



## The Levels of Oxidative Stress Biomarkers of Liver and Mammary Tissues of Apparently Healthy Mice

Sağlıklı Görünen Farelerin Karaciğer ve Meme Dokularında Oksidatif Stres Biyobelirteçlerinin Düzeyleri

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### ABSTRACT

**Purpose:** In the present study, the levels of antioxidant systems such as glucose-6-phosphate dehydrogenase (G6PDH), catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST) and, also reduced glutathione (GSH) in the liver and breast tissues were examined in order to obtain basal data for subsequent toxicological investigations. Additionally, the level of thiobarbituric acid reactive substance (TBARS) was measured in liver and breast tissues as an index of lipid peroxidation.

**Materials and Methods:** Sixty-six apparently healthy mice (36 males, 30 females) had normal liver and breast tissues (histopathological data) were taken into the study. The tissues were homogenised with ice-cold 1.15% KCl. The activities of antioxidant enzymes, the levels of GSH and TBARS were measured as spectrophotometric in the supernatant obtained from centrifugation at 14.000 rpm.

**Results:** The levels of antioxidant systems and TBARS in the liver were significantly higher than those found in the breast tissue of female and male mice. However, the levels of CAT, SOD, GST, GSH and TBARS in breast tissue except G6PDH enzyme were not significantly different between female and male mice. On the other hand, the levels of CAT, SOD, GST, GSH and TBARS in the male liver were higher than the female liver except for G6PDH enzyme.

**Conclusion:** Results suggest that antioxidant defense of the liver tissue was higher compared to breast tissue possibly due to a compensatory response to various toxic substances in the liver and thereby protects the cells against oxidative damage.

**Key Words:** Antioxidant systems, TBARS, liver, breast

### ÖZET

**Giriş:** Bu çalışmada ileride yapılacak toksikolojik araştırmalara temel veri sağlamak amacıyla karaciğer ve meme dokularında antioksidan sistemler [glukoz-6-fosfat dehidrogenaz (G6PDH), katalaz (CAT), süperoksit dismutaz (SOD), glutatyon-S-transferaz (GST), redükte glutatyon (GSH)] incelendi. Buna ek olarak, lipid peroksidasyonun bir göstergesi olarak karaciğer ve meme dokularında tiyobarbiturik asit reaktif maddesi (TBARS) ölçüldü.

**Materyal ve Metod:** Sağlıklı görünen, histopatolojik inceleme sonucu normal karaciğer ve meme dokusu bulgusu bulunan 66 fare (36 erkek, 30 dişi) çalışmaya alındı. Dokular soğuk % 1,15 KCl ile homojenize edildi. Antioksidan enzimler, GSH ve TBARS düzeyi 14.000 rpm'de santrifüjden sonra elde edilen süpernatant'ta spektrofotometrik olarak ölçüldü.

**Bulgular:** Erkek ve dişi farelerin karaciğer dokularındaki antioksidan sistemlerin ve TBARS düzeylerinin meme dokusundan yüksek olduğu gözlemlendi. Bununla birlikte, meme dokusundaki G6PDH hariç CAT, SOD, GST, GSH ve TBARS düzeylerinin erkek ve dişi fareler arasında farklılık göstermediği saptandı. Öte yandan, erkek fare karaciğerinde G6PDH hariç CAT, SOD, GST, GSH ve TBARS düzeylerinin dişi karaciğerine göre daha yüksek bulundu.

**Sonuç:** Sonuçlar, karaciğer dokusunda antioksidan savunmanın meme dokusuna oranla yüksek olmasının, karaciğerde olası çeşitli toksik maddelere karşı kompensatuar yanıtı bağlı olabileceğini ve bu şekilde hücreleri oksidatif hasara karşı koruyabileceğini göstermiştir.

**Anahtar Kelimeler :** Antioksidan sistemler, TBARS, karaciğer, meme.

## INTRODUCTION

Oxygen, a vital component of living organisms, produces reactive species known as oxygen free radicals which are detrimental to living cells. These are superoxide anion, hydrogen peroxide and hydroxyl radical and are formed during univalent reduction of oxygen to water. Reactive oxygen species (ROS) are produced during normal metabolism and this production causes damage in the cell. This possible damage is prevented by cellular antioxidant systems, which consist of glucose-6-phosphate dehydrogenase (G6PDH), superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and also reduced glutathione (GSH)<sup>1</sup>. Antioxidant enzyme activities are regulated by many factors<sup>2</sup>. Harris et al. (1992) have recently reviewed some parameters affecting eukaryotic antioxidant enzymes. These factors included tissue specificity, age and state of development, availability of cofactors, cytokines and xenobiotics<sup>3</sup>. These many factors clearly affect the ability of the cell to live in an aerobic environment.

