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Research Article

Effects of glutathione on mitochondrial DNA and antioxidant enzyme activities in *Drosophila melanogaster*

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Abstract: The free radical theory in aging assumes that the accumulation of macromolecular damage induced by toxic reactive oxygen species plays a central role in the aging process. The intake of nutritional antioxidants can prevent this damage by neutralizing reactive oxygen derivatives. Glutathione (GSH; en-L-Glutamyl-L-cysteinyl glycine) is the lowest molecular weight thiol in the cells and as a cofactor of many enzymes and a potent antioxidant plays an important role in maintaining normal cell functions by destroying toxic oxygen radicals. In this study, the effects of GSH on SOD, GST and catalase enzymes and mtDNA damage were investigated at various time intervals by giving reduced glutathione to Drosophila. It was observed that 3-week GSH administration did not have a statistically significant effect on SOD and GST activities whereas GSH application decreased the catalase enzyme activities significantly. Although the decrease in antioxidant capacity with age was observed in SOD and catalase enzymes, such a situation was not observed in GST enzyme activities. There was no statistically significant difference between the control and GSH groups in mtDNA copy number values, while in the GSH group, oxidative mtDNA damage was high. These results may be due to the prooxidant effect of GSH at the dose used in this study.

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1. INTRODUCTION

Reactive oxygen species (ROS) are by-products of exposure to cellular metabolism or xenobiotics. ROS production can be harmful because it may cause oxidative changes in cellular lipids, proteins, and DNA, or it may be beneficial by participating in intracellular signalling or cell regulation (El-Osta & Circu, 2016). Mitochondria, the centre of cellular respiration, is a quantitatively important source of ROS as a result of oxygen reduction. In addition, mitochondria are exposed to harmful reactive oxygen species that inhibit cellular antioxidant mechanisms such as SOD and glutathione (El-Osta & Circu, 2016; Collins, 2016).

Superoxide (O_2^-) is one of the free radicals that can exert oxidative effects on the cell. Superoxide is reduced by superoxide dismutase (SOD) enzyme to hydrogen peroxide (Abreu & Cabelli, 2010; Sheshadri & Kumar, 2016). There is evidence that ROS such as superoxide

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anion radical, hydrogen peroxide, carbon monoxide, and nitric oxide play an important role in various physiological processes such as cellular proliferation and differentiation, gene regulation, and anti-bacterial defense (Rebrin & Sohal, 2008). SOD can be considered as the first line of defense in ROS homeostasis (Abreu & Cabelli, 2010; Sheshadri & Kumar, 2016).

Catalase (CAT) is a common antioxidant enzyme found in almost all tissues using oxygen. This enzyme catalyzes the reduction of hydrogen peroxide (H_2O_2) to water and molecular oxygen to complete the detoxification process carried out by the SOD. The catalase is present in the cell in the peroxisome, not in the mitochondria, which shows that the separation of hydrogen peroxide to water and oxygen is carried out by another enzyme known as glutathione peroxidase in mammalian cell mitochondria (Ighodaro & Akinloye, 2018). Approximately 0.4% to 4% of the molecular oxygen metabolized in the mitochondrial electron transport chain is converted into ROS (Lim *et al*., 2006). Mitochondrial DNA (mtDNA) is a circular double helix DNA arranged in nucleotides. There is no histone in the mitochondrial genome and the genome is located very close to the mitochondrial electron transport system (ETS); therefore, mtDNA is very sensitive to oxidative damage (Circu & Aw, 2012).

