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The Differential of Glucocorticoid Bioavailability Modulators mRNA Expression and Neurotrophic Factors in Three Organs in Broiler Chicken under Immobilization Stress

Hakeem J. Kadhim [1,2]*

[1]*Department of Microbiology, College of Veterinary Medicine and Surgery, Shatarh University, Al-Shatrah, Thi-Qar, Iraq*
[2]*CEMB program, University of Arkansas, Fayetteville, Arkansas, USA*

*Corresponding author's Email: hakim.jawad@shu.edu.iq

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ABSTRACT

The hypothalamus-pituitary-adrenal (HPA) axis remains active despite the elevated corticosterone (CORT) levels during immobilization stress (IMS). This indicates that the HPA axis activity is dependent not only on CORT concentrations but also on the availability of free active CORT that is unbound of corticosteroid-binding globulin (CBG) and is activated by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzyme. The study examined the CORT levels in the blood and the mRNA expression of both *proopiomelanocortin* gene in the anterior pituitary gland (APit) and *brain-derived neurotrophic factor (BDNF)* in the septohypothalamus during IMS. Additionally, the expression of *glucocorticoid receptor (GR)*, *11β-HSD*, and *CBG* were analyzed in the septohypothalamus, APit, and liver. The experiment included three male Cobb 500 chicken groups, a control group, and two treatment groups exposed to 60 or 120 minutes of IMS. Blood, brain, APit, and liver were collected at 35 days of age (N= 12 samples/group). CORT concentrations in blood were quantified using radioimmunoassay, while reverse transcription-quantitative PCR was used to measure mRNA levels of *CBG*, *11β-HSD1*, *11β-HSD2*, and *BDNF* in the septohypothalamus, APit, and liver. The findings suggested that the IMS activated the HPA axis, as demonstrated by increased CORT levels and changes in *proopiomelanocortin* expression within the APit of stressed chickens compared to unstressed ones. The septohypothalamus of stressed chickens showed an increase in the *CBG*, *BDNF,* and *11β-HSD1* mRNA levels, whereas *11β-HSD2* and *GR*s expression remained stable compared to the control group. Although *CBG* and *BDNF* expression decreased from peak levels, their mRNA remained significantly elevated in the 120-minute group. In liver tissue, the treatment groups showed higher levels of *11β-HSD1* and *CBG* expression, but *11β-HSD2* expression decreased. Overall, CORT levels and the expression of *GR* and CORT modulators seemed to have a significant influence on the stress response. Notably, increased mRNA levels of *CBG* and *11β-HSD1* could improve the availability of free active CORT. Furthermore, a positive correlation between CORT levels and *BDNF* expression was demonstrated, highlighting the role of BDNF in neuronal protection during IMS. Additionally, the liver may contribute to stress regulation through the functions of *CBG* and *11β-HSD*, which are vital for CORT activation and transport.

Keywords: 11β-hydroxysteroid dehydrogenase, Brain-derived neurotrophic factor, Corticosteroid binding globulin, Corticosterone, Immobilization stress

INTRODUCTION

In vertebrates, the hypothalamus-pituitary-adrenal axis is a neuroendocrine system that controls homeostasis (Ulrich-Lai and Herman, 2009; Smulders, 2021). In avians, different stressors trigger neurons in the septal brain region and hypothalamus, causing activation of stress-related neurons that secret corticotrophin-releasing hormone (CRH) and arginine vasotocin (AVT) into the portal circulation. These hormones subsequently stimulate the anterior pituitary (APit) to cleave proopiomelanocortin (POMC) into a variety of peptides, including the adrenocorticotrophic hormone (ACTH, Kuenzel and Jurkevich, 2010; Bonfiglio et al., 2011). In avian, ACTH promotes CORT secretion from the adrenal cortex in response to both psychological and physiological stimulation (Romero, 2004; Herman et al., 2016). Furthermore, CORT interacts with glucocorticoid receptors (GRs) at various levels, including the APit, hypothalamus, and extrahypothalamic regions, to reduce the HPA axis stimulation and restore homeostasis (de Kloet et al., 2005; Vandenborne et al., 2005; Keller-wood, 2015).

According to studies, only unbound GCs can reduce the activity of the HPA axis because of their potential to cross the blood-brain barrier and interact with GRs in the target tissues (Willnow and Nykjaer, 2010; Groeneweg et al., 2011). Nevertheless, it is noteworthy that only 5% of GCs circulate freely in the blood, while 5-10% are binding non-specifically to albumin, and 80-90% are binding to corticosteroid-binding globulin (CBG; formally transcortin; Perogamvros et al., 2012; Hammond, 2016). CBG is primarily produced in the liver and many other tissues, including brain, heart, kidney, adipose tissue, lung, and pancreas (Tinnikov, 1999). Additionally, Chapman et al. (2013) demonstrated that local GCs can be modulated by two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD). The