Glucose-6-phosphate dehydrogenase is the first and the regulatory enzyme of the so-called pentose phosphate pathway. The main physiological role of G6PDH is to provide NADPH, a compound necessary for a number of detoxification and biosynthetic reactions, including fatty acid synthesis. Because of its biochemical role, and since it is found in all cell types and in all organisms thus far analyzed, G6PDH is regarded as the product of a typical "housekeeping" gene. At the same time, G6PDH is subject in some

tissues to physiologically important regulatory phenomenon<sup>4</sup>.

Superoxide dismutase enzyme catalyzes the dismutation of the highly reactive superoxide anion to O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>. In human, there are three forms of SOD; cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD and extracellular SOD. Cu/Zn-SOD is believed to play a major role in the first line of antioxidant defense and high SOD activities are correlated with high immune competence<sup>5</sup>.

Catalase is an enzyme virtually found in all aerobic cells that under certain conditions can play a critical role in detoxifying H<sub>2</sub>O<sub>2</sub>; in tissues such as liver and is mainly localized in peroxisomes, but may also be found in an unbound form in the cytoplasm<sup>6</sup>.

Glutathione-S-transferase protects tissues from the toxic effects of ROS as well as from the deleterious effects of lipid peroxidation<sup>7</sup>. A possible protective role of GST involving their selenium-independent glutathione peroxidase activity has been suggested<sup>8</sup>. However, GST catalyzes the conjugation of GSH with a wide variety of xenobiotics<sup>9</sup>. GST plays an important role in GSH metabolism and in detoxification process<sup>10</sup>.

Reduced glutathione is the most important low-molecular-weight nonenzymatic antioxidant in the cells. It serves as a substrate for GSH peroxidase and is also a scavenger of ROS. Decreased GSH levels occur in blood, liver, kidneys and intestinal mucosa of male CBF-1 mice as a function of age and a decreased cellular GSH with time may contribute to aging process as well as the increased incidence of neoplastic diseases

and greater susceptibility to drugs and foreign chemicals with aging<sup>11</sup>.

Lipid peroxidation is a normal phenomenon that occurs continuously at low levels in all living cells. These peroxidation reactions are in part toxic to cells and cell membranes; however, they are normally controlled by countervailing biologic mechanisms. Increased lipid peroxidation levels and decreased antioxidant protection generate epoxides that may spontaneously react with nucleophilic centers in the cell and thereby covalently bind to DNA, RNA and protein<sup>12</sup>.

In this study, we aimed to dissect out the antioxidant systems in liver and breast tissues in order to obtain basal data for subsequent toxicological investigations. Additionally, the level of thiobarbituric acid reactive substance (TBARS) was measured in the liver and breast tissues as an index of lipid peroxidation.

## MATERIALS and METHODS

All chemicals used in antioxidant enzyme and TBARS level assays were analytical grade and were provided from the Sigma Chemical Company (St. Louis, MO, USA).

### Animals

Non-inbred *Mus musculus* albino, sixty-six adult (30 females, 36 males), weighing between 23 and 40 g were obtained from the Medical Sciences Experimental Research Center of the Cukurova University. Medical Faculty Ethical Committee of Cukurova University approval was obtained in accordance with the principles of Declaration of Helsinki. Mice were fed with a standard laboratory diet and tap water and were maintained in 12-hour light/dark cycle with the room temperature of 22-24°C. The apparently healthy mice having normal liver and breast tissues (histopathological data) were included in the study.

### Preparation of Homogenate

The mice were weighed and sacrificed. The liver and breast tissues were quickly removed, weighed, blotted on a filter paper, and

homogenized with three volumes of ice-cold 1.15 % KCl. The activities of antioxidant enzymes, the levels of GSH and TBARS were measured in the supernatant obtained from centrifugation at 14.000 rpm.

### Biochemical Analysis

SOD activity was measured in the tissue samples according to the method described by Fridovich<sup>13</sup>. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with p-iodonitrotetrazlium violet (INT) to form a red formazan dye which was measured at 505 nm. Assay medium consisted 0.01 M phosphate buffer, CAPS (3-cyclohexilamino-1-propanesulfonicacid) buffer solution (50 mM CAPS, 0.94 mM EDTA, saturated NaOH) with pH 10.2, solution of substrate (0.05 mM xanthine, 0.025 mM INT) and 80 UL xanthine oxidase. SOD activity was expressed as U/mg protein.