Glutathione (GSH; γ-L-Glutamyl-L-cysteinyl glycine, the lowest molecular weight thiol in the cells, is composed of L-glutamate, L-cysteine, and glycine amino acids and is one of the most important antioxidant compounds in body fluids (Giustarini *et al*. 2016; Wu *et al*, 2004). GSH production is catalyzed by Gamma-glutamylcysteine synthetase (Gsh1p) and glutathione synthetase (Gsh2p) and synthesized in two steps dependent on ATP (Mannarino, 2008). Glutathione, an essential molecule and primary cellular redox buffer that participates in various biological processes, is often found in cells at millimolar concentrations (1-10 mM). Many processes in cells can use GSH as a cofactor and can be affected by changes in GSH level. The consequences of suboptimum GSH levels on all these processes are not yet clear (Ayer *et al.,* 2010). Glutathione is synthesized intracellularly and can be transferred from the cell. It then tends to be hydrolyzed to amino acids to be taken back by the cells and re-synthesized glutathione in the cell (Meister, 1991). When glutathione flows out of the cell as a tripeptide, a large amount cannot be absorbed back into the cell (Kern *et al*, 2011), which leads to basal glutathione content in serum with a normal range of 3.8-5.5μM and its half-life is reported as 14.1 ± 9.2 minutes. Cells that can absorb glutathione as a tripeptide are hepatocytes (HepG2), intestinal mucosal cells, and retinal cells (Aebi *et al*, 1991; Sze *et al*, 1993; Benard *et al*, 1993; Kannan *et al*, 1996). GSH is also an important source of cysteine and performs many physiological functions, including proliferation, cell cycle regulation, apoptosis, catabolism of xenobiotics, glutathionylation of proteins, and the production of some steroids, lipid compound, and deoxyribonucleotides (Bajic *et al*, 2019). Glutathione is present in a reduced form known as reduced glutathione (GSH) and an oxidized form (GSSG) after administration of antioxidant effects on targets. These two forms determine a ratio known as the GSH / GSSG ratio, and changes in this ratio indicate changes in cellular oxidative balance (Owen & Butterfield, 2010).

GSH, a cofactor of many enzymes and a potent antioxidant, plays an important role in maintaining normal cell functions by destroying toxic oxygen radicals. On the other hand, GSH level decreases in circulatory and tissue levels in chronic diseases such as diabetes and aging (Jain *et al*, 2016). The free radical theory in aging assumes that the accumulation of macromolecular damage induced by toxic reactive oxygen derivatives (ROS) plays a central role in the aging process (Lim *et al*, 2006).

Mitochondrial dysfunction in cells is closely related to formation of reactive oxygen species (ROS) and oxidative stress. Although ROS homeostasis and antioxidant enzymes are modulated by cellular mtDNA, the modulation of the cellular antioxidant defense system by changes in mitochondrial DNA (mtDNA) content is largely unknown (Min & Lee, 2019). The mitochondrial glutathione (mtGSH) pool is 10-15% of cellular glutathione and is derived from

the transport of cytosolic glutathione along mitochondrial membranes (Collins, 2016). Mitochondrial redox systems such as glutathione, thioredoxin, and pyridine nucleotide redox pairs participate in antioxidant defense by modulating mitochondrial functions, including apoptotic cell death. The imbalance between ROS and antioxidant defense causes oxidative stress and oxidative changes in cellular biomolecules. Increased oxidative stress also leads to loss of GSH (Choi *et al*, 2016). Furthermore, mitochondrial ROS can oxidize mitochondrial glutathione and cause the loss of intramitochondrial redox homeostasis since mitochondrial macromolecules including mitochondrial DNA (mtDNA) are exposed to irreversible oxidative modifications (El-Osta & Circu, 2016). In Min and Lee's studies (2019), expression and activity of glutathione peroxidase (GPx) and catalase were inversely proportional to mtDNA content in myoblasts. While the depletion of mtDNA slightly lowered both reduced glutathione (GSH) and oxidized glutathione (GSSG), the cellular redox status assessed by the GSH / GSSG ratio was similar to that of the control group. In their study, Min and Lee (2019) reported that ROS homeostasis and antioxidant enzymes are modulated by cellular mtDNA content and that increased Glutathione peroxidase (GPx) and catalase expression and activity through mtDNA depletion are closely related to the reduction of oxidative stress in myoblasts.

