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Fluoxetine and agomelatine mitigate anhedonic and hepatic changes in chronic restraint stress rat model

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Chronic stress, a common condition in modern life, is increasingly connected to a variety of medical concerns, including liver damage. Despite being one of the body's most resilient organs, the liver is yet susceptible to the harmful effects of chronic stress. This study aimed to investigate the therapeutic effects of fluoxetine and agomelatine on the liver after exposure to the chronic restraint stress (CRS) model in rats. Thirty-six male Sprague-Dawley rats were randomized into four groups: control, CRS + vehicle, CRS + fluoxetine, and CRS + agomelatine. Except for controls, all rats underwent CRS (2.5 h/day) for five weeks. During the last three weeks, groups received daily oral vehicle, fluoxetine, or agomelatine. Behavioral tests, forced swim test (FST) and sucrose preference test (SPT), were performed in the final week. Blood and liver samples were collected for biochemical and immunohistochemical analyses for liver enzymes, hepatic oxidative stress, and apoptotic and proliferative markers. The use of agomelatine for three consecutive weeks in CRS rats reversed immobility and climbing and slightly improved sucrose preference. No significant changes were detected in plasma liver transaminases compared to the control or treated groups. Nonetheless, agomelatine treatment significantly attenuated the oxidative stress induced by CRS, particularly in malondialdehyde (MDA) level (*$P \leq 0.05$). The histological examination of liver tissues revealed fluoxetine and agomelatine mitigated CRS-induced cellular infiltration and nuclear hyperchromasia. Additionally, both drugs significantly reduced the upregulation of caspase-3 (*$P \leq 0.001$) and proliferating cell nuclear antigen (PCNA, ***$P \leq 0.001$) in CRS rats. This study revealed for the first time the potential therapeutic role of fluoxetine and agomelatine on the liver during the CRS depression model by controlling oxidative stress and hepatic regeneration.**

Keywords Fluoxetine, Agomelatine, Liver function, Chronic restraint stress, Oxidative stress, Caspase-3, Proliferating cell nuclear antigen

Abbreviations

ABC	Avidin-biotin-complex
Ago	Agomelatine
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AR	Antigen retrieval
ARRIVE	Animal research: reporting of in vivo experiments
AST	Aspartate aminotransferase
BDNF	Brain-derived neurotrophic factor
CMS	Chronic mild stress

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CRS	Chronic restraint stress
CS	Chronic stress
CYP1A2	Cytochrome P450 Family 1 Subfamily A Member 2
DAB	Diaminobenzidine
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
Flx	Fluoxetine
FST	Forced swim test
GSH	Reduced glutathione
H&E	Hematoxylin and eosin
HO-1	Heme oxygenase-1
HPA	Hypothalamic-pituitary-adrenal
IHC	Immunohistochemistry
IVCs	Individually ventilated cages
Ki-67	Marker of proliferation Ki-67
KSU	King Saud University
MDA	Malondialdehyde
MT1	Melatonin receptor 1
MT2	Melatonin receptor 2
NAFLD	Nonalcoholic fatty liver disease
Nrf2	Nuclear factor erythroid 2-related factor 2
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
ROS	Reactive oxygen species
SA	Saudi Arabia
SD	Sprague-Dawley
SEM	Standard error of the mean
5-HT2C	Serotonin receptor 2 C
SOD	Superoxide dismutase
SPT	Sucrose preference test
TBA	Thiobarbituric acid
UDI	United diagnostics industry

Chronic stress (CS) is a common public health concern, it is consistently linked to various mental health issues, including depression and sleep disorders[1]. CS can become detrimental to the body's homeostasis, disrupting many organs, including the liver[2]. Studies indicate a strong correlation between liver disorders, depression, and stress[3–5]. Higher levels of psychological distress are associated with increased liver disease mortality rates[6]. The pathophysiological link between liver function and CS or depression is increasingly recognized as a critical factor in systemic health, with a bidirectional relationship evident between hepatic and neuropsychiatric dysfunction. CS can alter hepatic metabolic profiles and gene expression, affecting how it processes nutrients and toxins[7], and potentially contribute to metabolic disorders such as insulin resistance and fatty liver disease[8]. CS and depression may elevate the risk of developing hepatocellular carcinoma by epigenetically downregulating hypocretin (orexin), which has a role in stress control[9].

Interactions between the endocrine and sympathetic nervous systems are intricate in CS. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis can elevate cortisol levels and alter neurotransmitter systems. This imbalance may negatively affect hepatic tissue, leading to inflammation and disease progression[10,11]. Moreover, over recent years, the effects of CS on the exacerbation of liver diseases such as hepatitis and nonalcoholic fatty liver disease (NAFLD) have been acknowledged as a relevant factor[3,12–15]. Dysbiosis of the gut microbiome and enhanced intestinal permeability can increase systemic inflammation and disturb the gut-liver-brain axis, which contributes to the connection between depression and liver disorders[4,16]. In addition, soluble epoxide hydrolase, a liver enzyme that hydrolyzes epoxides derived from arachidonic acid, is pivotal in controlling behavioral and cellular impacts of prolonged stress[17]. Hepatic overexpression of this enzyme induces a depressive-like phenotype[17]. Thus, the liver may play a significant role in controlling mood. Zhou and his colleagues (2024) revealed a "brain-to-liver" neural connection that mediates the impairment of liver regeneration in a CRS model in mice. Notably, this study focused primarily on liver samples after confirming the stress model through anxiety-related behavior tests only[18]. Significant elevations in serum ALT and hepatic levels of inflammatory markers, as well as degeneration and necrosis of hepatocytes and inflammatory cell infiltration, have all occurred in the liver tissue of rats exposed to psychological CS[12]. The role of psychological CS in exacerbating hepatitis and promoting liver fibrosis has gained increasing recognition over recent years[14,19]. These findings provide a compelling rationale for investigating the liver's role in the pathophysiology of depression and anhedonia. They also emphasize the importance of managing depression and stress in patients with liver disease by incorporating antidepressant interventions.

Fluoxetine is a selective serotonin uptake inhibitor; it is widely used as a first-line treatment for depression and exerts its therapeutic effect by increasing serotonin availability in the brain. In addition to its antidepressant action, fluoxetine has been shown to ameliorate stress-induced oxidative damage[20]. It possesses neuroprotective activity by reducing oxidative stress, inflammation, and apoptotic responses in the peripheral and central nervous systems[21]. Agomelatine is a naphthalene analog of melatonin; it has a unique antidepressant with dual mechanisms of action, exhibiting melatonin receptor (MT_1 and MT_2) agonism and serotonin 5-HT_{2C} receptor antagonism. It has shown efficacy in treating both depression and anxiety disorders[22,23]. This dual

mechanism can resynchronize sleep rhythms that are commonly disturbed during depression and control core depressive symptoms[24–26]. Additionally, agomelatine can increase brain-derived neurotrophic factor (BDNF) expression, promote neurogenesis, and mitigate stress-induced glutamate release[26]. Some studies have shown that agomelatine is effective with a rapid onset of antidepressant effect and good tolerability[27,28]. Experimental depression models revealed that agomelatine reduced oxidative stress in the liver and other organs after chronic mild stress (CMS)[29]. It exhibits antioxidant and anti-inflammatory effects against paracetamol-induced liver injury[30]. Some experimental studies showed some potential protective effects of agomelatine in other models of tissue injuries[31–34].

Although considerable advances have been achieved in depression research, the mechanisms that underlie its association with liver pathogenesis remain obscure and necessitate further investigation. Here, we implement chronic restraint stress (CRS) because it causes anxiety- and depression-like behaviors after stress[35]. Assessing the effect of fluoxetine and agomelatine on the liver during CRS is preferred, as they are used in such conditions, and stress per se may affect liver function, as previously reported[29]. Therefore, this study aims to investigate the effects of subchronic use of fluoxetine and agomelatine on rat liver after exposure to CRS. It intentionally focuses on hepatic degeneration and regeneration parameters because these can serve as upstream indicators and mechanistic triggers of the systemic inflammation and metabolic disturbances that ultimately affect brain function. Chronic stress disrupts the gut-liver-brain axis, contributing to hepatic oxidative stress and apoptosis, which are understudied in depression models. Our study bridges this gap by examining liver-specific pathways (e.g., caspase-3, PCNA) influenced by stress-induced HPA axis dysregulation.

Materials and methods

All methods were performed in accordance with the relevant guidelines and regulations, including the ARRIVE guidelines[36]. The use of animals and experimental work followed the ethical principles and guidelines approved by the Research Ethics Committee at King Saud University (Ethics Reference No: KSU-SE-20-75).

Materials

Fluoxetine and agomelatine were obtained from a local pharmacy in Riyadh, Saudi Arabia. Liver enzyme kits for aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) were obtained from the United Diagnostics Industry (UDI, Dammam, Saudi Arabia). Primary antibodies were caspase 3 (ab4051, Abcam, Waltham, USA), Anti-Proliferating cell nuclear antigen (PCNA), and clone PC10 (CBL407, Sigma-Aldrich, Wicklow, Ireland). Avidin-biotin-complex (ABC) kit, diaminobenzidine kit (DAB), and secondary biotinylated antibodies were obtained from Vector Laboratories, Burlingame, CA, USA. Other Reagents, such as thiobarbituric acid, pyrogallol, and Ellman's reagent, were from Sigma Aldrich Chemicals Co. (St. Louis, MO, USA).

