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Depletion of hepatic glutathione and adenosine by glucocorticoid exposure in Wistar rats is pregnancy-independent

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ABSTRACT

Liver diseases have gained increasing attention due to their substantial impact on health, independently as well as in association with cardio-metabolic disorders. Studies have suggested that glutathione and adenosine assist in providing protection against oxidative stress and inflammation while glucocorticoid (GC) therapy has been associated with chronic inflammatory disorders, even in pregnancy. The implications of Glucocorticoid exposure on maternal health and fetal growth is a concern, however, the possible role of glutathione and adenosine has not been thoroughly investigated. The study therefore hypothesize that exposure to glucocorticoids leads to depletion of hepatic glutathione and adenosine levels, contributing to oxidative stress and tissue injury. Additionally, we aim to investigate whether the effects of glucocorticoids on hepatic health are pregnancy dependent in female rats. Twelve Pregnant and twelve age-matched non-pregnant rats were used for this study; an exogenous administration of glucocorticoid (Dex: 0.2 mg/kg) or vehicle (*po*) was administered to six pregnant and six non-pregnant rats from gestational day 14 to 19 or for a period of 6 days respectively. Data obtained showed that GC exposure led to a decrease in hepatic glucose-6-phosphate dehydrogenase, glutathione peroxidase, GSH/GSSG ratio and adenosine content in both pregnant and non-pregnant rats. In addition, increased activities of adenosine deaminase and xanthine oxidase, along with increased production of uric acid and increased levels of lactate dehydrogenase, aspartate aminotransferase, alanine transferase, alkaline phosphatase and gamma-glutamyl transferase were observed. In summary, the study indicates that GC-induced liver damage is underlined by depleted hepatic adenosine and glutathione levels as well as elevated markers of tissue inflammation and/or injury. Furthermore, the findings suggest that the effects of GC exposure on hepatic health are pregnancy independent.

1. Introduction

Liver disease is gaining significant global attention and has been experiencing a rapid increase in prevalence with rising rates of obesity and metabolic syndrome [1,2]. As the metabolic hub of the body, the liver plays a pivotal role in regulating energy status both in health and disease [3]. Cellular metabolism involves the generation of highly reactive biochemical wastes [reactive oxygen species (ROS) and free radicals from metabolic activities, particularly in the mitochondria] which are normally counterbalanced by the production of antioxidants [3,4]. However, excessive production of ROS and failure of clearance by innate antioxidant systems are suggested to be involved in the pathogenesis of metabolic syndrome-related hepatic damage [5,6].

The mitochondria tightly regulate cellular energy metabolism through fatty acid β-oxidation, the tri-carboxylic acid (TCA) cycle, and oxidative phosphorylation to generate ATP. Impaired mitochondrial function is a hallmark of oxidative stress-linked pathologies [7]. In addition, elevated lactate from glycolytic flux is a characteristic of

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mitochondrial dysfunction in metabolic disorders [8].

Glutathione (GSH), the major endogenously produced antioxidant enzyme present in all mammalian tissues, is synthesized from glutamate, cysteine and glycine in the presence of glutathione synthase [9]. Therapies modulating glutathione levels are crucial in the management of cardio-metabolic disorders [10]. Apart from its anti-oxidative effects, studies have shown that glutathione exerts anti-inflammatory effects through inhibition of Angiotensin- converting enzyme (ACE) activity and decrease of ROS production [11].

Glucose 6-phosphate dehydrogenase (G6PD), a key enzyme in the pentose phosphate pathway, generates and enriches cells with reducing equivalents (NADPH) for the synthesis and maintenance of GSH in the reduced form [12,13]. Excessive ROS production and/or altered redox state rapidly oxidize GSH to form glutathione disulfide (GSSG). A decrease in GSH levels has been reported in hepatocellular carcinoma patients [14]. Hence, the ratio of GSH to GSSG is a crucial determinant of the redox status of cells.