Reactive oxygen and nitrogen species can oxidize cellular glutathione or induce glutathione out of the cell, thereby reducing intracellular redox homeostasis and inhibiting the activation of the apoptotic signal cascade (Circu & Aw, 2012). Many defence mechanisms in the organism evolve reducing reactive oxidants and their damage (Ames *et al*, 1993). Apoptosis is a well-organized and important cell death pattern for tissue homeostasis, organ development, and aging. To date, exogenous (receptor-mediated) and intrinsic (mitochondrial-derived) apoptotic pathways have been characterized in mammalian cells. Reduced glutathione plays an important role in protecting the reduced intracellular environment. The mechanism in which oxidative mtDNA damage induces apoptotic signalling is unclear (Circu & Aw, 2012). Enzymes such as superoxide dismutase (SOD), catalase, and glutathione S-transferase (GST) neutralize reactive electrophilic mutagens. In addition to the protective effects of intrinsic enzymatic antioxidant defence mechanisms, studies suggest that antioxidant consumption in diet is of great importance for health. Consumption of fruits and vegetables as the main source of diet antioxidants reduces the risk of degenerative diseases. However, there are many arguments suggesting that antioxidant contents of fruits and vegetables have the greatest effect on their protective effects (Ames *et al*, 1993).

Studies on aging in Drosophila continue to provide new insights into the understanding of this complex process. Drosophila is well suited for experimental studies with its short life cycle, suitability for genetic manipulation, and its functionally preserved physiology (Shaw *et al*, 2008). In this article, however, the effects of glutathione on some antioxidant enzyme activities and mtDNA were investigated in *Drosophila melanogaster*.

2. MATERIAL and METHODS

Flies were produced in standard corn flour medium in an incubator at 25 °C. Flies of 1-3 days of age were included in experimental media. The control group included the standard Drosophila corn meal medium (corn meal, yeast, agar, sugar, water, and antifungal acid): in GSH group, GSH (Reduced L Glutathione 0.5%) was added in addition to this standard food medium.

Flies were evaluated once a week for enzyme activities. In each measurement, 12 flies were used from each group (GSH group and control group). Flies were homogenized in PBS buffer (pH: 7.4, with protease inhibitor) and then homogenized in ultrasonic homogenizer. The supernatant, which was centrifuged for 20 minutes at 20.000 g of homogenate, was used for enzyme activation measurement. GST activity was performed with CAYMAN GST activity assay kit and SOD analyzes were performed with CAYMAN SOD assay kit according to kit

instructions. BSA standards and total protein amount of samples were measured by Bradford method (SIGMA B6916 Bradford Reagent).

Oxidative mtDNA damage and mitochondrial copy number were measured by quantitative PCR method. The principle of this analysis is that a lesion in the template DNA prevents the progression of the thermostable polymerase. Thus, amplification in the damaged DNA template are reduced compared to that of the undamaged DNA. Considering that the number of mtDNA copies may be different in each sample, a short fragment of 100 bp was replicated for each sample to normalize the results (Yakes & Van Houten, 1997; Santos *et al*, 2002; Venkatraman *et al*, 2004). The ratio of long fragment results to that of short fragment results gives a relative amount of damage. In our study, SIGMA G1N350 Genomic DNA purification kit was used for total DNA isolation; Pico Green dsDNA quantitation dye and QUBIT 2.0 fluorometer were used for the quantitative analysis of template DNA and PCR products; 5 ng of template DNA was added to each tube; and Thermo Phire hot start II DNA polymerase was used for PCR processing, and 4% DMSO was added to the PCR mix.

For short fragment (100 bp) primers are as follows (Mutlu 2012a; Mutlu 2012b; Mutlu 2013):

11426 5'- TAAGAAAATTCCGAGGGATTCA - 3'

11525 5'- GGTCGAGCTCCAATTCAAGTTA - 3'

For long fragment (10629 bp) primers are as follows (Mutlu 2012a; Mutlu 2012b; Mutlu 2013):

1880 5'- ATGGTGGAGCTTCAGTTGATTT - 3'

12508 5'- CAACCTTTTTGTGATGCGATTA - 3'

Thermal conditions for long fragment amplification: At 98 °C for 1 minute and then for 21 cycles at 98 °C for 10 sec, at 52 °C for 45 sec and at 68 °C for 5 min, for a final elongation at 68 °C for 5 min. Thermal conditions for short fragment amplification: At 98 °C for 1 minute and then for 21 cycles at 98 °C for 10 sec, at 55 °C for 45 sec and 72 °C for 10 sec, for final elongation at 72 °C for 2 min. The statistical analysis of the data was performed using the Kruskal Wallis test in Minitab Release 13.0 statistics program. * Marked groups are statistically different from other groups (p<0.05).

