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Chronic Stress Enhances Hepatotoxic Effects of Sorbic and Benzoic Acids in a Rat Model

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Background: The individual effects of psychological stress and food preservatives on the liver—the body's primary detoxification organ—are well-documented; however, little is known about their combined impact. **The aim of this study** was to evaluate the hepatic effects of sorbic and benzoic acid exposure in male rats subjected to chronic stress.

Results: Over a 28-day period, four groups of male rats were studied: Control, Chronic Stress, Preservatives (sorbic acid at 500 mg/kg and benzoic acid at 100 mg/kg), and a Combined Exposure group. Gene expression analysis revealed increased *Sod1* expression under stress conditions, while *Nqo1* and *Hmox1* were significantly downregulated following preservative exposure and remained suppressed in the combined group. Biochemical analysis demonstrated reduced ALT, AST, and ALP activities across all experimental groups, with the most pronounced decreases observed under combined exposure. LDH activity was elevated under stress but declined when stress was coupled with preservative intake. Lipid metabolism was disrupted, as evidenced by decreased triglyceride levels and altered cholesterol concentrations. Total protein and albumin levels were significantly reduced only in the combined group. Despite preserved hepatic architecture, these molecular and biochemical changes suggest early signs of functional decompensation.

Conclusions: Co-exposure to chronic psychological stress and high doses of food preservatives resulted in a non-linear and potentially synergistic disruption of hepatic redox homeostasis, protein synthesis, and lipid metabolism. Further studies incorporating mitochondrial assessment, long-term exposure models, and quantitative interaction analysis are warranted to clarify the mechanisms underlying this toxicological synergy.

Key words: chronic stress, food preservatives, liver, gene expression, Sod1, Hmox1, Nqo1, rats

Chronic psychological stress represents an increasingly pervasive issue in modern society, with far-reaching consequences on both the individual and population levels. Its impact spans psychological well-being, social functioning, and quality of life (Ceccato *et al.,* 2018), as well as professional performance, healthcare outcomes, and public health metrics (Shchaslyvyic *et al.,* 2024; Lukan *et al.,* 2022; Bird *et al.,* 2024; Crielaard *et al.,* 2021).

Sustained activation of stress-response systems imposes a continuous burden on the body's adaptive capacity, leading to persistent biochemical, physiological, and frequently structural alterations. One of the primary targets of chronic stress is the liver, the central organ of metabolism and detoxification. Activation of the hypothalamic–pituitary–adrenal axis under stress results in elevated circulating glucocorticoids, which are predominantly metabolized in the liver via oxidation, reduction, and conjugation. While this detoxification process yields inactive metabolites, glucocorticoids have been shown to provoke inflammatory responses and promote hepatocyte apoptosis, notably through upregulation of the Fas antigen. Interestingly, studies have demonstrated that glucocorticoid exposure does not directly induce Fas expression in cultured hepatocytes (Chida *et al.,* 2004), suggesting that their hepatic effects in vivo are mediated indirectly through systemic mechanisms (Chida *et al*., 2004; de Sousa Rodrigues *et al.,* 2017). Chronic stress has also been shown to impair hepatic microcirculation, leading to decreased tissue perfusion and diminished vascular regulatory capacity (Potekhina *et al.,* 2024). These hemodynamic disturbances contribute to hypoxia, Kupffer cell activation, and increased leukocyte infiltration, amplifying local inflammation and disrupting hepatic homeostasis (Joung et al., 2019). Moreover, chronic stress is now recognized as a contributing factor in the progression of hepatitis, liver fibrosis, cirrhosis, and even hepatocellular carcinoma (Reichel *et al.,* 2018).

Food preservatives, while essential for extending shelf life and preventing microbial spoilage, may also pose toxicological risks. Potassium sorbate, a widely used preservative, has been associated with oxidative damage through its pro-oxidant potential (Taghavi et al., 2016), and sorbic acid may impair hepatic lipid metabolism (Chen et al., 2020). Benzoic acid, although not conclusively shown to induce oxidative stress, has been linked to metabolic cascades that accompany oxidative imbalance; elevated tissue levels of benzoic acid are associated with oxidative stress-related outcomes, albeit without direct evidence of reactive oxygen species generation (López-González et al., 2023).

We hypothesized that combined exposure to sorbic and benzoic acids under chronic stress would induce more pronounced structural and functional alterations in the liver than either factor alone. Given that both chronic stress and dietary preservatives can disrupt redox homeostasis and metabolic pathways, we expected additive or even synergistic interactions, particularly reflected in altered expression of antioxidant defense genes, serum biochemical markers, and hepatic histology. At the same time, we considered the possibility of antagonistic effects in selected parameters —particularly those reflecting protein synthesis, lipid metabolism, or morphological integrity—where co-exposure might attenuate or mask the individual effects of stress or preservative exposure, resulting in a non-linear or compensatory response pattern.

Accordingly, the aim of this study was to evaluate the hepatic effects of sorbic and benzoic acid exposure in male rats subjected to chronic stress, focusing on antioxidant gene expression, serum biochemical markers, and liver morphology.

MATERIALS AND METHODS

Ethical Approval. Experimental modeling of chemical exposure under chronic stress conditions was approved by the Institutional Bioethics Committee (Protocol No. 01-02, dated February 8, 2024). All procedures for animal housing, feeding, care, and euthanasia were carried out in accordance with the legislation of the Russian Federation on the treatment of laboratory animals, as well as the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No.

