Role of Exogenous Insulin in Modifying Stress and Carbohydrate Metabolic Gene Expression in Domestic Birds During Slaughter

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Physiology

Department of Physiology, Biochemistry and Pharmacology
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Chattogram-4225, Bangladesh

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I dedicate this thesis to my late **Grandmother, Mostafa Khatun**, whose unwavering commitment to her children's education in the face of extreme challenges, along with her heartfelt prayers, has paved the way for our academic pursuits today.

Authorization

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects and that all revisions required by the thesis examination committee have been made

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List of Abbreviations

Abbreviation	Full Form
ACTH	Adrenocorticotropic Hormone
ATP	Adenosine Triphosphate
CRH	Corticotropin-Releasing Hormone
CT	Threshold Cycle
cDNA	Complementary DNA
ELISA	Enzyme-Linked Immunosorbent Assay
FoxO1	Forkhead Box Protein O1
GLUT12	Glucose Transporter 12
H:L Ratio	Heterophil: Lymphocyte Ratio
HPA	Hypothalamo-Pituitary-Adrenal
IR	Insulin Receptor
IU	International Unit
IV	Intravenous
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
PBS	Phosphate-Buffered Saline
PI3K	Phosphoinositide 3-Kinase
PKB/Akt	Protein Kinase B
PDX-1	Pancreatic and Duodenal Homeobox 1
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SREBP-1c	Sterol Regulatory Element-Binding Protein

Summary

This thesis consists of two parts: the first explores the interplay between cortisol and insulin during the slaughter process in domestic birds, while the second investigates the effect of insulin on the mRNA expression of genes involved in glucose and energy metabolism. The initial study included three bird species: Broiler chickens, Sonali chickens, and Quail. The birds were administered either a control solution or varying doses of insulin (4 IU and 8 IU). Blood samples were collected before and after insulin administration to measure serum cortisol, glucose, and hemoglobin levels. Key findings indicate that insulin administration has species-specific effects on cortisol levels, with Sonali chickens demonstrating a significant reduction in cortisol, suggesting a stress-mitigating effect. This led to the second part of the study, which focused on a molecular level of analysis.

Using qPCR, tissues from Sonali chickens were examined for the mRNA expression of GLUT12 and the insulin receptor (IR) in the liver and GLUT12 and insulin genes in the pancreas. Molecular analysis revealed that insulin upregulated the mRNA expression of GLUT12, IR, and insulin in Sonali chickens' liver and pancreas, enhancing glucose and energy metabolism. The optimal insulin dose was determined to be 4 IU, which had the most pronounced effects on both hormonal and genetic parameters. These results highlight the potential of dose-dependent insulin and similar product administration to improve welfare and metabolic health in domestic birds.

***Keywords*:** insulin, cortisol, stress, metabolism, GLUT12

Chapter 1: Introduction

Understanding the physiological and molecular mechanisms that underlie stress responses in animals is critical, especially in relation to animal welfare and productivity. Stress triggers significant physiological changes that can affect both the well-being of the animal and the quality of its meat. In poultry, the interaction between hormones like cortisol and insulin is particularly important during stressful events such as slaughter. With global poultry consumption having doubled in the past decade and is projected to double again by 2050 (Alexandratos & Bruinsma, 2012), there have been major advances in chicken genetics to meet these growing demands. Insulin, as a key regulator of metabolism, has gained attention for its role in both energy homeostasis and stress response. Advances in molecular biology techniques now enable detailed examination of insulin's effects on gene expression at the mRNA level, offering new insights into how insulin regulates glucose and energy metabolism.

Stress is a complex phenomenon that can be challenging to define and measure. However, circulating glucocorticoid levels, particularly cortisol, are commonly used as a direct marker of stress in animals (Hofer & East, 1998). In vertebrates, stress responses are managed by the hypothalamic-pituitary-adrenal (HPA) axis, a highly conserved system. Upon exposure to stressors, glucocorticoid levels in the blood rise within minutes due to the activation of the HPA axis (Wingfield & Romero, 2001). Insulin can significantly influence the HPA axis, both centrally and peripherally, and these effects can occur independently of hypoglycemia. Research comparing the peak responses of the pituitary-adrenal system to various HPA activators—such as hyperinsulinemia, restraint, and hypoglycemia—has shown that defects in the HPA axis of diabetic animals result in an impaired stress response, with peak responses plateauing at lower levels than in normal animals. This suggests that insulin may play a key role in modulating stress reactivity, alongside its well-known role in energy metabolism.