Adenosine is an important regulator of cellular metabolism that is produced from adenosine triphosphate (ATP) in the intracellular space [15]. Studies have suggested adenosine to mediate anti-inflammatory, anti-oxidative and energy homeostatic effects via enhancement of glucose uptake and inhibition of lipolysis [16–18]. Adenosine receptors, expressed on various cells including hepatocytes modulate metabolic and inflammatory processes such as glutathione synthesis, glucose production and liver control of renal Na+/water balance [19]. Rodríguez-Aguilera et al. [20] also demonstrated adenosine treatment's protective effect against liver disease.

A depletion in cellular ATP/AMP ratio in hepatocytes may decrease adenosine content, leading to increased adenosine monophosphate deaminase (ADA) enzyme activity. ADA triggers uric acid production via the ADA/XO-catalyzed breakdown of purine nucleotides. Excessive uric acid is a well-documented initiator of inflammation and type 2 diabetes [21,22]. A recent study from our laboratory revealed a reduction in adenosine content that is accompanied by IR, oxidative stress, elevated ADA and XO activities and increased uric acid production in the hepatic tissue of pregnant rats given fructose-enriched drinks [23].

Glucocorticoid (GC) therapy is usually employed for patients that suffer from chronic inflammatory disorders due to their anti-inflammatory and immunosuppressive effects [24]. Pregnant women at risk of premature delivery may also receive GCs to improve pregnancy outcomes [25,26]. However, gestational GC administration may adversely impact maternal health and worsen intrauterine growth, as shown in our previous study [27]. Additionally, GCs can suppress the activity of the glutathione-antioxidant system through various mechanisms, including suppression of glutamate-cysteine ligase (Gclc) and nuclear factor erythroid 2-related factor 2 (Nrf2) activity and expression, and promotion of oxidative stress [59].

The late phase of pregnancy is characterized by reduced insulin sensitivity and breakdown of fat stores to support maternal and fetal nutrient needs, a physiological adaptation that is usually reversed after normal pregnancy [28]. However, this state could be worsened by factors including physical inactivity and high fat/sugar diet or drugs (corticosteroids: dexamethasone, betamethasone etc.) which can alter maternal metabolism, leading to gestational diabetes, preeclampsia and hampered fetal outcome [28,29]. The pathogenesis of liver disease in women exposed to GCs remains unclear. Therefore, this study hypothesized that GC exposure would disrupt hepatic tissue defense against oxidative stress and/or tissue injury, and that effects of GC are pregnancy dependent in the liver of female Wistar rats.

2. Materials and methods

2.1. Animals

The experiment was conducted in compliance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and it received approval by the University ethical review committee. Female Wistar rats weighing 130–150 g were used for the present study and all effort was made to reduce the number of animals utilized and their suffering. The animals were maintained under standard conditions of temperature (24 ± 0.2 °C), humidity (60 ± 10 %), ventilation and 12 h dark/light cycle. Also, the animals were provided with standard rat chow and tap water for a week to acclimate them. After this period, the animals were divided into four groups. Two groups consist of 12 non-pregnant rats which were randomly assigned into control non-pregnant rats (CT) and glucocorticoid-exposed non-pregnant rats (GC). The other two groups underwent vaginal smear analysis to determine their estrus phases. Following the identification of their respective estrus phases, these groups were then mated with male Wistar rats using a ratio of 3 female rats to 1 male rat (3:1), enabling the achievement of timed pregnancies. Afterwards, 12 age-matched pregnant rats were randomly assigned into untreated pregnant rats (PR) and glucocorticoid-exposed pregnant rats (PR + GC) respectively, (n = 6/group).

2.2. Treatments

The CT and PR groups received vehicle (*po*) daily for 6 days while the GC and PR + GC groups received glucocorticoid (dexamethasone; 0.2 mg/kg; *po*) daily from the 14th day to the 19th day of gestation.

2.3. Sample preparation

On gestational day 20, the rats were anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*) and killed by cervical dislocation. This is followed by immediate removal of their livers after which they were blotted and weighed. After weighing, a precise section of the tissue weighing 100 mg was carefully obtained and homogenized. The resulting homogenate was then utilized for consequent biochemical analysis.