123) and Directive 2010/63/EC of the European Parliament and of the Council on the protection of animals used for scientific purposes.

Animal Model and Group Allocation. The study design followed the ARRIVE guidelines to ensure high-quality reporting of animal experiments. Outbred male white rats (body weight 190–210 g, age 10–12 weeks at the start) were randomly assigned to four groups (n=6 per group). One group was exposed to stressors according to the protocol throughout the entire duration of the experiment ("Chronic Stress" or "Stress"). Another group received daily intragastric administrations of aqueous solutions of food preservatives – sorbic acid (500 mg/kg body weight, 100 mg/mL) and benzoic acid (100 mg/kg body weight, 20 mg/mL) – at the same time each day ("Sorbic and Benzoic Acids" or "SBA"). A third group was subjected to combined exposure to chronic stress and food preservatives ("Chronic Stress + Sorbic and Benzoic Acids" or "Comb"). The final group served as the control ("Control" or "Ctrl"), and animals received a daily intragastric administration of an equivalent volume of the preservative vehicle, which was distilled water.

The doses of sorbic and benzoic acids were set to ten times the average daily intake for an adult human, based on the list of food products approved by R2.1.10.3968-23 (Moscow, 2023) and TR TS 029/2012 (adopted by the Eurasian Economic Commission Council, No. 58 dated July 20, 2012). It is important to note that this study did not aim to replicate real-world consumption patterns of sorbic or benzoic acids for any specific population group; rather, it sought to identify potential toxic effects on the liver under chronic stress conditions at high-dose exposures to these widely used sorbic and benzoic acids as food preservatives. In summary, the study design evaluated (1) the impact of chronic stress, (2) the impact of food preservatives – sorbic and benzoic acids, and (3) their combined effects on the liver enzymes, morphology, gene expression in liver tissue. An overview of the experimental design is presented in Figure 1.

Chronic Stress Protocol. Chronic stress was induced following the method described by Matisz et al. (2021). Animals were subjected to mild, unpredictable stressors on a daily basis, comprising 2–3 randomly selected factors such as social isolation, immobilization, exposure to noise, continuous lighting during the dark phase, and restricted access to food and water for a limited period. Each combination of stressors was applied at random times over the course of a day, ensuring that the same stress factor was not repeated twice within a single day.

Evidence of chronic stress in the animals was confirmed by several behavioral and physiological signs, including reduced weight gain compared to the Control group, alterations in locomotor activity, and a tendency toward diminished exploratory behavior Gizatullina *et al.,* 2024 a; Gizatullina *et al.,* 2024 b), consistent with stress-induced changes described in related studies (Alexa *et al.,* 2023).

Measurements of biochemical stress markers (e.g., corticosterone or cortisol) were deliberately omitted due to the substantial influence of circadian rhythms and procedural interventions (blood sampling) on hormone levels, which reduces the specificity of such assessments (Kim *et al.,* 2018; Perhonen *et al.,* 1995). Moreover, while cortisol is the principal stress hormone in humans, corticosterone serves this role in rats, complicating direct comparisons of these results with clinical data focused on human physiology (Joëls *et al.,* 2019).

Euthanasia, Blood and Tissue Collection. After 28 days of exposure, the animals were euthanized by decapitation in accordance with the recommendations of the American Veterinary Medical Association (AVMA Guidelines for the Euthanasia of Animals: 2020 Edition). Blood samples were collected immediately at the time of decapitation, and necropsy was performed to harvest liver tissue samples.

Serum Biochemistry. Biochemical parameters reflecting metabolic status and liver function – including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) activity – were measured in serum using the Stat Fax 3300 photometer (Awareness Technology, USA). Diagnostic kits from Vector-Best (Vector-Best, Russia) were employed according to the manufacturer's instructions.

Histology. Liver samples for histomorphological evaluation were fixed immediately after necropsy in 10% neutral buffered formalin. Tissues were processed through graded isopropanol and embedded in paraffin using standard histological procedures. Sections 5–7 µm thick were prepared using an MS-2 microtome and stained with hematoxylin and eosin. Slides were subsequently imaged using a Celena X digital imaging system (Logos Biosystems, South Korea), and qualitative descriptive analysis was performed on the resulting histological preparations. Morphometric analysis was conducted using the open-source software QuPath-0.5.1 (The Queen's University of Belfast, United Kingdom). The nuclear and cytoplasmic areas of hepatocytes were measured, and the nuclear-to-cytoplasmic (N/C) ratio was calculated for 50 cells selected from five random fields of view on each histological slide obtained from a single animal. Additionally, the number of binucleated and anucleated hepatocytes was counted in 10 randomly selected fields of view per slide, with one slide analyzed per animal.

Gene Expression Analysis. To assess the expression of antioxidant defense genes, liver tissue samples were collected immediately after decapitation and necropsy. Small fragments of liver were rapidly frozen in liquid nitrogen and stored in ExtractRNA solution (Evrogen, Russia). Total RNA was isolated using a TRIzol-based extraction method, followed by reverse transcription and real-time PCR amplification using the Rotor-Gene Q system (QIAGEN, Germany).

Complementary DNA (cDNA) synthesis was performed from purified total RNA using the MMLV RT kit and oligo$(dT)_{15}$ primers (Evrogen, Russia). Specific oligonucleotide primers for real-time PCR were designed using PrimerQuest (Integrated DNA Technologies, USA) and synthesized by Evrogen (Russia). Amplification reactions were carried out in a total volume of 25 µL containing 2 µL of cDNA template and SYBR Green dye. The PCR conditions included an initial denaturation at 95°C for 3 minutes, followed by 45 cycles of 95°C for 15 seconds, 59°C for 25 seconds, and 72°C for 15 seconds.

