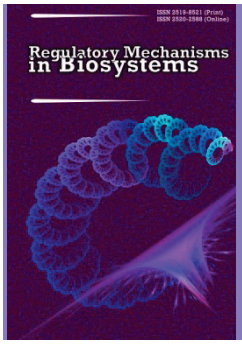

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.,
2024, 15(4), 951–956
doi: 10.15421/0224139

Correlations among oxidative DNA damage markers, enzymatic antioxidants and HSPs in stressed rats with H_2O_2 at different concentrations

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Article info

Received 09.10.2024
Received in revised form 19.11.2024
Accepted 15.12.2024

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Younis, M. A., Ali, S. M., & Alchalabi, A. S. (2024). Correlations among oxidative DNA damage markers, enzymatic antioxidants and HSPs in stressed rats with H_2O_2 at different concentrations. Regulatory Mechanisms in Biosystems, 15(4), 951–956. doi:10.15421/0224139

Oxidative stress is a normal metabolic byproduct and when produced in abnormal amounts it leads to cellular damage indicated by oxidative DNA damage and antioxidant enzyme reduction. The present study aimed to identify the mechanism by which DNA is destroyed. Blood samples were taken from rats exposed to oral 1% and 2% hydrogen peroxide and from the control group. RNA was extracted from blood, and transcription to cDNA synthesis was done. Primers for heat shock proteins and antioxidant enzymes were designed, PCR was conducted, and serum was analysed for 8-hydroxydeoxyguanosine and tumor protein 53 by ELISA techniques. Hydrogen peroxide significantly increased the expression of 8-hydroxydeoxyguanosine and tumour protein 53. The hydrogen peroxide positively increased the expression of heat shock protein (HSP) 27 while it negatively expressed HSP90. The damage associated with DNA also impacted the gene expression of antioxidant enzymes, namely catalase, superoxide dismutase, and glutathione peroxidase. Hydrogen peroxide has induced DNA damage and could potentially regarded as a model for genetic modulation of oxidative DNA damage.

Keywords: hydrogen peroxide; DNA damage; heat shock protein; antioxidant enzymes.

Introduction

When produced in minute amounts, free radical generations are part of normal physiological cellular functions to perform certain actions (Kohen & Nyska, 2002). Cytoplasm, cell membrane, and mitochondria are the main sites of generation of these free radical species; mainly reactive oxygen and nitrogen species are worthy of being mentioned for their participation in normal biochemical reactions, for instance, free radical production associated with ATP generation in mitochondria. Free radicals are an important part of cellular defence against infections, and gene activation (Sadasivam et al., 2022).

Free radical production is delivered through either intrinsic sources or induced through extrinsic sources. Intrinsic pathways include their production from the immune system during inflammatory response during infection, their excessive production from overgrowing cells in cancer. Stress induces reproduction of free radicals, the ageing process stimulates the overproduction of free radicals (Pizzino et al., 2017). The extrinsic production of free radicals includes the induction of free radicals through exposure to radiation (such as ultraviolet light) (De Jager et al., 2017), pollution exposure, chemicals, pesticides, xenobiotics, some drugs, and even food additives (Kohen & Nyska, 2002) (Fig. 1).

Excessive production or external free radical exposure, due to whatever causes, will lead to damage to endogenous biomolecules involved in cellular processing, such as DNA, neurotransmitters, and vital enzymes. DNA and RNA are delicate biomolecules, which are sensitive to excessive free radicals of hydroxyl radicals (HO•) and peroxynitrite (ONO^{2-}) (Christians et al., 2002) (Fig. 2).

Materials and methods

Animals and ethical approval. Following approval by the Scientific committee of the College of Pharmacy at the University of Mosul, the College of Veterinary Medicine at the University of Mosul completed the ethical approval process under the reference letter. Before being utilized in the experiment with unrestricted access to food and water, Sprague Dawley rats were kept in plexiglas cages in the Veterinary College's laboratory home for scientific and experimental animals for up to two weeks under typical conditions of heat, humidity, and light (12/12 hours of darkness and light). Forty-five male adult rats at eight weeks of age weighing 180–200 grams were split into three equal groups of 15 rats each: the control group, the H_2O_2 1% group, and the H_2O_2 2% group. Twice a day, every 12 hours, hydrogen peroxide was added to drinking water, while the control group of rats received tap water without restriction for 30 days in a row.