Animals

Adult male Sprague-Dawley (SD) rats, aged 6–7 weeks old, with weights ranging from 150 g to 220 g, were obtained from the experimental surgery and animal laboratory center at the Prince Naif bin Abdul-Aziz Health Research Center (King Saud University, Riyadh, Saudi Arabia). Each group of three rats was kept in individually ventilated cages of 18 × 25.5 × 38 cm dimensions (IVCs; Allentown LLC, US) at a temperature of 24 °C to 29 °C, a humidity of 25–48%, and a 12/12-hour light/dark cycle with the lights on at 4:00 am. Wood shavings were used as bedding, and access to pellets (1005, Saudi Grain organization, SA) and drinking water was *ad libitum* except during experimental procedures.

Experimental design

Randomization was used to divide the animals into 4 groups (6 rats/group). The experiment incorporates the use of CRS to establish a stress-induced depression rat model[37]. In this model, the rats were placed in transparent plexiglass restraints in their home cages for 2.5 h per day during the light cycle. The restraint dimensions were selected based on the rats' weights[38]. All rat groups, except the controls, underwent restraining stress for around 5 weeks. The selection of 2.5 h/day for 5 weeks to induce CRS is supported by several studies demonstrating that this protocol induces robust and reproducible depression-like behaviors in rats, such as anhedonia and behavioral despair. Seewoo et al. (2020) and Mao et al. (2022) validated a similar CRS protocol in young adult rats, demonstrating that daily restraint of 2 to 2.5 h for periods of 2–5 weeks reliably induces behavioral and neurobiological changes relevant to human depression[38–40].

The CRS protocol involved placing rats in transparent plexiglass restrainers within their home cages for 2.5 h per day during the light phase. This procedure was conducted daily for approximately five weeks, except for the control group. On day 14, they received different treatments by daily oral gavage for 3 weeks with CRS. **Group 1** (Control) consisted of neither stressed nor treated animals, left undisturbed in their home cages. Group 2 (CRS + vehicle) consisted of stressed animals that received a daily dose of normal saline (4 ml/kg). Group 3 (CRS + fluoxetine) consisted of stressed animals that received a daily dose of 14.4 mg/kg fluoxetine dissolved in 4 ml/kg saline. Lastly, Group 4 (CRS + agomelatine) consisted of stressed animals that received a daily dose of 18 mg/kg agomelatine dissolved in 4 ml/kg saline. Doses of fluoxetine and agomelatine were selected following a previously published work[41]. These doses were chosen because they have demonstrated their efficacy and safety in CS and depression models in rodents. The doses are within the effective range reported in the literature, and the administration protocols are consistent with standard preclinical practice.

All treatments were prepared daily and administered between 8:00–8:40 am before the restraining procedure. An interval of thirty minutes between treatments and restraining was allowed. Throughout the study period, all animals' body weight was measured twice weekly. All groups were subjected to sucrose preference tests (SPT) after the completion of treatments, and then a forced swim test (FST) was done one day after the SPT. To minimize observer bias, animal coding was implemented within the blinding procedures of the behavioral

experiments and molecular and biochemical measures. Ultimately, the animals were sacrificed twenty-four hours after the FST, and blood and liver tissue samples were collected. Figure 1 shows an illustration of the experimental design and timeline.

Behavioral studies

Sucrose preference test (SPT)

The SPT is used to evaluate the core symptom of depression (anhedonia)[42]. It was run as described previously[43] with some modifications. Before conducting each SPT, all rats underwent a 3-day adaptation training to ensure that sucrose neophobia was not a confounding variable. In the first 2 days, rats were habituated to drink from two identical bottles that contained filtered water. On the third day, water bottles were swapped with two fresh 1% sucrose bottles (200 ml). The SPT conducted the next day involved free access to a bottle containing water and an identical bottle containing an equal volume (200 ml) of fresh 1% sucrose solution (same water type and temperature) presented in the home cage. Rats were allowed to drink the sucrose solution for twenty hours, and the position of the bottles was switched halfway through this period to eliminate the potential effects of side preference. The timestamp for starting training and SPT was at 1:00 pm (3 h before the start of the dark cycle). Similar to the literature, the sucrose preference was calculated using the following formula: preference percentage (%) = sucrose solution consumption (ml)/ (sucrose solution consumption [ml] + water consumption [ml]) × 100%[44].

Forced swim test (FST)

FST assessed behavioral despair in rats[45], as previously detailed[46]. During the light phase, rats were exposed to a pre-swim test swim for 15 min by being placed individually in a glass cylinder (35 cm in diameter, 50 cm high) filled with 25 ± 2 °C water to a depth of 37 cm. The next day, rats were reintroduced to the cylinder for 5 min, and their behaviors were video recorded. The testing was carried out after thirty minutes in the experimental room to avoid novelty-induced behaviors. After each trial, animals were dried with absorbent paper, placed in a warm

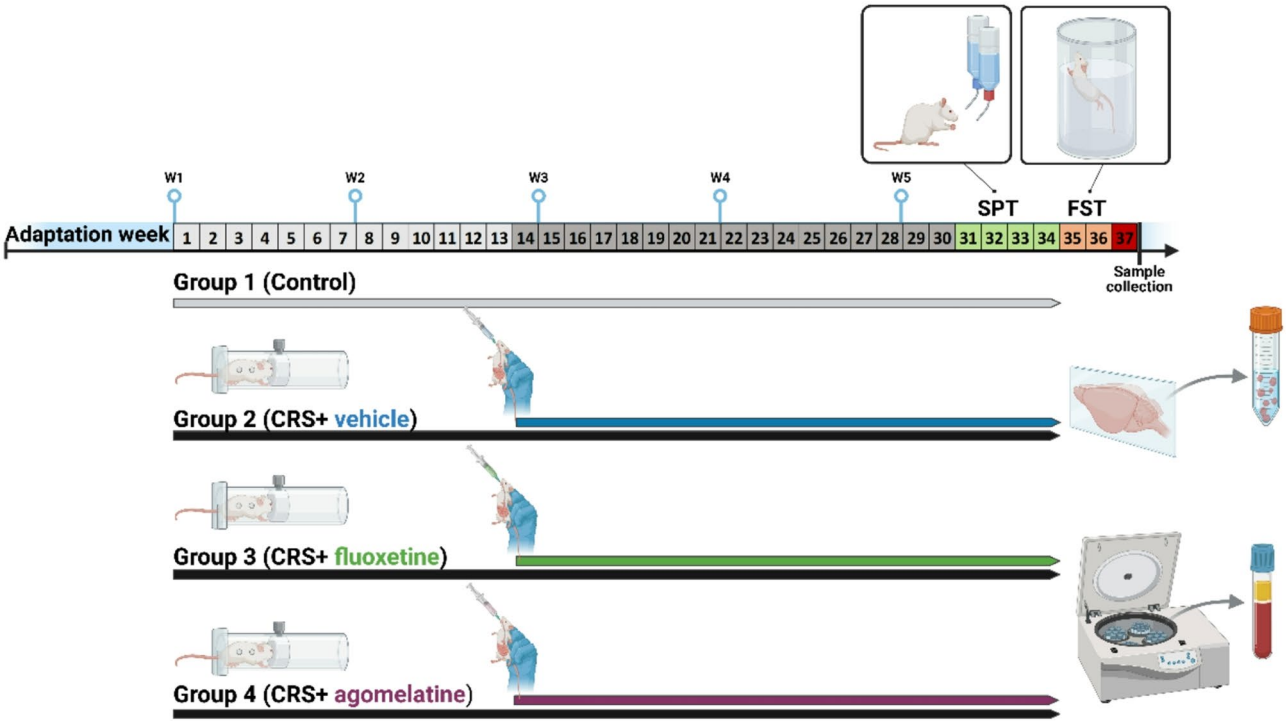

Fig. 1. Schematic representation of the experimental design and timeline. The experimental design illustrated in the figure spans five weeks. During the first week (days 1–7), all mice underwent an adaptation period to acclimate to their surroundings. For the subsequent three weeks (days 8–28), chronic restraint stress (CRS) was induced in three groups (Groups 2, 3, and 4), while the first group (Group 1) served as an unstressed control. Concurrently, the stressed groups received daily treatments: Group 2 received a vehicle, Group 3 was administered a daily dose of 14.4 mg/kg fluoxetine, and Group 4 received a daily dose of 18 mg/kg mg/kg agomelatine. In the final week, behavioral assessments were conducted, with the Sucrose Preference Test (SPT) taking place on days 31–33 and the Forced Swim Test (FST) on days 34–36. SPT consisted of a three-day training phase followed by a test day. During the training phase, animals were presented with two bottles containing 200 ml of drinking water and 1% sucrose solution. On the test day, their preference for the sucrose solution was assessed. FST consisted of a training phase followed by a test phase conducted over two days. On the last day of the experiment (day 37), samples, likely including liver tissue and blood, were collected from all four groups for subsequent analysis.

cage for fifteen minutes, and then returned to their home cage. The predominant behavior was rated manually every 5 s throughout the test session recordings. Immobility, swimming, and climbing were the main behaviors observed. Immobility comprised floating without struggling, and slight movements necessary for the rat to keep its head above water. Swimming was described as horizontal movements that included crossing into another quadrant and diving, and climbing was defined as upward movements of the forepaws against the cylinder wall.