Gene expression levels were normalized to Gapdh as a reference gene. Relative quantification was performed using the ΔΔCT method as described by Livak and Schmittgen (2001). The ΔΔCT value was calculated as the difference between the mean ΔCT of the experimental group and that of the control group. Fold change (FC) values were derived by exponential transformation of the ΔΔCT, reflecting the relative change in target gene expression compared to the control.

To evaluate molecular alterations, we selected genes involved in oxidative stress regulation, detoxification, and antioxidant defense mechanisms: *Superoxide dismutase 1 (Sod1), NAD(P)H quinone dehydrogenase 1 (Nqo1), Heme oxygenase 1 (Hmox1).*

The *Sod1* gene encodes the antioxidant enzyme superoxide dismutase 1 (SOD1), which plays a critical intracellular role in maintaining basal oxidative balance by catalyzing the dismutation of superoxide radicals generated in both the cytosol and mitochondria (Trist *et al.,* 2021). The *Nqo1* gene encodes NAD(P)H quinone dehydrogenase 1 (NQO1), a multifunctional cytoprotective enzyme with antioxidant properties that participates in redox cycling and cellular protection against electrophilic stress (Preethi *et al.,* 2022). The *Hmox1* gene encodes heme oxygenase-1 (HO-1), a stress-responsive heat shock protein (HSP32) that contributes to cellular resilience through its antioxidant, anti-inflammatory, and homeostatic functions (Kaszubowska *et al.,* 2024). The functional characteristics of the studied genes are summarized in Table 1.

The selection of these genes is based on their critical roles in the pathogenesis of oxidative liver injury induced by chronic stress and, as we hypothesize, by food preservatives–specifically, the widely used sorbic and benzoic acids. It is well established that chronic stress activates the hypothalamic-pituitary-adrenal axis, leading to elevated glucocorticoid production and, consequently, increased generation of reactive oxygen species (ROS). This promotes oxidative damage to lipids, proteins, and DNA, contributing to the development of various pathologies –including, as demonstrated in a 12-week mouse study, increased levels of triglycerides and cholesterol, as well as the onset of non-alcoholic fatty liver disease (NAFLD) (Liu *et*

al., 2014). NAFLD is currently one of the most prevalent causes of liver disease globally, though regional differences exist. The estimated global prevalence of NAFLD in adults is approximately 32%, with higher rates observed in males (40%) compared to females (26%) (Teng *et al.,* 2022). The overproduction of ROS requires a compensatory upregulation of endogenous antioxidant systems, particularly those involving SOD1 and HO-1 (Trist *et al.,* 2021; Kaszubowska *et al.,* 2024). Secondly, the liver – as the primary organ for xenobiotic metabolism – is especially vulnerable to toxic exposures. Benzoates and sorbates have been shown not only to inhibit cytochrome P450 activity but also to generate secondary free radicals (Piper *et al.,* 2017), reinforcing the importance of evaluating NQO1, a key phase II detoxification enzyme regulated by the Nrf2 pathway (Preethi *et al.,* 2022). Thirdly, the combined impact of these stressors may be additive or even synergistic, potentially exhausting the antioxidant reserves of hepatocytes. Therefore, monitoring the expression of Sod1, Nqo1, and Hmox1 is critical for assessing the adaptive capacity of liver cells under combined oxidative and xenobiotic stress.

Statistical Analysis. Statistical analyses were conducted using SPSS Statistics 21.0 (IBM, USA). Normality was assessed via the Kolmogorov–Smirnov test. For normally distributed data, one-way analysis of variance (ANOVA) followed by Tukey's and Tamhane's post-hoc tests were applied. Data are expressed as mean and standard error (SE). For non-normal distributions, the Kruskal–Wallis test (for comparisons of three or more groups) and the Mann–Whitney test (for comparisons between two groups) were used. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Expression of Antioxidant Defense Genes. The results are presented in Figure 2. The expression dynamics of genes involved in hepatic antioxidant defense displayed divergent trends across experimental groups.

Sod1 expression (Figure 2A) was significantly elevated in the Chronic Stress group (1.78 ± 0.30 arbitrary units vs. -0.12 ± 0.39 in the Control, $p = 0.0005$). In contrast, expression remained at control levels in the Sorbic and Benzoic Acids group and in the Combined Exposure group (-0.00 ± 0.36 and 0.39 ± 0.09, respectively; $p = 0.8194$ and $p = 0.5871$ vs. Control). However, pairwise comparisons revealed significant differences between the Stress group and the Sorbic and Benzoic Acids group ($p = 0.0004$), and between the Stress and Combined groups ($p = 0.0001$).

Nqo1 expression (Figure 2B) did not differ significantly from control in any experimental group: -0.79 ± 1.06 in Control, -1.66 ± 0.18 in Stress, -2.62 ± 0.19 in SBA, and -2.94 ± 0.35 in the Combined group ($p = 0.8118$, $p = 0.2565$, and $p = 0.2048$ vs. Control, respectively). However, intergroup comparisons revealed significant differences between the Stress and SBA groups ($p = 0.0012$), and between the Stress and Combined groups ($p = 0.0065$).