CAT activity was determined by measuring the decrease in hydrogen peroxide concentration at 230 nm by the method of Beutler<sup>14</sup>. Assay medium consisted 1 M Tris HCl, 5 mM Na<sub>2</sub>EDTA buffer solution (pH 8.0), 10 mM H<sub>2</sub>O<sub>2</sub> and tissue sample in a final volume of 1.0 ml. CAT activity was expressed as U/mg protein .

Activity of G6PDH was determined at 37°C in the tissue samples according to Beutler<sup>14</sup>. The reaction mixture contained 1M Tris-HCl pH 8.0, 6 mM G6P Na, 2 mM NADP, 0.1 M MgCl<sub>2</sub> and supernatant in a total volume of 3.0 ml. One unit of enzyme activity was the amount of enzyme catalyzing the reduction of 1mM of NADP per minute. G6PDH activity was expressed as U/mg protein.

GSH level was measured in the tissue samples according to Beutler<sup>14</sup>. The reaction mixture contained filtrate, phosphate buffer and DTNB (5,5'-dithiobis 2-nitrobenzoic acid) in a final volume of 10 ml. A blank was prepared by using precipitating reagent and distilled water instead of filtrate. The absorbance was read in a spectrophotometer immediately at 412 nm wave length before and after addition of DTNB. The

values were determined from the standard curve. GSH level was expressed as micromol/mg protein.

GST activity in tissues was determined in the presence of 1.0 mM GSH with 1.0 mM 1-chloro-2,4-dinitrobenzene as the substrate<sup>15</sup>. Assay was conducted in a thermostated cell compartment at 25 in 100 mM potassium phosphate buffer at pH 6.5.

TBARS level in the tissue samples was measured with the TBA test<sup>16</sup>. The reaction mixture contained 0.1 ml sample, 0.2 ml of 8.1 % sodium dodecyl sulphate (SDS), 1.5 ml of 20 % acetic acid and 1.5 ml of 0.8 % aqueous solution of TBA. The pH of the mixture was adjusted to 3.5 and the volume was finally brought up to 4.0 ml with distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1, v/v) was then added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm.

The protein concentration of the tissue samples was measured with Spectronic-UV 120 spectrophotometer by the method of Lowry<sup>17</sup>.

#### Histological Methods

The liver and breast tissues were fixed in 10% formaldehyde and processed routinely. They were embedded in paraffin. Five micrometer sections were obtained, stained with Harris hematoxyline-eosin and examined under light microscope<sup>18</sup>.

#### Statistical Analysis

The SPSS software (Version 9.05) was employed for student t-test. Results were expressed as the means  $\pm$  standard deviation (SD). The difference was considered significant when the probability was less than 0.05.

## RESULTS

Figure 1 and 2 showed the normal liver and breast tissues of *Mus musculus* according to the results of histopathological analysis. The levels of antioxidant systems and TBARS in the liver were

significantly higher than those found in breast tissue ( $P < 0.05$ ). On the other hand, the levels of breast tissue CAT, SOD, GST, GSH and TBARS except G6PDH activity were not significantly different between female and male mice ( $P > 0.05$ ). However, the levels of CAT, SOD, GST, GSH and TBARS in the male liver except for G6PDH enzyme were higher than female liver ( $P < 0.05$ ). These results were summarized in Table.

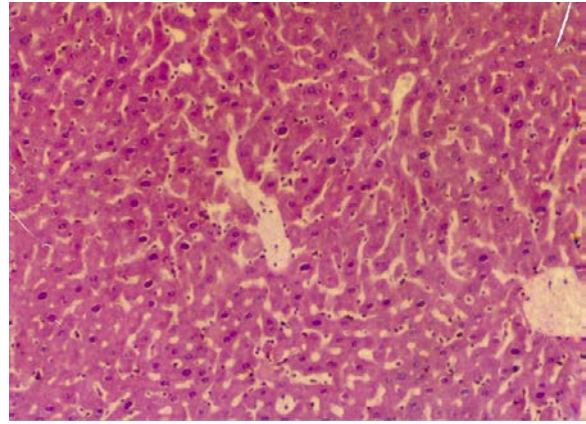


Figure 1. Normal histological observations of *Mus musculus* liver tissue. Tissue samples were fixed and then stained with hematoxyline-eosin x 200.