2.4. Biochemical assay

2.4.1. Evaluation of redox biomarkers

The enzymatic activity of Glucose-6-phosphate dehydrogenase (G6PD) was measured by monitoring the production of NADPH at 340 nm and 25 °C, using an assay kit obtained from Fortress diagnostics (Antrim, UK). The estimation of G6PD was done through the Kinetic enzyme method and assays were carried out in triplicate and the activities were followed up for 60 s [30]. The protein levels were estimated spectrophotometrically (595 nm) using the Bradford method [31]. According to an established procedure using H_2O_2 as a substrate, the activity of glutathione peroxidase (GPx) was determined by a standardized enzymatic colorimetric assay method [32]. Reduced and oxidized glutathione (GSH and GSSG) were also measured by standardized enzymatic colorimetric assay method according to an established procedure [33].

2.4.2. Evaluation of tissue injury markers

Lactate dehydrogenase (LDH) activity was estimated using an assay kit from Fortress Diagnostics (Antrim, UK). NAD was reduced to NADH by LDH to produce a color by interacting with a specific probe. The estimation of LDH was done at an optical density of 50 nm (T1) on a microplate reader in a kinetic mode, with readings recorded every 2–3 min, for a period of at least 30–60 min at room temperature, while ensuring it is protected from light. Also, lactate was estimated using an assay kit from Fortress Diagnostics (Antrim, UK). LDH oxidized lactate to generate a product that interacts with a probe to produce a colored optical density (OD) of 450 nm.

2.4.3. Estimation of uric acid production

Hepatic adenosine was estimated following a multi-step enzymatic

approach resulting in the generation of an intermediate that reacts with the adenosine probe to form a fluorescent product. The fluorescence formed was then immediately assessed using a microplate reader set at Ex/Em = 535/587 nm. The activity of adenosine deaminase (ADA) was determined using a standardized enzymatic colorimetric method that involves detecting the production of inosine resulting from the breakdown of adenosine through a multi-step reaction [34]. Measurement of xanthine oxidase was through a standardized enzymatic colorimetric method where xanthine was oxidized by XO to produce H_2O_2 which reacts subsequently with OxiRed Probe to generate color (ƛ = 570nm). Measurement of Uric acid was also conducted using a non-enzymatic colorimetric assay kit obtained from Oxford Biomedical Research, Inc. (Oxford, MI) following an established procedure [35].

2.4.4. Liver injury enzyme markers

Gamma-glutamyl transferase (GGT), alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were determined using a standardized enzymatic colorimetric method with an assay kit obtained from Fortress Diagnostics Limited, Antrim, UK.

2.4.5. Na^+-K^+ ATPase activity

Na^+-K^+-ATPase activity was determined by the spectrophotometric method using reagents from Randox Laboratory Ltd. (Co.Antrim, UK). The measurement of Na^+-K^+ ATPase activity in liver homogenate was conducted through an enzymatic assay that consists of two similar phases; one for the total ATPase activity estimation and another for the assessment of ouabain insensitive [36]. Na^+-K^+-ATPase activity was expressed as a micromole of inorganic phosphate released per milligram protein per hour. In addition, the concentration of protein was determined by the Biuret method [37].

2.5. Statistical analysis

Statistical analysis was performed using SPSS software (Version 22; SPSS Inc. IL., USA). The data were presented as mean ± SEM of 6 rats per group. Independent t-test was utilized to compare the mean values of variables among the groups. Statistically significant differences were accepted at $p < 0.05$.

3. Results

3.1. Effect of glucocorticoid on hepatic glutathione-dependent antioxidants in female rats

Glucocorticoid exposure led to a decrease in hepatic G6PD and GPx activities, as well as a decrease in GSH/GSSG ratio in both non-pregnant and pregnant groups (Fig. 1A–C).

3.2. Effect of glucocorticoid on hepatic tissue injury markers (lactate and lactate dehydrogenase) in female rats

The hepatic lactate level was not significantly affected by glucocorticoid exposure in both non- pregnant and pregnant groups (Fig. 2A). However, hepatic lactate dehydrogenase in GC-exposed non-pregnant and pregnant rats was elevated (Fig. 2B).