Blood and tissue sampling. The rats were euthanized by decapitation after receiving either ketamine or xylazine. Using a capillary tube, retro-orbital blood sampling was performed to collect 5–7 millilitres of blood in plain tubes, which were then allowed to clot overnight at 4 °C in the refrigerator. To get a clean serum sample, the coagulant was disposed of and the remaining material was centrifuged for 15 minutes at 1000 x g. Following aliquoting into 1 ml serum tubes, the serum was stored at –20 °C until it was needed for biochemical analysis. The liver samples were removed at the end of each experiment period. After being cleansed and rinsed with cold PBS, samples were stored at –80 °C until they were needed for molecular study on gene expression and marker estimation.

RNA extraction and transcription to cDNA synthesis. RNA extraction was performed on 50 mg of frozen liver tissues to determine the gene expression of specific heat shock proteins and antioxidant enzymes. As directed by the kit, RNA was extracted from rat liver samples using an RNA extraction kit (Invitrap® spin universal RNA small kit, Germany). The whole RNA that had been eluted was promptly put on ice. A nanophotometer (NanoPhotometer IMPLEN, Germany) was used to measure the purity and integrity of the RNA. RNA samples were stored at –80 °C until they were processed further after being aliquoted into 0.5 mL Eppendorf tubes. The Bio-Rad iScript reverse transcription Supermix (iScript™ Reverse Transcription Supermix for RT-qPCR, Bio-Rad, USA) was ap-

plied to convert RNA samples to cDNA. According to the instructions provided by the manufacturer, 5 µL of input RNA (1µg–1pg) was used to generate the reverse transcription master mix for ten reactions. For a total volume of 180 µL per reaction, 48 µL of iScript RT Supermix and 132 µL of nuclease-free water were combined. Using pipettes the prepared master mix solution was fully mixed. For every reverse transcription reaction, 5 µL of RNA was mixed with 15 µL of master mix, and the reaction mixture was then incubated using a heat cycler (C1000 Touch, Bio-Rad, Singapore). The following procedure was applied: Priming at 25 °C for 5 minutes. 20 minutes of reverse transcription at 46 °C. RT inactivation at 95 °C for 1 minute. Ultimately, cDNA samples were stored at –20 °C until they could be processed further.

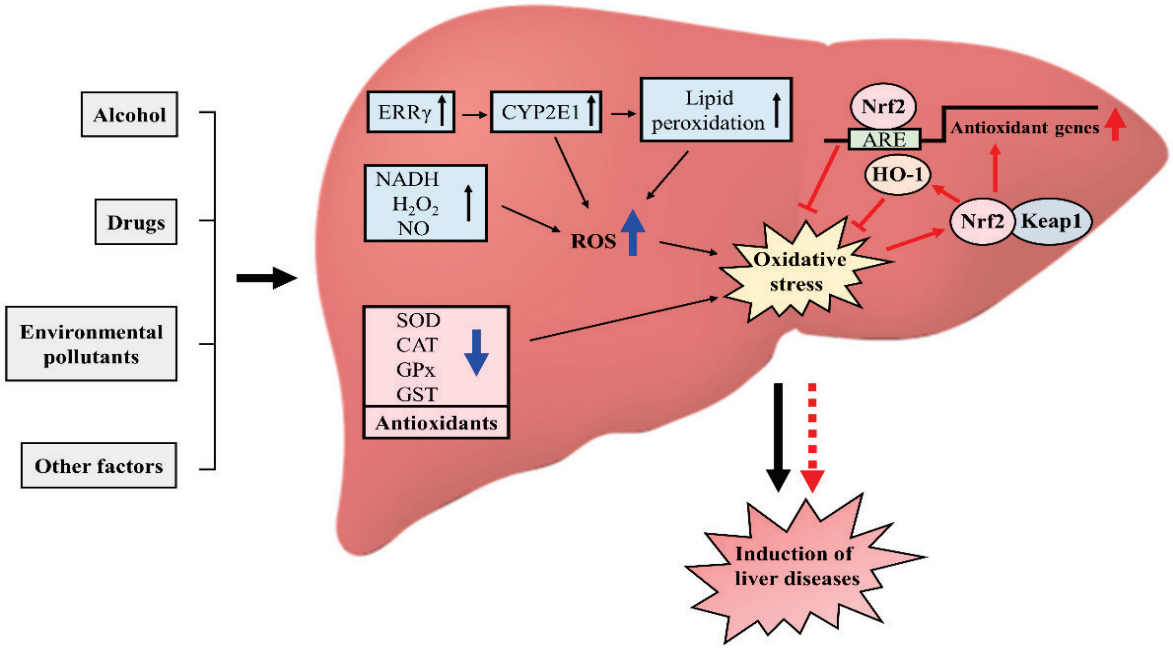


Fig. 1. Routes of liver damage by oxidative stress (Sadasivam et al., 2022)