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first isozyme, 11β-HSD1, converts inert 11-dehydrocorticosterone and cortisone into active CORT and cortisol, respectively (Tomlinson et al., 2004). In avian studies, non-significant alterations in the mRNA levels of *11β-HSD1* were found in the hypothalamus following one hour of restraint stress (Krause et al., 2021). In contrast, rodent studies indicated an elevation of 11β-HSD1 mRNA levels in the extrahypothalamic regions, which subsequently inhibited HPA axis functions during acute stress (Spiers et al., 2016). The second isozyme, 11β-HSD2, plays a crucial role in transforming active GCs (cortisol and CORT) back to the inactive form, which is the ketoform. Additionally, it is worth noting that cortisol is the term applied to GCs in both fish and mammals, including humans. In contrast, corticosterone (CORT) is the nomenclature used for these hormones in birds, reptiles, amphibians, and rodents (Romero, 2004; Herman et al., 2016).

As previously stated, the primary drivers of the HPA axis in chickens are the CRH and AVT hormones that are secreted by hypothalamic paraventricular nucleus (PVN) neurons. Researchers have found that these neurons were activated by a range of stressors, including feed deprivation (Nagarajan et al., 2017a), heat stress (Cramer et al., 2015), and immobilization stress (IMS, Aman et al., 2016). Specifically, IMS, defined as a restriction in movement and aggressiveness, is an effective strategy for inducing both physical and psychological stress, potentially affecting the HPA axis and increasing CORT levels. Notably, recent stress studies have shown that CRH and AVT-producing neurons in the septohypothalamic region remain active despite the rise in the CORT levels (Kadhim et al., 2019; 2021). Furthermore, previous research showed that the brain-derived neurotrophic factor (BDNF) is essential for the activation of CRH neurons during stress sessions. Specifically, *BDNF* gene expression preceded CRH gene expression, suggesting its significance in the stress response (Nagarajan et al., 2017b; Miranda et al., 2019; Notaras and van den Buuse, 2020). According to the literature, stress has a variety of negative impacts on vital organs (Yaribeygi et al., 2017; Bohler et al., 2021). However, the mechanism by which neurons are protected in stressful situations has remained ambiguous. Miao et al. (2020) have emphasized BDNF's protective role in reducing stress-related neuronal damage. Furthermore, the relationship between CORT levels and *BDNF* expression is currently under investigation. Consequently, it would be intriguing to explore the effects of glucocorticoid modulators on the HPA axis functions. Thus, the current study hypothesized that the regulation of stress responses in chickens is influenced not only by the actions of CORT through GRs but also by the levels of free active CORT. As a result, the research aimed to measure CORT concentration in the blood and P*OMC* mRNA in the APit during acute IMS as indicators of HPA axis activation. Additionally, the study sought to evaluate the expression of *BDNF* mRNA in the septohypothalamus to determine the correlation between *BDNF* mRNA expression and CORT levels. Furthermore, the mRNA expression of *GR*s, *11β-HSD*, and *CBG* in the septohypothalamus, APit, and liver were analyzed to investigate the role of GC modulators in regulating the HPA axis activity.

MATERIALS AND METHODS

Ethical Approval

The University of Arkansas Institutional Animal Care and Use Committee (IUCAC/USA) protocol # 19054 has approved all procedures used in this experiment, including housing conditions, handling, immobilization, and sampling.

Animals, stress procedure, and sample collection

Male broiler chickens (one-day-old, Cobb 500) were obtained from a commercial hatchery located in northwest Arkansas /Arkansas/USA on the day of the hatch. To eliminate any potential for gender-related variables, only males were used in the current study. The chickens were reared in controlled environments with unrestricted access to water and feed. During the initial three days, the chickens were subjected to continuous lighting to help them find feed and water easily. Following this period, chickens were transitioned to a photoperiod that consisted of 16 hours of light and 8 hours of darkness, with lights turned on at 6:00 AM. The chickens were fed an *ad libitum* standard diet containing 3050 kcal/kg metabolic energy and 23.3% crude protein (NRC, 1994). Additionally, the ambient temperature was reduced by 2.5 degrees per week from 32 °C to 24 °C, while the humidity was kept between 30% and 40%. Initially, the chickens were housed in a battery system for three weeks before being relocated to cages measuring 85 cm in height, 60 cm in weight, and 70 cm in length, where they were grouped in sets of three. The chickens were then categorized randomly into three distinct groups including, a control group referring to unstressed chickens and two treatment groups. Chickens in treatment groups were exposed to immobilization stress (IMS) for either 60 minutes called 60 minutes or 120 minutes and called 120 minutes. Each group included 14 chickens; thereafter, two samples were excluded from each group because of technical issues during sample collection and/or processing, resulting in 12 chickens per group. The IMS protocol was initiated in week 5, during which the stressed chickens were secured in a harness towel that restricted their wing movement and standing ability while still allowing them access to feed and water throughout the period of the immobilization (Aman et al., 2016). Upon completion of the stress session, the weight of chickens was taken (2316 ±