Blood and tissue sampling and Preparation

The day after FST, rats were anesthetized using an intraperitoneal injection of 91 mg/kg ketamine combined with 9.1 mg/kg xylazine. Blood samples were drawn by cardiac puncture and added immediately into heparinized vacutainers. The animals were sacrificed through exsanguination, followed by heart excision to ensure their deaths. Whole liver tissues were then carefully harvested and washed with ice-cold phosphate-buffered saline (PBS). Parts of liver samples were homogenized in 10 mM ice-cold Tris–HCl buffer (pH 7.4), centrifuged, and the supernatants were kept at -80 °C for further analyses. For histological and immunological studies, some parts of liver tissues were isolated and fixed in 4% phosphate-buffered formaldehyde (pH 7.4).

Determination of liver transaminases

Alanine Aminotransferase (ALT) and aspartate aminotransferase (AST) activity levels in plasma were measured spectrophotometrically by commercially available colorimetric kits from UDI, Dammam, Saudi Arabia. The rate of decrease in the measured absorbance at 340 nm was taken as directly proportional to enzyme activity.

Evaluation of oxidative stress markers

The level of malondialdehyde (MDA), lipid peroxidation end-product, in the liver samples was measured using the TBA reagent as previously described by Ohkawa et al.[47]. TBA reacts with MDA content in the tissue at 95 °C, generating pink to orange color products that are measured colorimetrically at 535 nm. In addition, the level of hepatic reduced glutathione (GSH), the main nonenzymatic antioxidant, was measured using the Ellman reagent as described by Ellman at 412 nm[48]. The activity of superoxide dismutase (SOD) in the liver tissue was measured using the Marklund method with pyrogallol reagent[49]. Rapid auto-oxidation of the pyrogallol in aqueous solution results in a yellow color visible at 430 nm.

Histopathology and immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) liver Sect. (5 µm thickness) were dewaxed by xylene and then placed in 100% ethanol, 95% ethanol, 70% ethanol, and distilled water for 10 min each. Some sections were stained with Hematoxylin and Eosin for histological evaluation. Antigen retrieval (AR) was performed on other sections for immunohistochemistry (IHC). AR was conducted by heating the sections in 10 mM citrate buffer (pH 6.0) for 8 min. The sections were then permeabilized by phosphate-buffered saline (PBS) /0.3% Triton X for one hour and then blocked by 10% FBS in the permeabilizing buffer for one hour. The sections were then probed overnight at 4 °C using primary antibodies for caspase-3 and PCNA at recommended dilutions. After being washed the next day, the sections were incubated for two hours with the secondary biotin-conjugated antibody. After washing, the sections were incubated for 30 min with an avidin-biotin-peroxidase combination (ABC, Vector Laboratories). The sections were treated with chromogen diaminobenzidine (DAB, Vector) and left in the dark until a brown precipitate developed. The sections were then washed, mounted, and examined under a light microscope.

Statistical analysis

The analysis was done using the Prism 9 Software (Graphpad, California, USA). The number of samples was calculated by Power G software[50], which determined a sample size of 24 rats (6 per group) at 80% power and 95% confidence, ensuring adequate statistical power to detect CRS effects while minimizing animal use. Data normality was evaluated using the Shapiro-Wilk test. If the data were normally distributed, parametric tests were used. Differences between the study groups and the degree of significance were determined using an unpaired Student's t-test for comparing two independent groups, one-way analysis of variance (ANOVA), or repeated measures of two-way ANOVA with Tukey's post hoc test for three or more groups. A P values of < 0.05 were deemed statistically significant.

Results

Effect of Fluoxetine and agomelatine on body weight during CRS

Rats showed a significant reduction in their body weight after twelve days of CRS exposure compared to the control rats ($P \leq 0.001$, F (5, 17) = 2.087, Fig. 2A). Restrained rats continued to weigh significantly less than controls throughout the 3 weeks of vehicle administration ($P \leq 0.05$, Fig. 2B). The percentage of body weight change remained significantly low in the chronically stressed group treated with agomelatine across weeks 3–5 compared to the control group ($P \leq 0.05$, F (3, 20) = 13.28). Agomelatine administration for 3 weeks in rats under restraint stress did not significantly improve body weight gain. However, a trend for increased body weight was observed following agomelatine administration compared to other treated groups. On the other hand, CRS depressive rats exhibited the most significant weight loss with fluoxetine compared to the control by the third week of treatment ($P \leq 0.001$, F (3, 20) = 13.28, Fig. 2B).

Effect of Fluoxetine and agomelatine on SPT in the CRS model of depression in rats

Rats exposed to CRS for 5 weeks and interspersed with 3 weeks of vehicle treatment exhibited the lowest sucrose consumption compared to other groups. However, this difference was not statistically significant ($P > 0.05$, F (3,

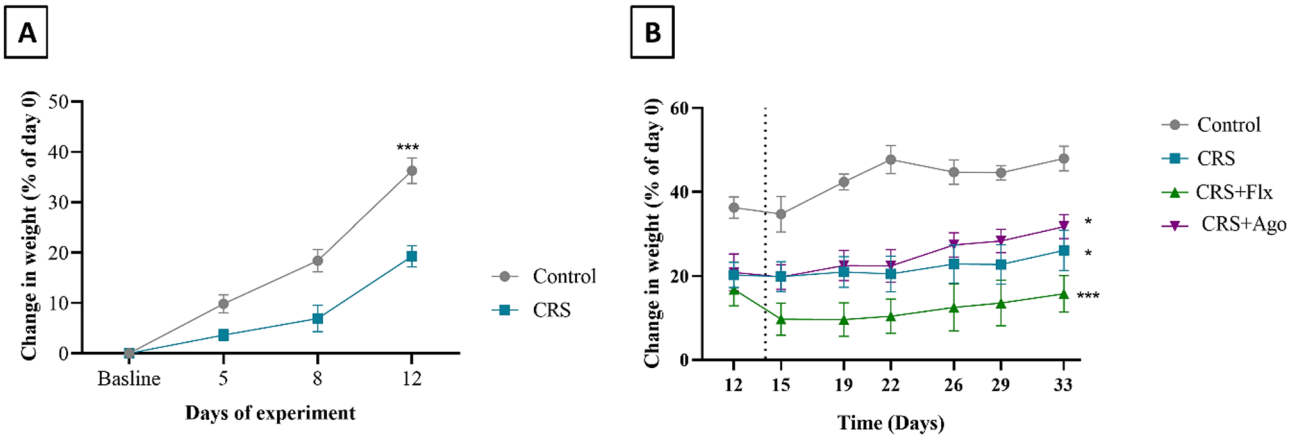

Fig. 2. Effect of CRS and pharmacological interventions on body weight changes depicted as a percentage of the initial weight. Data represent the group mean ± SEM. The vertical dotted line indicates the start of treatment. (**A**) Weight curves of Control ($n = 6$) and Stressed groups ($n = 18$) until day 12. An overall effect of stress was assessed using a two-tailed unpaired Student t-test. (**B**) Weight curves of control and stressed groups after applying treatments on day 14. Control: rats that were neither stressed nor treated; CRS: stressed rats treated with 4 ml/kg normal saline (vehicle); CRS + Flx: stressed rats treated with fluoxetine (14.4 mg/kg); CRS + Ago: stressed rats treated with agomelatine (18 mg/kg); $n = 6$ for all groups. The statistical significance of the differences in the mean values was assessed using repeated measures of two-way ANOVA, followed by Tukey multiple comparison tests. $^{*}P < 0.05$, $^{***}P < 0.001$ versus control group.

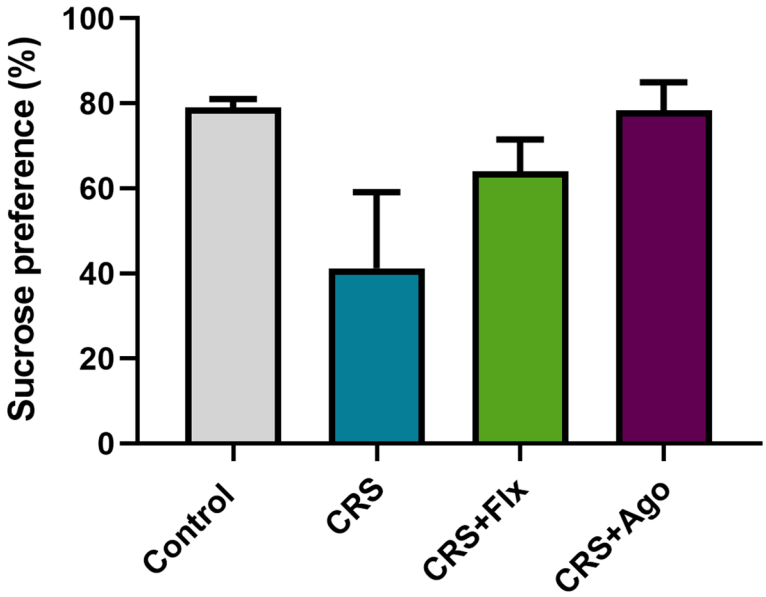

Fig. 3. Effect of CRS, fluoxetine and agomelatine on sucrose preference (SPT). Control: rats that were neither stressed nor treated; CRS: stressed rats treated with 4 ml/kg normal saline (vehicle); CRS + Flx: stressed rats treated with fluoxetine (14.4 mg/kg); CRS + Ago: stressed rats treated with agomelatine (18 mg/kg). Data represent the group mean ± SEM, and the differences between means were assessed using one-way ANOVA, followed by Tukey multiple comparison tests. $P > 0.05$, $n = 3$ for all groups, which referred to the number of cages carrying a group of animals, as testing the sucrose preference for a single rat was not possible.

