure 4.7).

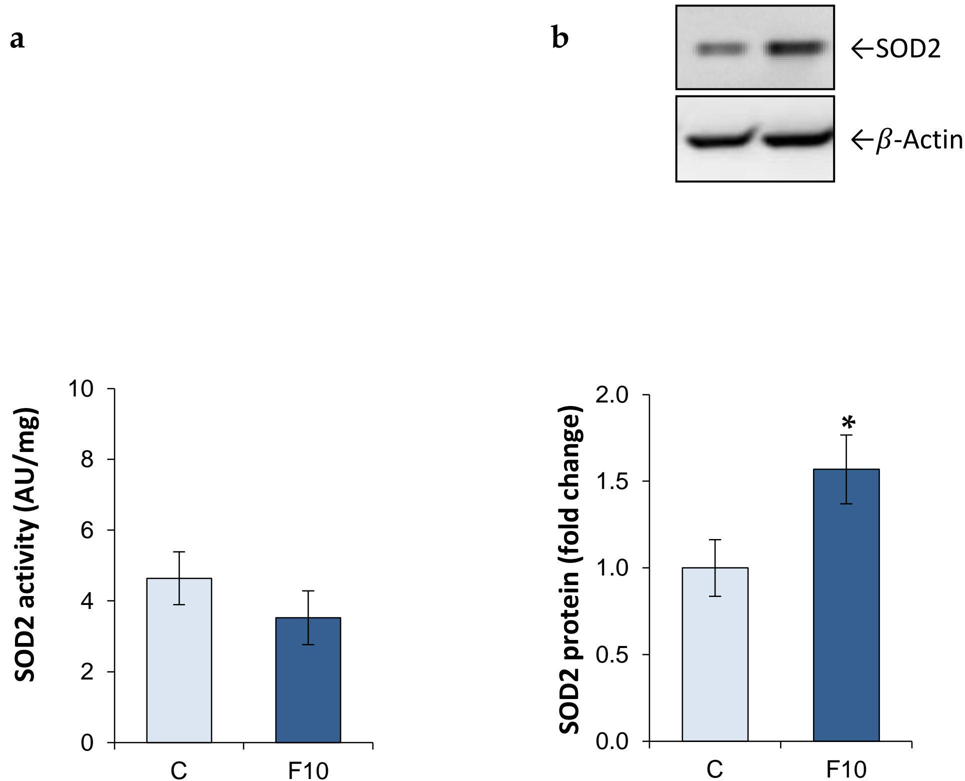

Figure 4.7. Activity and expression of SOD2 in the liver of male rats exposed to moderate-fructose diet over a period from weaning to adulthood. (**a**) SOD2 activity in hepatic whole cell extracts of control (C) and fructose-fed rats (F10) was determined spectrophotometrically, and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of SOD2 level in hepatic whole cell extracts (50 µg protein) of control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. β-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Asterisks indicate significant differences. *$P<0.05$.

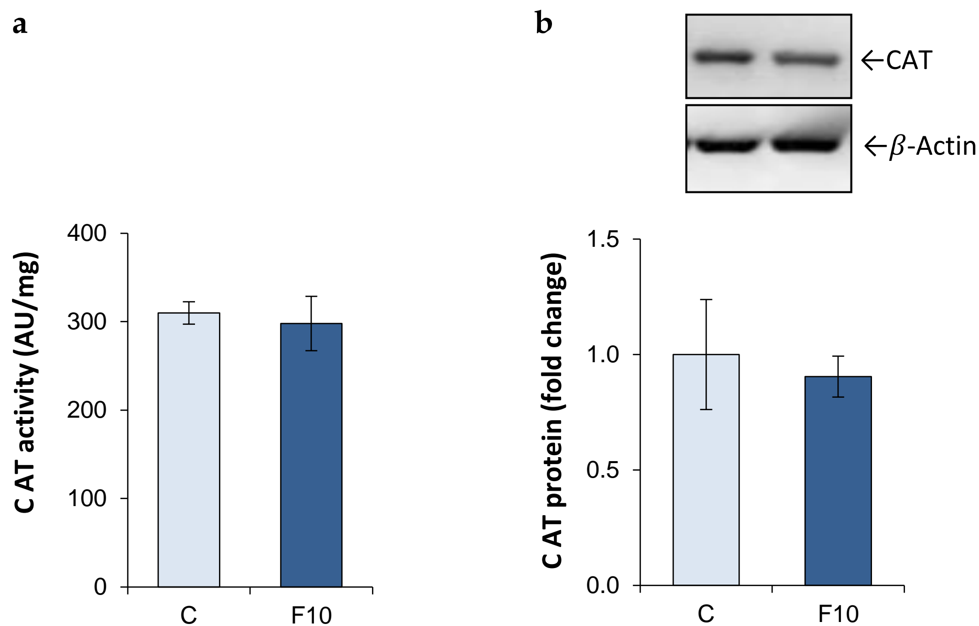

Figure 4.8. Activity and expression of CAT in the liver of male rats exposed to moderate-fructose diet over a period from weaning to adulthood. (**a**) CAT activity was determined spectrophotometrically and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of CAT level in hepatic whole cell extracts (50 µg protein) of control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. *β*-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

As shown on Figure 4.8 and 4.9, moderate fructose rich diet has not affected the activity and expression of CAT, GPx and GR in male rats. Similarly, the level of TBARS as marker of lipid peroxidation, did not differ between the groups (Figure 4.10a). The level of molecular chaperones Hsp70 and Hsp90, the expression of which is up-regulated in response to stress (Richter *et al.*, 2010), remained unchained in the fructose-fed group, suggesting the absence of the diet-related protein damage in the liver (Figure 4.10b and 4.10c).

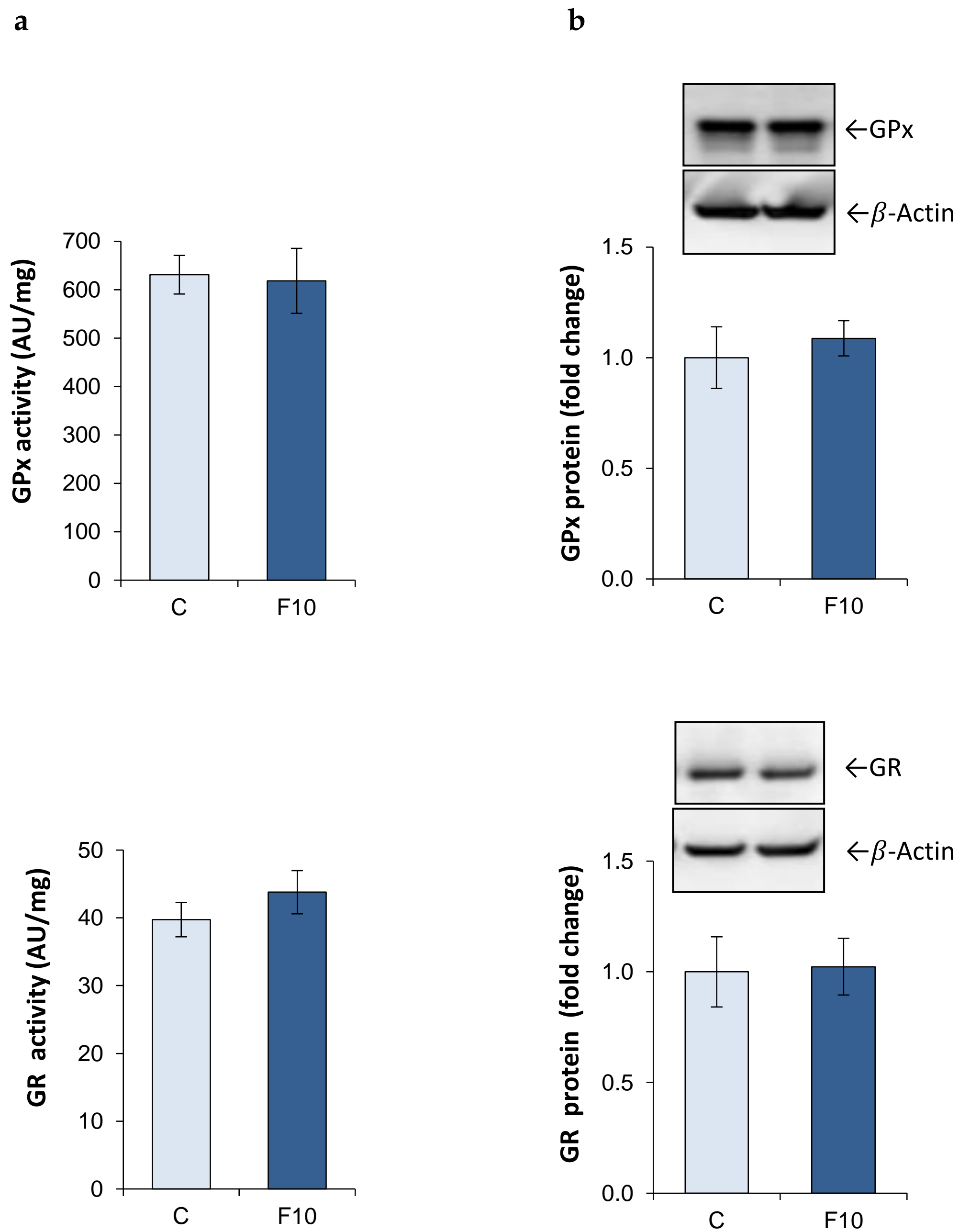

Figure 4.9. Activity and expression of GPx and GR in the liver of male rats exposed to moderate-fructose diet over a period from weaning to adulthood. (**a**) Enzyme activities were determined spectrophotometrically and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of GPx and GR level in hepatic whole cell extracts (50 µg protein) of control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. *β*-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

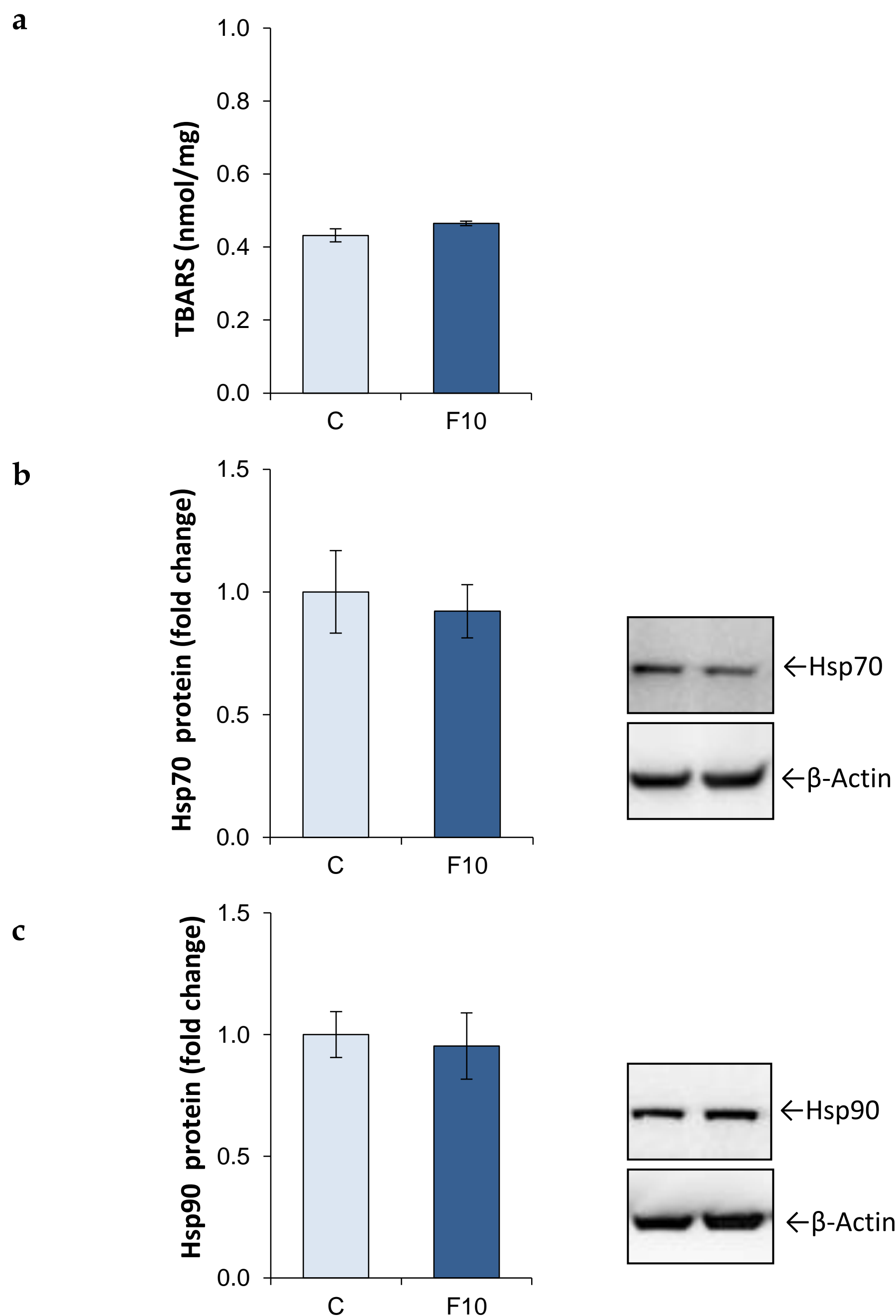

Figure 4.10. Levels of TBARS, Hsp70 and Hsp90 in liver of male rats subjected to moderate-fructose diet in a period from weaning to adulthood. **(a)** The level of TBARS was determined spectrophotometrically and expressed in arbitrary units per mg protein (AU/mg). Relative protein levels of Hsp70 **(b)** and Hsp90 **(c)** were determined by Western blotting as described for Figure 1. Representative Western blots of Hsp70 and Hsp90 in hepatic whole cell extracts (50 µg protein) of control (C) and fructose-fed rats (F10) are shown. Quantitative analysis was done by the ImageQuant software. *β*-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

4.3 The effects of high fructose-rich diet (60% fructose in drinking water) on physiological parameters and hepatic antioxidant enzymes function in male rats

4.3.1 Physiological and biochemical parameters of fructose-fed rats and rats on standard diet

Since males are shown to be more prone to development of diet-induced metabolic disturbances such as insulin resistance-related disorders and hepatic steatosis, we examined the effects of long-term high-fructose diet (60% fructose in drinking water) in male rats subjected to the dietary regime immediately after weaning. In order to prevent kidney hypertrophy, glomerular hypertension and cortical vasoconstriction, as possible consequences of disturbed water balance additional drinking water was provided for fructose-fed group.