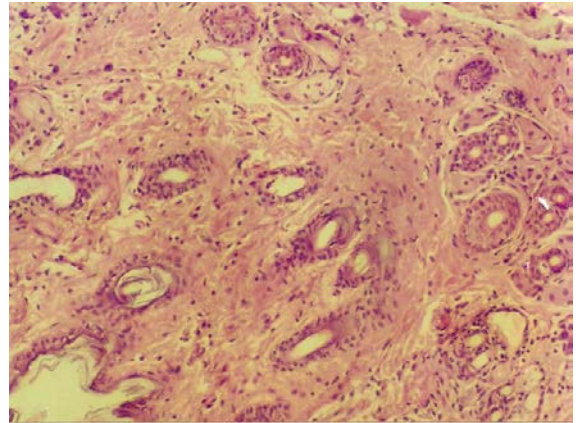


Figure 2. Normal histological observations of *Mus musculus* breast tissue. Tissue samples were fixed and then stained with hematoxyline-eosin x 200

## DISCUSSION

In this study, we obtained the basal data for oxidative stress biomarkers levels that are commonly measured in the laboratory when assessing oxidative stress including sex- or organ-related differences in mice. Liver is a uniform organ with the highest antioxidant enzyme activities (SOD, CAT). This could be related to the fact that the liver is the site of multiple oxidative reactions and maximal free radical generation<sup>19</sup>.

NADPH supply for xenobiotic detoxification and other cellular antioxidative defense mechanisms may be different in livers of female and male flounder. The strongly decreased supply of NADPH in hepatocytes of females may explain the reduced and/or delayed NADPH-dependent activity of xenobiotic biotransformative systems such as cytochrome P450 and a lower capacity of non-enzymatic defense systems such as GSH that is particularly observed in female flounder<sup>20</sup>.

In the current study, the levels of antioxidant systems against free radical activity that were formed during regular metabolism in the liver and breast tissues and the differences of TBARS levels in these tissues were demonstrated. In our study, G6PDH activity was found higher in the liver than in the breast tissue. Similar results were obtained by Martins et al<sup>21</sup>, who found the highest G6PDH activity in the liver compared to muscle, adipose and breast tissues. The results of our study suggest that lower G6PDH activity in the breast tissue causes a significant decrease in the reducing potential of pentose phosphate cycle. Indeed, GSH may be the key factor in lowering this potential. In support of this is the evidence that cellular GSH concentration may have a profound regulatory effect on the activity of pentose phosphate-cycle enzymes. Owing to decreased GSH concentrations, the NADPH coenzyme that is generated by the coupled enzyme system of glutathione reductase and glutathione peroxidase may become limited and less available to pentose phosphate-cycle enzymes. As a result, NADPH reducing equivalents may decrease and in turn

cause a lower biosynthetic activity<sup>22</sup>. In several organs of rat, G6PDH activity increases in response to nutritional or hormonal alterations. These changes are particularly large in the liver and breast tissues<sup>23</sup>. Grigor et al<sup>24</sup> reported that tissue mass and enzyme activity of the rat liver and breast tissues increased during lactation. On weaning, there was a concomitant decrease in both tissue mass and enzyme activity for the liver and breast tissues. Moreover, our study demonstrated that the liver G6PDH activity of the female mice was higher than the male mice. Since the G6PDH gene is located in the sub-telomeric region of the long arm of the X chromosome, the higher G6PDH activity in female mice may possibly be related to this phenomenon.

Schisler et al.<sup>25</sup> reported that CAT activity in mouse was the highest in the liver followed by the kidney, lung and blood hemolysate displaying similar, low levels of activity. These results are comparable favorably with those of Takenaka et al.<sup>26</sup>. In our study, the activity of CAT was found as ten-fold higher in the liver tissue than in the breast tissue which may be related to the higher production of superoxide radicals in the liver (Table).

Recent studies in rat indicate that SOD activity is present at high levels in the liver, whereas an intermediate activity is observed in the adrenals and the kidney, and a low activity is found in most of the other tissues, including brain, blood and lung<sup>27,28</sup>. Similar results were obtained in the present study, which indicated a higher SOD activity in the liver than in the breast tissue. An increased activity may be due to increased synthesis of the enzyme as a response to increased in vivo production of superoxide radical due to the increased metabolic activity in the liver. Takenouchi et al. reported that the plasma adiponectin level and the aortic Cu/Zn-SOD were increased in the female mice compared to male mice. Aortic total SOD activities were lower in the older male than in either younger male or older female mice<sup>29</sup>.