8) = 2.963, Fig. 3). After CRS rats' treatment with antidepressants, the consumption increased, particularly in rats treated with agomelatine, which was comparable to the control group.

Effect of Fluoxetine and agomelatine on the modified FST in the CRS model of depression in rats

Compared to the normal control, rats exposed to chronic stress and treated with vehicle only showed significant increases in the immobility (despair) behavior ($P \leq 0.01$, F (3, 20) = 7.509). Compared to vehicle-

CRS rats, agomelatine treatment significantly decreased the immobility behavior ($P \leq 0.05$). Likewise, fluoxetine significantly reduces immobility ($P \leq 0.01$, F (3, 20) = 7.509, Fig. 4A). No changes were observed in swimming scores among experimental groups ($P > 0.05$, F (3, 20) = 1.229, Fig. 4B). Moreover, CRS rats significantly decreased climbing behavior ($P \leq 0.01$, F (3, 20) = 7.572). This reduction in stressed rats was significantly reversed by fluoxetine ($P \leq 0.01$, F (3, 20) = 7.572), but not by agomelatine ($P \geq 0.05$, F (3, 20) = 7.572; Fig. 4C).

Effect of Fluoxetine and agomelatine on liver transaminases in CRS rats

To investigate the effect of CRS on liver function, AST and ALT were assessed. CRS rats showed slight increases in AST and ALT plasma levels compared to control groups. However, these elevations did not reach the level of significance ($P > 0.05$, Fig. 5). No significant differences were observed between all studied groups in plasma levels of ALT and AST.

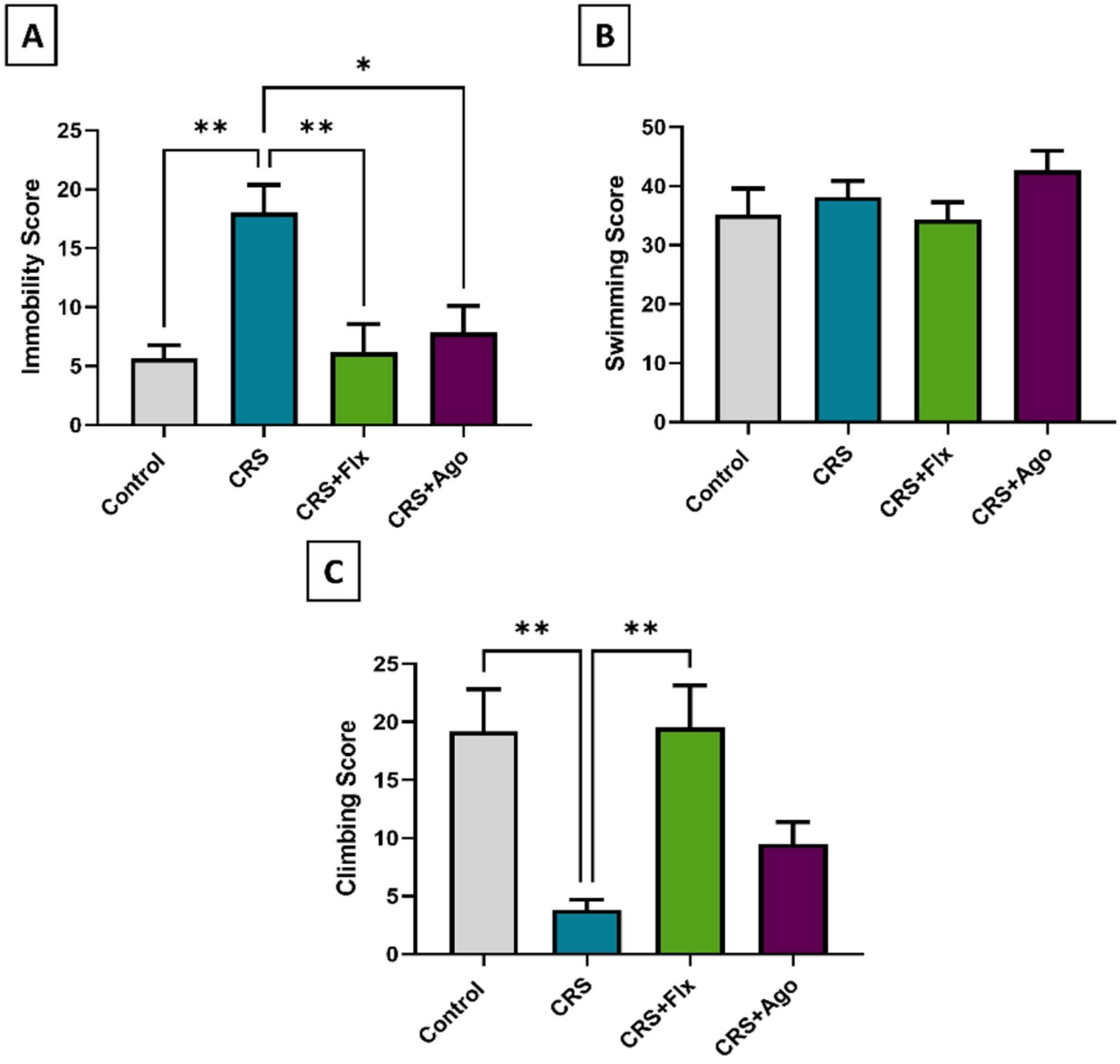

Fig. 4. Effect of CRS and pharmacological interventions, fluoxetine and agomelatine, on the scores of the three predominant behaviors in the modified FST. (**A**) Immobility, (**B**) Swimming, (**C**) Climbing. Control: rats that were neither stressed nor treated; CRS: stressed rats treated with normal saline (4 ml/kg); CRS + Flx: stressed rats treated with fluoxetine (14.4 mg/kg); CRS + Ago: stressed rats treated with agomelatine (18 mg/kg). Data represent the group mean ± SEM, and the differences between means were assessed using one-way ANOVA, followed by Tukey multiple comparison tests; $n = 6$ for all groups. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

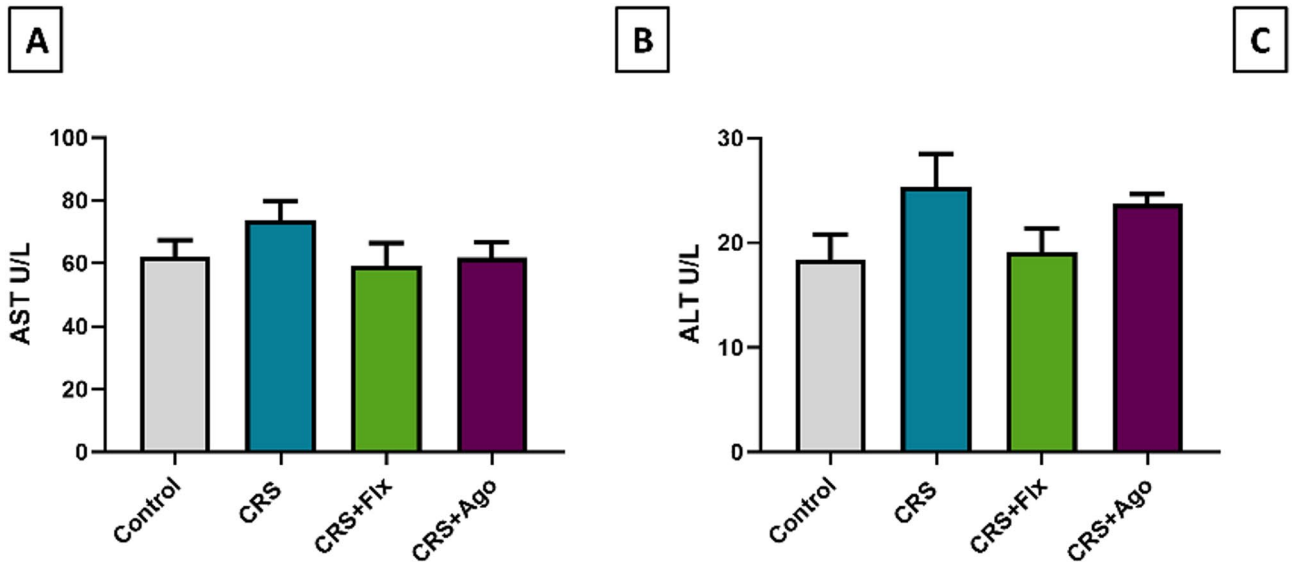

Fig. 5. Effect of fluoxetine and agomelatine use on liver transaminases during CRS. (**A**) AST and (**B**) ALT in CRS rat's model of depression. Control: rats that were neither stressed nor treated; CRS: stressed rats treated with normal saline (4 ml/kg); CRS + Flx: stressed rats treated with fluoxetine (14.4 mg/kg); CRS + Ago: stressed rats treated with agomelatine (18 mg/kg). Data represent the group mean ± SEM, and the differences between means were assessed using one-way ANOVA, followed by Tukey multiple comparison tests; $n = 6$ for all groups.