Table 4.5. Food, liquid and energy intake, and physiological parameters in male rats subjected to high fructose-rich diet.

	Control	60% Fructose
Food intake (g/day/animal)	21.73 ± 0.73	16.99 ± 2.71 *
Liquid intake (mL/day/animal)	36.82 ± 4.05	24.79 ± 3.76 * Fru 13,32 ± 1.66 H_2O
Total energy intake (kJ/day/animal)	239.08 ± 8.05	324.35 ± 23.79 **
Body mass (g)	333.50 ± 32.34	345.64 ± 32.95
Mass of liver (g)	11.37 ± 1.57	12.80 ± 1.40
Liver-to-body ratio (x100)	3.40 ± 0.19	3.70 ± 0.32 *
Mass of visceral adipose tissue (g)	4.37 ± 1.73	6.26 ± 1.62 *
Adiposity index (%)	1.33 ± 0.51	1.82 ± 0.14 *

The data are presented as means ± SD (n = 9 animals per group). Comparisons between fructose-fed and control rats were made by unpaired Student's t-test. A value of $P<0.05$ was considered statistically significant. Asterisks indicate significant differences. *$P<0.05$, **$P<0.01$.

As shown in Table 4.5, a decrease in chow intake and increase in liquid and energy intake were observed in high fructose-fed rats, as compared to controls. Despite increased energy intake observed in fructose-fed rats, body mass and liver mass remained unaltered. However, visceral adipose tissue mass, adiposity index and liver to body ratio were significantly increased in high fructose-fed group, as compared to control group (Table 4.5).

Table 4.6. Biochemical and hormonal parameters in male rats subjected to high fructose-rich diet

	Control	60% Fructose
Glucose (mmol/l)	4.36 ± 0.52	3.88 ± 0.24
Insulin (mIU/l)	17.12 ± 6.63	18.35 ± 11.10
NEFA (mmol/l)	0.69 ± 0.18	0.70 ± 0.32
Triglycerides (mmol/l)	1.13 ± 0.25	1.55 ± 0.32**
Triglycerides in the liver (mmol/l)	1.37 ± 0.70	1.20 ± 0.31

Blood glucose and triglyceride levels were determined at the end of the 9-week application of high fructose-rich diet, after overnight fasting of animals. Insulin and NEFA levels were measured in isolated plasma samples. Liver triglycerides were isolated from fresh liver tissue. The data are presented as means ± SD (n = 9 animals per group). Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test. A value of $P<0.05$ was considered statistically significant. Asterisks indicate significant differences. **$P<0.01$.

There were no significant differences in fasting glucose level and plasma insulin level between high fructose-fed and control rats (Table 4.6). An increase in blood triglyceride level was observed in male rats subjected to high fructose-rich diet as compared to rats on standard diet, while plasma NEFA level and hepatic triglyceride level remained unchanged (Table 4.6).

4.3.2 *Activity and expression of antioxidant enzymes in the liver of male rats exposed to high-fructose rich diet*

To further examine possible dose-related differences in pro-oxidative effects of long term fructose consumption we determined the activity of antioxidant enzymes SOD1, SOD2, CAT, GSH-Px, and GR in the livers of male rats subjected to high fructose-enriched diet in the period from weaning to adulthood, since males in comparison to females were shown to be more prone to development of diet-induced metabolic disturbances including insulin resistance-related disorders and hepatic steathosis, all being related to oxidative stress.

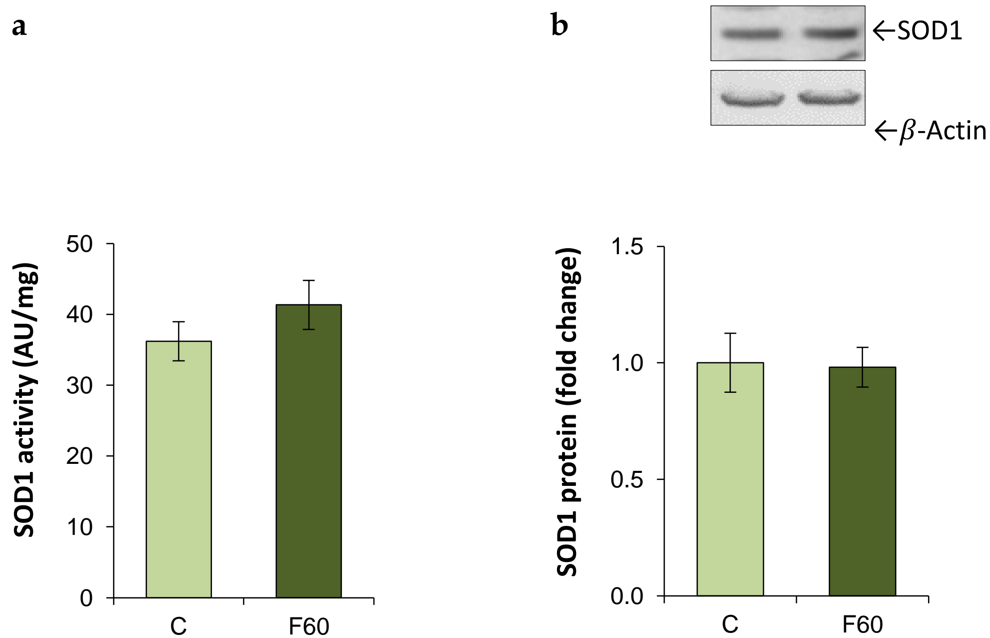

Figure 4.11. Activity and expression of SOD1 in the liver of male rats exposed to high-fructose diet over a period from weaning to adulthood. (**a**) SOD1 activity in hepatic whole cell extracts of control (C) and high fructose-fed rats (F60) was determined spectrophotometrically, and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of SOD1 level in hepatic whole cell extracts (50 µg protein) of control (C) and high fructose-fed rats (F60) are shown. Quantitative analysis was done by the ImageQuant software. *β*-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

High fructose diet had no effect on activity and expression of SOD1 (Figure 4.11). A statistically significant increase in SOD2 activity was observed in high fructose-fed group, as compared to controls, while its expression remained unaltered (Figure 4.12).

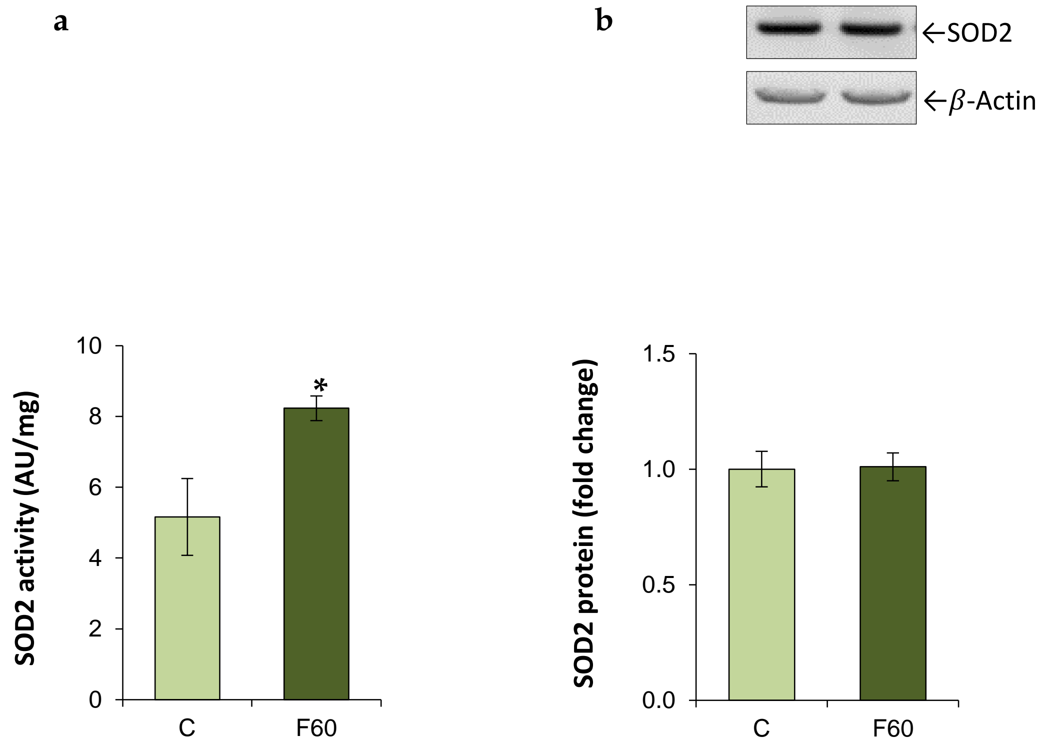

Figure 4.12. Activity and expression of SOD2 in the liver of male rats exposed to high-fructose diet over a period from weaning to adulthood. (**a**) SOD2 activity in hepatic whole cell extracts of control (C) and high fructose-fed rats (F60) was determined spectrophotometrically, and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of SOD2 level in hepatic whole cell extracts (50 μg protein) of control (C) and high fructose-fed rats (F60) are shown. Quantitative analysis was done by the ImageQuant software. β-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Asterisks indicate significant differences. $^{*}P<0.05$. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

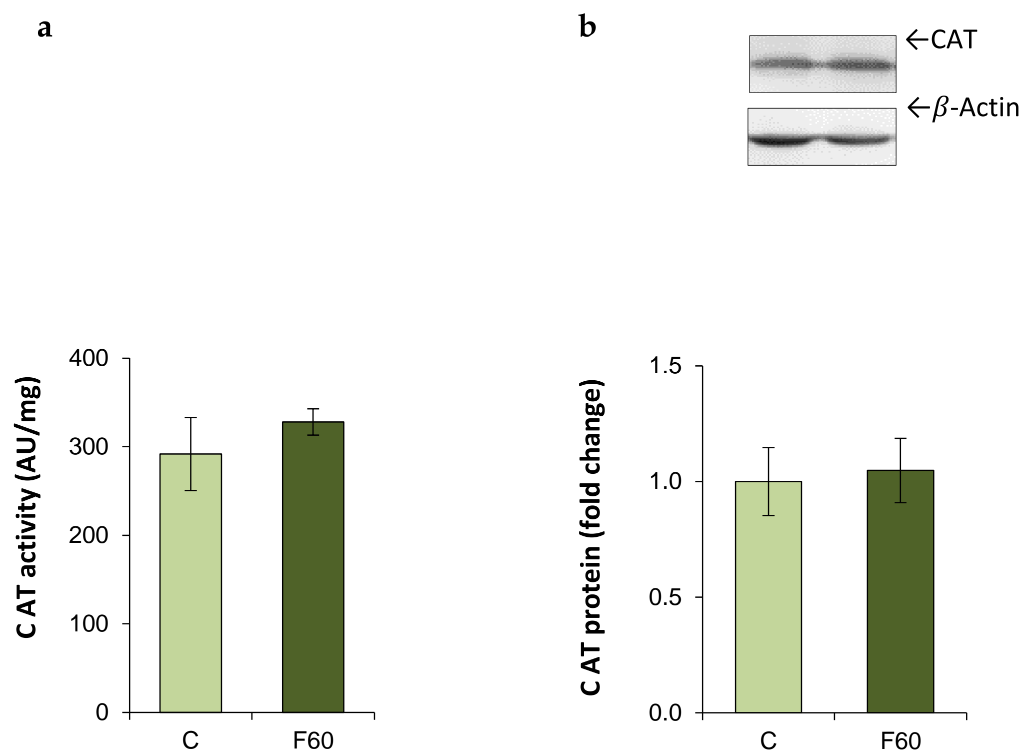

Figure 4.13. Activity and expression of CAT in the liver of male rats exposed to high-fructose diet over a period from weaning to adulthood. (**a**) CAT activity was determined spectrophotometrically and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of CAT level in hepatic whole cell extracts (50 µg protein) of control (C) and high fructose-fed rats (F60) are shown. Quantitative analysis was done by the ImageQuant software. β-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

As shown on Figure 4.13 and 4.14, high fructose diet has not affected the activity and expression of CAT, GPx and GR in male rats. The level of TBARS as marker of lipid peroxidation, and levels of Hsp70 and Hsp90 as markers of accumulation of damaged proteins, did not differ between the groups (Figure 4.15).