Though superoxide radicals have limited chemical reactivity, they can generate more dangerous species, such as highly reactive hydroxyl radicals *in vivo* in the presence of transition metal ions, and lead to biomolecular peroxidation. TBARS measures the three-carbon-compound malondialdehyde, which is derived from free radical-induced peroxides of lipids, amino acids, carbohydrates and nucleic acids<sup>30</sup>. In our study, TBARS levels in the liver tissue were higher than the breast tissue. The higher TBARS in the liver tissue suggests that liver may be related to a higher peroxidation of lipids and other biomolecules, or to a higher susceptibility to free radical-induced peroxidation.

In our study, GSH level was found to be higher in liver tissue than in breast tissue. Decreased GSH concentrations could have a marked effect on the detoxification capacity of the breast tissue, since a major function of GSH is in the detoxification of peroxides produced by normal metabolism and of xenobiotics via GST. Sharma et al. reported that GSH level showed difference among tissues such as liver, lung, kidney, spleen, heart, brain and muscle<sup>31</sup>. They found the highest GSH level in the liver compared to other tissues. The increased susceptibility to drugs and foreign chemicals with advanced age may not only be due to a decrease in the availability of GSH but also to a decrease in GST. However, results of the present study showed that the activities of CAT, SOD and the levels of GSH and TBARS in male liver were higher than female liver. These differences were elicited to be mainly related to the higher metabolic activity in males compared to females.

Some studies indicated that the GST activity of the male rats was only slightly higher than the female rats confirming prior studies<sup>2</sup>. Singhal et al. reported sex-related differences in the expression

of GST in the mouse liver and about 2-fold higher activity of GST in male as compared to that in female mice. Similar results were obtained in this study as the GST activity of the male mice liver was about 2-fold higher than the female mice<sup>7</sup>. When GST activity was compared in the liver and breast tissues, it was found to be 5-6 fold higher in the male liver and 3-4 fold higher in the female liver tissues of mice. The GSH/GST system is one of the major protection mechanisms against toxic electrophilic xenobiotics. GSH and GST levels in various organs, particularly in the liver, of male and female mice are remarkably different. This would suggest that in toxicological studies with the mouse model, the possibilities of sex-related differential responses to various toxicants have to be carefully considered. Lower levels of GST in the female mouse liver and to some extent in the breast tissue account for the possibility of females being more susceptible in these organs to electrophilic compounds, which require GSH/GST system detoxification.

Another result of the present study showed that the breast tissue G6PDH activity in female was higher than male. No remarkable difference exists between male and female mice in the number and localization of breasts. However, the breast tissue of female mice is functional even though it is not in males. Both gross and microanatomy, and also biochemical properties (hormonal changes) of breast tissue differ from each other in male and female mice<sup>32</sup>. We thought that the higher level of G6PDH activity in female was due to their hormonal status.

Our preliminary results suggest that antioxidant defense of the liver tissue was higher compared to breast tissue possibly due to a compensatory response to various toxic substances in the liver and thereby protects the cells against oxidative damage. Moreover, the levels of antioxidant systems and TBARS in the liver and breast tissues will provide basal data for subsequent toxicological investigations.

**Table.1. The levels of oxidative stress biomarkers in liver and breast tissues of mice**

	Male Mice (n:36)		Female Mice (n:30)	
	Liver tissue	Breast tissue	Liver tissue	Breast tissue
G6PDH (U/mg protein)	0.022 ± 0.008	0.007 ± 0.001**	0.034 ± 0.010	0.016 ± 0.004*** <sup>b</sup>
CAT (U/mg protein)	265.2 ± 71.1*	23.2 ± 5.7	199.2 ± 50.7*	21.2 ± 4.9
SOD (U/mg protein)	7.4 ± 2.4*	4.2 ± 1.6	4.9 ± 1.1*	3.9 ± 1.5
GST (nmol/mg protein)	1645.3 ± 523.2 <sup>a</sup>	285.1 ± 63.5	721.0 ± 187.1*	252.1 ± 48.7
GSH (µmol/mg protein)	1.99 ± 0.727*	0.006 ± 0.003	1.01 ± 0.347 *	0.005 ± 0.002
TBARS (nmol/mg protein)	4.4 ± 1.15*	2.6 ± 0.8	3.2 ± 0.98*	2.4 ± 0.7

\*Significant differences in liver tissues between male and female (P < 0.05)

\*\*Significant differences in breast G6PDH activity between male and female (P < 0.05)

<sup>a,b</sup> Higher in liver GST activity and breast G6PDH activity in female group than male group (P < 0.05).

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