A similar pattern was observed for *Hmox1* expression (Figure 2C): while no significant differences were detected relative to Control (-0.18 ± 0.68 in Control, 0.34 ± 0.22 in Stress, -1.73 ± 0.22 in SBA, and -1.50 ± 0.26 in Combined; $p = 0.9358$, $p = 0.1176$, and $p = 0.2025$ vs. Control), significant differences emerged between the Stress and SBA groups, as well as between the Stress and Combined groups ($p = 0.0001$ for both).

Liver Enzyme Activity. The results of the biochemical analysis are presented in Figure 3. ALT activity (Fig. 3A) was significantly reduced in the Stress group (75.60 ± 2.87 U/L, $p = 0.0060$) and in the Sorbic and Benzoic Acids group (76.95 ± 3.12 U/L, $p = 0.0112$) compared to the Control group (92.42 ± 4.63 U/L). A further decrease was observed in the combined exposure group (62.12 ± 3.02 U/L, $p = 0.0001$). The combined exposure group also differed significantly from both single exposure groups ($p = 0.0044$ vs. Stress and $p = 0.0006$ vs. SBA). AST activity showed a similar trend (Fig. 3B), with a decrease in the Stress group (189.56 ± 6.30 U/L) that did not reach statistical significance vs. Control (215.32 ± 10.27 U/L, $p = 0.1032$). However, AST activity was significantly reduced in the SBA group (175.17 ± 11.41 U/L, $p = 0.0372$), with a further significant reduction in the combined group (153.68 ± 10.50 U/L, $p = 0.0001$). A significant difference

was also observed between the Stress and combined exposure groups ($p = 0.0170$). In contrast, LDH activity increased only in the Stress group (2265.69 ± 86.57 U/L vs. 1777.02 ± 93.94 U/L in Control, $p = 0.0006$) and remained at control levels in the SBA (1757.74 ± 129.78 U/L) and combined exposure (1470.71 ± 180.06 U/L) groups (Fig. 3C). ALP activity (Fig. 3D) was significantly reduced in the Stress group (240.08 ± 29.77 U/L vs. 477.35 ± 22.31 U/L in Control, $p = 0.0001$), remained unchanged in the SBA group (469.50 ± 51.76 U/L, $p = 0.8884$), and was again significantly decreased in the combined group (268.20 ± 30.64 U/L, $p = 0.0001$).

Assessment of Protein Metabolism Parameters. Total serum protein concentration exhibited a decreasing trend in all experimental groups (Fig. 3E); however, no statistically significant differences from the Control group were detected ($p > 0.05$). A near-significant difference was observed between the Stress group (63.38 ± 1.58 g/L) and the combined group (57.56 ± 1.57 g/L, $p = 0.0582$). Albumin levels (Fig. 3F) showed a slight, non-significant increase in the Stress (33.33 ± 0.76 g/L, $p = 0.2566$) and SBA (34.73 ± 0.73 g/L, $p = 0.1292$) groups compared to Control (31.75 ± 1.21 g/L). However, a marked decrease was recorded in the combined exposure group (28.72 ± 0.76 g/L), which differed significantly from both isolated exposure groups ($p = 0.0001$ for both comparisons) but not from the Control group ($p = 0.105$).

Assessment of Lipid Metabolism Parameters. Triglyceride levels in blood serum (Fig. 3G) were significantly decreased in all experimental groups compared to Control (1.19 ± 0.08 mmol/L), with the most pronounced reduction observed in the combined group (0.56 ± 0.04 mmol/L in Stress, $p = 0.0001$; 0.79 ± 0.10 mmol/L in Comb, $p = 0.0001$ vs. Control and vs. each isolated group, $p = 0.0001$). Conversely, total cholesterol concentrations (Fig. 3H) were significantly elevated in the single exposure groups (0.56 ± 0.14 mmol/L in Stress, $p = 0.0070$; 1.73 ± 0.34 mmol/L in SBA, $p = 0.0001$) compared to Control (1.39 ± 0.11 mmol/L). However, in the combined exposure group, cholesterol levels (1.00 ± 0.36 mmol/L) did not significantly differ from Control ($p = 0.5908$).

Histomorphology. Representative liver tissue micrographs are shown in Figure 4. In rats exposed to chronic stress, numerous small, immature hepatocytes with double nuclei were observed, along with visually detectable anucleated hepatocytes. In the Sorbic and Benzoic Acids group, the liver preserved its typical radial arrangement of hepatocyte cords relative to the central vein, which appeared mildly congested. Hepatocyte nuclei were clearly defined and rounded, and the cytoplasm was uniform with no signs of degeneration. In the portal tracts, mild vascularization and occasional foci of leukocytic infiltration were observed, while hemorrhages and necrotic areas were absent. In the group subjected to combined exposure to preservatives and chronic stress, the hepatic histoarchitecture remained intact. The radial trabecular pattern was preserved; hepatocytes across all zones exhibited round nuclei with prominent nucleoli and homogeneous cytoplasm. Central veins and sinusoids were moderately congested, and bile ducts were unobstructed. In the fields of view, clusters of small immature hepatocytes were noted alongside areas containing anucleated hepatocytes. No signs of inflammatory infiltration, necrosis, or atypical cell proliferation were detected.