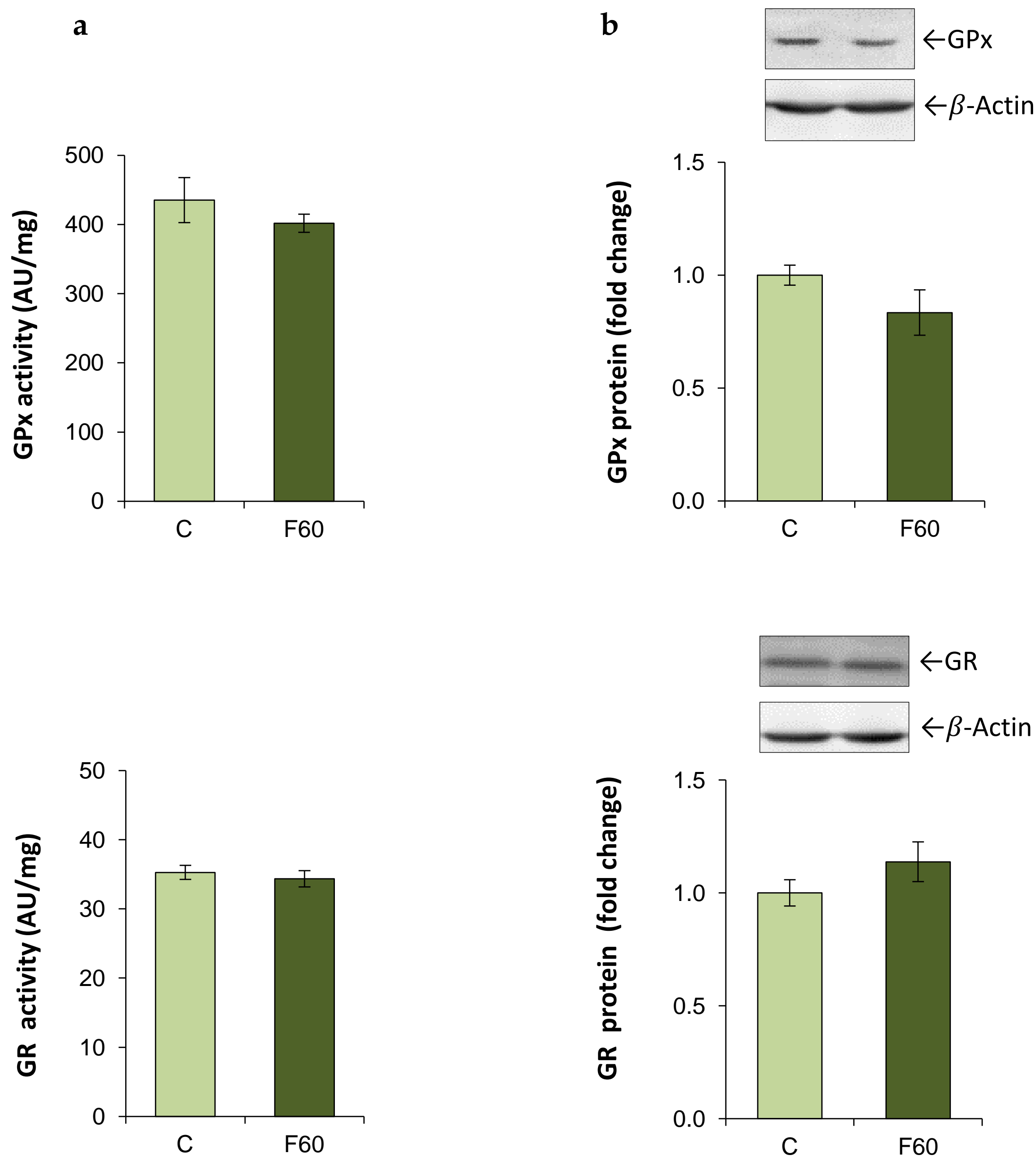

Figure 4.14. Activity and expression of GPx and GR in the liver of male rats exposed to high-fructose diet over a period from weaning to adulthood. (**a**) Enzyme activities were determined spectrophotometrically and expressed in arbitrary units per mg protein (AU/mg). Values are means ± SEM (n=9). (**b**) Representative Western blots and relative quantification of GPx and GR level in hepatic whole cell extracts (50 µg protein) of control (C) and high fructose-fed rats (F60) are shown. Quantitative analysis was done by the ImageQuant software. β-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

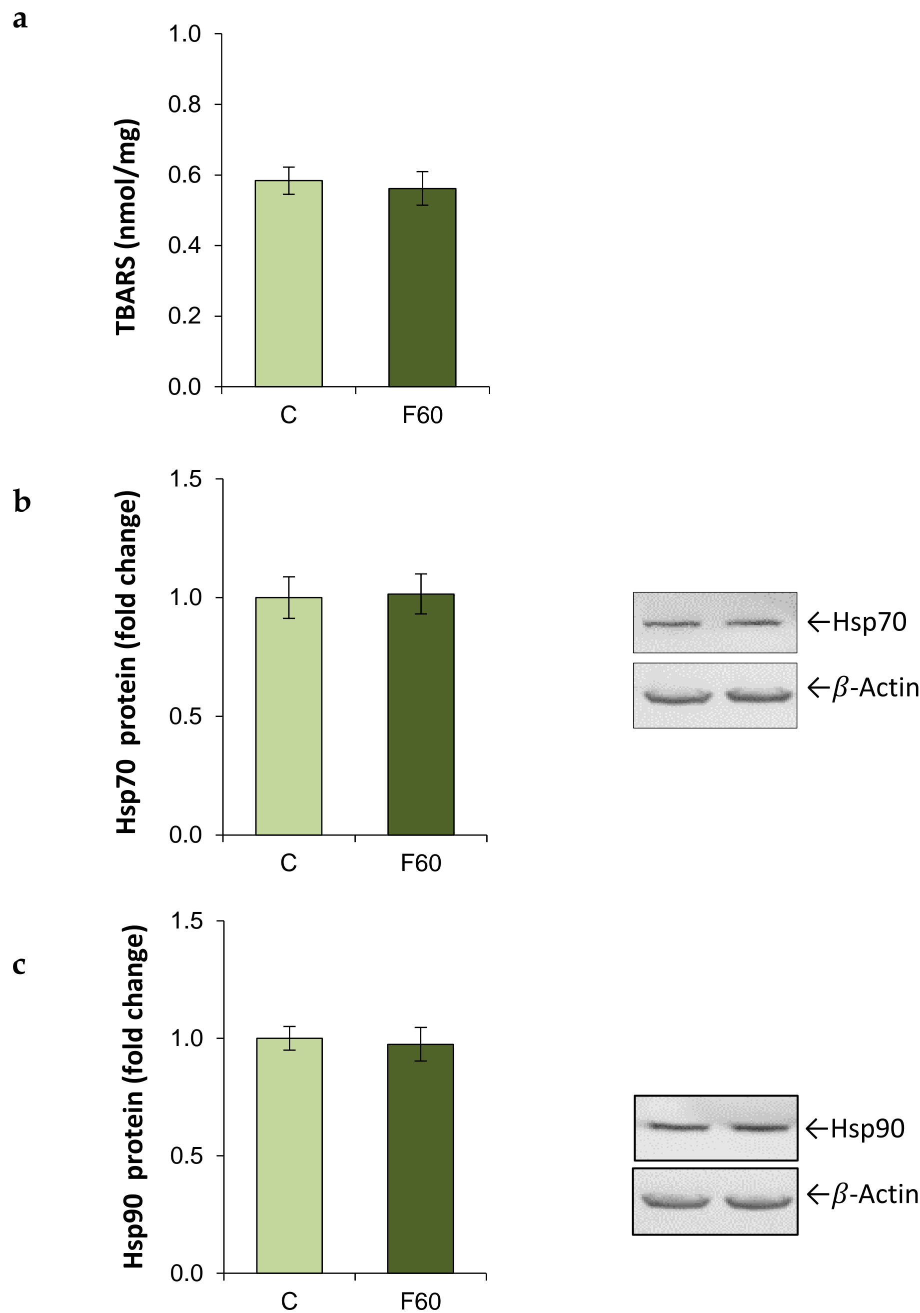

Figure 4.15. Levels of TBARS, Hsp70 and Hsp90 in liver of male rats subjected to high-fructose diet in a period from weaning to adulthood. **(a)** The level of TBARS was determined spectrophotometrically and expressed as nmol per mg of protein. Relative protein levels of Hsp70 **(b)** and Hsp90 **(c)** were determined by Western blotting as described for Figure 1. Representative Western blots of Hsp70 and Hsp90 in hepatic whole cell extracts (50 μg protein) of control (C) and high fructose-fed rats (F60) are shown. Quantitative analysis was done by the ImageQuant software. *β*-Actin was used as loading control. Values are means ± SEM (n=9) and are expressed relative to Control taken as 1. Comparisons between fructose-fed and control rats were made by unpaired Student's *t*-test.

5. DISCUSSION

5 DISCUSION

Since the introduction of high-fructose corn syrup in 1970s its daily intake has largely increased. The usage of high-fructose corn syrup in food industry was encouraged due to its functional advantages over sucrose such as greater sweetness and palatability, better solubility, better preservative features, easier handling, as well as low cost and high production efficiency. Due to these favourable properties it became one of the most versatile cost-effective ingredients on the market (Hanover & White, 1993). However, increase in fructose consumption coincided with the rising incidence of obesity, metabolic syndrome and type 2 diabetes, which has drawn the attention of medical professionals and scientists on the metabolic effects of this sugar (Dekker *et al.*, 2010; Bray, 2013). Soon it became clear that fructose affects lipid metabolism, and being a highly lipogenic sugar it can trigger a cascade of events originating in the liver and adipose tissue that can lead to hyperglycemia, dyslipidemia, hepatic steatosis, abdominal adiposity and insulin resistance, as well as hypertension, hyperuricemia and weight gain (Havel, 2005).

Fructose-fed rat represents a commonly used animal model for studying diet-induced metabolic disturbances (Tran *et al.*, 2009). Previous studies performed on animals have shown that fructose-rich diet can induce most features of metabolic syndrome, including hypertension, insulin resistance, abdominal obesity, hepatic steatosis, endothelial dysfunction and inflammation (Dekker *et al.*, 2010; Tappy *et al.*, 2010). Also, it was suggested that oxidative stress participates in the development and progression of these metabolic disturbances (Grattagliano *et al.*, 2008; Rains & Jain, 2011).

In addition, gender differences in the susceptibility and progression of metabolic disturbances have been reported (Regitz-Zagrosek *et al.*, 2006; Denzer *et al.*, 2009; Varlamov *et al.*, 2014). Males and females differ in distribution of adipose tissue. Namely, in males it is located predominantly in the abdominal region and carries much greater metabolic risk than adipose tissue distributed subcutaneously, as in females (Wajchenberg, 2000). Visceral adipose tissue is metabolically active, produces adipokines and inflammatory mediators, and is associated with insulin resistance and higher cardiovascular risk, in a gender specific manner (Pradhan, 2014). Animal studies have shown that males exhibit a greater resistance to increase in body mass than females. On the other hand, female rats seem to be less susceptible to carbohydrate- or lipid-induced insulin resistance (Horton *et al.*, 1997; Busserolles *et al.*, 2002; Riant *et al.*, 2009). Male rats are more prone to diet-induced hypertension and hyperinsulinemia (Roberts *et al.*, 2001), while female rats develop these symptoms only after ovariectomy (Galipeau *et al.*, 2002).

However, most of the previous studies investigated fructose-induced metabolic disturbances in adults, while data regarding the effects of fructose rich diet on young individuals are lacking. The immature young organism largely differs in the metabolic and physiological sense from adult, and the link between development of metabolic disorders in adulthood and increased fructose consumption in childhood is still not clear.

In this study we have examined the effects of moderate fructose rich diet applied over the period from weaning to adulthood on metabolic parameters. After 9-week application of the diet, an increase in caloric intake was noticed, both in male and female rats. This increase can be attributed to passive overconsumption of fructose, since drinking a palatable solution is not causing a feeling of fullness in the way that calories from food do. One can assume that increase in total energy intake originating from fructose would lead to escalation of body mass, since body mass is crucially dependent on energy balance. Nevertheless, in spite of increased caloric intake observed herein, total body mass remained unaffected by the applied diet regardless of sex, while absolute and relative visceral adipose tissue mass showed marked increase in female rats and only a trend towards an increase in male rats subjected to moderate fructose-rich diet. These findings could suggest that energy expenditure was higher in fructose-fed rats. Based on our unpublished results, we could propose that energetically expensive hepatic lipogenesis, as well as adipose tissue low-grade inflammation (Kovacevic *et al.*, 2015) contribute to enhanced energy expenditure. Previously observed increase in hepatic *de novo* lipogenesis (unpublished results) was followed by triglyceridemia, but not by lipid accumulation in the liver, both in males and females. However we have observed gender differences in plasma NEFA levels and adiposity index of rats subjected to moderate fructose enriched diet immediately after weaning (Milutinovic *et al.*, 2014). Namely, in males fructose-rich diet led to an increase in plasma NEFA levels, while the mass of visceral adipose tissue remained unaltered, suggesting that fructose-rich diet stimulated lipolysis in visceral adipose tissue (Bursac *et al.*, 2013). On the other hand, fructose-rich diet induced adiposity in young female rats, without the rise in plasma NEFA (Kovacevic *et al.*, 2014). Females, in general, due to a larger fat mass as compared to males, might have a more efficient way of removal, processing and storage of excess of lipids, thereby preventing and/or delaying the onset of further diet-induced metabolic disturbances (Votruba & Jensen, 2006; Couchepin *et al.*, 2008).