215gm/chicken) for the control and treated groups. Then, without anaesthesia, blood (2-3 ml) was immediately drawn from the wing vein into heparinized tubes and refrigerated (4°C). On the same sampling day, plasma was isolated through the centrifugation of blood at 3000 rpm for 20 min at 4 °C, followed by storage at -20 °C for subsequent analysis of CORT concentration by radioimmunoassay. Following blood collection, the chickens were subjected to cervical dislocation, and their liver, APit, and brain were immediately sampled and frozen in liquid nitrogen (-196 °C). For brain samples, the septohypothalamus was isolated from frozen brains as described in previous studies (Kadhim et al., 2019; 2020). Importantly, throughout the eight hours of each sampling day (8:00 AM to 4:00 PM) at the Poultry Farm of the University of Arkansas, an equal number of chickens were sampled from each group to minimize variations in plasma CORT levels.

Quantification of corticosterone concentrations

Radioimmunoassay (RIA) was used to determine plasma CORT levels and performed in the duplicate assay for each sample, as documented previously (Madison et al., 2008; Kadhim et al., 2021). At room temperature, 200 µl of plasma was combined with 2 ml of ethyl ether in borosilicate tubes and vortexed for 30 min. Subsequently, the tubes were transferred to a bath of methanol/dry ice (-20 °C), and the soluble part was aspirated into the new tube and dried in a 37 °C evaporator. Thereafter, an assay buffer (400 µl) was added to the dried extracts and incubated overnight at 4 °C for equilibrations. Subsequently, the samples were mixed with two hundred microliters of ^{125}I CORT tracer (MP Biochemical Inc. Orangeburg, NY, USA) and one hundred of polyclonal rabbit anti-CORT (Fitzgerald Inc., Concord, MA, USA) and incubated at 4 °C. After 24 hours of incubation, two hundred microliters of sheep anti-rabbit (MP Biochemical Inc. Orangeburg, NY, USA) were added to the mixture, and bound tracers were precipitated using polyethylene glycol (6%). After removing the supernatant from each sample, the residual liquid was air-dried. Finally, the CORT concentration in samples was measured using a gamma counter (Perkin Elmer Wizard gamma counter, NY, USA).

RNA extraction, reverse transcription, and quantitative polymerase chain reaction

Total RNA was extracted from the septohypothalamus, APit, and liver using the TRIzol-chloroform (Life Technologies, CA, USA) protocol, as previously described (Kadhim et al., 2019, Kang et al., 2020). Then, deoxyribonuclease I (Ambion, Austin, TX, USA) was used to maintain RNA free from genomic DNA. Subsequently, the RNeasy mini kit (Qiagen, Valencia, CA, USA) was used to purify the extracted RNA. The RNA concentration in the samples was quantified using nanodrop spectrophotometry (Biotek, Winooski, VT, USA). Then, 1µg of extracted RNA was utilized for cDNA synthesis in 40 µl reaction volume using Superscript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The primer sets for the examined genes and a housekeeping gene (GAPDH) are listed in Table 1.

Table 1. Primer set sequences for the real-time-quantitative PCR technique in the present study for evaluation of gene expression changes in chickens

Genes	Gene bank #	Primer sequence (5'__3')	Amplicon size (bp)	Annealing Temp. (°C)	Reference
GR	NM_001037826	F: GCCATCGTGAAAAGAGAAGG R: TTT CAACCACATCGTGCAT	95	54	(Kang et al., 2020)
CBG	KU_180444	F: CTTTTGCCTATGGCCAGCTT R: GGTCTTTAGGTTCATTTGGATCGT	66	58-59	(Vashchenko et al., 2016)
BDNF	NM_001031616	F: GACATGGCAGCTTGGCTTAC R: GTTTTCCTCA CTGGGCTGGA	167	58-60	(Kadhim et al., 2019)
11β-HSD1	XM_417988	F: CTGGGAACTGTCTGCACAAC R: GATTGCGAGGAACCATTTACAG	96	56	(Kang et al., 2020)
11β-HSD2	XM_040680958	F: TGGACACGTTCCGCAGTG R: CGTAGTCCTCGCCGTAAGC	60	168	(Wang et al., 2014)
POMC	NM_001031098	F: GCCAGACCCCGCTGATG R: CTTGTAGGCGCTTTTGACGAT	56	58-60	(Kadhim et al., 2019)
GAPDH	NM_204305	F: CTTTGGCATTGTGGAGG GTC R: ACGCTGGGATGATGTTCTGG	128	58-60	(Kadhim et al., 2019)