Effect of Fluoxetine and agomelatine on oxidative stress markers in CRS rats

Oxidative stress was evaluated by measuring hepatic MDA and GSH levels as well as SOD enzymatic activity. CRS significantly increased MDA levels in the liver relative to unstressed rats ($P \leq 0.05$, F (3, 20) = 4.854). Agomelatine significantly reduced MDA levels ($P \leq 0.05$, F (3, 20) = 4.854), whereas fluoxetine produced a non-significant reduction in the levels of MDA. Furthermore, the antioxidant markers; GSH level and SOD activity were significantly reduced during CRS relative to control ($P \leq 0.01$, F (3, 20) = 4.928, and $P \leq 0.05$, F (3, 20) = 3.948, respectively), these changes were mitigated after fluoxetine or agomelatine administration, however, the effect was not significant (Fig. 6).

Effect of fluoxetine and agomelatine on liver histology in CRS rats

The stained liver sections from control rats showed normal control liver with normal classic hepatic lobules and portal areas, while the liver section from the CRS rat showed leukocyte infiltration. In addition, the sizes of the hepatocytes and their nuclei were reduced as nuclei appeared more closely spaced. The spaces between hepatocytes (sinusoids) were blood-filled and appeared distended and some pyknotic (dark) nuclei were also present. The liver section from CRS rats that received fluoxetine or agomelatine revealed almost normal hepatocytes and hepatic lobules between regenerated portal areas with no leukocyte infiltration. Figure 7 represents the effect of CRS on liver histology and after interventions.

Effect of Fluoxetine and agomelatine on the liver expression of caspase 3 and PCNA in CRS rats

Caspase 3

The cell death and proliferation responses were investigated in liver tissue using caspase 3 immunostaining. Liver sections from rats that received normal saline showed normal immunoreactive signals of caspase 3. On the contrary, liver sections from CRS rats showed strongly positive immune reactivity in comparison to controls. Fluoxetine or agomelatine administration during CRS mitigated the upregulation of caspase 3 protein expression and normalized it to a control group level (***$P < 0.001$, F (3, 20) = 32.10, Fig. 8).

PCNA

The hepatic cell proliferation response was assessed using PCNA immunostaining. Liver sections from rats that received normal saline showed normal immunoreactive signal and PCNA. In contrast, liver sections from CRS rats showed a strongly positive immune reactivity signal relative to controls. The use of fluoxetine or agomelatine during CRS reversed the increase in PCNA protein expression after CRS exposure (***$P < 0.001$, F (3, 20) = 56.98, Fig. 9).

Discussion

Chronic stress has a profound negative impact on liver health and function. Patients with NAFLD exhibit high rates of depression, anxiety, and stress[13]. Depression is particularly prevalent in patients with chronic liver diseases, with shared underlying mechanisms involving inflammation and stress[51]. It is established that

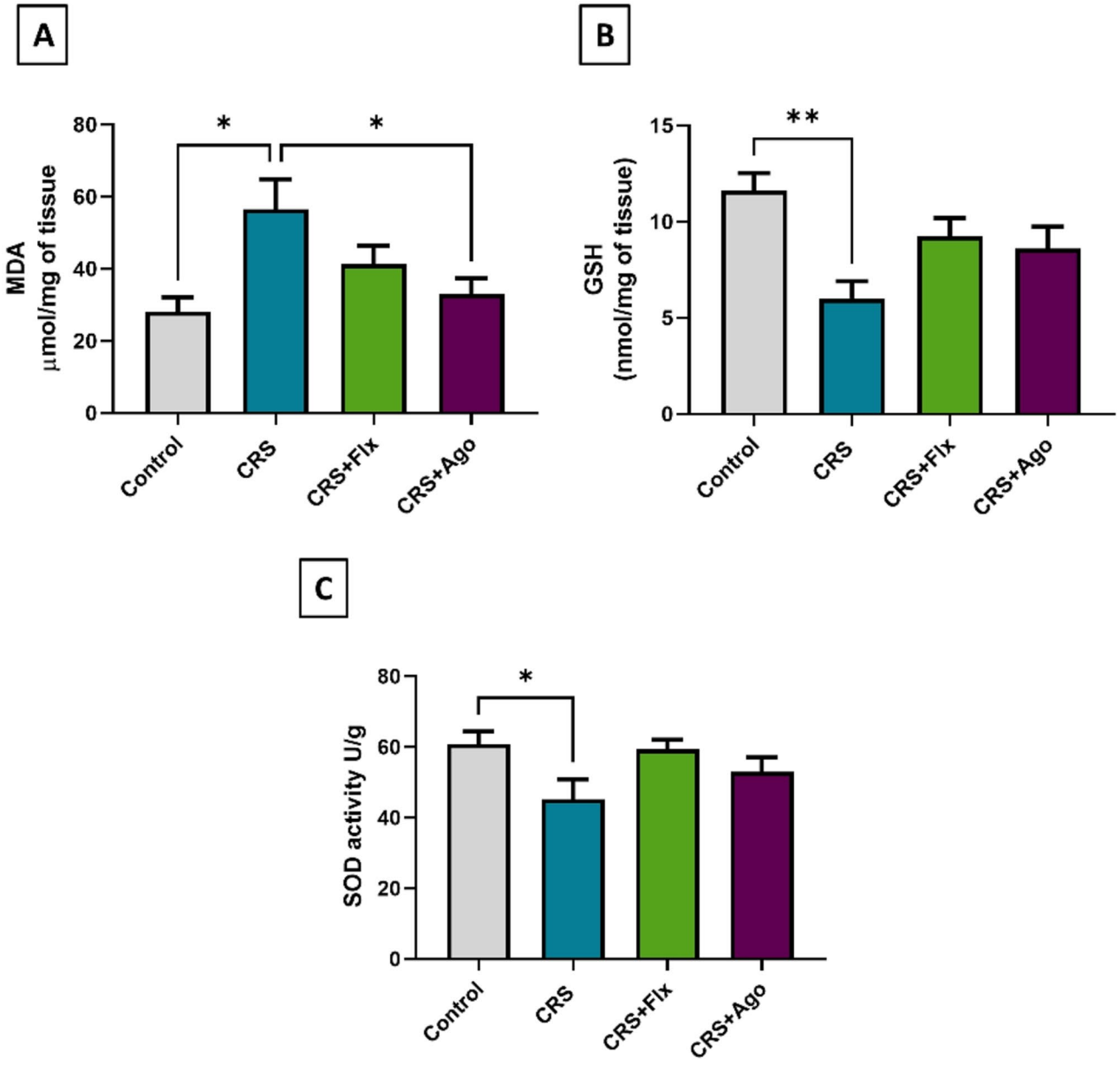

Fig. 6. Effect of fluoxetine and agomelatine on oxidative stress markers in liver tissue during CRS. (**A**) MDA, (**B**) GSH and (**C**) SOD activity in CRS rat's model of depression. Control: rats that were neither stressed nor treated; CRS: stressed rats treated with normal saline (4 ml/kg); CRS + Flx: stressed rats treated with fluoxetine (14.4 mg/kg); CRS + Ago: stressed rats treated with agomelatine (18 mg/kg). Data represent the group mean ± SEM, and the differences between means were assessed using one-way ANOVA, followed by Tukey multiple comparison tests; $n = 6$ for all groups. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

chronic stress is one of the causative factors of depression. There is a growing recognition of the bidirectional relationship between mental health and peripheral organ function, particularly the liver. Chronic liver disease and hepatic inflammation are frequently accompanied by depressive symptoms, fatigue, and social withdrawal, suggesting that liver pathology may actively contribute to neuropsychiatric manifestations rather than being a mere consequence of brain dysfunction[52,53]. While numerous studies have explored hippocampal and other brain changes in depression models, there is a relative paucity of research examining the hepatic consequences of chronic stress and antidepressant treatment. Our study addresses this gap by providing novel insights into the peripheral effects of depression and its treatments, specifically focusing on the liver.

Accordingly, the CRS model in rats was used to resemble some anhedonic and hepatic alterations of depression and to investigate underlying mechanisms. CRS is a well-known model of stress-induced depression in rodents. CRS is a robustly validated depression model that emphasizes predictable chronic stress exposure via one simple and easily reproducible procedure[37]. The CRS has been shown to recapitulate depression phenotypes in rodents, such as body weight loss, despair, and anhedonia[38,54–57]. Herein, we examine oxidative stress, apoptosis, and