In line with these, we have previously observed a decrease in insulin sensitivity in the liver of male (Vasiljevic *et al.*, 2013; Velickovic *et al.*, 2013), but not female rats (unpublished results) subjected to moderate fructose-enriched diet immediately after weaning. One of the possible explanations of gender differences in fructose-induced alterations in hepatic insulin signalling might be the difference in the plasma NEFA levels and adipose tissue storage function, since increased influx of NEFA, derived from the adipose tissue, was shown to affect hepatic insulin sensitivity (Boden *et al.*, 2005; Solinas & Karin, 2010). It is possible to assume that enhanced adipose tissue storage function might protect the liver from adverse effects of NEFA pressure, thus playing an additional role in later onset of hepatic insulin resistance in young female rats on moderate fructose-rich diet. On the other hand, high influx of NEFA into the liver can contribute to disturbances in hepatic insulin signalling in males. Another possible explanation might be related to hepatic low-grade inflammation which was observed in male (Velickovic *et al.*, 2013), but not in female rats (unpublished results). Finally, changes in redox environment could also contribute to these gender differences in hepatic insulin signalling.

In line with previous studies, we have recently reported gender-related differences in fructose-induced systemic insulin signalling using the same animal model. Herein we show that fructose-rich diet led to hypoglycemia in female rats, which might indicate enhanced insulin activity. However, a rise of plasma insulin concentration was not observed, implying preserved systemic insulin signalling. Unaltered plasma insulin level was also observed in male rats, however, we have previously reported higher area under the curve (AUC) and longer glucose disposal halftime in male but not female fructose-fed rats, indicating a decrease of insulin sensitivity in male rats subjected to fructose-rich diet after weaning (Koricanac *et al.*, 2013; Vasiljevic *et al.*, 2013). In line with previous results obtained on adults, our results suggest that young male rats are more prone to fructose-induced disturbances in insulin signalling,

while female rats develop adiposity. A rise in plasma NEFA was observed only in males, and can be attributed to enhanced lipolysis in visceral adipose tissue (Bursac *et al.*, 2013). Long-term consumption of moderate dose of fructose increases plasma triglycerides, regardless of sex. Overall, our results show that moderate fructose-rich diet induces gender-specific metabolic disturbances in young rats.

For the purpose of this study, the choice of fructose concentration used for moderate fructose-rich diet was based on the data that 10% fructose solution closely resembles the intake of sweet solutions characteristic for Western diet (Ventura *et al.*, 2011). We have hypothesized that 60% fructose solution would induce more prominent effects on metabolism of young male rats, since males are shown to be more prone to development of diet-induced metabolic disturbances such as insulin resistance-related disorders. Interestingly, the effects of high fructose diet were not as intense as we have anticipated, regarding the insulin sensitivity. Namely, we have observed an increase in caloric intake in high fructose-fed rats, however both body weight and liver mass were unaffected by the applied diet regime, which is in accordance with results obtained from another animal study using 60% fructose enriched diet (Kelley *et al.*, 2004). Although high fructose-fed group had significantly higher liver-to-body ratio than the control group, we have not observed elevated lipid accumulation in the liver. However, the diet has induced hypertriglyceridemia and increased *de novo* lipogenesis in the liver of male rats subjected to high fructose diet (Teofilovic *et al.*, 2016). Moreover, both adipose tissue mass and relative visceral adipose tissue to body ratio were increased in the high fructose-fed male rats, pointing to visceral adiposity. The observed visceral adiposity was not followed by increased plasma NEFA level, which resembles the situation observed in female rats subjected to moderate fructose-rich diet. However, while female rats preserved systemic and hepatic insulin signalling, without low-grade inflammation, a slight disturbance in hepatic, but not

systemic insulin sensitivity was recorded in male rats subjected to high fructose diet (Vasiljevic *et al.*, 2014). Since plasma NEFA levels in high-fructose fed male rats remained unaltered, this decrease in hepatic insulin signalling could be attributed to low-grade inflammation (Vasiljevic *et al.*, 2014), and possibly to oxidative stress. Namely, besides low-grade inflammation, oxidative stress was suggested as possible mediator that could induce and/or aggravate diet-provoked metabolic abnormalities including insulin resistance, obesity and fat accumulation in the liver (Wellen & Hotamisligil, 2005; Keane *et al.*, 2015; Rani *et al.*, 2016).

Previous studies have revealed the link between nutritional excess and oxidative stress, suggesting that redox disbalance might participate in the development and progression of metabolic disturbances including insulin resistance-related disorders (Houstis *et al.*, 2006; Grattagliano *et al.*, 2008; Rains & Jain, 2011). However relation between fructose consumption and oxidative stress appear to be rather complex, since fructose was shown to produce both pro- and anti-oxidative effects, depending on the dose, duration of consumption and (patho)physiological milieu (Semchyshyn, 2013). It was suggested that fructose exhibits negative effects mostly after long-term consumption; however our results on young animals do not support this view. Namely, 9-week fructose rich diet applied after weaning did not induce marked oxidative stress in the rat liver, regardless of sex. However, the diet has induced gender-specific alterations in function of major mitochondrial antioxidant enzyme – SOD2. Namely, an increase in SOD2 protein level was observed in male, but not in female fructose-fed rats.

Males were shown to be more prone to insulin resistance-related disorders. The later onset and less severe metabolic phenotypes in females were related to a better antioxidative capacity of females (Busserolles *et al.*, 2002; Borras *et al.*, 2003; Baba *et al.*, 2005). Namely, females exhibit better antioxidative capacity as compared to males (Borras *et al.*, 2003), and this was even correlated

with longer life span of females (Vina *et al.*, 2005b). Gomez-Perez et al (Gomez-Perez *et al.*, 2008), reported that female rats on high fat diet, in spite of having a greater excess of body mass than males, showed a less marked insulin resistance profile than males, and better oxidative and inflammatory profile, thus a better capacity to cope with oxidative stress-related disturbances in insulin signalling. In addition, only ovariectomized female sucrose-fed rats displayed higher susceptibility to lipid peroxidation as compared to intact females or ovariectomized females supplemented with estradiol (Busserolles *et al.*, 2002), implying the protective role of estrogens. Recently Valencia et al (2016) have reported that overiectomy increases hepatic mitochondrial H_2O_2 production, which further exacerbates with ageing. Furthermore, mitochondrial lipid peroxidation was highest in the aged mice and exacerbated by ovariectomy (Valencia *et al.*, 2016). The authors have suggested that ovarian secretory function is necessary for the maintenance of hepatic ROS buffering capacity in the mitochondria, while age significantly influences mitochondrial respiration. These findings imply that when age is coupled with loss of ovarian function there is an increased risk for developing hepatic mitochondrial dysfunction, which may influence the onset of metabolic disease in females.

However, most of the previous studies were performed on adults while studies on young individuals are still scarce. The ability of antioxidant defence system to react efficiently in conditions of disturbed homeostasis is largely defined by the age. A large body of evidence correlates dysfunction of antioxidant system and increased ROS production with ageing (Zhang *et al.*, 2015). The results presented herein show that fructose-rich diet did not induce oxidative stress in the liver of female and male rats subjected to the dietary regime immediately after weaning. Importantly, moderate fructose rich diet affected only SOD2 function in male but not in female rats.

Oxidative stress and cellular site-specific alterations in redox settings were related to development and progression of metabolic disturbances,

(Houstis *et al.*, 2006; Grattagliano *et al.*, 2008; Rains & Jain, 2011). Disturbed balance between formation and removal of the ROS can lead to damage of cellular macromolecules, result in diverse functional changes and thus contribute to genesis and progression of several pathophysiologies including insulin resistance. Namely, the excess of ROS can lead to deregulation of insulin signalling by impairing serine/threonine phosphorylation of insulin receptor substrate 1 (IRS-1), and by disturbing cellular redistribution of insulin downstream signalling components (Bloch-Damti & Bashan, 2005; Al-Lahham *et al.*, 2016). In addition, it was found that antioxidant supplementation leads to a decrease in ROS generation and improves insulin sensitivity, pointing to the role of ROS in progression and aggravation of insulin-resistance related disorders (Faure *et al.*, 1997; Reddy *et al.*, 2009). We have previously shown that moderate fructose–rich diet applied immediately after weaning affects hepatic insulin sensitivity and induces hypertension, hyperlipidaemia and low grade inflammation only in male rats (Koricanac *et al.*, 2013; Vasiljevic *et al.*, 2013; Velickovic *et al.*, 2013), while females developed adiposity and triglyceridemia (Kovacevic *et al.*, 2014). The results presented herein show that moderate fructose-rich diet did not induce oxidative stress in the liver of female and male rats subjected to the dietary regime immediately after weaning. Namely, the level of TBARS as marker of lipid peroxidation, remained unaltered after fructose consumption, and the absence of increased accumulation of damaged proteins in the liver was confirmed by the unchanged level of Hsp70 and Hsp90. Although the dietary regime has affected SOD2 function only in male rats, the absence of oxidative stress in the cell suggests that young males still have the capacity to maintain redox homeostasis when challenged by the energy overload. However, altered SOD2 function points to mitochondria as possible mediators between fructose overconsumption and deregulation in hepatic insulin signalling in males.

Mitochondria are the major source of ROS in cells, where ROS formation results from imperfectly coupled electron transport (Murphy, 2009). Unchanged mitochondrial SOD2 protein level observed in young females, could be related to preserved activity of electron transport chain. Namely the results of our laboratory show unaltered mitochondrial beta-oxidation of fatty acids in the liver of female rats subjected to fructose-enriched diet immediately after weaning (unpublished results). Namely, the level of Carnitine palmitoyltransferase I α (CPT-1α) which transports acyl-CoA intermediates into mitochondria, thereby representing the rate-limiting initial step in mitochondrial beta-oxidation of fatty acids, remained unchanged (unpublished results). Besides mitochondrial antioxidative enzymes, mitochondrial uncoupling proteins (UCPs) play an important role in the antioxidant defence mechanism. UCPs are proton carrier proteins located in the mitochondrial inner membrane, and UCP-induced proton leak across the membrane can cause partial depolarization of the mitochondrial transmembrane potential (Dulloo & Samec, 2001). Previous results have shown an increased expression of UCP2 in liver of male fructose-fed rats, and it was assumed that it could be an adaptive mechanism to the metabolic overload caused by fructose administration (Castro *et al.*, 2011). On the other hand, the results of our laboratory show unaltered UCP2 expression in the liver of young fructose-fed female rats (unpublished results). Unchanged UCP2 level is in line with unchanged mitochondrial beta-oxidation of fatty acids and unaltered SOD2 protein level the liver of female rats. In addition, the results of our laboratory have shown that moderate fructose-rich diet increases hepatic *de novo* lipogenesis in female rats (unpublished results). Altogether, these results could imply that young female rats use anabolic processes such as *de novo* lipogenesis rather that catabolic processes, such as mitochondrial beta-oxidation of fatty acids, to cope with fructose overload in liver. In addition, the rate of hepatic mitochondrial beta-oxidation can also be altered by NEFA absorbed from the blood (Boden *et al.*, 2005). Our results show that female rats subjected to moderate fructose-rich diet

develop adiposity which was paralleled with increase in blood triglycerides without the rise in NEFA level, suggesting that fructose-fed females adsorb and process fructose in the liver, and transport it in the form of triglycerides to adipose tissue for storage. In line with these, our previously published results show that fructose overconsumption stimulates lipogenic rather than lipolytic processes in visceral adipose tissue of young fructose-fed female rats (Kovacevic *et al.*, 2014). Furthermore, fructose-rich diet led to an increase in the mass of liver but did not elevated lipid deposition in the liver cells. Lipids accumulated in the liver might serve as substrates in harmful chain reactions such as lipid peroxidation, thereby contributing to development and progression of metabolic disorders. However, our results show that fructose-rich diet did not elevated lipid deposition, induced lipid peroxidation and led to an oxidative stress in the liver. Thereby, it is possible to assume that enhanced adipose tissue storage function in young female rats might protect the liver by having a role in preserving hepatic redox environment, and consequently contribute to later onset of metabolic disturbances including hepatic insulin resistance.

In contrast to fructose-fed females, an increase in adipose tissue lipolysis followed by increased plasma concentration of NEFA was observed in male rats subjected to moderate fructose-rich diet immediately after weaning (Bursac *et al.*, 2013). Also, the diet led to a deregulation of hepatic insulin signalling (Vasiljevic *et al.*, 2013). An increase in hepatic SOD2 expression observed in fructose-fed male rats, might be attributed to the enhanced mitochondrial activity and most likely represents an adaptation to site-specific alterations in redox conditions. In line with this, our unpublished results showed increased mitochondrial beta-oxidation of fatty acids in the liver of male rats subjected to moderate fructose-rich diet immediately after weaning. Namely, facilitated activity of electron transport chain inevitably leads to promoted electron leakage and superoxide radical anion production (Murphy, 2009).