3.3. Effect of glucocorticoid on hepatic Na^+-K^+ ATPase in female rats

Hepatic Na^+-K^+ ATPase caused no significant changes with GC exposure in both non-pregnant and pregnant rats (Fig. 3).

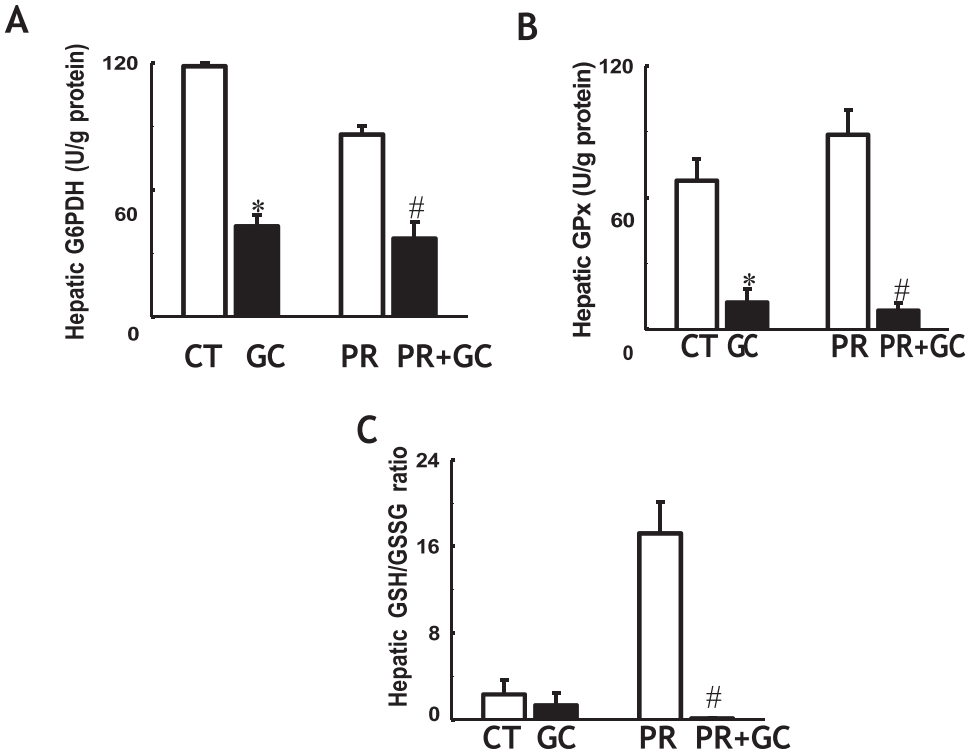

Fig. 1. Effect of GC exposure on hepatic G6PDH (A), GPx (B), and GSH/GSSG ratio (C) in pregnant and non-pregnant rats. Glucocorticoid exposure decreased hepatic G6PDH and GPx activities in both pregnant and non-pregnant groups. However, glucocorticoid exposure decreased the GSH/GSSG ratio only in the pregnant group. The data were analyzed by student's t-test and the values are expressed as mean ± SEM of 6 rats per group (*$p < 0.05$ vs CT; #$p < 0.05$ vs PR), G6PDH represents glucose-6-phosphate dehydrogenase, GPx represents glutathione peroxidase, GSH/GSSG ratio indicates the ratio of reduced to oxidized glutathione, CT represents non- pregnant control, PR represents pregnant control, and GC represents glucocorticoid.