Quantitative morphometric analysis revealed a series of statistically significant differences. The mean nuclear area of hepatocytes in the control group was 33.42 ± 0.52 μm². In the chronic stress group, this parameter was significantly reduced (26.53 ± 0.54 μm², $p < 0.0001$), whereas in the group treated with sorbic and benzoic acids, a significant increase was observed (37.12 ± 0.80 μm², $p = 0.0002$). Notably, in the combined exposure group, the nuclear area remained comparable to control values, although a trend toward an increase was detected (35.25 ± 0.78 μm², $p = 0.0537$). No significant differences were found between the combined group and the preservative-only group ($p = 0.0971$), while a statistically significant difference was observed between the combined group and the stress group ($p < 0.0001$).

The cytoplasmic area of hepatocytes in the control group was 200.57 ± 4.67 μm². This value was significantly decreased in the stress group (168.83 ± 3.29 μm², $p < 0.0001$). In the preservative

group, cytoplasmic area did not differ from the control (207.96 ± 3.93 μm², p = 0.2289), whereas in the combined group, it was significantly reduced (180.84 ± 5.14 μm², p = 0.0054). A significant difference was found between the combined and preservative-only groups (p = 0.0001), but not between the combined and stress groups (p = 0.0520).

Given that nuclear and cytoplasmic areas alone do not fully capture the cellular response, a nuclear-to-cytoplasmic (N/C) ratio was calculated. In the control group, the N/C ratio was 0.17 ± 0.01. In the stress group, this ratio was 0.16 ± 0.01, which did not differ from control (p = 0.0741). In the preservative group, it was slightly higher at 0.18 ± 0.01, but not statistically significant (p = 0.1562). However, in the combined exposure group, the N/C ratio was significantly increased (0.20 ± 0.01, p = 0.0005 vs. control), likely due to a decrease in cytoplasmic area along with a moderate increase in nuclear size. This ratio also differed significantly from the preservative group (p = 0.0291) and the stress group (p < 0.0001).

The number of binucleated hepatocytes was significantly increased in the chronic stress group (15.90 ± 1.67 vs. 9.30 ± 1.14 in control, p = 0.0043), but did not differ significantly from control in either the preservative group (8.70 ± 0.50, p = 0.6341) or the combined exposure group (9.00 ± 0.70, p = 0.8246). A statistically significant difference was found between the stress and combined exposure groups (p = 0.0013).

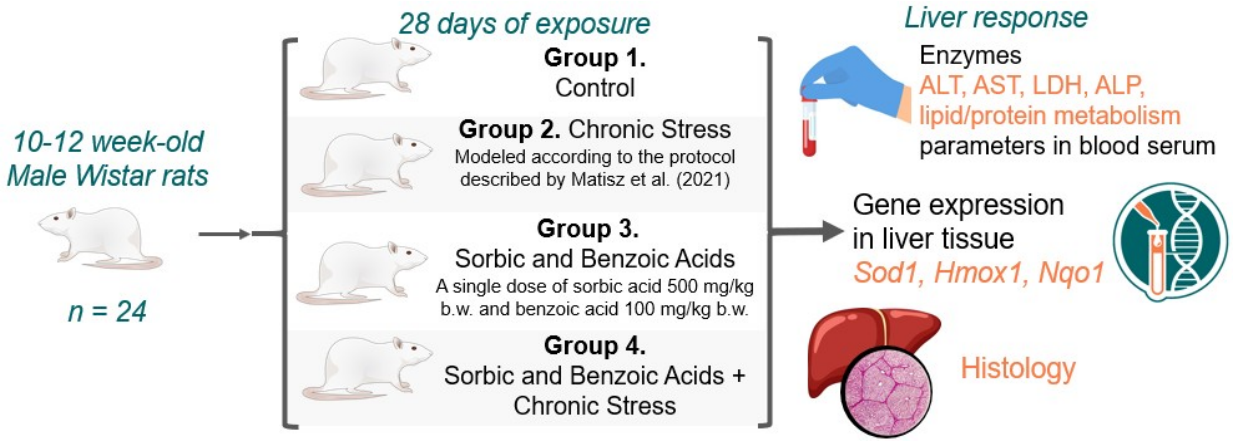

Figure 1: Schematic diagram of the experimental design.