Mitochondrial SOD2 rapidly removes superoxide anion radicals produced during oxidative phosphorylation. The product of this reaction is H_2O_2. It is important to point that in our study fructose consumption increased SOD2 expression, but rendered its activity unaffected, and we can only assume that in this situation posttranslational regulation SOD2 function serves to protect mitochondria from short living superoxide anion radical, but without steady rise in H_2O_2 level. However, these assumptions need confirmation.

A plethora of data relates mitochondrial dysfunction to insulin resistance (Montgomery & Turner, 2015). However, there is an ongoing debate whether superoxide anion radical or H_2O_2 is the key player. Most of the studies propose mitochondrial H_2O_2 rather than superoxide anion radical as the link to insulin resistance. It is well known that long term exposure of cells to high levels of H_2O_2 leads to insulin resistance (Anderson *et al.*, 2009; Iwakami *et al.*, 2011). However, Hoehn et al. have demonstrated that overexpressed mitochondrial SOD2 has significant insulin sensitizing properties under various cellular and physiological stresses (Hoehn *et al.*, 2009). Since SOD selectively decreases superoxide anion radical levels at the expense of increased H_2O_2 production, the authors pointed to a critical role for superoxide anion radical rather than H_2O_2. Our results cannot offer a direct answer to this question, but we can propose that in our model fine tuning of SOD2 function represents an adaptation aimed to restore hepatic insulin signalling. Namely, although hepatic insulin signalling was deregulated after fructose-rich diet, a clear state of insulin resistance was not induced. Since low doses of hydrogen peroxide can enhance insulin sensitivity *in vitro* and *in vivo* (Loh *et al.*, 2009; Iwakami *et al.*, 2011), while long term exposure of cells to high levels of H_2O_2 leads to insulin resistance (Anderson *et al.*, 2009; Iwakami *et al.*, 2011) , we can assume that under the conditions of increased production of superoxide anion radical, highly expressed SOD2 enables fast switches in the enzyme activity, where

pulsatile rapid dismutation of its membrane impermeable substrate to permeabile H_2O_2 could provide positive effect on insulin action.

In this study fructose consumption increased SOD2 expression, but rendered its activity unaffected. The majority of studies described transcriptional regulation of SOD2 level suggesting that oxidative stress-induced SOD2 gene expression represents an important cellular defence mechanism (Miao & St Clair, 2009). In line with this, elevated expression of SOD2 in the liver of male rats subjected to moderate fructose-rich diet most likely represents an adaptation aimed to protect mitochondria against elevated superoxide radical anion, produced by enhanced mitochondrial activity. The lack of correlation between SOD2 activity and its protein level, could be related to inactivation of the enzyme by glycation (Jabeen & Saleemuddin, 2006). However, our previously published results show that the level of protein carbonyl groups remained unaltered in fructose-fed *vs.* control rats suggesting that other mechanisms might regulate SOD2 activity (Nestorov *et al.*, 2014). It has been reported that tyrosine nitration leads to significant inactivation of SOD2 (Yamakura *et al.*, 1998; MacMillan-Crow & Thompson, 1999). Peroxynitrite produced by the coupling reaction between nitric oxide and superoxide anion radical, causes nitration of proteins. Our previously published results show that the expression of inducible nitric oxide synthase (iNOS) was not affected by the fructose consumption implying involvement of other posttranslational modifications in the regulation of SOD2 function (Nestorov *et al.*, 2014). Perhaps a more plausible explanation may be the regulation of SOD2 activity by acetylation/deacetylation, which occurs in response to changes in mitochondrial nutrient and/or redox status. It was found that SOD2 acetylation decreased the enzyme activity, while deacetylation by mitochondrial deacetylase Sirtuin-3 (SIRT3) had an opposite effect (Qiu *et al.*, 2010; Chen *et al.*, 2011).

Overall, our results on the effect of moderate fructose rich diet on hepatic antioxidant enzymes activity show that long-term moderate fructose consumption did not induce oxidative stress in the liver of female and male rats subjected to diet in period from weaning to adulthood. The function of antioxidant enzymes and markers of general redox conditions and lipid peroxidation remained unaltered in female rats, while in males the diet led to an increase in the expression of SOD2, but did not affect its activity or activity and expression of all other examined antioxidant enzymes. Since only fructose fed males exhibited disturbed hepatic insulin signalling our results suggest a mediating role of SOD2, however further research is needed.

Our results raised the question whether consumption of higher doses of fructose in young age might induce oxidative stress in the liver as the main fructose-metabolizing tissue, and consequently contribute to development and aggravation of metabolic disturbances in later adulthood. In light of previously reported sex differences in antioxidative capacity, it is possible to assume that high doses of fructose might lead to more pronounced redox-related disturbances particularly in males. To answer this question, we have subjected male rats to long term high-fructose diet regime.

The results show that high fructose-rich diet (60% fructose solution) did not induce oxidative stress in the liver of male rats subjected to the dietary regime immediately after weaning. Namely, the level of TBARS and Hsp70 and Hsp90, as markers of lipid peroxidation and protein damages, remained unaltered after the treatment. In support to our results obtained on male rats subjected to moderate-fructose rich diet, a disruption of hepatic insulin signalling in young male high-fructose-fed rats previously observed in our laboratory (Vasiljevic *et al.*, 2014), which occurred in the absence of oxidative stress, demonstrates that fructose-provoked impairment of insulin signalling is not induced by oxidative stress, at least not at young age. In line with our observation that moderate fructose-rich diet alters only mitochondrial SOD2

function in male rats, we have observed increased SOD2 activity after high-fructose diet. However, moderate fructose-rich diet increased SOD2 expression while rendered its activity unaltered, which may represent an adaptation to enhanced mitochondrial activity, with possible role in insulin signalling. On the other hand, high fructose-rich diet increased only mitochondrial SOD2 activity while the expression of the enzyme remained unaltered. This enhanced SOD2 activity coupled with unaltered expression most likely represents transient modulation of the enzyme function in response to site-specific alterations in redox conditions, which might reflect current energy demands of the cell. In support, the results of our laboratory show unaltered mitochondrial beta-oxidation of fatty acids in high-fructose fed rats (Teofilovic *et al.*, 2016). Also, high fructose-rich diet induced adiposity and triglyceridemia, while plasma NEFA levels remained unchanged, thus implying to stimulated anabolic, rather than catabolic processes. A lack of correlation between SOD2 expression and activity might be ascribed to the posttranslational regulation of the enzyme activity. Although most of the posttranslational modifications of SOD2 have inhibitory effects on the enzyme activity (Yamakura & Kawasaki, 2010), it was found that deacetylation increases SOD2 activity (Qiu *et al.*, 2010; Tao *et al.*, 2010; Chen *et al.*, 2011; Zhu *et al.*, 2012; Tao *et al.*, 2014). Since the increase in SOD2 activity in high fructose-fed rats was not accompanied by increased expression or enhanced activity of other antioxidant enzymes or damage of cellular macromolecules, we can assume that the expected rise in concentration of hydrogen peroxide represents transient and regulated stage during the adaptation to nutritional excess. Using the same animal model, we have previously observed that high-fructose diet impairs hepatic insulin sensitivity (Vasiljevic *et al.*, 2014); therefore the absence of oxidative stress observed herein suggests that the rise in hydrogen peroxide concentration in the liver of high fructose-fed rats might represent a cellular response aimed to restore hepatic insulin sensitivity at the early stage of the disease. However, the possibility that prolonged fructose overconsumption might finally lead to oxidative stress and

consequently contribute to progression and aggravation of metabolic disturbances in later adulthood, should also be investigated.

Overall, moderate fructose-rich diet did not altered hepatic insulin signalling and induced oxidative stress in the liver of female rats subjected to dietary regime immediately after weaning. The diet has not affected antioxidant enzymes function, nor induced lipid peroxidation and protein damages in the liver. Unaffected insulin signalling and absence of oxidative stress suggests that concept of later onset and less severe pathophysiology of insulin-resistance related disorders in females expands to young age.

The lack of oxidative stress observed after both moderate (Nestorov *et al.*, 2014) and high-fructose diets imply that young male rats are also capable to maintain the redox homeostasis challenged by the energy overload and protect the cell from oxidative damages. The proposed causative role of oxidative stress in induction of fructose-induced metabolic disturbances was not confirmed in this study, since both moderate and high-fructose diet decreased hepatic insulin sensitivity and induced low grade inflammation in males without the occurrence of oxidative stress. However modulation of SOD2 function was observed in both dietary regimes implying a mediatory role of mitochondria.

Although the majority of previous studies reported that long-term fructose consumption disrupts antioxidant mechanisms, a large discrepancies in the course and intensity of fructose-induced alterations can be found in the literature (Francini *et al.*, 2010; Kannappan *et al.*, 2010; Pasko *et al.*, 2010; Botezelli *et al.*, 2012; Crescenzo *et al.*, 2013; Glban *et al.*, 2014). For example, fructose rich diet was found to induce a reduction of hepatic CAT and SOD mRNA, (Cavarape *et al.*, 2001), elevation of SOD2 expression and decline in enzyme activity (Kizhner *et al.*, 2007) an increase in SOD1 and GPx activities (Girard *et al.*, 2006), although SOD expression remained unaltered while the expression of GPx decreased in the liver of fructose-fed rats (Girard *et al.*, 2006),

etc. Furthermore, some authors reported that fructose rich diet reduced the antioxidant capacity and caused oxidative damages in the liver (Kannappan *et al.*, 2010; Botezelli *et al.*, 2012; Crescenzo *et al.*, 2013), while others reported the absence of oxidative stress (Pasko *et al.*, 2010), or adaptation to modest oxidative stress (Francini *et al.*, 2010) in fructose fed rats. Finally, Girard et al. reported that fructose rich diet enhanced the total antioxidant capacity of the liver (Girard *et al.*, 2006). The inconsistency of the experimental data might stem from the differences in the treatment duration (ranging from 3 weeks to 17 months), as well as in the fructose concentration (ranging from 10% to 60%), and the age of the animals at the beginning of the treatment. Importantly, none of these studies was performed on juvenile rodents. Our results indicate that the role of oxidative stress and mitochondrial function in the pathogenesis of metabolic disturbances induced by the fructose overconsumption in childhood and adolescence should be further examined.

Our results have raised an important question regarding the prooxidative and antioxidative effects of fructose after long-term consumption. Namely current literature data suggest that fructose exhibits antioxidative and protective effects after short-term application, while its long-term consumption exerts mostly negative effects. Herein, we show that both moderate and high fructose diet induced metabolic disturbances in the young rat. However, we have expected oxidative stress and more aggravated metabolic disturbances after high fructose diet, such as marked state of insulin resistance in the liver, accompanied by liver steatosis, and aggravated inflammation. However, high-fructose diet did not induce oxidative stress after 9-week consumption, which in part, could be attributed to young age of the animals. Another possible explanation could be the unique metabolism of fructose. Namely, fructose and its metabolic derivatives showed high antioxidative capacities and fructose appeared to be better antioxidant as compared to other sugars such as mannose and glucose (Spasojevic *et al.*, 2009a; Spasojevic *et al.*, 2009b). In addition to high

antioxidative capacity and cytoprotective effects, fructose promotes the production of reducing agent NADPH by pentose phosphate pathway. It appears that fructose stimulates anabolic pathways such as lipogenesis, but the absence of more aggravated metabolic disturbances in high fructose fed rats might arise from the absence of marked oxidative stress due to antioxidative properties of fructose and its metabolic derivatives.

At the end, we could propose that the rapid increase in worldwide prevalence of metabolic disorders could not be attributed only to fructose overconsumption. It rather reflects global change in modern lifestyle which includes not only dietary changes, but also a switch to processed food rich in fat, fructose and salts, as well as markedly reduced physical activity.

6. CONCLUSIONS

6 CONCLUSIONS

1. Both male and female rats subjected to moderate (10%) and high (60%) fructose-rich diet exhibited an increase in caloric intake which can be attributed to passive overconsumption of fructose.

2. Also, moderate (10%) and high (60%) fructose-rich diet had no effect on insulin and glucose blood levels, suggesting preserved systemic insulin signalling in the rats of both genders.

3. Long term moderate (10%) fructose-rich diet induced triglyceridemia in both male and female rats. However, it induced gender-specific metabolic disturbances in young rats: in males the diet led to an increase in plasma NEFA levels, while in female rats it caused adiposity without a rise in plasma NEFA levels. These gender differences might be related to differences in activation of lipolytic or lipogenic pathways in the adipose tissue.

4. In male rats, high (60%) fructose diet induced triglyceridemia and adiposity without a rise in plasma NEFA levels.

5. Fructose overconsumption in the period from weaning to adulthood did not induce oxidative stress, as evidenced by unaltered lipid peroxidation as well as accumulation of damaged proteins in the liver of female and male rats. The result suggests that young organisms, due to a higher capacity of antioxidant system, have an increased ability to maintain the redox homeostasis, regulate ROS concentration within non-toxic homeostatic levels and protect the cells from oxidative stress.

6. Moderate (10%) fructose-rich diet applied over a period from weaning to adulthood did not affect the activities, nor the protein levels of hepatic antioxidant enzymes in female rats.