3. RESULTS

In our study, it was observed that 3-week GSH administration did not have a statistically significant effect on SOD and GST activities (Table 1) whereas GSH application decreased the catalase enzyme activity significantly (p<0.05). This decrease in catalase enzyme activities may be due to the prooxidant effect of GSH at the dose used in this study. Although the decrease in antioxidant capacity with age was observed in SOD and catalase enzymes, such a situation was not observed in GST enzyme activities.

Table 1. Results of Cat, GST and SOD enzyme analysis.

Groups	Cat (IU SA /mg pro ± SE)	GST (IU SA /mg pro ± SE)	SOD (IU SA /mg pro ± SE)
GSH 8 days	115.2±8.41	4.6±0.8	16.72±4.66
Control 8 days	161.14±9.20*	4.34±0.81	20.91±3.94
GSH 15 days	106.63±6.06	4.42±0.71	17.27±3.71
Control 15days	146.62±5.54*	4.20±0.76	15.09±4.65
GSH 22 days	99.21±5.92	4.71±0.79	14.6±3.01
Control 22days	125.46±6.95	4.39±0.69	13.91±2.28

*Marked groups are statistically different from other groups (p<0.05). (Cat: Catalase, GST: Glutathione S-transferase, GSH: Glutathione, SOD: Superoxide dismutase, IU:International unit, SA: Specific activity, mg pro: miligram protein, SE: standard error).

There was no statistically significant difference between the control and GSH groups in mtDNA copy number values, but in the GSH group oxidative mtDNA damage was relatively high (Table 2). This may be due to the prooxidant effect of GSH at the dose used in this study.

Table 2. Results of oxidative mtDNA damage and mtDNA copy number analysis (Low relative amplification indicates high mtDNA damage)

Groups	Relative Amplification	mtDNA copy number
GSH 22 days	0.996±0.26	245.56±21.36
Control 22days	1.214±0.33	251.11±22.28

4. DISCUSSION and CONCLUSION

The level of glutathione (GSH) decreases with age or in chronic diseases such as diabetes (Jain *et al*, 2016). The presence of mitochondrial GSH (mtGSH) is necessary for the protection of mitochondrial DNA. In mouse embryonic fibroblasts, aging mice and rats have been reported to have a direct relationship between decreasing mtGSH and increased mtDNA damage in brain and kidneys (Circu & Aw, 2012). In their study, Dannenman *et al*, (2015) reported that GSH depletion significantly increased mtDNA lesions induced by H_2O_2 in fibroblasts.

Since glutathione is a small peptide molecule, it is exposed to hydrolysis with ɤ-glutamyltransferase in the small intestine (Garvey *et al*, 1976). However, it can also be absorbed as a tripeptide. Although the glutathione given orally to the rats causes an increase in serum and tissue glutathione, the general glutathione activity in humans does not correlate with dietary glutathione (Iantomasi *et al*, 1997; Hagen *et al*, 1990; Aw *et al*, 1991). Witschi *et al*, (1992) reported that oral intake of 3g glutathione (0.15 mM/kg) in healthy subjects failed to increase circulating glutathione concentrations for 270 minutes.

In most foods, glutathione is present in a wide range of 13-110 mg. More than half of the nutritional glutathione comes from fruits and vegetables, and less from meats. However, glutathione content of diet does not correlate with systemic glutathione activity in humans (Flag *et al*, 1994).

The GSH: GSSG ratio, which is the primary determinant of the cellular redox state, shifts more and more to oxidation in the aging process due to an increase in GSSG content or a decrease in de novo GSH biosynthesis. The Km of Glutamate-Cysteine Ligase (GCL), a speed-limiting enzyme in de novo GSH biosynthesis, increases significantly during aging. Particularly under stressful conditions, the speed of GSH biosynthesis is adversely affected. Experimental studies suggest that the accumulation of homocysteine, an intermediate in the trans-sulfation pathway, may cause loss of affinity between the GCL and its substrates. Over-expression of GCL has been shown to prolong the life of Drosophila by up to 50%, suggesting that irregularities in glutathione metabolism play a causative role in the aging process (Rebrin & Sohal, 2008).