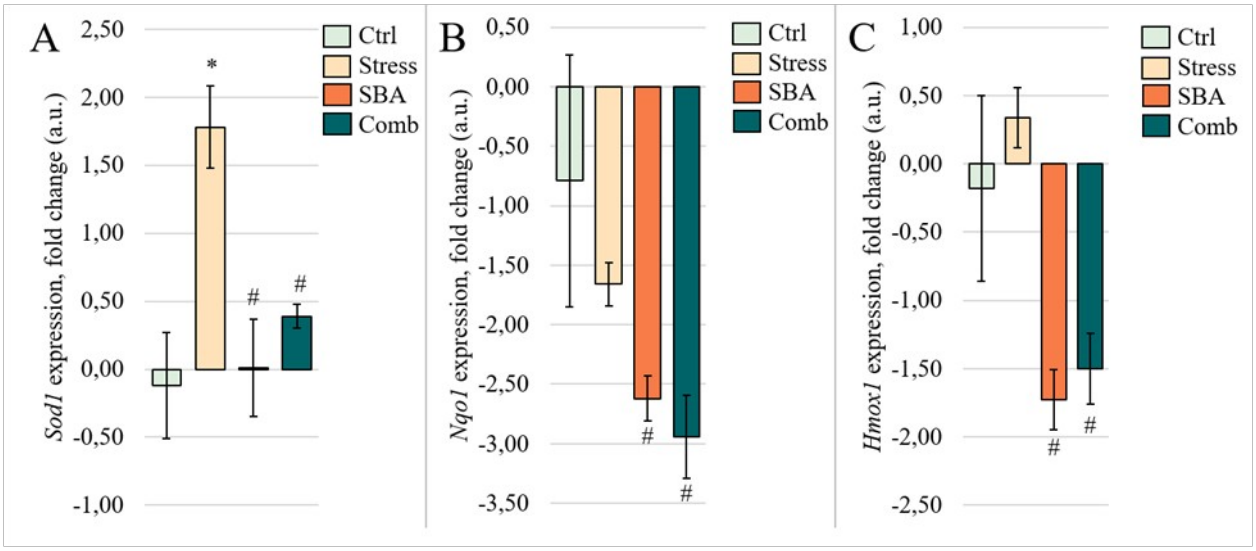

Figure 2: Fold change in expression of Sod1 (A), Nqo1 (B), and Hmox1 (C) genes in liver tissue from animals in the Control group and groups exposed to chronic stress (Stress), food preservatives (sorbic and benzoic acids, SBA), or their combination (Comb). The Y-axis shows the fold change relative to control; the X-axis indicates the experimental groups. Asterisks (*) denote significant differences vs. the Control group; hash marks (#) vs. the Stress group.

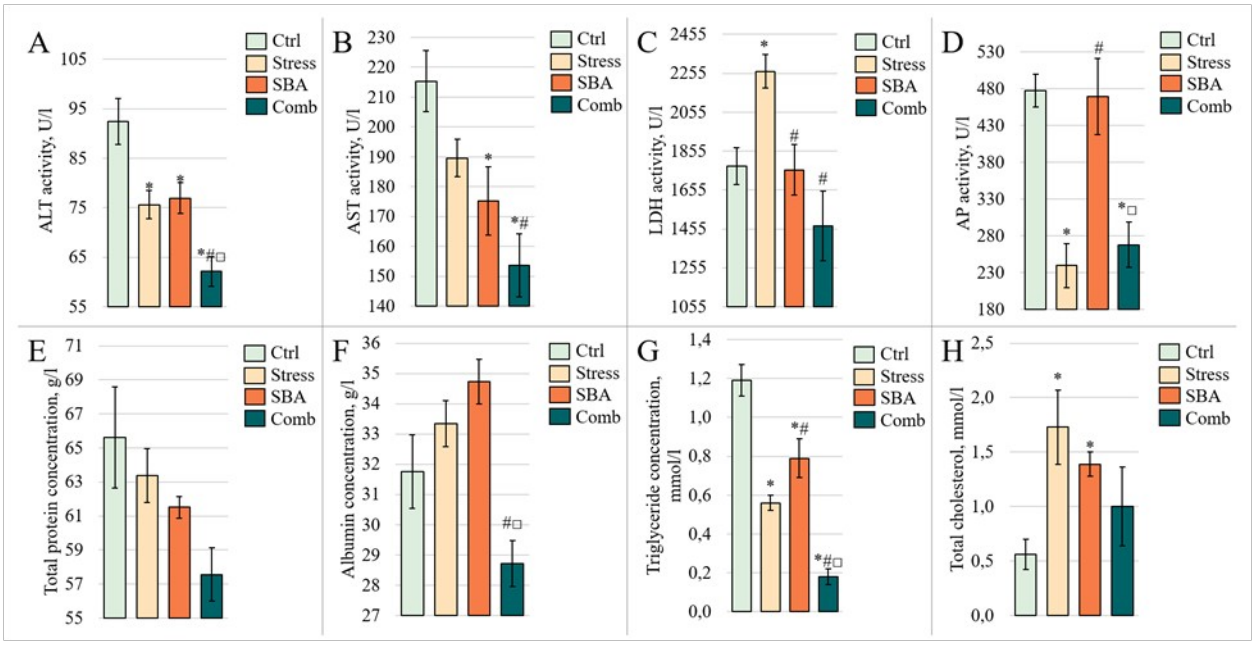

Figure 3: Activity levels of liver enzymes ALT (A), AST (B), LDH (C), and ALP (D), as well as concentrations of total protein (E), albumin (F), triglycerides (G), and total cholesterol (H) in the blood serum of animals from the Control group and those exposed to chronic stress (Stress), food preservatives (sorbic and benzoic acids, SBA), or their combination (Comb). The Y-axis shows the measured parameter values; the X-axis indicates the experimental groups. Asterisks (*) denote significant differences vs. the Control group; hash marks (#) vs. the Stress group; and open squares (□) vs. the SBA group ($p < 0.05$).