7. In male rats, both moderate (10%) and high (60%) fructose diet altered only mitochondrial SOD2 function, but did not affect the activities, nor the level of expression of other examined antioxidant enzymes.

8. Previously observed fructose-induced disturbances in hepatic insulin signalling in males could not be attributed to oxidative stress, at least not at the young age. Nevertheless, a possible mediatory role of mitochondrial SOD2 in development of metabolic disorders should be further investigated. The later onset of metabolic disturbances, including hepatic insulin resistance, in young females as compared to males, was confirmed in our study.

7. LITERATURE

7 LITERATURE

Al-Lahham, R., Deford, J.H. & Papaconstantinou, J. (2016) Mitochondrial-generated ROS down regulates insulin signaling via activation of the p38MAPK stress response pathway. *Molecular and cellular endocrinology*, **419**, 1-11.

Anderson, E.J., Lustig, M.E., Boyle, K.E., Woodlief, T.L., Kane, D.A., Lin, C.T., Price, J.W., 3rd, Kang, L., Rabinovitch, P.S., Szeto, H.H., Houmard, J.A., Cortright, R.N., Wasserman, D.H. & Neufer, P.D. (2009) Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *J Clin Invest*, **119**, 573-581.

Armutcu, F., Ataymen, M., Atmaca, H. & Gurel, A. (2008) Oxidative stress markers, C-reactive protein and heat shock protein 70 levels in subjects with metabolic syndrome. *Clinical chemistry and laboratory medicine*, **46**, 785-790.

Aruoma, O.I., Kaur, H. & Halliwell, B. (1991) Oxygen free radicals and human diseases. *Journal of the Royal Society of Health*, **111**, 172-177.

Baba, T., Shimizu, T., Suzuki, Y., Ogawara, M., Isono, K., Koseki, H., Kurosawa, H. & Shirasawa, T. (2005) Estrogen, insulin, and dietary signals cooperatively regulate longevity signals to enhance resistance to oxidative stress in mice. *The Journal of biological chemistry*, **280**, 16417-16426.

Baena, M., Sanguesa, G., Davalos, A., Latasa, M.J., Sala-Vila, A., Sanchez, R.M., Roglans, N., Laguna, J.C. & Alegret, M. (2016) Fructose, but not glucose, impairs insulin signaling in the three major insulin-sensitive tissues. *Scientific reports*, **6**, 26149.

Balmus, I.M., Ciobica, A., Antioch, I., Dobrin, R. & Timofte, D. (2016) Oxidative Stress Implications in the Affective Disorders: Main Biomarkers, Animal Models Relevance, Genetic Perspectives, and Antioxidant Approaches. *Oxid Med Cell Longev*, **2016**, 3975101.

Barclay, T., Ginic-Markovic, M., Johnston, M.R., Cooper, P. & Petrovsky, N. (2012) Observation of the keto tautomer of D-fructose in D(2)O using (1)H NMR spectroscopy. *Carbohydrate research*, **347**, 136-141.

Bloch-Damti, A. & Bashan, N. (2005) Proposed mechanisms for the induction of insulin resistance by oxidative stress. *Antioxid Redox Signal*, **7**, 1553-1567.

Boden, G., Homko, C., Barrero, C.A., Stein, T.P., Chen, X., Cheung, P., Fecchio, C., Koller, S. & Merali, S. (2015) Excessive caloric intake acutely causes oxidative stress, GLUT4 carbonylation, and insulin resistance in healthy men. *Science translational medicine*, **7**, 304re307.

Boden, G., She, P., Mozzoli, M., Cheung, P., Gumireddy, K., Reddy, P., Xiang, X., Luo, Z. & Ruderman, N. (2005) Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver. *Diabetes*, **54**, 3458-3465.

Bomback, A.S., Derebail, V.K., Shoham, D.A., Anderson, C.A., Steffen, L.M., Rosamond, W.D. & Kshirsagar, A.V. (2010) Sugar-sweetened soda consumption, hyperuricemia, and kidney disease. *Kidney international*, **77**, 609-616.

Borras, C., Sastre, J., Garcia-Sala, D., Lloret, A., Pallardo, F.V. & Vina, J. (2003) Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free radical biology & medicine*, **34**, 546-552.

Botezelli, J.D., Cambri, L.T., Ghezzi, A.C., Dalia, R.A., Voltarelli, F.A. & de Mello, M.A. (2012) Fructose-rich diet leads to reduced aerobic capacity and to liver injury in rats. *Lipids Health Dis*, **11**, 78.

Bray, G.A. (2013) Energy and fructose from beverages sweetened with sugar or high-fructose corn syrup pose a health risk for some people. *Adv Nutr*, **4**, 220-225.

Bray, G.A., Nielsen, S.J. & Popkin, B.M. (2004) Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. *The American journal of clinical nutrition*, **79**, 537-543.

Bruun, J.M., Maersk, M., Belza, A., Astrup, A. & Richelsen, B. (2015) Consumption of sucrose-sweetened soft drinks increases plasma levels of uric acid in overweight and obese subjects: a 6-month randomised controlled trial. *European journal of clinical nutrition*, **69**, 949-953.

Bursac, B.N., Djordjevic, A.D., Vasiljevic, A.D., Milutinovic, D.D., Velickovic, N.A., Nestorovic, N.M. & Matic, G.M. (2013) Fructose consumption enhances glucocorticoid action in rat visceral adipose tissue. *J Nutr Biochem*, **24**, 1166-1172.

Busserolles, J., Mazur, A., Gueux, E., Rock, E. & Rayssiguier, Y. (2002) Metabolic syndrome in the rat: females are protected against the pro-oxidant effect of a high sucrose diet. *Experimental biology and medicine*, **227**, 837-842.

Castro, M.C., Massa, M.L., Del Zotto, H., Gagliardino, J.J. & Francini, F. (2011) Rat liver uncoupling protein 2: changes induced by a fructose-rich diet. *Life Sci*, **89**, 609-614.

Cavarape, A., Feletto, F., Mercuri, F., Quagliaro, L., Daman, G. & Ceriello, A. (2001) High-fructose diet decreases catalase mRNA levels in rat tissues. *J Endocrinol Invest*, **24**, 838-845.

Ceriello, A. & Motz, E. (2004) Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arteriosclerosis, thrombosis, and vascular biology*, **24**, 816-823.

Chaudiere, J. & Ferrari-Iliou, R. (1999) Intracellular antioxidants: from chemical to biochemical mechanisms. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, **37**, 949-962.

Chen, Y., Zhang, J., Lin, Y., Lei, Q., Guan, K.L., Zhao, S. & Xiong, Y. (2011) Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. *EMBO Rep*, **12**, 534-541.

Claiborne, A. (1985) Catalase activity. In Greenwald, R.A. (ed) *CRC Handbook of Methods for Oxygen Radical Research*. CRC Press, Boca Raton, FL, pp. 283-284.

Commoner, B., Townsend, J. & Pake, G.E. (1954) Free radicals in biological materials. *Nature*, **174**, 689-691.

Couchepin, C., Le, K.A., Bortolotti, M., da Encarnacao, J.A., Oboni, J.B., Tran, C., Schneiter, P. & Tappy, L. (2008) Markedly blunted metabolic effects of fructose in healthy young female subjects compared with male subjects. *Diabetes care*, **31**, 1254-1256.

Couto, N., Wood, J. & Barber, J. (2016) The role of glutathione reductase and related enzymes on cellular redox homoeostasis network. *Free Radic Biol Med*, **95**, 27-42.

Crescenzo, R., Bianco, F., Falcone, I., Coppola, P., Liverini, G. & Iossa, S. (2013) Increased hepatic de novo lipogenesis and mitochondrial efficiency in a model of obesity induced by diets rich in fructose. *Eur J Nutr*, **52**, 537-545.

Das, P., Biswas, S., Mukherjee, S. & Bandyopadhyay, S.K. (2016) Association of Oxidative Stress and Obesity with Insulin Resistance in Type 2 Diabetes Mellitus. *Mymensingh medical journal : MMJ*, **25**, 148-152.

Dekker, M.J., Su, Q., Baker, C., Rutledge, A.C. & Adeli, K. (2010) Fructose: a highly lipogenic nutrient implicated in insulin resistance, hepatic steatosis, and the metabolic syndrome. *Am J Physiol Endocrinol Metab*, **299**, E685-694.

Demir, B., Demir, E., Aciksari, G., Uygun, T., Utku, I.K., Gedikbasi, A., Caglar, I.M., Pirhan, O., Tureli, H.O., Oflar, E., Ungan, I., Ciftci, S. & Karakaya, O. (2014) The Association between the Epicardial Adipose Tissue Thickness and Oxidative Stress Parameters in Isolated Metabolic Syndrome Patients: A Multimarker Approach. *International journal of endocrinology*, **2014**, 954045.

Denzer, C., Thiere, D., Muche, R., Koenig, W., Mayer, H., Kratzer, W. & Wabitsch, M. (2009) Gender-specific prevalences of fatty liver in obese children and adolescents: roles of body fat distribution, sex steroids, and insulin resistance. *J Clin Endocrinol Metab*, **94**, 3872-3881.

Dulloo, A.G. & Samec, S. (2001) Uncoupling proteins: their roles in adaptive thermogenesis and substrate metabolism reconsidered. *Br J Nutr*, **86**, 123-139.

Duncombe, W.G. (1964) The Colorimetric Micro-Determination of Non-Esterified Fatty Acids in Plasma. *Clin Chim Acta*, **9**, 122-125.

Ervin, R.B., Kit, B.K., Carroll, M.D. & Ogden, C.L. (2012) Consumption of added sugar among U.S. children and adolescents, 2005-2008. *NCHS data brief*, 1-8.

Ervin, R.B. & Ogden, C.L. (2013) Consumption of added sugars among U.S. adults, 2005-2010. *NCHS data brief*, 1-8.

Faienza, M.F., Francavilla, R., Goffredo, R., Ventura, A., Marzano, F., Panzarino, G., Marinelli, G., Cavallo, L. & Di Bitonto, G. (2012) Oxidative stress in obesity and metabolic syndrome in children and adolescents. *Hormone research in paediatrics*, **78**, 158-164.

Faure, P., Rossini, E., Lafond, J.L., Richard, M.J., Favier, A. & Halimi, S. (1997) Vitamin E improves the free radical defense system potential and insulin sensitivity of rats fed high fructose diets. *The Journal of nutrition*, **127**, 103-107.

Fletcher, M.J. (1968) A colorimetric method for estimating serum triglycerides. *Clinica chimica acta; international journal of clinical chemistry*, **22**, 393-397.

Folch, J., Lees, M. & Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry*, **226**, 497-509.

Forman, H.J. (2016) Redox signaling: An evolution from free radicals to aging. *Free Radic Biol Med*, **97**, 398-407.

Forman, H.J., Maiorino, M. & Ursini, F. (2010) Signaling functions of reactive oxygen species. *Biochemistry*, **49**, 835-842.

Francini, F., Castro, M.C., Schinella, G., Garcia, M.E., Maiztegui, B., Raschia, M.A., Gagliardino, J.J. & Massa, M.L. (2010) Changes induced by a fructose-rich diet on hepatic metabolism and the antioxidant system. *Life Sci*, **86**, 965-971.

Frenzel, J., Richter, J. & Eschrich, K. (2002) Fructose inhibits apoptosis induced by reoxygenation in rat hepatocytes by decreasing reactive oxygen species via stabilization of the glutathione pool. *Biochim Biophys Acta*, **1542**, 82-94.

Fridovich, I. (1995) Superoxide radical and superoxide dismutases. *Annual review of biochemistry*, **64**, 97-112.

Fujita, K., Nishizawa, H., Funahashi, T., Shimomura, I. & Shimabukuro, M. (2006) Systemic oxidative stress is associated with visceral fat accumulation and the metabolic syndrome. *Circulation journal : official journal of the Japanese Circulation Society*, **70**, 1437-1442.

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M. & Shimomura, I. (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. *The Journal of clinical investigation*, **114**, 1752-1761.

Galipeau, D., Verma, S. & McNeill, J.H. (2002) Female rats are protected against fructose-induced changes in metabolism and blood pressure. *American journal of physiology. Heart and circulatory physiology*, **283**, H2478-2484.

Garawi, F., Devries, K., Thorogood, N. & Uauy, R. (2014) Global differences between women and men in the prevalence of obesity: is there an association with gender inequality? *European journal of clinical nutrition*, **68**, 1101-1106.

Geer, E.B. & Shen, W. (2009) Gender differences in insulin resistance, body composition, and energy balance. *Gender medicine*, **6 Suppl 1**, 60-75.

Gerschman, R., Gilbert, D.L., Nye, S.W., Dwyer, P. & Fenn, W.O. (1954) Oxygen poisoning and x-irradiation: a mechanism in common. *Science*, **119**, 623-626.

Giergiel, M., Lopucki, M., Stachowicz, N. & Kankofer, M. (2012) The influence of age and gender on antioxidant enzyme activities in humans and laboratory animals. *Aging Clin Exp Res*.

Girard, A., Madani, S., Boukortt, F., Cherkaoui-Malki, M., Belleville, J. & Prost, J. (2006) Fructose-enriched diet modifies antioxidant status and lipid metabolism in spontaneously hypertensive rats. *Nutrition*, **22**, 758-766.