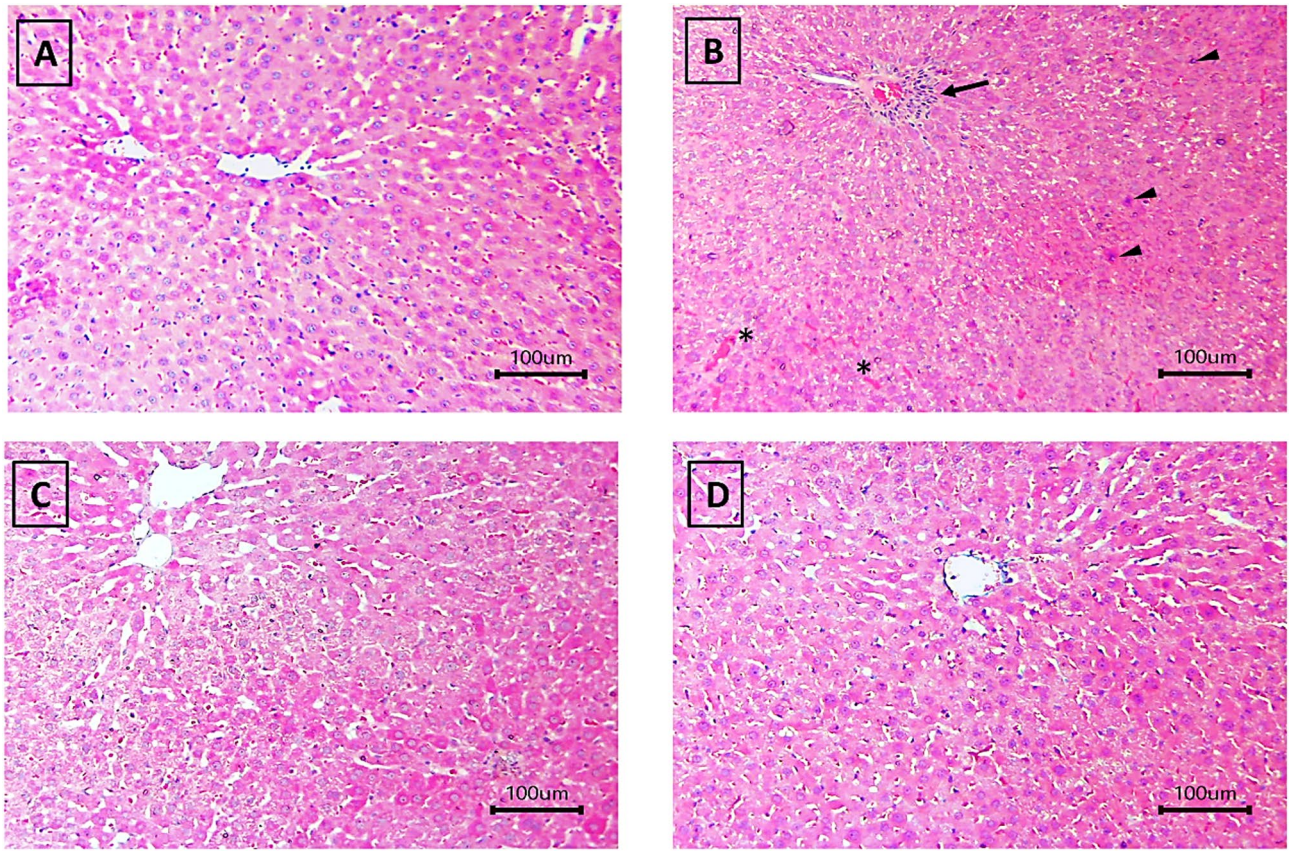

Fig. 7. Effect of CRS and pharmacological interventions, fluoxetine and agomelatine, on liver histology. (**A**) Control rats showing normal hepatocytes and hepatic architecture (**B**) CRS: stressed rats treated with normal saline showing some changes in hepatocytes and hepatic architecture, leukocyte infiltration (arrow), darkened nuclei (arrowhead), and interstitial blood (asterisk). (**C**) CRS + Flx: stressed rats treated with fluoxetine and (**D**) CRS + Ago: stressed rats treated with agomelatine showing improvement in hepatic architecture with no sign of leukocyte infiltration and pyknosis, ($n = 6$), scale bar 100 µm, and amplification ×200.

regenerative capacity in hepatic tissue to elucidate the peripheral biological changes that may precede or drive central neurobiological alterations in depression and anhedonia.

Our results confirmed that the CRS rat model was established, as indicated by the significant decrease in body weight gain, increased immobility in FST, and numerically reduced sucrose solution consumption while performing the SPT. The reduction in sucrose consumption did not reach significance due to variability in CRS duration, test intensity, methodological inconsistencies, and SPT protocols[58,59]. The protocol variability in habituation and deprivation methods may explain inconsistent findings regarding the effects of CS on sucrose consumption[60,61]. This highlighted the need for detailed testing protocols and experimental design. In addition, measuring the sucrose consumption in a group of rats, not individually, may influence the significance. Measuring sucrose preference at the cage (group) level may mask individual variability and reduce the sensitivity to detect small differences between experimental groups. However, housing animals individually to assess sucrose consumption also presents challenges. While individual housing eliminates the confounding effects of dominance hierarchies and social influences on sucrose drinking behavior, it can introduce other confounding factors, notably, social isolation. Social isolation is known to increase stress and depressive-like behaviors in rats, particularly when it is prolonged, as in the 20-hour sucrose preference assay. This additional stressor could confound the effects of CRS and influence the interpretation of the results. Given these reasons, both group and individual housing approaches have limitations. Future studies are recommended to directly compare group versus individual sucrose consumption measurements and determine the most reliable and valid method for assessing anhedonia in this model. Furthermore, we minimized sucrose neophobia and side preference in the experimental design by following the recommended procedure. Of note, previous studies on CS in rodent models have yielded mixed results regarding the impact of CS on sucrose preference. While a marked reduction in sucrose consumption was seen in mice[40,62]. Recent systematic research indicated that some CS-induced depression in rodents exhibited significant variability, leading to inconsistent behavioral results, including the SPT[63].

In the current study, the antidepressant agomelatine did not reverse the weight reduction, but its outcomes were better than those of fluoxetine. It has been demonstrated that high doses of agomelatine increased food intake and body weight, counteracting the effects of restraint stress in mice, which is often linked to depressive

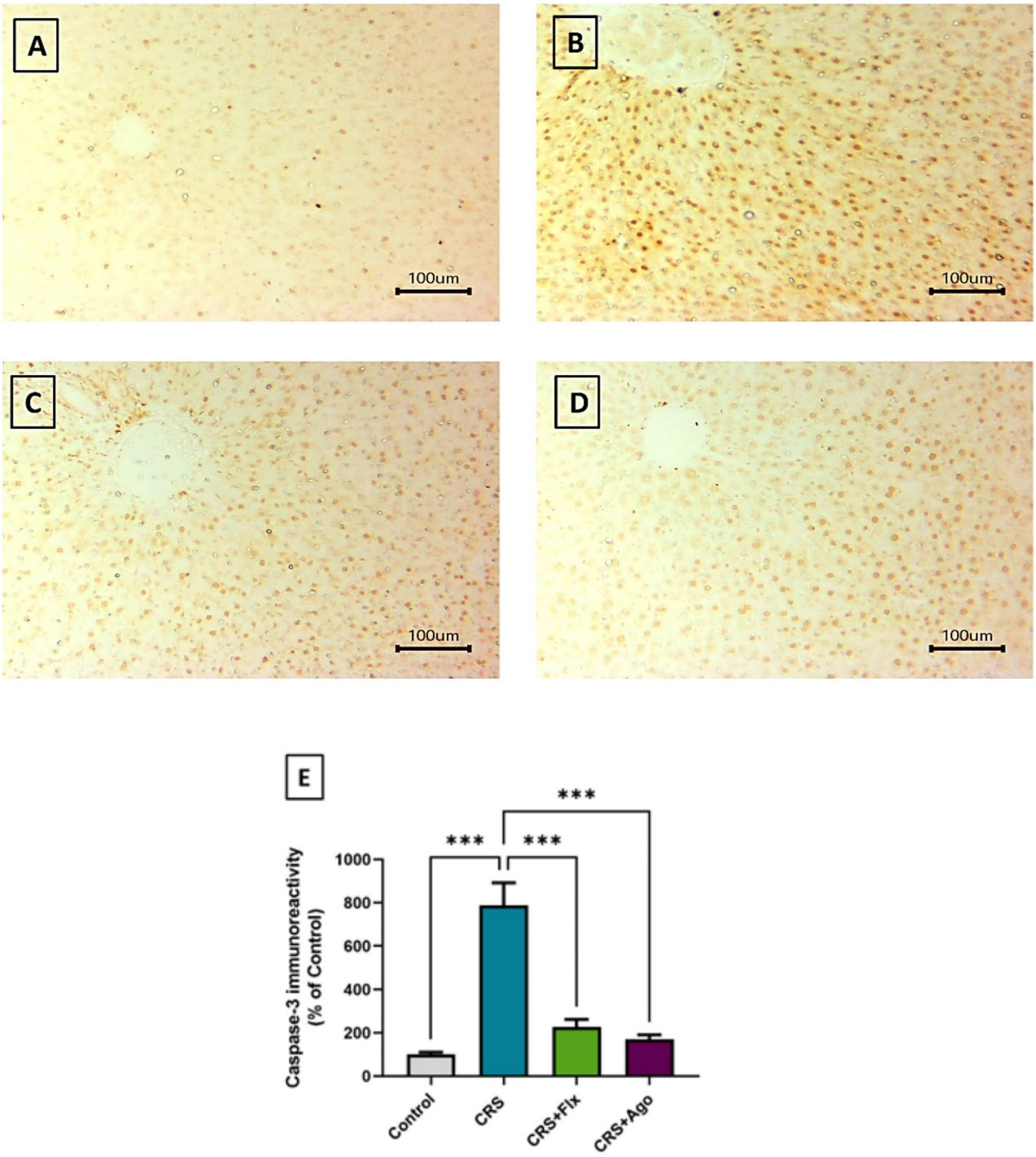

Fig. 8. Caspase 3 immunoreactivity in the liver of CRS rats and after fluoxetine and agomelatine interventions. (**A**) Control rats showing normally low expression of caspase 3. (**B**) CRS: stressed rats treated with normal saline showing an apparent increase in immunoreactive signals of caspase 3. (**C**) CRS + Flx: stressed rats treated with fluoxetine, and (**D**) CRS + Ago: stressed rats treated with agomelatine showing reductions in the immunoreactivities, (**E**) Quantification of caspase 3 expressions within study groups. Data represent the group mean ± SEM, and the differences between means were assessed using one-way ANOVA, followed by Tukey multiple comparison tests; $n = 6$, $^{***}P < 0.001$, scale bar 100 µm, and amplification ×200.

symptoms and changes in feeding behavior[64]. Fluoxetine might induce weight loss in obese adults[65]Yet the exact effect is not well understood and needs further investigation.