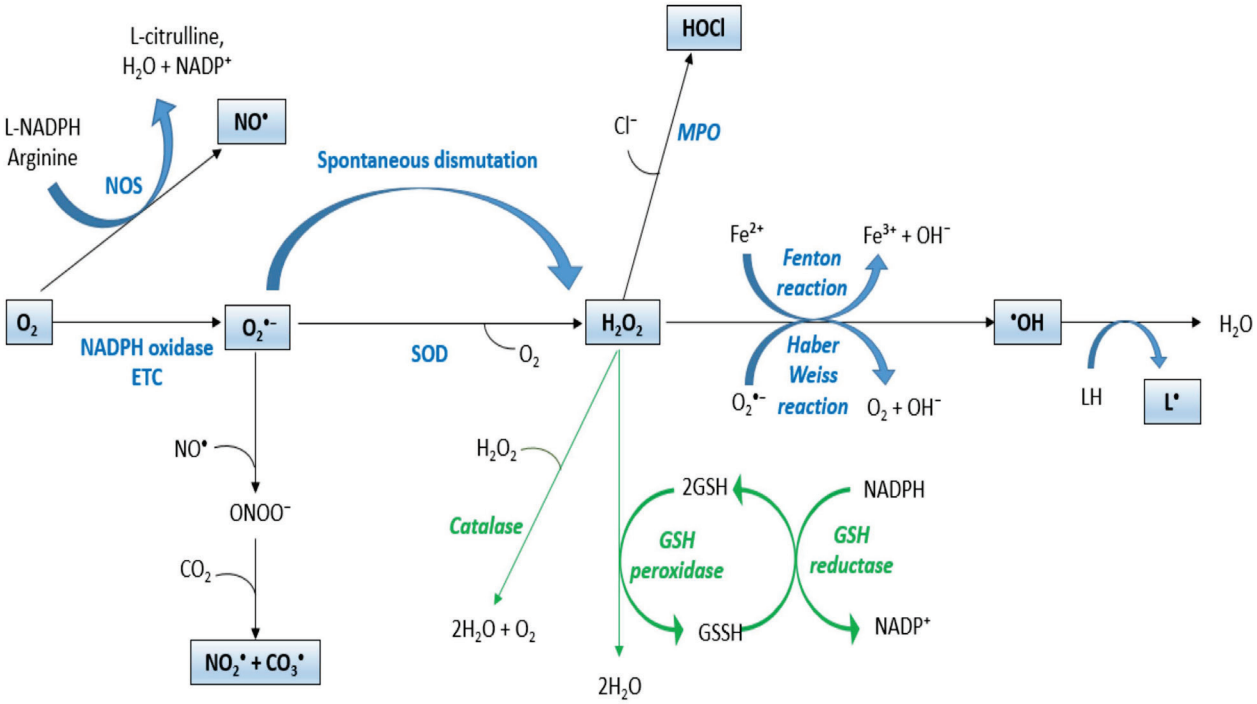


Fig. 2. Impacts of ROS at molecular levels

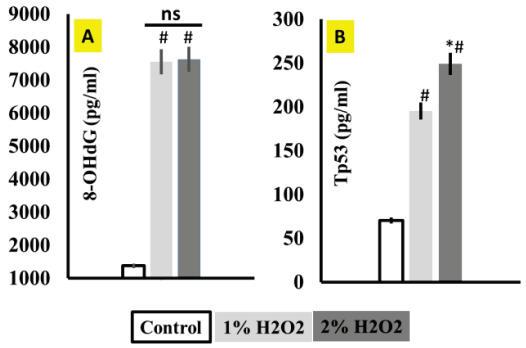
Protocol of RT-qPCR. Using the FastStart Universal SYBR Green Master (Rox) kit (Roche's Diagnostics GmbH, Germany) and a step-one applied biosystem (USA), real-time PCR was used to analyze the gene expression of antioxidant enzymes and HSPs. Using the comparative CT approach (the number of cycles needed for the fluorescent signal to cross the threshold), relative qPCR was used to evaluate relative gene expression. All samples underwent the following RT-qPCR procedure, which was carried out in a 25 µL reaction mixture volume: 8.5 µL of sterile water, 12.5 µL of FastStart Universal SYBR Green Master (Rox), 1 µL of forward primer (20 pmol/µL), 1 µL of reverse primer (20 pmol/µL), and 2 µL of cDNA (templates). Initial denaturation at 95 °C for three minutes was followed by 35 cycles of annealing at 95 °C for one minute, 60 °C for one minute, and extension at 72 °C for one minute. The relative quantitative PCR was analyzed using the step-one software, and the values derived from each sample were standardized to the expression of actin-ß. The following antioxidant enzymes and HSPs genes, which were provided by Integrated DNA Technologies, Singapore, were analyzed using quantitative polymerase chain reaction (qPCR) (Alchalabi, 2019) (Table 1).