Glatzle, D., Vuilleumier, J.P., Weber, F. & Decker, K. (1974) Glutathione reductase test with whole blood, a convenient procedure for the assessment of the riboflavin status in humans. *Experientia*, **30**, 665-667.

Glban, A.M., Vasiljevic, A., Velickovic, N., Nikolic-Kokic, A., Blagojevic, D., Matic, G. & Nestorov, J. (2014) The expression and activity of antioxidant enzymes in the liver of rats exposed to high-fructose diet in period from weaning to adulthood. *J Sci Food Agric*.

Gomez-Perez, Y., Amengual-Cladera, E., Catala-Niell, A., Thomas-Moya, E., Gianotti, M., Proenza, A.M. & Llado, I. (2008) Gender dimorphism in high-fat-diet-induced insulin resistance in skeletal muscle of aged rats. *Cell Physiol Biochem*, **22**, 539-548.

Gonzalez-Muniesa, P., Marrades, M.P., Martinez, J.A. & Moreno-Aliaga, M.J. (2013) Differential proinflammatory and oxidative stress response and vulnerability to metabolic syndrome in habitual high-fat young male consumers putatively predisposed by their genetic background. *International journal of molecular sciences*, **14**, 17238-17255.

Grattagliano, I., Palmieri, V.O., Portincasa, P., Moschetta, A. & Palasciano, G. (2008) Oxidative stress-induced risk factors associated with the metabolic syndrome: a unifying hypothesis. *J Nutr Biochem*, **19**, 491-504.

Hanover, L.M. & White, J.S. (1993) Manufacturing, composition, and applications of fructose. *The American journal of clinical nutrition*, **58**, 724S-732S.

Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry. *Journal of gerontology*, **11**, 298-300.

Havel, P.J. (2005) Dietary fructose: implications for dysregulation of energy homeostasis and lipid/carbohydrate metabolism. *Nutrition reviews*, **63**, 133-157.

Henriksen, E.J., Diamond-Stanic, M.K. & Marchionne, E.M. (2011) Oxidative stress and the etiology of insulin resistance and type 2 diabetes. *Free radical biology & medicine*, **51**, 993-999.

Hewitt, F.G. (1940) Augustin-Pierre Dubrunfaut—An early sugar chemist. *Journal of Chemical Education*, **17**.

Hoehn, K.L., Salmon, A.B., Hohnen-Behrens, C., Turner, N., Hoy, A.J., Maghzal, G.J., Stocker, R., Van Remmen, H., Kraegen, E.W., Cooney, G.J., Richardson, A.R. & James, D.E. (2009) Insulin resistance is a cellular antioxidant defense mechanism. *Proc Natl Acad Sci U S A*, **106**, 17787-17792.

Hopps, E., Noto, D., Caimi, G. & Averna, M.R. (2010) A novel component of the metabolic syndrome: the oxidative stress. *Nutrition, metabolism, and cardiovascular diseases : NMCD*, **20**, 72-77.

Horton, T.J., Gayles, E.C., Prach, P.A., Koppenhafer, T.A. & Pagliassotti, M.J. (1997) Female rats do not develop sucrose-induced insulin resistance. *The American journal of physiology*, **272**, R1571-1576.

Hostmark, A.T. (2010) The Oslo Health Study: a Dietary Index estimating high intake of soft drinks and low intake of fruits and vegetables was positively associated with components of the metabolic syndrome. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme*, **35**, 816-825.

Houstis, N., Rosen, E.D. & Lander, E.S. (2006) Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature*, **440**, 944-948.

Imlay, J.A. (2003) Pathways of oxidative damage. *Annual review of microbiology*, **57**, 395-418.

Iwakami, S., Misu, H., Takeda, T., Sugimori, M., Matsugo, S., Kaneko, S. & Takamura, T. (2011) Concentration-dependent dual effects of hydrogen peroxide on insulin signal transduction in H4IIEC hepatocytes. *PLoS One*, **6**, e27401.

Jabeen, R. & Saleemuddin, M. (2006) Polyclonal antibodies inhibit the glycation-induced inactivation of bovine Cu,Zn-superoxide dismutase. *Biotechnol Appl Biochem*, **43**, 49-53.

Johnson, J.L., Slentz, C.A., Duscha, B.D., Samsa, G.P., McCartney, J.S., Houmard, J.A. & Kraus, W.E. (2004) Gender and racial differences in lipoprotein subclass distributions: the STRRIDE study. *Atherosclerosis*, **176**, 371-377.

Johnson, R.J., Nakagawa, T., Sanchez-Lozada, L.G., Shafiu, M., Sundaram, S., Le, M., Ishimoto, T., Sautin, Y.Y. & Lanaspa, M.A. (2013) Sugar, uric acid, and the etiology of diabetes and obesity. *Diabetes*, **62**, 3307-3315.

Kannappan, S., Palanisamy, N. & Anuradha, C.V. (2010) Suppression of hepatic oxidative events and regulation of eNOS expression in the liver by naringenin in fructose-administered rats. *Eur J Pharmacol*, **645**, 177-184.

Katsuki, A., Sumida, Y., Urakawa, H., Gabazza, E.C., Murashima, S., Nakatani, K., Yano, Y. & Adachi, Y. (2004) Increased oxidative stress is associated with serum levels of triglyceride, insulin resistance, and hyperinsulinemia in Japanese metabolically obese, normal-weight men. *Diabetes care*, **27**, 631-632.

Keane, K.N., Cruzat, V.F., Carlessi, R., de Bittencourt, P.I., Jr. & Newsholme, P. (2015) Molecular Events Linking Oxidative Stress and Inflammation to Insulin Resistance and beta-Cell Dysfunction. *Oxidative medicine and cellular longevity*, **2015**, 181643.

Kelley, G.L., Allan, G. & Azhar, S. (2004) High dietary fructose induces a hepatic stress response resulting in cholesterol and lipid dysregulation. *Endocrinology*, **145**, 548-555.

Kelly, A.S., Steinberger, J., Kaiser, D.R., Olson, T.P., Bank, A.J. & Dengel, D.R. (2006) Oxidative stress and adverse adipokine profile characterize the metabolic syndrome in children. *Journal of the cardiometabolic syndrome*, **1**, 248-252.

Kizhner, T., Shilovizki, O. & Werman, M.J. (2007) Long-term fructose intake reduces oxidative defense and alters mitochondrial performance in mice. *Nutrition Research*, **27**, 423-431.

Koricanac, G., Djordjevic, A., Zakula, Z., Vojnovic-Milutinovic, D., Tepavcevic, S., Velikovic, N., Milosavljevic, T., Stojiljkovic, M., Romic, S. & Matic, G. (2013) Gender Modulates Development of the Metabolic Syndrome Phenotype in Fructose-Fed Rats. *Arch Biol Sci*, **65**, 455-464.

Kotani, K. & Yamada, T. (2012) Oxidative stress and metabolic syndrome in a Japanese female population. *Australasian journal on ageing*, **31**, 124-127.

Kovacevic, S., Nestorov, J., Matic, G. & Elakovic, I. (2014) Dietary fructose-related adiposity and glucocorticoid receptor function in visceral adipose tissue of female rats. *Eur J Nutr*.

Kovacevic, S., Nestorov, J., Matic, G. & Elakovic, I. (2015) Fructose-enriched diet induces inflammation and reduces antioxidative defense in visceral adipose tissue of young female rats. *Eur J Nutr*.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Loh, K., Deng, H., Fukushima, A., Cai, X., Boivin, B., Galic, S., Bruce, C., Shields, B.J., Skiba, B., Ooms, L.M., Stepto, N., Wu, B., Mitchell, C.A., Tonks, N.K., Watt, M.J., Febbraio, M.A., Crack, P.J., Andrikopoulos, S. & Tiganis, T. (2009) Reactive oxygen species enhance insulin sensitivity. *Cell Metab*, **10**, 260-272.

Lushchak, V.I. (2014) Classification of oxidative stress based on its intensity. *EXCLI journal*, **13**, 922-937.

MacMillan-Crow, L.A. & Thompson, J.A. (1999) Tyrosine modifications and inactivation of active site manganese superoxide dismutase mutant (Y34F) by peroxynitrite. *Arch Biochem Biophys*, **366**, 82-88.

Malik, V.S. & Hu, F.B. (2015) Fructose and Cardiometabolic Health: What the Evidence From Sugar-Sweetened Beverages Tells Us. *Journal of the American College of Cardiology*, **66**, 1615-1624.

Manna, P. & Jain, S.K. (2015) Obesity, Oxidative Stress, Adipose Tissue Dysfunction, and the Associated Health Risks: Causes and Therapeutic Strategies. *Metabolic syndrome and related disorders*, **13**, 423-444.

Marshall, R.O. & Kooi, E.R. (1957) Enzymatic conversion of D-glucose to D-fructose. *Science*, **125**, 648-649.

Mates, J.M., Perez-Gomez, C. & Nunez de Castro, I. (1999) Antioxidant enzymes and human diseases. *Clinical biochemistry*, **32**, 595-603.

Meier, B., Scherk, C., Schmidt, M. & Parak, F. (1998) pH-dependent inhibition by azide and fluoride of the iron superoxide dismutase from Propionibacterium shermanii. *Biochem J*, **331 (Pt 2)**, 403-407.

Meyer, M.R., Clegg, D.J., Prossnitz, E.R. & Barton, M. (2011) Obesity, insulin resistance and diabetes: sex differences and role of oestrogen receptors. *Acta physiologica*, **203**, 259-269.

Miao, L. & St Clair, D.K. (2009) Regulation of superoxide dismutase genes: implications in disease. *Free Radic Biol Med*, **47**, 344-356.

Miljkovic, D. & Spasojevic, I. (2013) Multiple sclerosis: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal*, **19**, 2286-2334.

Milutinovic, D.V., Nikolic, M., Dinic, J., Dordevic, A., Velickovic, N., Elakovic, I., Matic, G. & Nestorov, J. (2014) Leptin and Glucocorticoid Signaling Pathways in the Hypothalamus of Female and Male Fructose-Fed Rats. *Arch Biol Sci*, **66**, 829-839.

Misra, H.P. & Fridovich, I. (1972) The generation of superoxide radical during the autoxidation of hemoglobin. *J Biol Chem*, **247**, 6960-6962.

Montgomery, M.K. & Turner, N. (2015) Mitochondrial dysfunction and insulin resistance: an update. *Endocrine connections*, **4**, R1-R15.

Moreno, J.A. & Hong, E. (2013) A single oral dose of fructose induces some features of metabolic syndrome in rats: role of oxidative stress. *Nutr Metab Cardiovasc Dis*, **23**, 536-542.

Murphy, M.P. (2009) How mitochondria produce reactive oxygen species. *Biochem J*, **417**, 1-13.

Nestorov, J., Glban, A.M., Mijuskovic, A., Nikolic-Kokic, A., Elakovic, I., Velickovic, N. & Matic, G. (2014) Long-term fructose-enriched diet introduced immediately after weaning does not induce oxidative stress in the rat liver. *Nutrition research*, **34**, 646-652.

Nomura, K. & Yamanouchi, T. (2012) The role of fructose-enriched diets in mechanisms of nonalcoholic fatty liver disease. *The Journal of nutritional biochemistry*, **23**, 203-208.

Paglia, D.E. & Valentine, W.N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med*, **70**, 158-169.

Pajovic, S.B. & Saicic, Z.S. (2008) Modulation of antioxidant enzyme activities by sexual steroid hormones. *Physiol Res*, **57**, 801-811.

Pasko, P., Barton, H., Zagrodzki, P., Izewska, A., Krosniak, M., Gawlik, M. & Gorinstein, S. (2010) Effect of diet supplemented with quinoa seeds on oxidative status in plasma and selected tissues of high fructose-fed rats. *Plant Foods Hum Nutr*, **65**, 146-151.

Perez-Pozo, S.E., Schold, J., Nakagawa, T., Sanchez-Lozada, L.G., Johnson, R.J. & Lillo, J.L. (2010) Excessive fructose intake induces the features of metabolic syndrome in healthy adult men: role of uric acid in the hypertensive response. *International journal of obesity*, **34**, 454-461.

Pradhan, A.D. (2014) Sex differences in the metabolic syndrome: implications for cardiovascular health in women. *Clinical chemistry*, **60**, 44-52.

Qiu, X., Brown, K., Hirschey, M.D., Verdin, E. & Chen, D. (2010) Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. *Cell Metab*, **12**, 662-667.

Rains, J.L. & Jain, S.K. (2011) Oxidative stress, insulin signaling, and diabetes. *Free Radic Biol Med*, **50**, 567-575.

Rani, V., Deep, G., Singh, R.K., Palle, K. & Yadav, U.C. (2016) Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. *Life sciences*, **148**, 183-193.

Rao, V.S., Nagaraj, R.K., Hebbagodi, S., Kadarinarasimhiah, N.B. & Kakkar, V.V. (2010) Association of inflammatory and oxidative stress markers with metabolic syndrome in asian indians in India. *Cardiology research and practice*, **2011**, 295976.

Reddy, S.S., Ramatholisamma, P., Karuna, R. & Saralakumari, D. (2009) Preventive effect of Tinospora cordifolia against high-fructose diet-induced insulin resistance and oxidative stress in male Wistar rats. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, **47**, 2224-2229.

Regitz-Zagrosek, V., Lehmkuhl, E. & Weickert, M.O. (2006) Gender differences in the metabolic syndrome and their role for cardiovascular disease. *Clin Res Cardiol*, **95**, 136-147.