The interplay between insulin and the HPA axis has primarily been studied with regard to energy homeostasis. Glucocorticoids are believed to create an environment

conducive to the development of obesity and insulin resistance, whereas insulin acts centrally to suppress food intake and prevent the onset of obesity (Baskin et al., 1999). Additionally, studies on humans have shown that insulin can stimulate pituitary-adrenal activity under specific conditions. For example, Fruehwald-Schultes et al. (1999) demonstrated that intravenous infusion of high doses of insulin in the presence of euglycemia increases plasma ACTH and cortisol concentrations. However, at lower insulin doses, pituitary-adrenal activity remains unchanged (Fruehwald-Schultes et al., 2001). These findings suggest that insulin's ability to stimulate HPA function may be dose-dependent. Stress hormone cortisol is found to be release during slaughter of animal as well as in birds (Ismail et al., 2019). Insulin has an antagonistic effect on cortisol secretion (Varlamov et al., 2021).

In addition to its influence on stress responses, insulin plays a central role in regulating glucose and energy metabolism. It exerts its effects through a coordinated action on various tissues, including the liver, pancreas, adrenal glands, and muscles. Molecular biology techniques have made it possible to study insulin’s effects on gene expression at the mRNA level, leading to new insights into how insulin regulates glucose uptake, utilization, and lipid metabolism. For example, insulin increases the expression of the glucose transporter gene GLUT4 in muscle, promoting glucose uptake and utilization. In the liver, insulin regulates the expression of genes involved in glycogen synthesis and gluconeogenesis. Studies have also shown that insulin regulates the expression of genes involved in steroid synthesis and metabolism in the adrenal gland. Additionally, insulin’s effects extend to the pituitary gland, where it regulates the expression of genes encoding key hormones like growth hormone and corticotropin-releasing hormone (CRH), both of which influence glucose and energy metabolism.

This research aims to explore the dual role of insulin in modulating both stress responses and metabolic gene expression in poultry during slaughter. By investigating the interaction between cortisol and insulin, the study seeks to determine whether exogenous insulin can reduce stress by lowering cortisol secretion, contributing to improved animal welfare practices in live bird markets. Simultaneously, it examines the effect of insulin on the mRNA expression of genes involved in glucose and energy metabolism, offering insights into the molecular mechanisms behind insulin's regulatory functions across different tissues.

The findings from this study will contribute to a deeper understanding of whether insulin can mitigate stress during slaughter by influencing cortisol release, which could enhance both the welfare of birds and the quality of their meat. Furthermore, by understanding insulin's role in regulating metabolic processes at the molecular level, this research may offer critical insights into managing metabolic diseases like diabetes in poultry and potentially in other species.

Objectives of the Study:

1. Investigate the interplay between cortisol and insulin during the slaughter of birds.

2. Determine whether insulin can mimic or suppress the release and effect of cortisol during slaughter.

3. Evaluate the glucose and hemoglobin levels during slaughter, with and without exogenous insulin exposure.

4. Assess the effect of insulin on the mRNA expression of genes involved in glucose and energy metabolism in the liver and pancreas of chickens.

5. Compare the mRNA expression of genes involved in glucose and energy metabolism between insulin-treated and control chickens to identify differences in gene regulation.

This thesis aims to provide a comprehensive analysis of insulin's role in stress management and metabolic regulation in poultry, with potential implications for improving animal welfare and optimizing poultry production and health management.

Chapter 2: Literature Review

2.1 Introduction

The interplay between cortisol and insulin during stressful events, such as the slaughter of birds, is crucial to understanding how stress responses can be managed to enhance both animal welfare and meat quality. Cortisol, a glucocorticoid hormone, plays a vital role in stress responses by affecting glucose metabolism, while insulin is essential for maintaining glucose homeostasis. These hormones interact in complex ways that influence energy balance and metabolic health. Insulin's effect on the mRNA expression of genes related to glucose and energy metabolism is particularly important for maintaining metabolic balance and preventing metabolic disorders. This chapter explores the dynamics of cortisol and insulin and their influence on the metabolic responses of domestic birds, particularly during stress-inducing events like slaughter.