Temp: Temprature

The ABI 7500 system (Applied Biosystems 7500 Real-Time PCR System, CA, USA) was used in this experiment to measure the mRNA levels for *POMC, GRs, CBG, BDNF, 11β-HSD1, and 11β-HSD2* in the dissected tissues. The real-time quantitative PCR assay was conducted in duplicate within a 30 µl volume, following specific thermal cycling conditions. This included an initial denaturation step at 95 °C for 10 minutes, followed by 40 amplification cycles comprising 30 seconds at 95 °C, 1 minute at 60 °C, and 30 seconds at 72 °C. In the context of RT-qPCR, the average cycle threshold (Ct) and delta Ct values were calculated for all genes in each sample. Then, fold changes in mRNA expression were determined through normalization against the housekeeping gene using the $2^{-\Delta\Delta Ct}$ equation (Schmittgen and Livak, 2008). In detail, the initial delta Ct was computed by determining the difference in the Ct values between the target and housekeeping genes for each sample, expressed as [delta Ct = Ct (target gene) – Ct (housekeeping gene)]. Subsequently, delta Ct values were determined by calculating the differences between delta Ct in the treated groups and control group for each sample, represented as [delta Ct = delta Ct (treated sample # 1) – delta Ct (control sample #1)]. Then, fold changes were calculated by using the $2^{-\Delta\Delta Ct}$ equation. It is important to note that the delta Ct value for the control group is equal to 0, thereby 2^0 is equivalent to 1. Therefore, relative gene expression in the control group is consistently maintained at a value of 1.

Statistical analysis

The results of the experiment were analyzed using John's Macintosh Project (JMP) Pro 18.0 (SAS Institute Inc., NC, USA). The Shapiro-Wilk test was used initially to validate the normal distribution. Subsequently, variations across three independent groups were assessed for the blood samples and examined tissues. The analyses started with a one-way ANOVA first, followed by Tukey's HSD test to determine the relative variations in mRNA levels for each gene across groups as well as for changes in the CORT levels. The findings were reported as the mean ± standard deviation (SD), with a p-value less than 0.05 indicating statistical significance. Additionally, the coefficients of variation for RIA were calculated, revealing inter and intra CV were 13% and 9%, respectively.

RESULTS

The corticosterone levels and pro-opiomelanocortin gene expression

In the samples examined, a significant rise in CORT levels was observed in the blood plasma of IMS groups when compared with the control group ($p < 0.05$). This increase was correlated with changes in the mRNA levels of *POMC* in the APit (Figure 1). Specifically, CORT levels peaked in the 60-minute group, showing a significant elevation compared to the control group ($p < 0.05$). Following this peak, CORT concentrations decreased in the 120-minute group yet remained significantly higher than those in the control group (Figure 1A). In the APit, *POMC* gene expression exhibited a significant downregulation in the 60-minute group ($p < 0.05$; Figure 1B). conversely, *POMC* mRNA levels were significantly elevated in the 120-minute group compared to the control groups ($p < 0.05$). The variations in CORT levels and *POMC* mRNA expression between stressed and unstressed groups were statistically significant ($p < 0.05$).

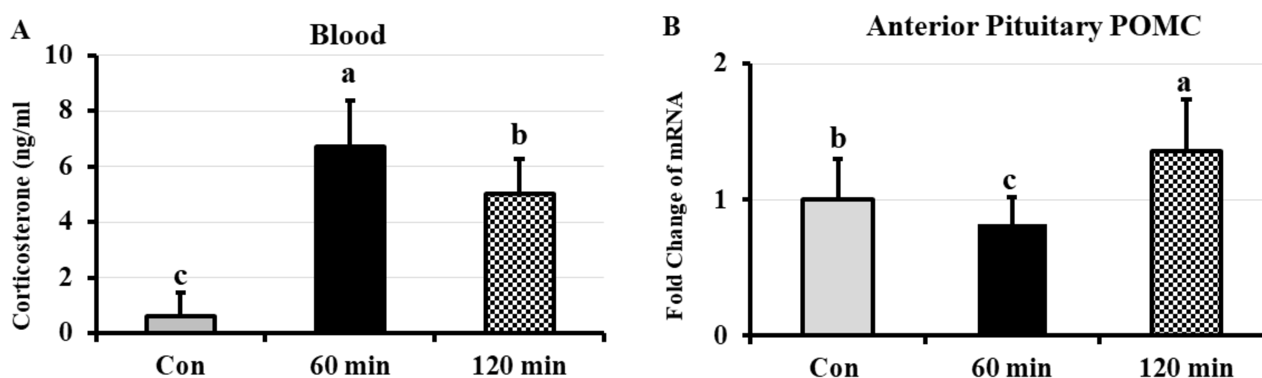

Figure 1. Plasma CORT levels and POMC mRNA expression during immobilization stress. **A**: CORT concentrations in the stressed groups compared with the control group (non-stressed chickens). **B**: Changes in the relative mRNA expression of POMC in the anterior pituitary were normalized with the housekeeping gene (GAPDH) and quantified using $2^{-\Delta\Delta Ct}$ equation. Data were presented as mean ± the standard deviation (SD) and expressed as fold changes. Significant differences ($p < 0.05$) between groups were verified by different letters above the bars with a > b > c. (CORT = Corticosterone, POMC: Proopiomelanocortin, Con: Control group; 60 min: 60 minutes of immobilization stress; 120 min: 120 minutes of immobilization stress).