Although GSH has an antioxidant effect, it can provide a pro-oxidant effect in some cases. During GSH catabolism, removal of γ-glutamate residue from the cysteine residue causes a prooxidant effect and may induce lipid peroxidation of the plasma membrane and some cellular damage associated with it (Bajic *et al*, 2019). It is thought that anti-oxidant defenses should be developed in the aging process or ROS production will be reduced. In addition, ROS can be useful as well as dangerous. For this reason, it has become quite problematic to establish a causal and effective relationship between ROS and its participation in the aging process. For example, although mitochondrial hydrogen peroxide production decreases with overexpression of Mn superoxide dismutase and ectopic catalase in mitochondrial matrix, Drosophila's life span is shortened instead of elongation. However, it is a fact that antioxidant defenses decrease

with aging. The balance between ROS and anti-oxidants increasingly shifts to a pro-oxidant state (Rebrin & Sohal, 2008).

There are scientific data showing that calorie restriction (CR) delays the onset and progression of age-related changes based on oxidative stress. Rebrin *et al*, (2003) reported that calorie restriction did not affect the concentration of GSH in mitochondria except for the increase in heart and eye, the GSSG concentration was significantly reduced in all tissues except the brain and the GSH and GSSG ratio increased significantly in all tissues.

Glutathione was generally considered to be safe for use as a dietary supplement, but in an oral acute toxicity study of GSH in mice, fatal dose 50 (LD50) was observed to be 5 g/kg (Weschawalit *et al*, 2017). Richie *et al*, (2014) reported that the increase in blood was dose and time dependent, and that levels returned to baseline after a period of 1 month in oral Glutathione intake. Glutathione is used orally or intravenously to whiten skin in various countries. Studies have shown that supplements in reduced and oxidized glutathione forms have skin lightening efficacy in humans; however, in humans, very good absorption in oral use does not occur. Furthermore, side effects, including colds, stomach disorders, headache, back pain, hot flashes, soft stool, eye twitching, ear infection, urinary tract infection, and constipation may be observed while intravenous applications are prohibited in some countries because of serious side effects including anaphylaxis (Arjınpathana & Asawanonda, 2012; Richie *et al*, 2014; Weschawalit *et al*, 2017).

There is a direct relationship between glutathione oxidation and mtDNA damage in apoptosis. However, one of the earliest and most noticeable events during apoptosis is a decrease in the concentration of GSH (Esteve *et al*, 1999; Franco *et al*, 2007). Buthionine sulfoximine (BSO) is a specific inhibitor of GSH biosynthesis (Pallardó *et al*, 2009). Marengo *et al*, (2008) demonstrated that acute treatment with BSO induces a significant GSH depletion that causes excessive production of radical oxygen species (ROS) and DNA damage and thus apoptosis. It is known that there is a decrease in cellular and mitochondrial glutathione during apoptosis, and this decrease is known to induce apoptosis inducing permeability transition pores (Armstrong & Jones 2002).

Allen and Bradley (2011) did not observe any significant changes in their study with oral glutathione supplementation, in healthy individuals, lipid peroxidation biomarkers, DNA, and glutathione. Although GSH is a very important antioxidant that plays a role in the elimination of oxygen radicals in the body, it has been demonstrated in some studies that it has prooxidant properties (Solov'eva *et al*, 2007). Glutathione may act as a pro-oxidant that damages DNA although it is present as a cleanser in millimolar levels (Fucassi *et al*, 2006).

In our study, the prooxidant properties of GSH were observed. In this case, it should be considered that intake of GSH and its derivatives as nutritional supplements in the form of pills or capsules may be harmful. The complexity of antioxidants or the ability of antioxidants such as glutathione to have prooxidant activity depending on the dose is a complex situation called "antioxidant paradox" by Halliwell (2013). In fact, the redox balance can be the cause or the result of a disease, and in some cases it is difficult to know the level at which an antioxidant becomes a prooxidant. Therefore, there is still much to understand about the role of glutathione levels in health (Minich & Brown, 2019).

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Hulya Yildiz: Investigation, Resources, Formal Analysis, Writing. **Duygu Dus:** Investigation, Resources, Formal Analysis. **Ayse Gul Mutlu:** Investigation, Resources, Formal Analysis, Statistics, Supervision.

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