The regulation of glucose and energy metabolism by insulin involves a range of genetic interactions. In birds, insulin signaling pathways show distinctive differences from those in mammals, especially in the expression of glucose transporters (GLUTs). This section reviews how insulin affects the mRNA expression of genes involved in glucose metabolism, with a particular focus on avian species.

2.2 The Growing Importance of the Poultry Industry

The global poultry industry has expanded rapidly in response to increasing demand for poultry meat and eggs. Poultry meat is highly valued for its low saturated fat content and richness in protein, vitamins, and minerals (Marangoni et al., 2015). Poultry eggs are also a cost-effective source of animal protein, rich in vitamins, minerals, proteins, and antioxidants such as lutein and zeaxanthin, which benefit eye health (Zaheer, 2015). Given these nutritional benefits, global consumption of poultry products has doubled over the past decade and is expected to double again by 2050 (Alexandratos & Bruinsma, 2012).

2.3 Stress Response and the HPA Axis

The hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) are key components of the body's response to stress (Guilliams & Edwards, 2010). When the brain detects stress, it triggers a hormonal response, with cortisol being the primary hormone involved in this process. Cortisol, acting through glucocorticoid receptors, shifts the body's focus toward survival by altering metabolic processes (Heim & Nemeroff, 2001). However, chronic stress and prolonged exposure to cortisol can impair glucose tolerance and disrupt metabolic regulation (Di Dalmazi et al., 2012).

Cortisol and insulin interact in regulating energy balance. Insulin inhibits food intake, while cortisol stimulates it. This relationship is mediated in part by their effects on hypothalamic neuropeptide Y (NPY) expression, where insulin suppresses NPY mRNA and cortisol stimulates it (Strack et al., 1995). Chronic exposure to cortisol, particularly during prolonged stress, can suppress insulin secretion by binding to glucocorticoid receptors on pancreatic β-cells, reducing glucose uptake and impairing insulin release (Scaroni et al., 2017). Additionally, cortisol-induced insulin resistance disrupts insulin signaling pathways, decreasing insulin receptor sensitivity and inhibiting glucose transporter activity (Stulnig & Waldhausl, 2004).

2.4 Cortisol and Insulin: Mechanisms of Action

Cortisol is a key regulator of stress responses, directing the body's resources toward immediate survival while affecting long-term metabolic processes. Prolonged exposure to cortisol can impair glucose tolerance (Di Dalmazi et al., 2012). In contrast, insulin promotes anabolic processes such as glycogenesis and lipogenesis, while inhibiting catabolic actions like gluconeogenesis and glycogenolysis (Saltiel & Kahn, 2001). Insulin also reduces hypothalamic neuropeptide Y (NPY) expression, while cortisol increases it, explaining their opposing roles in energy regulation (Strack et al., 1995).

Insulin's interaction with the HPA axis shows that different insulin infusion rates influence cortisol secretion. Higher insulin levels have been found to increase cortisol concentrations, suggesting that insulin can directly stimulate the HPA axis and influence cortisol release through central and pituitary mechanisms (Fruehwald-

Schultes et al., 1999). Hyperinsulinemia, combined with chronic stress, may lead to hypercortisolism, contributing to the development of metabolic disorders like the metabolic syndrome (Fruehwald-Schultes et al., 2001).

2.5 Heterophil to Lymphocyte (H/L) Ratio as a Stress Indicator

In poultry, the heterophil to lymphocyte (H/L) ratio is a reliable indicator of stress. This ratio tends to increase in response to environmental stressors, providing a long-term measure of stress exposure (Hofer & East, 1998). Higher H/L ratios have been linked to reduced survival in birds and poorer immune function, particularly during extreme stress (Wingfield & Romero, 2001). Conversely, severe stress can sometimes result in lymphocytosis, leading to a decrease in the H/L ratio (Maxwell, 1993).