Relative mRNA expression for septohypothalamic genes

The examination of mRNA expression levels for target genes within the septohypothalamus revealed notable differences across the experimental groups. Specifically, the 60-minute group displayed a significant increase in the mRNA levels of *BDNF* and *CBG* in comparison to the control group, followed by a decrease from this peak in the 120-minute group. Nevertheless, the mRNA levels of *BDNF* and *CBG* in the immobilized groups remained significantly elevated (Figure 2A; $p < 0.05$ for both genes). In contrast, the mRNA levels of *GR* exhibited a non-significant decrease in the 60-minute group ($p > 0.05$), which was followed by a significant increase in the 120-minute group when compared to the control group (Figure 2A; $p < 0.05$). Additionally, the mRNA levels of 11β-HSD1 and 11β-HSD2 showed opposing responses to IMS. In the 60-minute group, there was a non-significant downregulation of 11β-HSD1, accompanied by a significant upregulation of 11β-HSD2. In the 120-minute group, 11β-HSD1 mRNA levels were upregulated, while a significant downregulation of 11β-HSD2 was detected (Figure 2B; $p < 0.05$ for both genes).

Relative mRNA expression for anterior pituitary genes

The expression of *GR* and *CBG* genes in the APit demonstrated a diverse response during IM stress (Figure 3A). In the 60-minute group, there was a significant decrease in *GR* mRNA levels ($p < 0.05$), which subsequently recovered to baseline levels in the 120-minute group ($p > 0.05$). In contrast to *GR* expression, *CBG* mRNA levels exhibited a significant increase in immobilized groups compared to the control group ($p < 0.05$). Remarkably, the mRNA levels of *11β-HSD1* and *11β-HSD2* maintained a consistent pattern throughout the different treatment conditions (Figure 3B). Specifically, the stressed groups showed significantly elevated mRNA levels of *11β-HSD1* and reduced mRNA levels of *11β-HSD2* compared to the control group ($p < 0.05$ for both genes).

Relative mRNA expression for liver genes

Gene expression data for multiple genes in the liver showed distinct variations in response to different times of IM stress. In detail, glucocorticoid receptors (GRs) displayed a significant downregulation in the 60-minute group compared to the control group (Figure 4A, $p < 0.05$), followed by a quick, considerable increase in the 120-minute group ($p < 0.05$). In contrast, *CBG* mRNA levels demonstrated a gradual increase, reporting a peak response in the 120-minute group (Figure 4A, $p < 0.05$). Compared with the control group, both *11β-HSD1* and *11β-HSD2* mRNA levels showed significant increases in the 60-minute group, followed by sustaining the upregulation of *11β-HSD1,* while *11β-HSD2* mRNA levels decreased from their peak in the 120-minute group (Figure 4B, $p < 0.05$ for 11β-HSD1 and 11β-HSD2).