Table 1
Primer designed for genes

Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
HSP27	GAG GAG CTC ACA GTT AAG ACC AA	TTC ATC CTG CCT TTC TTC GT
HSP 90α	TTT CGT GCG TGC TCA TTC T	AAG GCA AAG GTT TCG ACC TC
SOD	TAA GAA ACA TGGCGGTCC A	TGG ACA CAT TGG CCA CAC
Catalase	CAG CGA CCA GAT GAA GCA	GGT CAG GAC ATC GGG TTT C
GSH-Px	CGA CAT CGA ACC CGA TAT AGA	ATG CCT TAG GGG TTG CTA AGG
Actin-ß	GACCTGTTCTTTGAGGCTGAC	TTCATCTCGAAGCCTGCAGTG

Estimation of oxidative DNA damage enzyme and TP 53 marker. The 8-hydroxydeoxyguanosine (8-OHdG) enzyme ELISA kit (Cloud-Clone Corp, USA) was used to measure oxidative DNA damage. The procedure as outlined in the kit's instructions was followed, and the reagents were meticulously prepared. The TP53 ELISA kit (Cloud-Clone Corp, USA) was used to measure the amount of TP53 in the serum. Every reagent in the kit was made according to the instructions.

Statistical analysis. IBM SPSS Statistics 22 (SPSS Inc., Chicago, USA) was used to analyze the data. One-way and two-way analysis of variance (ANOVA) were applied to analyze the data, which was presented as mean ± standard error (x ± SE). A post hoc multiple comparison LSD was used to assess the differences within groups after the differences between experimental groups were assessed using $P < 0.05$, which was deemed statistically significant in comparison to the control group.



Fig, 3. The serum levels of measured DNA oxidative enzymes before and after exposure to stressful stimuli by hydrogen peroxide (*A*) 8-hydroxydeoxyguanosine (*B*) tumor protein 53: the histogram bar represents the mean and standard deviation and the way the ANOVA test was conducted to find differences between groups followed by the Bonferroni post hoc test to identify the significant differences at $P < 0.05$; *# $P < 0.05$, * – indicates the differences between 2% H_2O_2 and other groups (control and 1% H_2O_2 groups), # – as compared to the control group

Results

In the rats used in the experiment, oral H_2O_2 at varying dosages for up to 30 days had an extensive effect on the oxidative DNA enzyme by raising its serum level in contrast to control rats provided with tap water ($P = 0.003$, Fig. 1). More importantly, rats offered oral H_2O_2 demonstrated considerably higher serum levels of the tumour protein 53 markers versus normal rats receiving tap water ($P = 0.001$, Fig. 2).

Once heat stress protein 27 gene expression was analyzed in the liver samples of the stressed rats, it proved to be five to nine times more prominent than in the liver samples of normal rats. At a $P = 0.002$, 11.40 and 18.91 folds have been calculated in contrast with 2.22 fold, respectively. Stated differently, the amplification plot of the target gene under analysis in stressed hepatic tissues showed downward regulation of HSP27.

Stressed hepatic tissues demonstrated a reduction in their calculated fold change in contrast to those samples in normal rats (1.64, 1.43, and 5.80 respectively) at $P = 0.005$, according to the results of a gene expression analysis of HSP90α using step-one software and the comparative 2^-(ΔΔ Ct) method to obtain fold change of the studied gene. Figure 4 demonstrated that tissue samples from stressed rats had higher levels of HSP90α than tissue samples from normal rats.