Regarding the effect of agomelatine on depression phenotypes, anhedonia, and despair, we demonstrated that agomelatine and fluoxetine slightly increased sucrose consumption, but their effects on FST were more significant as they decreased immobility score and increased climbing score, indicating reduced despair-like

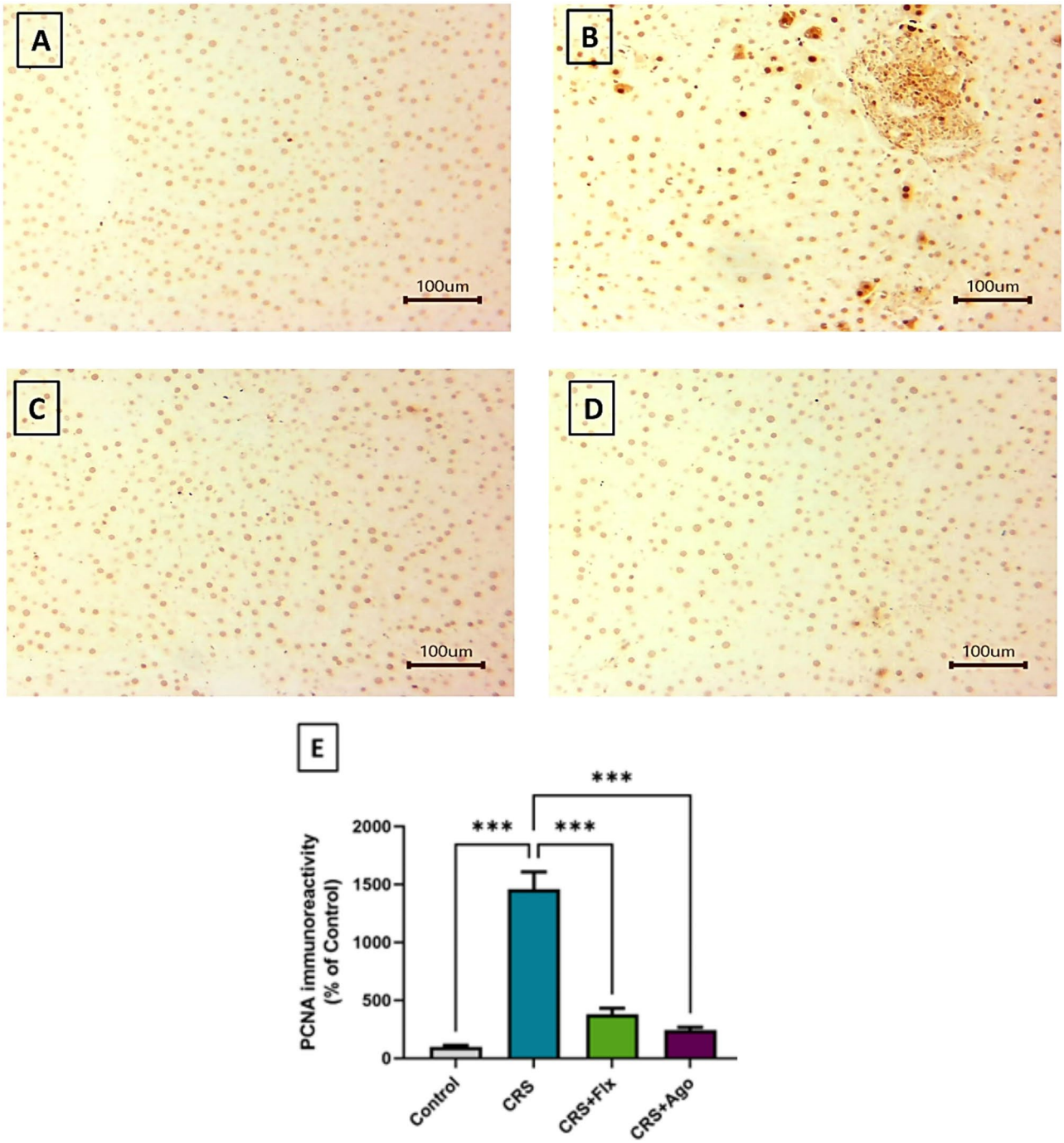

Fig. 9. Proliferating cell nuclear antigen (PCNA) immunoreactivity in the liver of CRS rats and after fluoxetine and agomelatine interventions. (**A**) Control rats showing normal expression of PCNA. (**B**) CRS: stressed rats treated with normal saline showing upregulation of PCNA expression, (**C**) CRS + Flx: stressed rats treated with fluoxetine, and (**D**) CRS + Ago: stressed rats treated with agomelatine showing downregulation in protein expression. (**E**) Quantification of PCNA expression within study groups. Data represent the group mean ± SEM, and the differences between means were assessed using one-way ANOVA, followed by Tukey multiple comparison tests; $n = 6$, $^{***}P < 0.001$, scale bar 100 µm, and amplification ×200.

behavior in stressed rats. Agomelatine has shown antidepressant activity in several animal models of depression, such as chronic mild stress, forced swimming, and psychosocial stress, and its effectiveness was comparable to traditional antidepressants like imipramine and fluoxetine[66–68]. In CRS, agomelatine administration has been shown to alleviate depressive-like behaviors, as evidenced by improved performance in the FST[69]. The positive effect of agomelatine on depression phenotypes was consistent with the FST findings from previous studies that employed chronic constant light model[70] and unpredictable CMS model[71]. This may be attributed to

agomelatine's ability to promote neurogenesis and restore normal neuronal activity in stressed rats[72], suggesting neuroprotective action.

In this work, we assessed the effect of CRS on liver transaminases, ALT, and AST. The activity of these enzymes in the blood is usually proportional to the severity and cause of liver injury. ALT is a more precise biomarker for liver injury, but other enzymes, such as AST, are less specific to liver injury, as they also rise with heart or kidney injury[73]. CRS has been shown to influence liver function, indicated by elevations in liver transaminases, although these changes did not reach statistical significance. Research has demonstrated that CS can induce subtle, non-clinical increases in biochemical liver parameters, including ALT and AST. Of greater concern, CS may contribute to the progression of liver diseases such as NAFLD[15,74]. The liver is capable of adapting to stress-induced changes in liver enzymes. Our findings suggested that the liver might initially respond to CRS by increasing liver enzyme levels. However, these levels might gradually decrease over 5 weeks of CRS as the liver attempts to restore homeostasis. This hypothesis aligns with previous research demonstrating that the return of corticosterone levels to baseline in chronically stressed rats indicates a potential adaptive response to prolonged stress[75]. Indeed, the overall impact of CS on liver transaminases remains complex, necessitating further investigation.

On the other hand, agomelatine failed to ameliorate the CRS-induced elevation in ALT level significantly, and no significant difference was observed in ALT activity between CRS and CRS + agomelatine groups. This might be related to the used dose, as the incidence of enzyme elevation after agomelatine consumption is dose-dependent[76], and could be linked to gene polymorphism in CYP1A2, the main metabolic enzyme of agomelatine[77].

Regarding oxidative stress, chronic stress has been linked to liver injury through mechanisms involving the gut microbiota and the activation of the HPA, which can exacerbate liver conditions[11,12]. Activation of the HPA axis and subsequent cortisol release can elevate oxidative stress in the liver. This increase in oxidative stress leads to a surplus of free radicals, such as reactive oxygen species (ROS), overwhelming the liver's antioxidant capacity and consequently impairing its detoxification function. CRS caused hepatic imbalance in oxidant/antioxidant markers, as evidenced by significantly elevated MDA level but decreased GSH level and SOD activity. MDA is one of the final products of the peroxidation process of cell membrane lipids, it is generally considered the most sensitive indicator of oxidative stress. This is because MDA is a direct product of lipid peroxidation, which is a major cause of oxidative damage. SOD and GSH are important antioxidants, but they can also be affected by other factors besides oxidative stress. A previous study reported a positive correlation between corticosterone and liver MDA[75]. Some previous studies reported that 21 days of CRS caused similar alterations in oxidant/antioxidant markers[78,79]. Other studies utilizing CMS have been shown to induce oxidative stress in the liver, characterized by increased levels of ROS and MDA, alongside decreased activity of antioxidant enzymes like glutathione peroxidase and catalase[29,80].