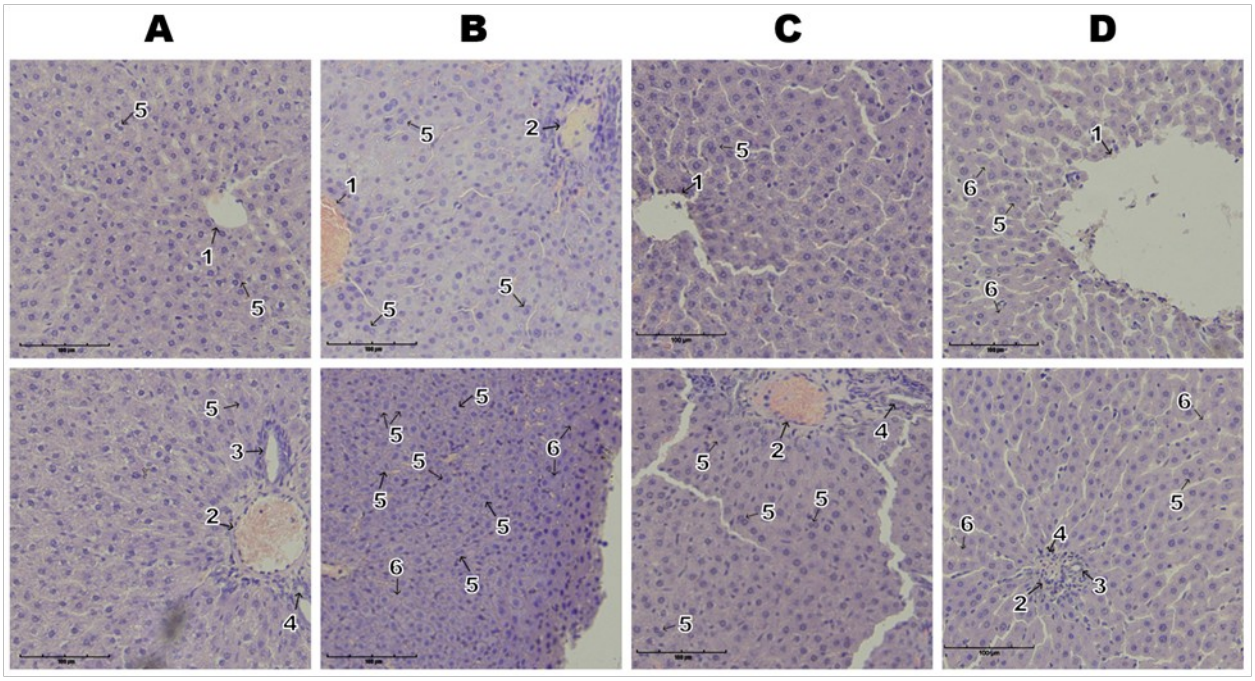

Figure 4: Representative micrographs of liver tissue sections from animals in the Control group (A) and those exposed to chronic stress (B), food preservatives – sorbic and benzoic acids (C), or their combination (D). Labeled structures include: central vein (1) in the liver parenchyma; interlobular vein (2), bile duct (3), and interlobular artery (4) within the hepatic triad; binucleated young hepatocytes (5); and anucleated hepatocytes (6). Hematoxylin and eosin staining; original magnification ×200.

Table 1: Characteristics of the Analyzed Genes

Gene Name	NCBI Gene ID	Reference mRNA Sequence (GenBank)	Oligonucleotide Primers for PCR Amplification
Superoxide dismutase 1 (Sod1)	24786	NC_086029.1	F-5'-CTT-CTG-TCG-TCT-CCT-TGC-TT-3' R-5'-CTC-CAA-CAT-GCC-TTG-TGT-ATT-G-3'
NAD(P)H quinone dehydrogenase 1 (Nqo1)	24314	NC_086037.1	F-5'-GCT-GCA-GAC-CTG-GTG-ATA-TT-3' R-5'-AGA-ATC-CTG-CCT-GGA-AGT-TTA-G-3'
Heme oxygenase 1 (Hmox1)	24451	NM_012580.2	F-5'-ATT-TCA-GAA-GGG-CCA-GGT-GA-3' R-5'-GGA-AGT-AGA-CAG-GGG-CGA-AGA-3'

The number of anucleated hepatocytes did not differ significantly from control in any experimental group. The values were 3.30 ± 0.37 in the control group, 3.00 ± 0.39 in the stress group, 3.00 ± 0.39 in the preservative group, and 3.60 ± 0.34 in the combined exposure group.

DISCUSSION

Our findings are broadly consistent with previously published data demonstrating stress-induced hepatic dysfunction and metabolic disruption. For instance, Xu et al. (2022) showed that a four-week exposure to chronic psychological stress alone can initiate inflammatory responses and impair liver function in rats, including hepatocyte necrosis. However, the changes observed in our study appeared more compensatory in nature: in the chronic stress group, we detected a significant upregulation of *Sod1* expression, while *Nqo1* and *Hmox1* expression levels remained comparable to the control.

This expression pattern may indicate an early stage of mitochondrial dysfunction, characterized by activation of the primary antioxidant defense without the engagement of phase II detoxification mechanisms (Trist *et al.,* 2021; Kaszubowska *et al.,* 2024; Piper *et al.,* 2017; Preethi *et al.,* 2022). This hypothesis is supported by the findings of Komar et al. (2021), who reported that in obesity, hepatic SOD1 expression and mitochondrial DNA copy number are reduced, correlating with the severity of steatosis. Given the high sensitivity of mitochondria to ROS, chronic stress accompanied by ROS overproduction may selectively induce *Sod1* expression as a mitochondrial adaptive response to superoxide anions. In contrast, the lack of *Nqo1* and *Hmox1* induction may result from transcriptional suppression under persistent hypercortisolemia, as previously described by Joëls et al. (2019).