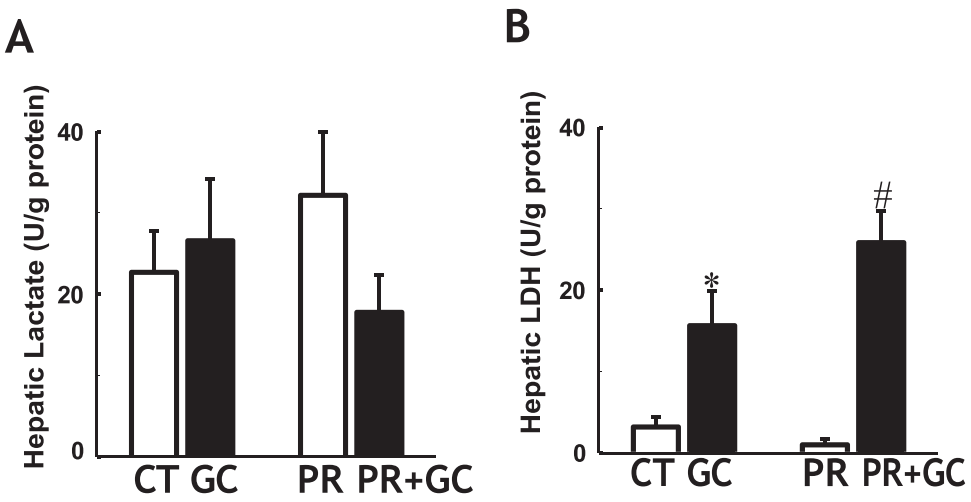

Fig. 2. Effect of GC exposure on hepatic lactate (A) and LDH (B) in pregnant and non-pregnant rats. Glucocorticoid exposure resulted in elevated hepatic LDH in both pregnant and non-pregnant groups. However, glucocorticoid exposure did not have an impact on the hepatic lactate levels. The data were analyzed by student's t-test and the values are expressed as mean ± SEM of 6 rats per group (*$p < 0.05$ vs CT; #$p < 0.05$ vs PR), LDH represents lactate dehydrogenase, CT represents non-pregnant control, PR represents pregnant control, and GC represents glucocorticoid.

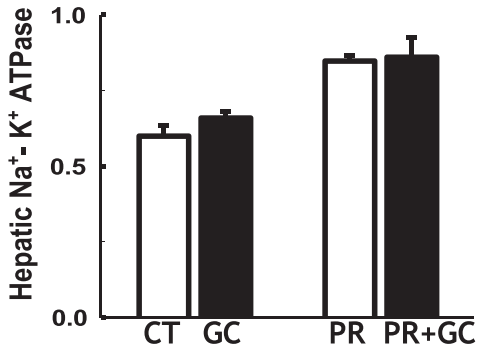

Fig. 3. Effect of GC exposure on hepatic Na^+-K^+-ATPase activity in pregnant and non-pregnant rats. Glucocorticoid exposure did not have any impact on the hepatic Na^+-K^+-ATPase activity in both pregnant and non-pregnant groups. The data were analyzed by student's t-test and the values are expressed as mean ± SEM of 6 rats per group (*$p < 0.05$ vs CT; #$p < 0.05$ vs PR), CT represents non-pregnant control, PR represents pregnant control, and GC represents glucocorticoid.

3.4. Effect of glucocorticoid on hepatic adenosine, ADA/XO/UA pathway in female rats

Glucocorticoid exposure resulted in decreased levels of hepatic adenosine in non-pregnant and pregnant groups (Fig. 4A). Consequently, there were elevated levels of hepatic ADA, XO and UA levels in GC-exposed non-pregnant and pregnant rats (Fig. 4B–D).

3.5. Effect of glucocorticoid on liver injury enzyme markers in female rats

Glucocorticoid exposure resulted in elevated hepatic aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine transferase (ALT), and gamma-glutamyl transferase (GGT) levels in both non-pregnant and pregnant rats (Fig. 5A–D).

4. Discussion

The present study shows that GC exposure caused hepatic impairment that is accompanied by depleted glutathione and adenosine content in the non-pregnant and pregnant rats but did not affect hepatic Na^+-K^+ ATPase.