2.6 Insulin's Role in Glucose and Energy Metabolism

Insulin is widely recognized as the master regulator of glucose metabolism. It promotes anabolic processes like glycogenesis and lipogenesis, while inhibiting gluconeogenesis and glycogenolysis (Saltiel & Kahn, 2001). In the liver, insulin inhibits glucose-6-phosphatase activity and regulates enzymes such as phosphofructokinase and glycogen synthase, which reduces hepatic glucose production (HGP) (Ortmeyer et al., , 1997). Insulin also indirectly suppresses HGP by inhibiting lipolysis in adipose tissue, thereby lowering circulating free fatty acids and glycerol, which reduces hepatic glucose output (Sindelar et al., 1997). In skeletal muscle, insulin facilitates glucose uptake by converting glucose into glycogen and promoting fatty acid uptake into adipocytes, which are stored as triglycerides for long-term energy use (Kohn et al., 1996)

The stimulation of glucose uptake by insulin is mediated through both phosphatidylinositol (PI) 3-kinase–dependent and independent pathways, which regulate the translocation of glucose transporters like GLUT12 to the plasma membrane (Watson, Kanzaki, & Pessin, 2004; Saltiel & Pessin, 2003). These processes are essential for maintaining energy balance and ensuring a steady supply of ATP for cellular functions. Insulin’s effects also extend to the central nervous system, where it regulates energy balance and increases energy expenditure, especially during hyperinsulinemic conditions. However, prolonged exposure to glucocorticoids like cortisol can impair insulin's action by reducing insulin receptor sensitivity. Cortisol

promotes gluconeogenesis in the liver, which can lead to insulin resistance and metabolic disorders.

2.7 Role of GLUT12 in Skeletal Muscle and Insulin Sensitivity

Insulin regulates glucose uptake by target tissues through specific glucose transporters (GLUTs), with GLUT1, GLUT3, and GLUT8 being expressed in skeletal muscles (Kono et al., 2005). GLUT12 has emerged as an alternative glucose transporter in chickens, which lack GLUT4, the primary insulin-sensitive GLUT in mammals (Seki et al., 2003).

Further studies show that insulin stimulation leads to GLUT12's translocation to the plasma membrane in skeletal muscle, similarly to GLUT4 in mammals (Purcell et al., 2011). This translocation enhances glucose uptake, making GLUT12 crucial for maintaining glucose homeostasis in birds. Insulin-stimulated glucose transport has been observed in chickens, but primarily in skeletal muscle, not in cardiac muscle or adipose tissue, indicating a specialized role for GLUT12 (Chadt & Al-Hasani, 2020). Exercise training in mammals has also been shown to increase GLUT12 expression, suggesting that this transporter plays a key role in glucose transport under energy-demanding conditions (Stuart et al., 2010).

2.8 Breed-Specific Variations in Insulin Regulation and Glucose Metabolism in Birds

Broilers and Silky fowls respond differently to exogenous insulin injections. After insulin administration, serum insulin levels in broilers decrease significantly, whereas Silky fowls show stronger recovery (Ji et al., 2020). Real-time PCR analysis of mRNA expression indicates that GLUT2 is primarily expressed in the liver, while GLUT12 is predominantly found in skeletal muscle (Ji et al., 2020).

Moreover, insulin receptor (IR) and neuropeptide Y (NPY) mRNA expression also varies between the two breeds. Broilers show higher insulin sensitivity in the liver, while Silky fowls have elevated IR mRNA levels in the pancreas (Ji et al., 2020). These variations underscore the genetic differences in insulin regulation and glucose metabolism between breeds.

2.9 Conclusion

The interplay between cortisol and insulin during stress, particularly during slaughter, significantly impacts metabolic regulation and animal welfare. Cortisol's role in modulating energy balance, coupled with insulin's function in glucose metabolism, creates a complex dynamic that influences meat quality and overall health outcomes in poultry. Understanding these hormonal interactions can lead to improved practices in poultry management and slaughter, enhancing both welfare and productivity. Additionally, insulin's regulation of glucose metabolism through glucose transporters like GLUT12, especially in the absence of GLUT4, highlights key metabolic adaptations in chickens. The interaction between insulin and the HPA axis adds complexity to energy balance regulation, with breed-specific differences in insulin sensitivity further emphasizing the importance of genetics in metabolic health.

Chapter 3: Materials and Methods

This chapter outlines the materials and methods used in two consecutive studies: the first examining the interplay between cortisol and insulin during slaughter in birds, and the second investigating the effect of insulin on the mRNA expression of genes involved in glucose and energy metabolism.