Biochemical data further support this interpretation. Elevated LDH activity combined with reduced ALT, AST, and ALP levels may indicate developing mitochondrial dysfunction in the absence of overt cytolysis—considering that LDH release is associated with reduced tissue oxygenation and progressing hypoxia (Kotoh et al., 2011), while ALT and AST are classic markers of hepatocellular injury (Bonventre & Yang, 2010; Botros & Sikaris, 2013; Sookoian & Pirola, 2015; de Vos et al., 2019). These findings align with histological observations showing no necrosis but the presence of binucleated hepatocytes, which may reflect compensatory regeneration or a protective response to stress (Darmasaputra et al., 2024). Notably, binucleated hepatocytes are the main proliferative cell type in regenerating liver, including after toxic injury (Hammad et al., 2014). Total protein and albumin levels remained unchanged, indicating preserved synthetic liver function. Dysregulated lipid metabolism, with reduced triglycerides and elevated cholesterol, is consistent with stress-induced dyslipidemia (Dille et al., 2022; Xu et al., 2022). Interestingly, despite the preservation of the nucleus-to-cytoplasm ratio in the chronic stress group, a significant reduction in both nuclear and cytoplasmic areas of hepatocytes was observed. In combination with an increased number of binucleated cells, this may indicate a predominance of young, functionally active hepatocytes involved in regenerative processes. It is plausible that by the end of the 28-day exposure period,

the morphological picture reflected not the acute phase of injury, but rather the ongoing recovery phase of the liver.

Under isolated exposure to preservatives (SBA group), we observed signs of xenobiotic-induced metabolic disruption. The decrease in ALT and AST activity, in the absence of cytolysis, may suggest transaminase suppression (Cornell et al., 1984), although we did not find direct evidence of such effects specifically for sorbic and benzoic acids. LDH and ALP levels remained stable, whereas *Nqo1* and *Hmox1* expression was significantly suppressed—possibly indicating depletion or downregulation of antioxidant systems following an initial activation. These alterations occurred alongside dyslipidemia, consistent with toxic liver effects of food preservatives (Taghavi et al., 2016; Chen et al., 2020). Histological examination of the group exposed to food preservatives revealed signs of moderate hepatocellular hypertrophy, in contrast to the cellular reduction observed under chronic stress. The simultaneous enlargement of both nuclear and cytoplasmic compartments, while maintaining a stable nucleus-to-cytoplasm ratio, may suggest functional overload of mature hepatocytes, particularly in light of the accompanying biochemical alterations. A slight decrease in the number of binucleated cells and the unchanged count of anucleated cells indicate the absence of acute cytotoxic injury. These findings point to functional strain without overt cellular destruction, though a risk of subsequent decompensation remains with prolonged xenobiotic exposure.

The combined exposure group deserves particular attention. Here, a marked reduction was observed in all enzyme activities (ALT, AST, ALP, LDH), accompanied by a significant decrease in albumin levels—indicative of impaired energy metabolism and protein synthesis. Serum triglycerides were also sharply reduced, potentially reflecting impaired β-oxidation and lipid mobilization. At the molecular level, *Sod1* expression remained unchanged, while *Nqo1* and *Hmox1* were downregulated, suggesting a breakdown in the antioxidant defense system. In the setting of preserved hepatic histoarchitecture and stable numbers of binucleated and anucleated hepatocytes, a statistically significant reduction in cytoplasmic area was detected, accompanied by an increased nucleus-to-cytoplasm ratio. This morphological pattern may represent an early manifestation of cellular stress in the absence of overt tissue damage. The observed cytoplasmic reduction is likely related to diminished cellular energy reserves and contraction of metabolically active organelles, such as mitochondria and the endoplasmic reticulum. Preservation of nuclear size may suggest maintained or even compensatorily increased transcriptional activity. Collectively, these findings support the interpretation of an adaptive remodeling phase preceding the onset of functional hepatic decompensation.

Taken together, our findings suggest that chronic stress modifies the hepatotoxic effects of food preservatives, intensifying molecular and functional alterations even in the absence of overt structural liver damage. However, definitive conclusions regarding the nature of this interaction—whether additive, synergistic, or antagonistic—require quantitative assessment and the development of mathematical approaches to combined toxicity evaluation. This represents an important avenue for future investigation.

Several methodological limitations must be considered in interpreting our results. First, antioxidant gene expression (Sod1, Nqo1, Hmox1) was assessed only at the transcriptional level, without corresponding protein quantification or functional enzymatic activity, and without measurement of pro- and antioxidant metabolites. Second, the 28-day duration of the experiment captures subacute effects only, leaving potential long-term outcomes unexplored. Third, mitochondrial status was not directly assessed, despite the proposed involvement of mitochondrial dysfunction in the mechanisms of stress and toxicant action. Fourth, the absence of a quantitative framework for analyzing the interaction between stress and preservative exposure (e.g., models of additivity, synergy, or antagonism) limits the interpretation and risk prediction of combined exposure. These aspects form the basis for future research aimed at in-depth molecular, morphometric, and computational validation of our findings.

CONCLUSIONS

Our findings demonstrate that chronic psychological stress can modulate the hepatotoxic effects of food preservatives in a 28-day experimental model in male rats. The combined exposure resulted in amplified alterations in hepatic molecular and biochemical parameters. Despite preserved liver histoarchitecture, signs of functional and antioxidant system decompensation were observed in the group exposed to preservatives under chronic stress conditions.

The mechanisms underlying this modification remain insufficiently understood; however, they are likely to involve mitochondrial dysfunction, suppression of phase II detoxification gene expression, and impaired lipid regulation.

The lack of a quantitative assessment of the nature of interaction between the studied factors limits our ability to accurately predict health risks and interpret the findings. This highlights the urgent need for the development and validation of a methodological approach to quantitatively evaluate the type of combined action—whether additive, synergistic, or antagonistic—between psychological stress and chemical exposure.

AUTHOR CONTRIBUTION

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CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

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