In a previous experiment from our laboratory, GC facilitates the accumulation of reactive oxygen species (ROS), which have been linked to vascular inflammation in non-pregnant rats [38]. The liver is a target of many oxidative insults that can result in hepatic diseases [39]. Therefore, it is equipped with antioxidant compounds such as glutathione (GSH) to serve as a protection mechanism against oxidative damage [40,41]. Alterations in the ratio of reduced to oxidized glutathione (GSH/GSSG), which is the primary determinant of the cellular redox state, are associated with alcoholic liver cirrhosis [42]. Our results showed that G6PD and GPx activities, enzymes that are responsible for the maintenance of GSH, are reduced in the livers of GC-exposed rats. This is due to the inhibition of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates the expression of antioxidant genes, including those encoding GPx and G6PD. Suppression of Nrf2 by glucocorticoids can lead to decreased expression of these antioxidant enzymes, compromising the overall antioxidant defense [59]. In addition, studies have showed that Glucocorticoids can affect the expression and activity of enzymes involved in glutathione synthesis, such as glutamate-cysteine ligase (Gclc), the rate-limiting enzyme in glutathione biosynthesis. Down regulation of Gclc can lead to reduced production and activity of glutathione [59].

Moreover, an elevation in glutathione levels and GSH/GSSG ratio was noted during early pregnancy to mitigate the oxidative stress associated with gestation and facilitate fetal growth [60]. However, this protective effect diminishes as pregnancy progresses [60]. Our study corroborates these findings, as we observed a significant increase in GSH/GSSG ratio in the pregnant group, which was reversed in the glucocorticoid-exposed pregnant group due to the mechanism discussed above. Therefore, GC exposure in both non-pregnant and pregnant rats led to diminished hepatic GSH/GSSG ratio which suggests that GC exposure with or without gestation is capable of depleting the glutathione system in the hepatocytes of rats, thus, exposing the liver to oxidative damage.

Lactate dehydrogenase (LDH), plays an important role in anaerobic cellular metabolism but it is elevated when tissues are damaged by injury or disease [43]. Previous study has shown that LDH production in hepatocytes is increased at an early stage of acute liver failure in humans [44] and elevation in the LDH enzyme activity in the serum was significantly correlated with an increase in oxidative stress [45]. Our findings of significant GC exposure with or without gestation is capable of depleting the glutathione system in the hepatocytes of rats under chronic hypoxic conditions.