Part 1: Interplay Between Cortisol and Insulin During Slaughter in Broiler, Sonali Chicken and Quail

3.1 Study Area

The study was conducted in the Physiology Laboratory of Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh. A total of 45 healthy birds were used, divided equally among three species (broiler chickens, Sonali chickens, and quail, 15 each). The birds, collected from the live bird market, had no more than a 10% body weight variation within each species. The birds were kept in cages for eight hours in the Physiology laboratory of CVASU.

3.2 Experimental Design

3.3 Sample Collection and Processing

Blood Sample Collection:

Using proper restraining techniques, the blood samples were collected from 8 AM to 10 AM to minimize diurnal variation. Venipuncture of the jugular vein was performed under aseptic conditions using a sterile syringe and transferred into EDTA-coated vacutainers (for hematology) and serum vacutainers (for biochemistry). Approximately 5 ml of blood was collected per animal.

The procedure was as follows:

Control group: Blood was collected before and 30 minutes after NaCl administration.

Treatment groups: Blood was collected before and 30 minutes after insulin administration.

The collected blood was processed as follows:

Serum Preparation: Blood was allowed to clot at room temperature for 2 hours, then centrifuged at 3000 rpm for 15 minutes. The serum was aliquoted and stored at -20°C until further analysis.

EDTA-treated Samples: Blood collected in EDTA tubes was used to estimate hemoglobin and make blood smears to evaluate the heterophil: lymphocyte (H: L) ratio.

3.4 Biochemical and Hormone Analysis

Glucose: Serum glucose levels were measured using a commercial kit (Glucose-GOD PAP, Randox) and a biochemical analyzer (Humalyzer-3000, Germany).

Hemoglobin: Hemoglobin levels were measured using a Sahli hemoglobinometer, following standard procedures.

Cortisol: Serum cortisol levels were measured using a commercial ELISA kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

3.5 Heterophil:Lymphocyte (H:L) Ratio

Direct blood smears were prepared, stained with Wright-Giemsa stain, and examined under a microscope. The H:L ratio was determined by counting 100 leukocytes and categorizing them as heterophils or lymphocytes.

3.6 Data Analysis

Data were stored in MS Excel 2007 and analyzed using STATA-11 software. Then, the levels of glucose, hemoglobin, and cortisol were expressed as mean and standard error and compared against the insulin-administered and non-administered groups.

3.7 Ethical Considerations

All experimental procedures were conducted in accordance with the ethical guidelines of the CVASU Animal Ethics and Experimentation Committee (AEEC). The

committee approved the study protocol, ensuring that the welfare and humane treatment of the birds were maintained throughout the study.

Part 2: Effect of Insulin on the mRNA Expression of Genes Involved in Glucose and Energy Metabolism

3.8 Study Area and Animals

This phase specifically focused on Sonali chickens (age 65 days, body weight 900±50 gm) which were collected from contact farm.

3.9 Experimental Design

The chickens were randomly assigned to three groups (n=5 per group) and fasted for 8 hours prior to treatment:

Control Group: The control group was administered phosphate-buffered saline (PBS) subcutaneously to serve as a neutral vehicle, ensuring no pharmacological effect on the animals while maintaining experimental consistency. This choice allowed for proper comparison with the treatment groups.

Treatment Group 1: Subcutaneously administered 4 IU of insulin (Ansulin 30/70, Square Pharmaceuticals Ltd., Bangladesh).

Treatment Group 2: Administered 8 IU of insulin subcutaneously.

3.10 Sample Collection

Thirty minutes after injection, blood was collected from the birds of these three groups by decapitating them at the atlanto-occipital joint. The blood was collected into vacutainers, and serum was separated by centrifuging the coagulated blood at 3000 rpm for 30 minutes. The serum was stored at -80°C prior to the cortisol assay.

Tissue Sample Collection:

Liver and pancreas samples were collected immediately after decapitation. The tissues were snap-frozen in liquid nitrogen and stored at -80°C until further processing.

3.11 RNA Extraction and cDNA Synthesis

Total RNA was extracted from the liver and pancreas using an easy-BLUE™ Total RNA extraction kit (intronbio, USA) according to the manufacturer's protocol, and the RNA concentration was measured using a spectrophotometer (Nanodrop, Thermoscientific, USA).