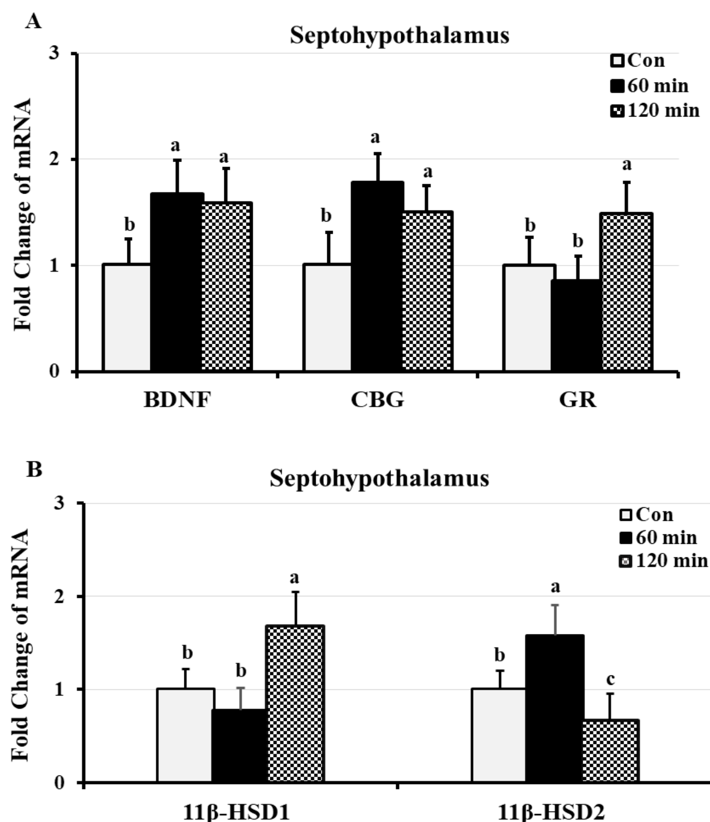

Figure 2. Alterations in the mRNA expression of septohypothalamus genes in immobilized chickens in comparison to control chickens. **A**: Brain-derived neurotrophic factor (BDNF), corticosteroid-binding globulin (CBG), and glucocorticoid receptor (GR). **B**: 11β-hydroxysteroid dehydrogenase 1 and 2 (11β-HSD1 and 11β-HSD2). The relative mRNA expression of these genes was normalized with the housekeeping gene (GAPDH) and quantified using the $2^{-\Delta\Delta Ct}$ equation. Data were presented as mean ± the standard deviation (SD) and expressed as fold changes. Significant differences ($p < 0.05$) between groups were verified by different letters above the bars with a > b > c (Con: control group; 60 min: 60 minutes of immobilization stress; 120 min: 120 minutes of immobilization stress).

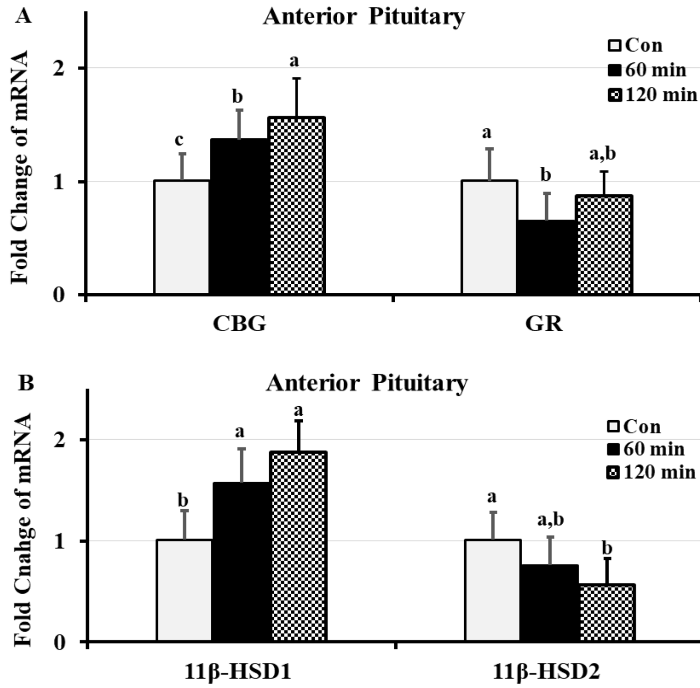

Figure 3. Alterations in the mRNA expression of anterior pituitary genes in immobilized chickens in comparison to control chickens. **A**: Corticosteroid-binding globulin (CBG) and glucocorticoid receptor (GR); **B**: 11β-hydroxysteroid dehydrogenase 1 and 2 (11β-HSD1 and 11β-HSD2). The relative mRNA expression of the genes was normalized with the housekeeping gene (GAPDH) and quantified using the $2^{-\Delta\Delta Ct}$ equation. Data were presented as Mean ± the standard deviation (SD) and expressed as fold changes. Significant differences ($p < 0.05$) between groups were verified by different letters above the bars with a > b > c. While a,b is not different statistically from a or b (Con: Control group; 60 min: 60 minutes of immobilization stress; 120 min: 120 minutes of immobilization stress).