Herein, agomelatine has antioxidant effects by significantly reducing MDA in CRS rats. Additionally, the antioxidants GSH and SOD were increased with agomelatine treatment, although to a lesser extent than the reduction in MDA. The dose of agomelatine in this study has a more pronounced effect on lipid peroxidation, as indicated by the significant decrease in MDA, a primary marker of oxidative stress. While agomelatine also positively influences antioxidant markers like GSH and SOD, its impact on lipid peroxidation may be more substantial. Agomelatine exerts hepatoprotective effects by decreasing MDA and increasing antioxidants like SOD and GSH in liver tissue after CMS[29] paracetamol acute toxicity[30] or after partial hepatectomy[81]. Besides that, it reversed oxidative stress induced by cisplatin toxicity in the liver and kidney; however, this effect was observed with a dose of 20 mg/kg but not 40 mg/kg[31]. It also showed positive effects in CRS rats by enhancing catalase activity and halting oxidative stress[82]. The antioxidant and hepatoprotective effects of agomelatine are related to its activation of melatonin receptors, which upregulate antioxidant expression[83,84]. In addition, it may exert its antioxidant effects by activating melatonin receptors, which scavenge ROS and promote antioxidant activity[85], or by modulating the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway[86,87]. This pathway is a crucial cellular defense mechanism against various stressors, primarily oxidative stress, but also inflammation and other cellular insults.

The liver, despite its remarkable regenerative capacity, is particularly susceptible to stress-induced injury. This vulnerability stems from its primary role as a metabolic and detoxification organ, making it the first line of defense against various toxins and stressors, especially after chronic exposure. Here, we utilized H&E staining to evaluate the impact of CRS on liver tissue architecture. Consistent with the biochemical evidence of oxidative stress, the liver histopathological results revealed signs of hepatic congestion and more leukocytes infiltrating the liver tissue. Additionally, hepatocytes displayed reduced cell and nuclear size, with nuclei appearing more densely packed. Pyknotic nuclei, indicative of cell death, were also present. In support of such results, Xu et al. reported that the use of an unpredictable CMS model caused inflammatory cell infiltration and degeneration of hepatocytes[12]. Besides that, others reported that high-stress model cold exposure, immobilization, and starvation showed hepatic parenchymal vacuolization, necrosis, hemorrhage, cell infiltration, sinusoidal dilatation, and pyknotic nuclei[85]. These histopathological alterations may be attributed to the generation of ROS and oxidative stress, hepatic inflammation, and impaired fatty acid oxidation[88,89]. CRS can trigger the overproduction of ROS, such as hydroxyl radicals and superoxide anions. These highly reactive molecules with unpaired electrons can disrupt cellular components like lipids, proteins, and DNA, leading to cell death and abnormal liver architecture. Furthermore, the release of pro-inflammatory cytokines may further increase hepatic histological changes[15,89].

Alongside the histological findings and to further investigate the cellular response to CRS, we assessed the expression of caspase-3 and PCNA, two key markers of cell death and regeneration, respectively. Our results indicated that CRS induces hepatocyte turnover as a stress response, characterized by increased expression of both proteins. Caspase-3 is a crucial executioner caspase involved in programmed cell death (apoptosis), while PCNA is a protein involved in DNA replication and cell cycle progression, essential for cell proliferation. Our

findings indicate that, although CRS triggers hepatic cell death, the liver retains its regenerative capacity. This novel observation has not been previously reported which such stress model. Following CRS exposure, the liver responded by initiating apoptosis in damaged cells. Caspase-3 can facilitate the efficient clearance of these cells, a process known as 'apoptotic clearance'[90,91]. Subsequently, PCNA expression increases in hepatocytes as they enter the cell cycle and divide to regenerate lost liver tissue. PCNA levels can serve as a marker for assessing liver regenerative capacity[92]. This delicate balance between hepatocyte apoptosis and proliferation is crucial for maintaining liver homeostasis during regeneration[93].

The interplay between oxidative stress, inflammation, and apoptosis forms a critical triad in the pathogenesis of stress-induced hepatic injury. Our data suggest that oxidative stress plays a central role in the hepatic alterations observed following CRS. Elevated hepatic MDA levels and reduced levels of GSH and SOD activity indicate enhanced lipid peroxidation and compromised antioxidant defense. These findings are consistent with prior evidence showing that activation of the HPA axis under chronic stress conditions leads to excessive ROS production, contributing to cellular damage in peripheral organs, including the liver[12,52]. It has also been demonstrated that stress leads to a reduction in hepatic blood flow, which is mediated by the hypothalamus–hepatic sympathetic nerve–norepinephrine axis and hypothalamus–adrenal medulla–epinephrine axis[94]. Hypoxia in hepatic tissue aggravates the production of ROS, leading to endoplasmic reticulum stress and cell death[95]. This stress-mediated hypoxic condition hinders the oxidative phosphorylation, leading to a depletion in ATP production and subsequently to necrotic changes and increased severity of liver injury[96].

In addition, hypoxic conditions release various tissue-derived inflammatory mediators, which are followed by the recruitment of macrophages, resulting in inflammatory injury[97]. Furthermore, oxidative stress can also lead to the overproduction of proinflammatory cytokines that induce the infiltration of inflammatory cells (as observed by H&E staining in our study). These inflammatory cells produce more ROS, which exacerbates the oxidative stress as well as triggers inflammation and hepatic cell death[98]. Moreover, the stress-induced increase in gut permeability augments the influx of gut-derived lipopolysaccharides (LPS) and alien substances into the liver[99]. This over-influx of LPS leads to liver inflammation, and the increased levels of alien substances easily damage the liver[100].

Oxidative stress is closely linked to apoptotic signaling. The observed upregulation of caspase-3 in CRS rats suggests the activation of intrinsic apoptotic pathways, potentially driven by mitochondrial dysfunction and ROS accumulation. Additionally, stress-induced ROS can impair mitochondrial membrane potential and promote cytochrome c release, further amplifying caspase cascade activation. Simultaneously, increased expression of PCNA indicates enhanced hepatocyte turnover, likely reflecting a compensatory regenerative response to oxidative and apoptotic insults. This balance between cell death and proliferation is critical for maintaining hepatic homeostasis under stress.

Agomelatine administration attenuated oxidative stress markers, attenuated the inflammatory cell infiltration, and reduced the expression of both caspase-3 and PCNA. The normalization of these molecular markers suggests that agomelatine may modulate oxidative and apoptotic stress while preserving regenerative capacity in hepatic tissue exposed to CRS. The protective effects of agomelatine mirror its known anti-inflammatory and antioxidative properties observed in other organ systems. As far as we know, this work revealed for the first time that agomelatine improved liver histopathology changes associated with CRS and restored normal expression of caspase 3 and PCNA. These positive effects were reported in other disease or toxicity models; for example, Cankara et al. showed that agomelatine decreases liver histopathological alterations such as liver necrosis, inflammation and congestion that occurred after cisplatin exposure[31]. Agomelatine was reported to decrease the number of Ki-67, another cell proliferation marker, after partial hepatectomy[81]. In paracetamol-induced liver injury, agomelatine was revealed to enhance hepatic regeneration, evidenced by binucleated hepatocytes, indicating more cell proliferation and potential for liver regeneration[30]. Notably, these effects were seen with 18 mg/kg dose, which was within the level of therapeutic doses. Higher doses of this drug can show negative effects on the liver[31].

The findings from this study have important clinical implications, particularly in the context of the growing recognition of the bidirectional relationship between chronic stress, depression, and liver health. Chronic stress is not only a major risk factor for the development and exacerbation of psychiatric disorders such as depression, but it is also increasingly recognized as a contributor to liver pathology, including NAFLD and hepatic inflammation[13]. The observed attenuation of oxidative stress and histopathological changes in the liver by agomelatine in the CRS rat model suggests a potential therapeutic benefit for patients suffering from comorbid depression and liver dysfunction. Future clinical studies are warranted to investigate the effects of agomelatine on liver function and structure in patients with chronic stress or depression, particularly those with comorbid metabolic or liver diseases. Understanding how these drugs alter the liver under stress conditions is crucial for optimizing clinical care.

While this study successfully achieved its aims and objectives, it is important to acknowledge certain limitations. The relatively small sample size may have limited our ability to detect statistically significant variations between the study groups, even in the presence of true effects. Future studies with larger sample sizes are essential to increase statistical power. Measuring inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) would provide additional insights into the connection between stress-induced depression, inflammation, and liver changes. Assessing the expression of stress-related genes such as glucocorticoid receptor (NR3C1) and hypocretin/orexin (HCRT) can further elucidate the molecular mechanisms linking depression and liver dysfunction. Additionally, frequent blood sampling to monitor liver enzymes and corticosterone levels throughout the experimental period would offer valuable data on the time-dependent effects of CRS on liver function. Correlating corticosterone levels with changes in liver enzymes and oxidative markers like MDA would further elucidate the dynamic relationship

between stress and liver changes. Subsequent studies could investigate the long-term impact of agomelatine on the liver by extending treatment durations and using different doses.

Conclusion

This study revealed for the first time the potential therapeutic role of antidepressants, fluoxetine and agomelatine, in liver damage that occurs during the CRS depression model. These drugs highlight the intricate link between mental health and liver function by regulating mood, reducing oxidative stress, and promoting liver regeneration. Future research is needed to elucidate the underlying mechanisms of fluoxetine and agomelatine in the liver and to assess their long-term efficacy and safety in this context.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

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Declarations

Competing interests

The authors declare no competing interests.

Animal ethics

All experimental procedures were reviewed and authorized by the Research Ethics Committee at King Saud University (Ethics Reference No: KSU-SE-20-75), and performed following the ARRIVE guidelines.

Additional information

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