For cDNA synthesis:

- 500 ng of total RNA was reverse-transcribed using the ABScript II cDNA first-strand synthesis kit (Abclonal, USA).
- The reverse transcription was achieved by 42°C for 60 min, and 80°C for 5 min.

3.12 Quantitative Real-Time PCR (qPCR)

The mRNA expression levels of target genes (GLUT12 and IR in the liver; GLUT12 and insulin in the pancreas) were quantified using a 7500 Fast Real Time PCR System (Applied Biosystems, USA). The reaction mixture was prepared using GoTaq qPCR Master Mix (Promega, Japan) according to the manufacturer's protocol in a 25 µl reaction volume.

The qPCR conditions were:

- Initial denaturation at 95°C for 10 minutes.
- 40 cycles of 95°C for 5 seconds, annealing at 60°C for 30 seconds, and elongation at 90°C for 15 seconds.

The ß-actin/ribosomal protein s17 (s17) was used as the reference gene to normalize the expression of the target gene. The relative quantification of the target genes and reference genes was evaluated according to standard curves. The relative gene expression was calculated using the calibration curve method, where CT is the threshold cycle. The primer sequences used are listed below:

Gene	Accession No	Forward Primer	Reverse Primer	Amplicon (bp)
GLUT12	XM_419733.7	TGGGGTCTCACA CAGAGAGT	GGACGAGCCAAG ACATTGGT	120
IR	XM_001233398.7	CAGTGATGTGTA CGTTCCCGA	CCAGCTCTCCCTT CACGATG	131
Insulin	NM_205222.3	GGAGAGCGTGGC TTCTTCTA	CAAGGGACTGCT CACTAGGGG	72
β-actin	NM_205518.2	TGGCAATGAGAG GTTCAGGT	ACCACAGGACTC CATACCCAA	70

3.13 Cortisol Assay

Serum cortisol levels were measured using an immunofluorescence analyzer (Anbio Biotech, China) according to the manufacturer's protocol. Briefly, 100 µl of serum was loaded into the respective well of the cortisol kit and incubated for 15 minutes. After incubation, the cortisol concentration was measured by the analyzer.

3.14 Statistical Analysis

The data were stored and managed using MS Excel 2007 and analyzed using STATA 11 and R software. The following statistical tests were employed:

- Descriptive Statistics: Mean and standard error were calculated for all measured parameters.
- Comparative Analysis: Differences between groups (control, 4 IU insulin, 8 IU insulin) were analyzed using Tukey's HSD test.
- Significance: Statistical significance was set at $p < 0.05$.

3.15 Ethical Considerations

As in Part 1, all procedures were conducted in accordance with AEEC guidelines.

Chapter 4: Results

This chapter presents the findings from both studies: the first on the interplay between cortisol and insulin during bird slaughter and the second on the effect of insulin on the mRNA expression of genes involved in glucose and energy metabolism.

Part 1: Interplay Between Cortisol and Insulin During Slaughter in Birds

4.1 Changes in Cortisol and Insulin Levels Post-Insulin Administration

4.1.1 Broiler Chickens

Cortisol levels in broiler chickens increased significantly with the administration of 4 IU insulin compared to the control group and 8 IU insulin group (Figure 1)