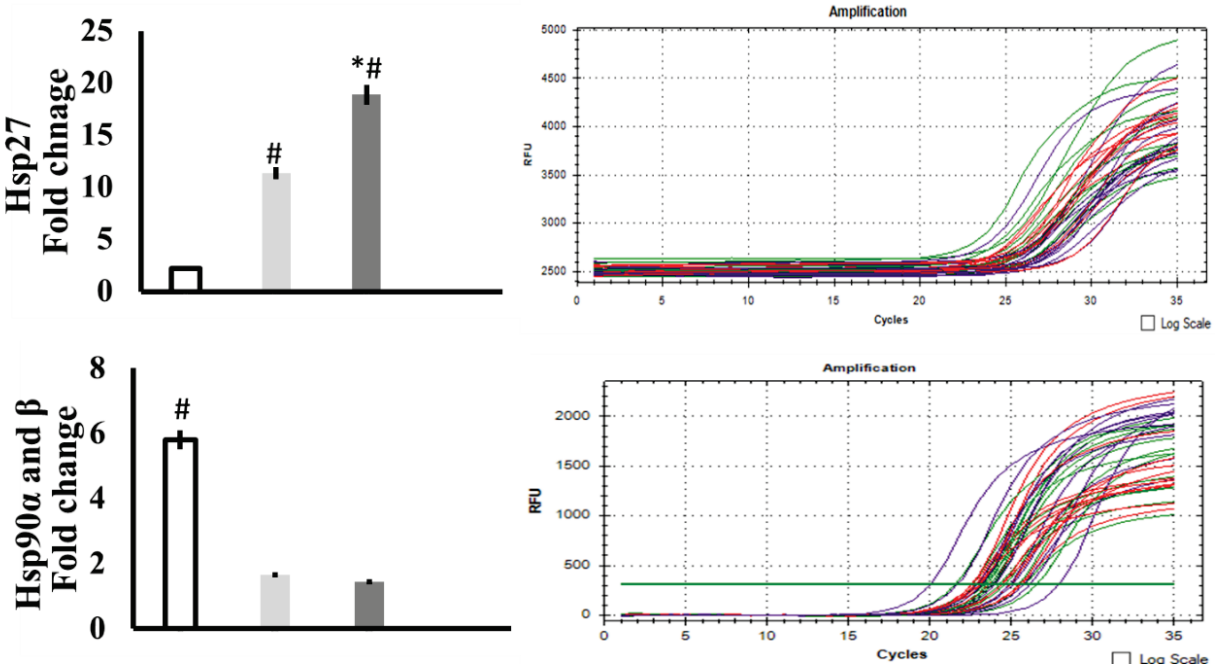


Fig. 4. Fold change of heat shock proteins in experimental hepatic tissue samples before and after exposure to stressful stimuli by hydrogen peroxide: the histogram bar represents the mean and standard deviation, one way ANOVA test conducted to find differences between groups followed by Bonferroni post hoc test to identify the significant differences at $P < 0.05$; *# – $P < 0.05$, * – indicates the differences between 2% H_2O_2 and other groups (control and 1% H_2O_2 groups), # – as compared to control group

In liver tissue samples from both rats receiving oral H_2O_2 with varying concentrations, gene expression analysis of the antioxidant enzyme catalase revealed a significant decrease in the fold change protein of this enzyme up to 62.50, 95.83, and 2% in rats of H_2O_2 1% and 2% concentration, respectively, compared to normal rats 178.47 at $P < 0.001$ (Fig. 3). In comparison to normal animals, Figure 4 demonstrated an overexpression of the catalase enzyme in the hepatic tissues of oral H_2O_2 rat groups.

In the rats that were tested, the expression of the superoxide dismutase enzyme demonstrated a drop in the fold change of protein synthesis of 35.56 and 37.64 fold for the two stressed groups, as opposed to 83.26 fold in the hepatic tissues of normal rats ($P = 0.006$, Fig. 5). The RT-PCR system's catalase gene amplification plot shows that the enzyme was highly expressed in contrast to the undamaged liver tissues of the control animals (Fig. 6).

The GSH-Px enzyme gene analysis stated a significant increase in protein fold change synthesis in the hepatic samples of stressed rats with varying H_2O_2 concentrations (85.97, 90.45 fold change) compared to 42.44 fold change of the same enzyme in the hepatic tissues of normal rats, which is roughly twice as much, at $P = 0.004$ (Fig. 5). Figure 5 illustrates how the RT-PCR amplification plot in stressed hepatic tissues with varying H_2O_2 concentrations clearly showed downregulation of the GSH-Px enzyme.