Rehncrona, S., Smith, D.S., Akesson, B., Westerberg, E. & Siesjo, B.K. (1980) Peroxidative changes in brain cortical fatty acids and phospholipids, as characterized during Fe2+- and ascorbic acid-stimulated lipid peroxidation in vitro. *J Neurochem*, **34**, 1630-1638.

Reuter, S., Gupta, S.C., Chaturvedi, M.M. & Aggarwal, B.B. (2010) Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med*, **49**, 1603-1616.

Riant, E., Waget, A., Cogo, H., Arnal, J.F., Burcelin, R. & Gourdy, P. (2009) Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice. *Endocrinology*, **150**, 2109-2117.

Richter, K., Haslbeck, M. & Buchner, J. (2010) The heat shock response: life on the verge of death. *Mol Cell*, **40**, 253-266.

Roberts, C.K., Vaziri, N.D. & Barnard, R.J. (2001) Protective effects of estrogen on gender-specific development of diet-induced hypertension. *Journal of applied physiology*, **91**, 2005-2009.

Rochlani, Y., Pothineni, N.V. & Mehta, J.L. (2015) Metabolic Syndrome: Does it Differ Between Women and Men? *Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy*, **29**, 329-338.

Sabir, A.A., Bilbis, L.S., Saidu, Y., Jimoh, A., Iwuala, S.O., Isezuo, S.A., Kaoje, A.U. & Abubakar, S.A. (2016) Oxidative stress among subjects with metabolic syndrome in Sokoto, North-Western Nigeria. *Nigerian journal of clinical practice*, **19**, 128-132.

Sanchez-Lozada, L.G., Tapia, E., Jimenez, A., Bautista, P., Cristobal, M., Nepomuceno, T., Soto, V., Avila-Casado, C., Nakagawa, T., Johnson, R.J., Herrera-Acosta, J. & Franco, M. (2007) Fructose-induced metabolic syndrome is associated with glomerular hypertension and renal microvascular damage in rats. *Am J Physiol Renal Physiol*, **292**, F423-429.

Semchyshyn, H.M. (2013) Fructation in vivo: detrimental and protective effects of fructose. *Biomed Res Int*, **2013**, 343914.

Semchyshyn, H.M. & Lozinska, L.M. (2012) Fructose protects baker's yeast against peroxide stress: potential role of catalase and superoxide dismutase. *FEMS Yeast Res*, **12**, 761-773.

Shallenberger, R.S. (1978) Intrinsic Chemistry of Fructose. *Journal of Chemical Education*, **50**, 1409-1420.

Sies, H. & Chance, B. (1970) The steady state level of catalase compound I in isolated hemoglobin-free perfused rat liver. *FEBS letters*, **11**, 172-176.

Solinas, G. & Karin, M. (2010) JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction. *FASEB J*, **24**, 2596-2611.

Spasojevic, I., Bajic, A., Jovanovic, K., Spasic, M. & Andjus, P. (2009a) Protective role of fructose in the metabolism of astroglial C6 cells exposed to hydrogen peroxide. *Carbohydr Res*, **344**, 1676-1681.

Spasojevic, I., Mojovic, M., Blagojevic, D., Spasic, S.D., Jones, D.R., Nikolic-Kokic, A. & Spasic, M.B. (2009b) Relevance of the capacity of phosphorylated fructose to scavenge the hydroxyl radical. *Carbohydr Res*, **344**, 80-84.

Spector, T. (1978) Refinement of the coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for less than or equal to 0.5 to 50 microgram of protein. *Anal Biochem*, **86**, 142-146.

Stanhope, K.L., Medici, V., Bremer, A.A., Lee, V., Lam, H.D., Nunez, M.V., Chen, G.X., Keim, N.L. & Havel, P.J. (2015) A dose-response study of consuming high-fructose corn syrup-sweetened beverages on lipid/lipoprotein risk factors for cardiovascular disease in young adults. *The American journal of clinical nutrition*, **101**, 1144-1154.

Stanhope, K.L., Schwarz, J.M. & Havel, P.J. (2013) Adverse metabolic effects of dietary fructose: results from the recent epidemiological, clinical, and mechanistic studies. *Current opinion in lipidology*, **24**, 198-206.

Sun, S.Z. & Empie, M.W. (2012) Fructose metabolism in humans - what isotopic tracer studies tell us. *Nutrition & metabolism*, **9**, 89.

Tao, R., Coleman, M.C., Pennington, J.D., Ozden, O., Park, S.H., Jiang, H., Kim, H.S., Flynn, C.R., Hill, S., Hayes McDonald, W., Olivier, A.K., Spitz, D.R. & Gius, D. (2010) Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. *Mol Cell*, **40**, 893-904.

Tao, R., Vassilopoulos, A., Parisiadou, L., Yan, Y. & Gius, D. (2014) Regulation of MnSOD enzymatic activity by Sirt3 connects the mitochondrial acetylome signaling networks to aging and carcinogenesis. *Antioxid Redox Signal*, **20**, 1646-1654.

Tappy, L. (2012) Q&A: 'toxic' effects of sugar: should we be afraid of fructose? *BMC biology*, **10**, 42.

Tappy, L., Le, K.A., Tran, C. & Paquot, N. (2010) Fructose and metabolic diseases: new findings, new questions. *Nutrition*, **26**, 1044-1049.

Teofilovic, A., Bursac, B., Djordjevic, A., Vojnovic Milutinovic, D., Matic, G. & Velickovic, N. (2016) High dietary fructose load aggravates lipid metabolism in the liver of Wistar rats through imbalance between lipogenesis and fatty acid oxidation. *Turk J Biol*, **40**, DOI:10.3906/biy-1512-3940.

Tran, L.T., Yuen, V.G. & McNeill, J.H. (2009) The fructose-fed rat: a review on the mechanisms of fructose-induced insulin resistance and hypertension. *Mol Cell Biochem*, **332**, 145-159.

Urakawa, H., Katsuki, A., Sumida, Y., Gabazza, E.C., Murashima, S., Morioka, K., Maruyama, N., Kitagawa, N., Tanaka, T., Hori, Y., Nakatani, K., Yano, Y. & Adachi, Y. (2003) Oxidative stress is associated with adiposity and insulin resistance in men. *The Journal of clinical endocrinology and metabolism*, **88**, 4673-4676.

Valencia, A.P., Schappal, A.E., Morris, E.M., Thyfault, J.P., Lowe, D.A. & Spangenburg, E.E. (2016) The presence of the ovary prevents hepatic mitochondrial oxidative stress in young and aged female mice through glutathione peroxidase 1. *Experimental gerontology*, **73**, 14-22.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. & Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, **39**, 44-84.

Van Guilder, G.P., Hoetzer, G.L., Greiner, J.J., Stauffer, B.L. & Desouza, C.A. (2006) Influence of metabolic syndrome on biomarkers of oxidative stress and inflammation in obese adults. *Obesity*, **14**, 2127-2131.

Varlamov, O., Bethea, C.L. & Roberts, C.T., Jr. (2014) Sex-specific differences in lipid and glucose metabolism. *Frontiers in endocrinology*, **5**, 241.

Vasiljevic, A., Bursac, B., Djordjevic, A., Milutinovic, D.V., Nikolic, M., Matic, G. & Velickovic, N. (2014) Hepatic inflammation induced by high-fructose diet is associated with altered 11betaHSD1 expression in the liver of Wistar rats. *Eur J Nutr*, **53**, 1393-1402.

Vasiljevic, A., Velickovic, N., Bursac, B., Djordjevic, A., Milutinovic, D.V., Nestorovic, N. & Matic, G. (2013) Enhanced prereceptor glucocorticoid metabolism and lipogenesis impair insulin signaling in the liver of fructose-fed rats. *J Nutr Biochem*.

Velickovic, N., Djordjevic, A., Vasiljevic, A., Bursac, B., Milutinovic, D.V. & Matic, G. (2013) Tissue-specific regulation of inflammation by macrophage migration inhibitory factor and glucocorticoids in fructose-fed Wistar rats. *Br J Nutr*, **110**, 456-465.

Ventura, E.E., Davis, J.N. & Goran, M.I. (2011) Sugar content of popular sweetened beverages based on objective laboratory analysis: focus on fructose content. *Obesity (Silver Spring)*, **19**, 868-874.

Vina, J., Borras, C., Gambini, J., Sastre, J. & Pallardo, F.V. (2005a) Why females live longer than males: control of longevity by sex hormones. *Sci Aging Knowledge Environ*, **2005**, pe17.

Vina, J., Borras, C., Gambini, J., Sastre, J. & Pallardo, F.V. (2005b) Why females live longer than males? Importance of the upregulation of longevity-associated genes by oestrogenic compounds. *FEBS letters*, **579**, 2541-2545.

Vistisen, D., Witte, D.R., Tabak, A.G., Brunner, E.J., Kivimaki, M. & Faerch, K. (2014) Sex differences in glucose and insulin trajectories prior to diabetes diagnosis: the Whitehall II study. *Acta diabetologica*, **51**, 315-319.

Votruba, S.B. & Jensen, M.D. (2006) Sex-specific differences in leg fat uptake are revealed with a high-fat meal. *American journal of physiology. Endocrinology and metabolism*, **291**, E1115-1123.

Wajchenberg, B.L. (2000) Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocrine reviews*, **21**, 697-738.

Wang, L., Szklo, M., Folsom, A.R., Cook, N.R., Gapstur, S.M. & Ouyang, P. (2012) Endogenous sex hormones, blood pressure change, and risk of hypertension in postmenopausal women: the Multi-Ethnic Study of Atherosclerosis. *Atherosclerosis*, **224**, 228-234.

Wellen, K.E. & Hotamisligil, G.S. (2005) Inflammation, stress, and diabetes. *The Journal of clinical investigation*, **115**, 1111-1119.

Wittekind, A. & Walton, J. (2014) Worldwide trends in dietary sugars intake. *Nutrition research reviews*, **27**, 330-345.

Yamakura, F. & Kawasaki, H. (2010) Post-translational modifications of superoxide dismutase. *Biochim Biophys Acta*, **1804**, 318-325.

Yamakura, F., Taka, H., Fujimura, T. & Murayama, K. (1998) Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by

exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J Biol Chem*, **273**, 14085-14089.

Yubero-Serrano, E.M., Delgado-Lista, J., Pena-Orihuela, P., Perez-Martinez, P., Fuentes, F., Marin, C., Tunez, I., Tinahones, F.J., Perez-Jimenez, F., Roche, H.M. & Lopez-Miranda, J. (2013) Oxidative stress is associated with the number of components of metabolic syndrome: LIPGENE study. *Experimental & molecular medicine*, **45**, e28.

Zhang, H., Davies, K.J. & Forman, H.J. (2015) Oxidative stress response and Nrf2 signaling in aging. *Free Radic Biol Med*, **88**, 314-336.

Zhu, Y., Park, S.H., Ozden, O., Kim, H.S., Jiang, H., Vassilopoulos, A., Spitz, D.R. & Gius, D. (2012) Exploring the electrostatic repulsion model in the role of Sirt3 in directing MnSOD acetylation status and enzymatic activity. *Free Radic Biol Med*, **53**, 828-833.

BIOGRAPHY

Alhadi Mohamed Ali Glban was born in Rayayna, Libya, on Febuary 12th 1966. He started his Bachelor studies in 1983 at The Department of Animal Production, Faculty of Agriculture, University of Omar – Almokhtar and got his Bachelor degree in 1987 with grade point average C (70%). In 1990 he started working for a veterinary clinic belonging to the Ministry of Agriculture of Libya and worked there until 1997. In 1999 he started his Master studies at The Department of Animal Production, Faculty of Agriculture, University of Tripoli, and got his Master degree on April 2004. From 2005 to 2008 Alhadi Mohamed Ali Glban worked as a lecturer at the Faculty of Agriculture, University of Al-Jabal Al-Gharbi. In 2008 he got a scholarship to study English language and do his PhD studies. He studied English language at the University of Guelph, Ontario, Canada and in 2012 came to Serbia to start his PhD program at the Faculty of Biology, University of Belgrade.

Изјава о ауторству

Име и презиме аутора ______Алхади М. Глбан____________________________

Број индекса ______________М3014/2012________________________________

Изјављујем

да је докторска дисертација под насловом

Експресија и активност ензима антиоксидативне заштите у јетри мужјака и женки пацова након исхране обогаћене фруктозом

- резултат сопственог истраживачког рада;
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Потпис аутора

У Београду, ______12. 09. 2016.___

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Алхади М. Глбан

Број индекса M3014/2012

Студијски програм Молекуларна биологија

Наслов рада **Експресија и активност ензима антиоксидативне заштите у јетри мужјака и женки пацова након исхране обогаћене фруктозом**

Ментор др Јелена Несторов, научни сарадник, Институт за биолошка истраживања „Синиша Станковић", Универзитет у Београду

Проф. др Гордана Матић, редовни професор, Биолошки факултет, Универзитет у Београду; научни саветник, Институт за биолошка истраживања „Синиша Станковић", Универзитет у Београду

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањена у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, 12. 09. 2016.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић" да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

<u>Експресија и активност ензима антиоксидативне заштите у јетри мужјака и женки пацова након исхране обогаћене фруктозом</u>

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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