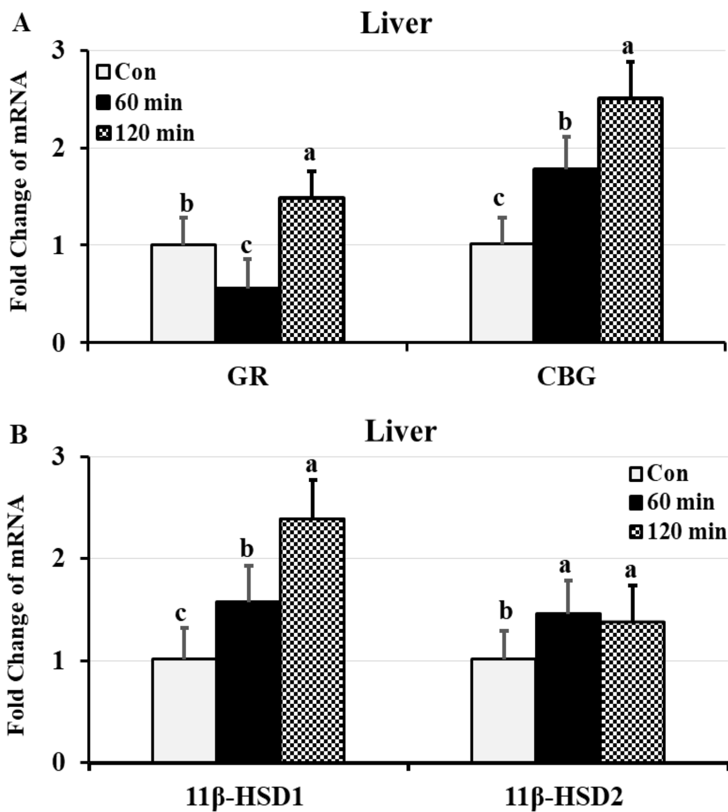

Figure 4. Alterations in the mRNA expression of liver genes in immobilized chickens in comparison to control chickens. **A**: Corticosteroid-binding globulin (CBG) and glucocorticoid receptor (GR); **B**: 11β-hydroxysteroid- dehydrogenase 1 and 2 (11β-HSD1 and 11β-HSD2). The relative mRNA expression of the genes was normalized with the housekeeping gene (GAPDH) and quantified using the $2^{-\Delta\Delta Ct}$ equation. Data were presented as mean ± the standard deviation (SD) and expressed as fold changes. Significant differences ($p < 0.05$) between groups were verified by different letters above the bars with a > b > c. (Con: Control group; 60 min: 60 minutes of immobilization stress; 120 min: 120 minutes of immobilization stress).

DISCUSSION

The study utilized classical acute immobilization stress to examine the relationship between CORT levels and *BDNF* expression in the chicken hypothalamus. It also explored the changes in the mRNA expression of enzymes that influence the local bioavailability of CORT in stress-related organs during IMS. The findings revealed a significant increase in CORT levels across both stress groups. Supporting the finding of this study, Scanes et al. (2024) reported that restraint stress led to elevated CORT concentrations in the blood plasma of chickens, thereby activating both the HPA axis and the opioid system, which are integral to carbohydrate metabolism. Additionally, it was demonstrated that exposing chickens to IMS for one hour resulted in a fivefold increase in CORT levels in comparison with unstressed chickens (Aman et al., 2016). The high CORT levels in the 60-minute group, coupled with the downregulation of *GR* mRNA in the APit and septohypothalamus, may serve to inhibit CORT binding to GRs and facilitate continued CORT release. In this context, it has been noted that the release of GCs ceases once CORT binds to GRs (Lane et al., 2021). In contrast, the upregulation of *GR* expression in liver tissues may mediate the effects of CORT on liver function, thereby promoting the release of energy necessary for the maintenance of homeostasis. Feng et al. (2020) indicated that GCs influence liver functions, such as gluconeogenesis, through GRs. Concurrently, the mRNA levels of *POMC* within the APit exhibited a slight decrease in the 60-minute group, followed by an increase in the 120-minute group. Consistent with the results of the current study, Løtvedt et al. (2017) reported that a one-hour exposure to restraint stress did not significantly decline *POMC* expression in the APit of chickens. Notably, the observed CORT levels and POMC expression in the 60-minute group were mismatched phenomena that remained unexplained in avian species. Fortunately, research conducted by Harno et al. (2018) on rodents revealed that the precursor peptide of POMC is sequestered in vesicles within the corticotropic cells of the APit. Upon activation of corticotrophs, pro-hormone convertase enzymes cleave POMC into several peptides, including ACTH, which typically leads to an increase in plasma CORT levels within 60 minutes or less (Kadhim et al., 2021). Following the depletion of POMC in vesicles, changes in POMC gene expression occur, which appear to contribute to the maintenance of elevated CORT levels at 120 minutes of IM stress and beyond.

In the septohypothalamus, the mRNA levels of *BDNF* and *CBG* experienced a significant increase in the IMS groups. Despite the upregulation of both *BDNF* and *CBG*, their roles appear to be opposite. BDNF is essential for protecting neurons from the negative impacts of IMS. Furthermore, BDNF plays a pivotal role in the stimulation of CRH neurons, potentially facilitating their rapid response (Jeanneteau et al., 2012; Miranda et al., 2019; Miao et al., 2020). The elevation in *BDNF* mRNA levels coincided with an increase in CORT levels triggered by IM stress, displaying a positive correlation. Previous studies have shown that exposure to CORT can lead to neuronal damage in brain structures such as the hippocampus. Specifically, the administration of BDNF reduced neuronal death associated with CORT exposure (Nitta et al., 1999). Regarding CBG expression, its expression was upregulated significantly in the 60-minute group correlated with a downregulation of GR, which serves to mitigate the negative feedback effects of elevated CORT on CRH-producing neurons. Additionally, the increase in *CBG* mRNA levels within brain regions may reduce the capacity of CORT to penetrate the blood-brain barrier and sustain its release (Groeneweg et al., 2011), while the upregulation of *BDNF* mRNA levels during IMS might attenuate CORT-induced neuronal death (Miao et al., 2020). Remarkably, in the 120-minute group, there was an observed increase in the expression of *11β-HSD1,* accompanied by a reduction in the mRNA levels of *11β-HSD2*. This pattern of expression indicates that *11β-HSD1* plays a crucial role in converting CORT to its active form and aids the transport of active CORT into target cells within the APit tissue (Tomlinson et al., 2004). Consequently, *CBG and 11β-HSD1* appear to work together in order to enhance the regulation of the HPA axis through the action of CORT on GRs. Previous studies have shown that the peak expression of *CRH* mRNA in PVN occurs at one hour following the initiation of IMS (Kadhim et al., 2021), with a subsequent decline in expression in the 120-minute group. This suggests that both CBG and GR are involved in the regulation of CRH-producing neurons located in the septohypothalamus.