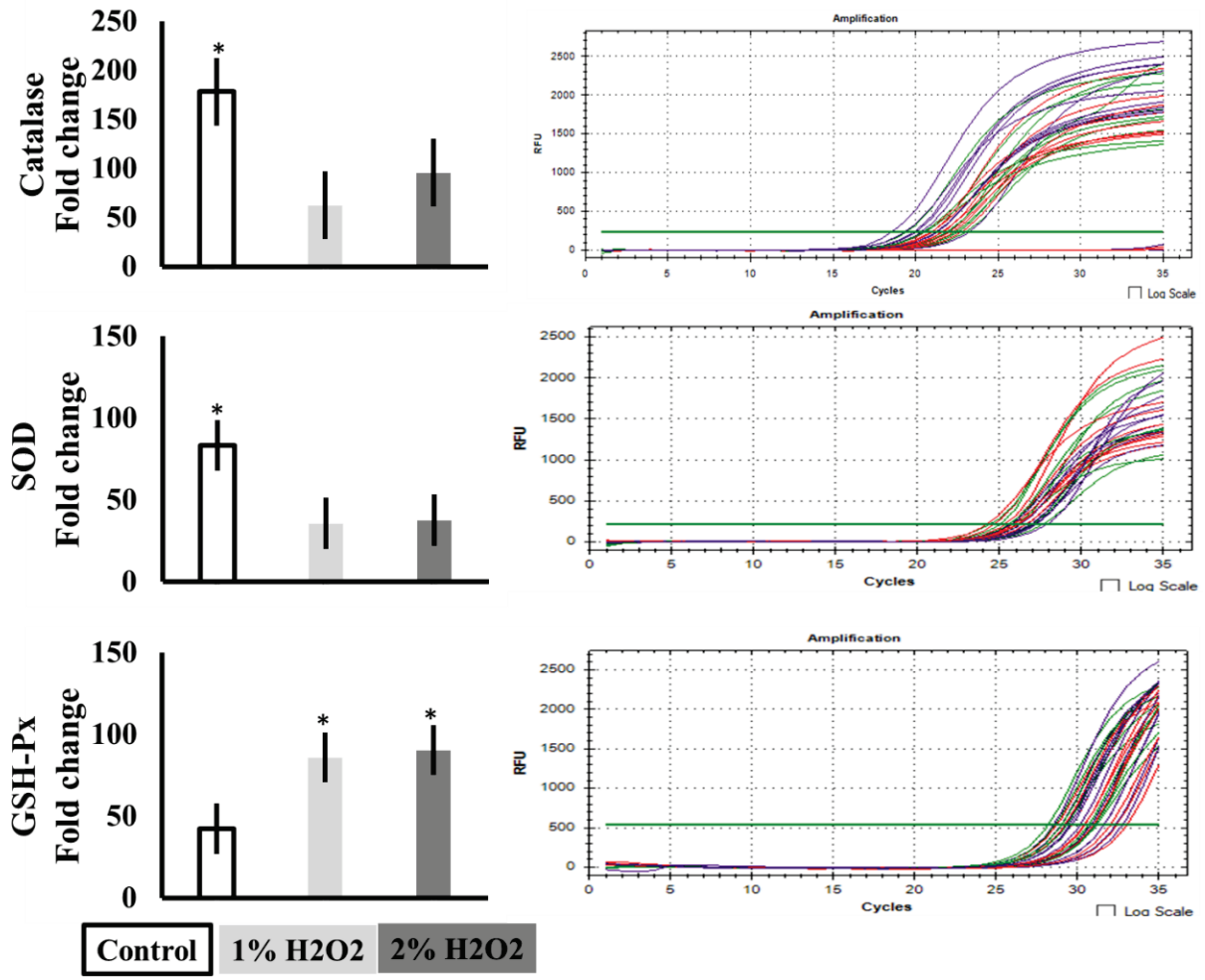


Fig. 5. Fold change of antioxidant enzymes in experimental hepatic tissue samples before and after exposure to stressful stimuli by hydrogen peroxide: the histogram bar represents mean and standard deviation, one way ANOVA test was conducted to find differences between groups followed by the Bonferroni post hoc test to identify the significant differences at a $P < 0.05$; * – $P < 0.05$, * – indicates the differences as compared to the control group

Table 2
Correlations among studied parameters in experimented animals

Parameters		8-OHdG	CAT	SOD	GSH-Px	HSP27	HSP90α	HSP90ß
Tp53	r	0.540**	0.404*	0.526**	–0.532**	0.457**	–0.488**	–0.016
	P	0.001	0.015	0.001	0.001	0.005	0.003	0.925
8-OHdG	r	–	0.385*	0.499**	–0.432**	0.411*	–0.413**	0.101
	P	–	0.017	0.001	0.007	0.010	0.010	0.545
CAT	r	–	–	0.570**	–0.707**	0.456**	–0.636**	–0.042
	P	–	–	0.0001	0.0001	0.004	0.0001	0.802
SOD	r	–	–	–	–0.676**	0.628**	–0.657**	–0.150
	P	–	–	–	0.0001	0.0001	0.0001	0.370
GSH-Px	r	–	–	–	–	–0.601**	0.678**	0.237
	P	–	–	–	–	0.0001	0.0001	0.153
HSP27	r	–	–	–	–	–	–0.660**	–0.079
	P	–	–	–	–	–	0.0001	0.638
HSP90α	r	–	–	–	–	–	–	0.095
	P	–	–	–	–	–	–	0.572