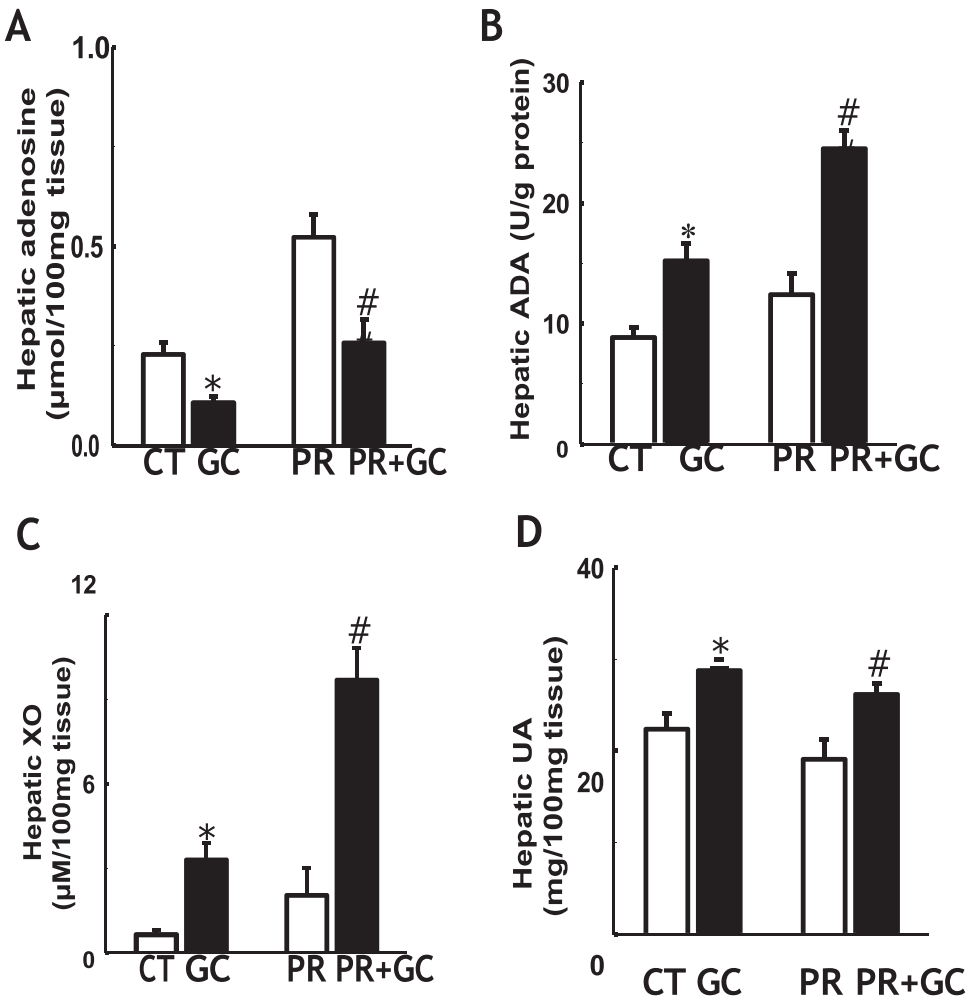

Fig. 4. Effect of GC exposure on hepatic adenosine (A), ADA (B), XO (C) and UA (D) in pregnant and non-pregnant rats. Glucocorticoid exposure resulted in elevated hepatic ADA, XO and UA levels in both pregnant and non-pregnant groups. However, glucocorticoid exposure led to a decrease in hepatic adenosine levels in both pregnant and non-pregnant groups. The Data were analyzed by student's t-test and the values are expressed as mean ± SEM of 6 rats per group (*$p < 0.05$ vs CT; #$p < 0.05$ vs PR), ADA represents adenosine deaminase, XO represents xanthine oxidase, UA represents uric acid, CT represents non-pregnant control, PR represents pregnant control, and GC represents glucocorticoid.

Another imperative finding of the present study was decreased adenosine production in the GC- exposed non-pregnant and pregnant rats. Adenosine is produced during conditions of limited oxygen availability to confer tissue protection [46] and also acts as an endogenous activator of the cellular antioxidant defense system for cytoprotection during ischemic cell injuries [47,48]. Xanthine oxidase, the enzyme that catalyzes the last two steps in purine metabolism [49], is an enzyme that generates ROS, participates in oxidative stress [50] and has been found to be 10–20-fold higher than that found in healthy liver tissue [51]. Also, increased ADA has been linked to hepatic dysfunction [52]. Excess uric acid production has been linked to oxidative stress in nonalcoholic fatty liver disease (NAFLD) conditions [53,54]. In the present study, limited oxygen availability as evident from elevated hepatic LDH and reduced hepatic adenosine levels that are supposed to offer tissue protection in the presence of oxygen deficit suggests hepatic injury in GC- exposed pregnant and non-pregnant rats. Furthermore, our study indicated an elevation in hepatic ADA and XO with a resulting increase in hepatic uric acid, suggesting the involvement of disrupted ADA/XO/uric acid pathway in GC-induced hepatic ROS and liver injury in pregnant and non-pregnant rats.

An earlier study in humans reported raised ADA with increased ALT in alcohol-induced liver damage [55]. Likewise, increased serum uric acid levels have been independently associated with elevated ALT [56]. The finding of this study is that during gestation and non-gestation, GC-induced hepatic ROS resulted in elevated hepatic ALT, AST, and ALP thereby confirming liver function impairment.

It has been documented that regulation of Na^+-K^+-ATPase may be one of the therapies for alcoholic fatty liver disease [57]. Previous studies also show that GCs increased liver Na^+-K^+-ATPase activity [58]. However, our finding shows that GC exposure seems to cause an unchanged Na^+-K^+-ATPase activity in pregnant and non-pregnant rats. This result implies that GC-induced hepatic ROS injury does not involve Na^+-K^+-ATPase activity.

In conclusion, our study for the first time provided evidence that reduced glutathione and adenosine content play a crucial role in hepatic injury in GC-exposed non-pregnant or pregnant rats. Notably, the effects of glucocorticoids appear to be more pronounced in the pregnant group, suggesting a heightened susceptibility to hepatic injury during gestation.