In the anterior pituitary (APit), a reduction in the expression *of GRs and 11β-HSD1* genes may facilitate the activation of corticotropic cells during IMS, as evidenced by the elevated levels of CORT in the blood of stressed chickens. Supporting the finding of this study, Vodička et al. (2014) stated that the downregulation of *GR*s and *11β-HSD1* expression could reduce the negative feedback of high CORT concentrations by limiting the accessibility of GRs within APit and maintaining CORT in its inert form. Furthermore, Michael et al. (2019) reported that the decrease in *GR*s and *11β-HSD1* expression functions as a protective response to elevated CORT concentrations. Additionally, the upregulation of local *11β-HSD2* and *CBG* genes is crucial for sustaining APit activation, as the main function of 11β-HSD2 is to inactivate GCs and convert them into their inactive ketoform (Cooper and Stewart, 2009; Sattler et al., 2018). Consequently, the APit remained activated, as only free CORT can cross the cell membrane and modulate the function of the HPA axis.

In the current study, the liver showed a significant increase in the mRNA levels of *CBG and 11β-HSD1* in the IMS groups. This elevation in *11β-HSD1* mRNA levels may reduce or lessen cells' capacity to neutralize oxidants, potentially resulting in cellular death (Kratschmar et al., 2012; Chen et al., 2020). Furthermore, the upregulation of *CBG* and ***11β-HSD1*** in the liver matched with elevated CORT levels at both stress time points, indicating that CBG plays a crucial role in the CORT distribution to target organs through blood circulation. Supporting the findings of the current study, Satter et al. (2018) indicated that the elevation of *11β-HSD1* mRNA improved the translocation of active GCs into cells. On the other hand, there was a significant reduction in the expression of the *11β-HSD2* gene during IMS, suggesting a reduced ability to prevent CORT activation. Nonetheless, the decline in *GR*s may mitigate the effects of the active form of GCs (Cooper and Stewart, 2009; Sattler et al., 2018). Research suggests that elevated levels of *11β-HSD1* and *GR*s within the hepatic tissue may contribute to the development of metabolic disorders, a condition that can be exacerbated by elevated GC levels (Livingstone et al., 2000; Candia et al., 2012). Collectively, the findings of increased circulating CORT, the upregulation in *11β-HSD1* and *CBG* mRNA levels, alongside the downregulation of *11β-HSD2* expression, suggest that the hepatic tissue is adversely affected by excessive GC levels, leading to a reduction of *GR* expression during periods of stress.

CONCLUSION

The results of the current study indicated that immobilization stress influenced glucocorticoid bioavailability by modulating local mediators such as *CBG* and *11β-HSD*. Moreover, IMS was associated with elevated levels of *BDNF* mRNA and increased concentrations of CORT in chickens. Notably, there was a positive correlation between *BDNF* and CORT levels; the increase in BDNF appeared to be essential for neuronal protection and for mitigating neuronal damage induced by CORT during IM stress. Furthermore, the interaction between GCs with GRs within the HPA axis, along with the availability of free active GCs, played a significant role in the regulation of the HPA axis activity. Enzymes such as CBG and 11β-HSDs in the examined structures were vital for the generation of bioactive GCs. Furthermore, the modulators of CORT bioavailability, namely CBG, and 11β-HSD, were identified in the studied tissues, which include the brain, APit, and liver. Moreover, it is recommended to investigate the direct action of ACTH on the liver tissue and determine which ACTH receptors are expressed in the liver. Additionally, investigating the alterations in liver enzyme activity during stress episodes is of considerable importance.

DECLARATIONS

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Authors' contributions

Hakeem Kadhim conceptualized the study, collected the samples, analyzed the data, conducted the study, and prepared the initial draft of the manuscript. Furthermore, Hakeem Kadhim has read, improved, and approved the submitted version of the manuscript.

Competing interests

There is no conflict of interest to declare.

Availability of data and materials

The original data presented in the study are available in the article.

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Ethical considerations

The author confirms that the author has reviewed and submitted the manuscript to this journal for the first time. Additionally, the author checked the originality of data and sentences via plagiarism checkers.

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