Note: * – correlation is significant at the P = 0.05 level (2-tailed); ** – correlation is significant at the P = 0.01 level (2-tailed).

The Pearson correlation test showed very interesting results represented by strong correlation between Tp53 and each of 8-OHdG, CAT, SOD, and HSP27 and inversely correlated with GSH-Px and HSP90α. Meanwhile, 8-OHdG is strongly linked to CAT, SOD and Hsp27 and inversely linked to GSH-Px and HSP90α. CAT showed strong correlation with both SOD and HSP27 and inversely with GSH-Px and Hsp90α. At the same time, SOD exhibited a strong correlation with HSP27 only and a strong inverse link with GSH-Px and HSP90α. Furthermore, GSH-Px showed a strong direct link with HSP90α and inversely link with HSP27. Meanwhile, HSP27 is inversely linked to HSP90α (Table 2).

Discussion

In the present study, rats exposed to hydrogen peroxide exhibited oxidative liver tissue damage represented by damaged liver tissues indicated by elevated serum enzymes of 8-hydroxydeoxyguanosine and tumour protein 53 and modulated heat shock proteins, alongside reduced antioxidant pools indicated by genetically measured antioxidant enzymes (catalase, superoxide dismutase, and glutathione peroxidase). Remarkably effective correlations existed between the antioxidant pool and the genetic markers of antioxidant systems, especially catalase and superoxide dismutase, moreover, heat shock protein correlated variably with oxidant parameters, negative correlation existed between HSP90ß and antioxidant genes, similarly, negative correlation exists between HSP90α and antioxidant genes except for GSH-Px, which is positively correlated with HSP90α. Moreover, a positive correlation existed between Tp53 and 8-OHdG.

Oxidative stress plays a great role in liver cells resulting in liver disease (Poli & Parola, 1997). The free radicals impact all liver cell types including liver cells, stellate cells, vessels endothelial cells, and immune cells (Kolios et al., 2006; Luangmonkong et al., 2018; Zapotoczny et al., 2019), via oxidative genetic damage of these cells especially hepatocytes (Kang, 2002; Loguercio & Federico, 2003). Even at cellular oxidative stress provoked by cellular production of hydrogen peroxide, the induced DNA damage can invoke genetic mutation due to attacks by free radicals (Helbock et al., 1998; Kawanishi et al., 2001; Dominissini & He, 2014), alongside the normal physiological function of induction of intracellular signalling via the capacity of these free radicals in stimulation of certain phosphorylation pathway and inhibition of others (Szumiel, 2015).

Exposure to hydrogen peroxide was associated with liver oxidative damage reflected by serum increase of more than one fold with Tp53 and 8-OHdG. This parameter was associated with endogenous DNA damage resulting in the formation of damage to the structure of DNA and loss of its functionality. Similarly, a study conducted by Huang et al. (1999) reported that hydrogen peroxide induces liver injury and hepatocellular oxidative damage (Huang et al., 1999). The mechanism of damage has involved free radical production and subsequent initiation of inflammation. These actions have been noticed in in vitro cell culture of L02 liver cell line and in vivo in mouse model, confirmed via measured oxidative stress parameters and apoptosis markers (Huang et al., 1999).These free radicals were mainly produced by liver cells, Kupffer cells, and neutrophils. Hence, the use of H_2O_2 has led to the induction of liver damage by oxidative reactions (Abdellatif et al., 2004).