CRediT authorship contribution statement

Abdullahi Adejare: Formal analysis, Investigation. **Mary Ologe:** Visualization, Writing – review & editing. **Taofeek O. Usman:** Investigation, Visualization, Writing – original draft. **Kamaldeen O. Abdullahi:** Formal analysis, Investigation, Validation, Writing – review &

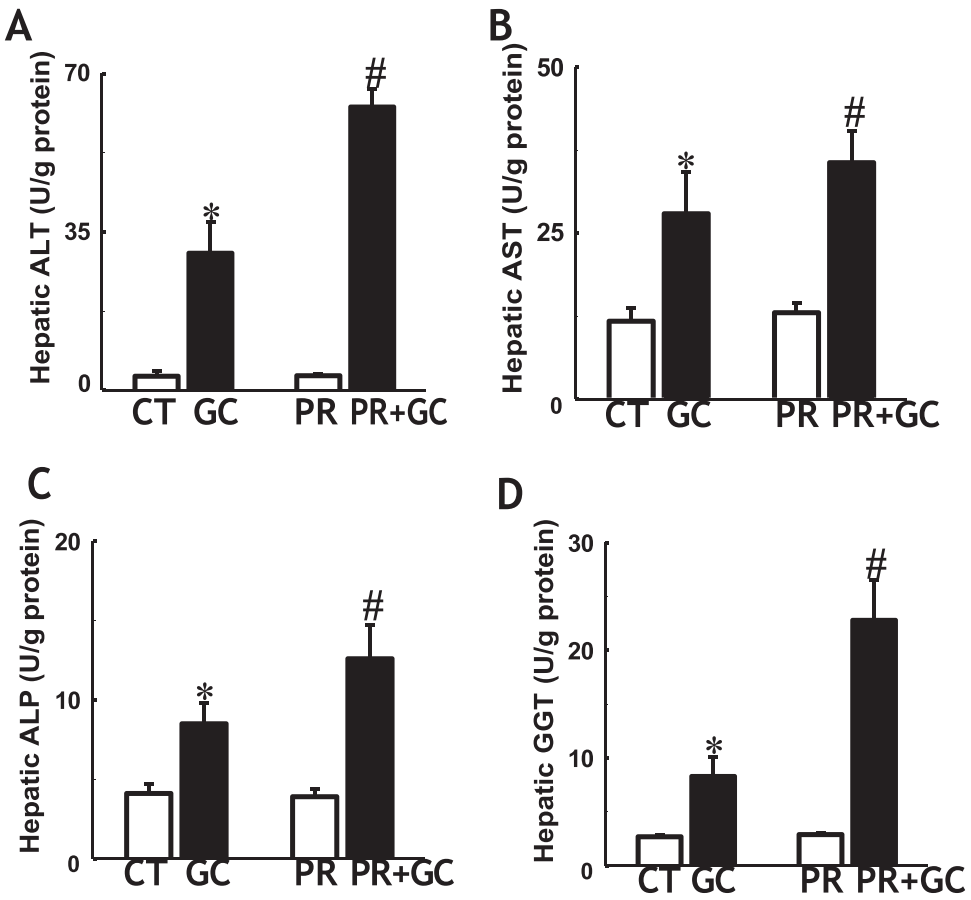

Fig. 5. Effect of GC exposure on hepatic ALT (A), AST (B), ALP (C) and GGT (D) in pregnant and non-pregnant rats using hepatic homogenate samples. Glucocorticoid exposure led to increased hepatic injury markers in both pregnant and non-pregnant groups. The data were analyzed by student's t-test and the values are expressed as mean ± SEM of 6 rats per group (*p < 0.05 vs CT; #p < 0.05 vs PR), ALT represents alanine transferase, AST represents aspartate aminotransferase, ALP represents alkaline phosphatase, GGT represents gamma-glutamyl transferase, CT represents non-pregnant control, PR represents pregnant control, and GC represents glucocorticoid.

editing. **Olufunto O. Badmus:** Formal analysis, Funding acquisition, Investigation, Resources, Validation, Writing – original draft. **Lawrence Olatunji:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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