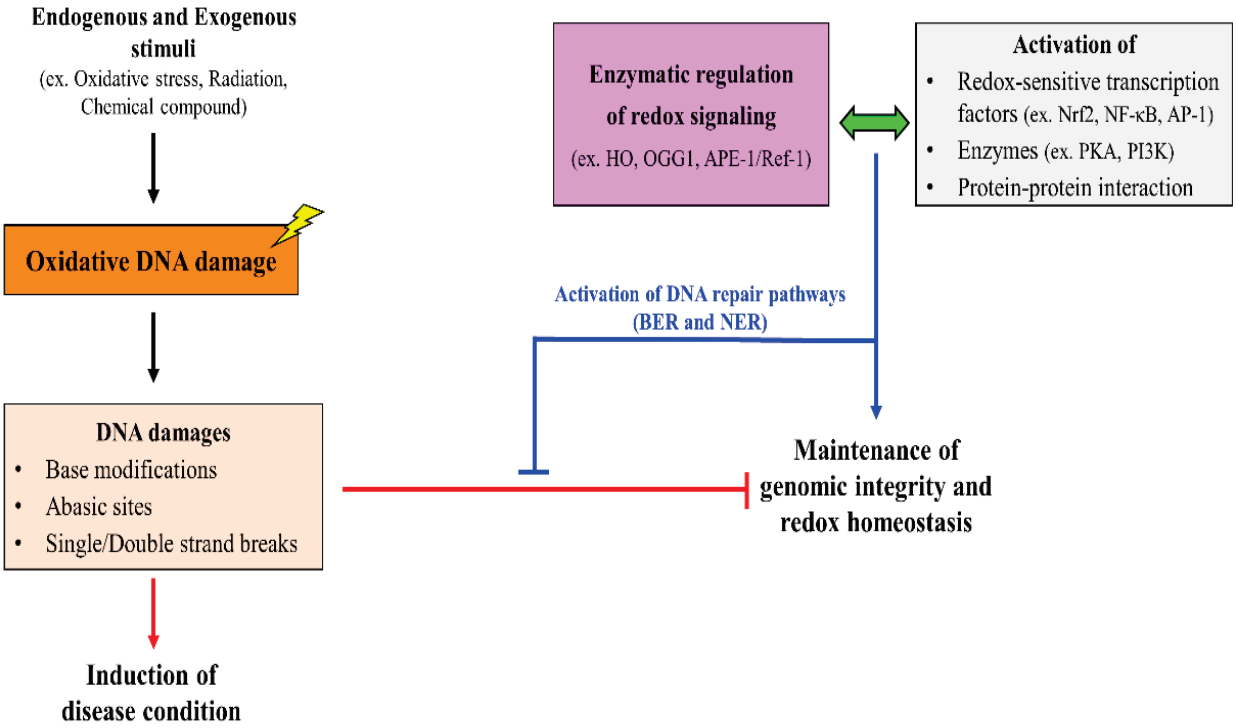


Fig. 6. Genetic stability and DNA damage during oxidative reactions (Sadasivam et al., 2022)

HSP90α and HSP27 have been modulated with exposure to H_2O_2, HSP90α is reduced while HSP27 is elevated by H_2O_2 in concentration concentration-dependent way. HSP is a protein that maintains cellular homeostasis in normal and abnormal conditions (Snoeckx et al., 2001). In line with our study, the ROS associated with ischemia resulted in an increase in the generation of heat shock protein (Christians et al., 2002). Oxidative stress has been remarkably associated with increased HSP70 with subsequent association with reduced catalase and superoxide dismutase. Moreover, HSP is associated with increased oxidative stress associated with physical activity (Starnes et al., 2005) – physical activity on the treadmill increased the expression of HSP70 in the muscle tissue of rats (Krause et al., 2015). The antioxidant in dragon fruit has shown decreased oxidative stress in rats and subsequently blunted the HSP70 production (Harahap et al., 2019). The higher the intensity of exercise, the higher the association with HSP expression (Liu et al., 2006; Yamada et al., 2008), moreover, HSP is also reciprocally expressed with metabolic oxidative stress (Khassaf et al., 2001). The protective stress response of HSP provides an indicator of oxidative stress (Samali et al., 2001; Ogawa et al., 2011). This action could be down-regulated by red dragon antioxidant providing protein protection of HSP70 expression from denaturation (Latchman, 2001), the same action which mimics red dragon was achieved by vitamin C reducing oxidative stress and thereby HSP70 (Khassaf et al., 2003). Similar to our results, the depletion of GSH has been associated with modulated HSP27 and HSP90 (Katsuki et al., 2004). The free radical generation associated with the oxidation of protein biomolecules leads to the oxidation of protein moiety affecting HSP (Kiang, 1998). Free radical generation is associated with increased HSP synthesis (Calabrese et al., 2006).

Conclusion

The study identified that exposure of rats to oxidative agents such as hydrogen peroxide provides good indication for changing oxidative reactions leading to DNA damage and modulating oxidative parameters resulting in reduced antioxidant enzymes and heat shock protein.

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