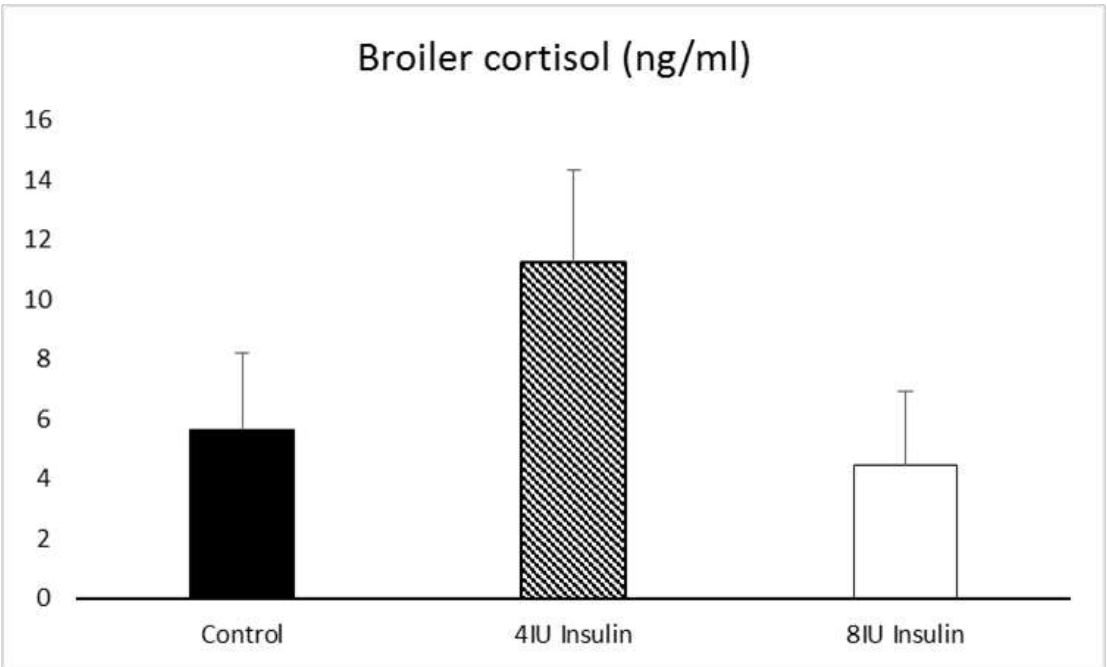

Figure 1: Cortisol level in broiler chicken after insulin administration

4.1.2 Sonali Chickens

Cortisol levels in Sonali chickens decreased significantly with both 4 IU and 8 IU insulin administration, indicating a stress-mitigating effect of insulin in this species (Figure 2).

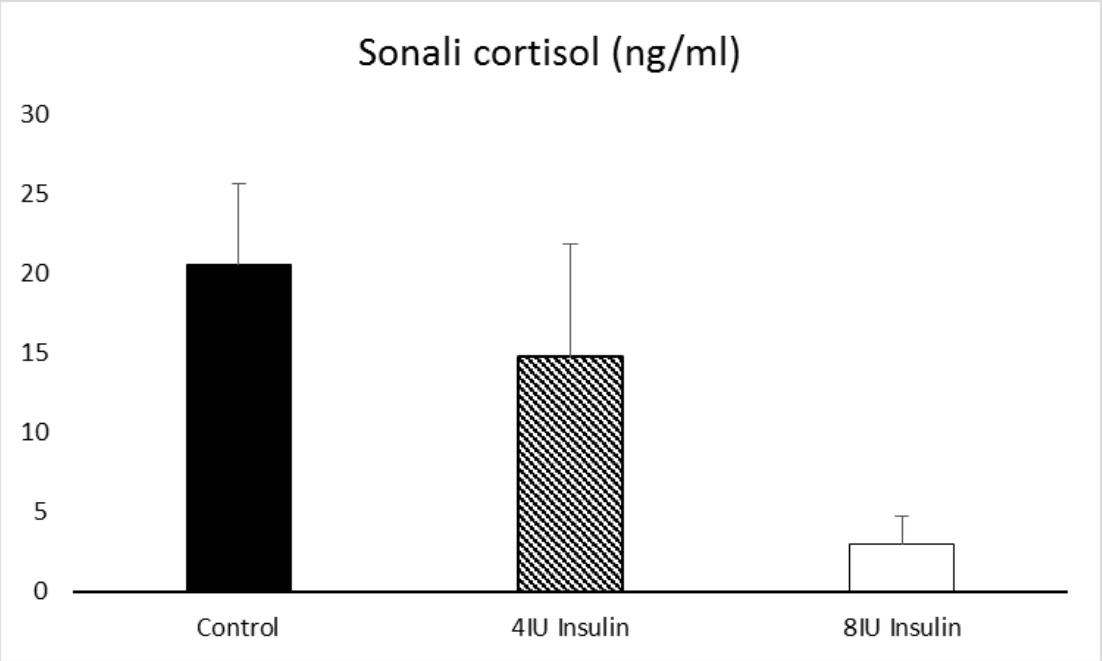

Figure 2: Cortisol level in Sonali chicken after insulin administration

4.1.3 Quail

Cortisol levels in quail increased with 4 IU and 8 IU insulin administration (Figure 3).

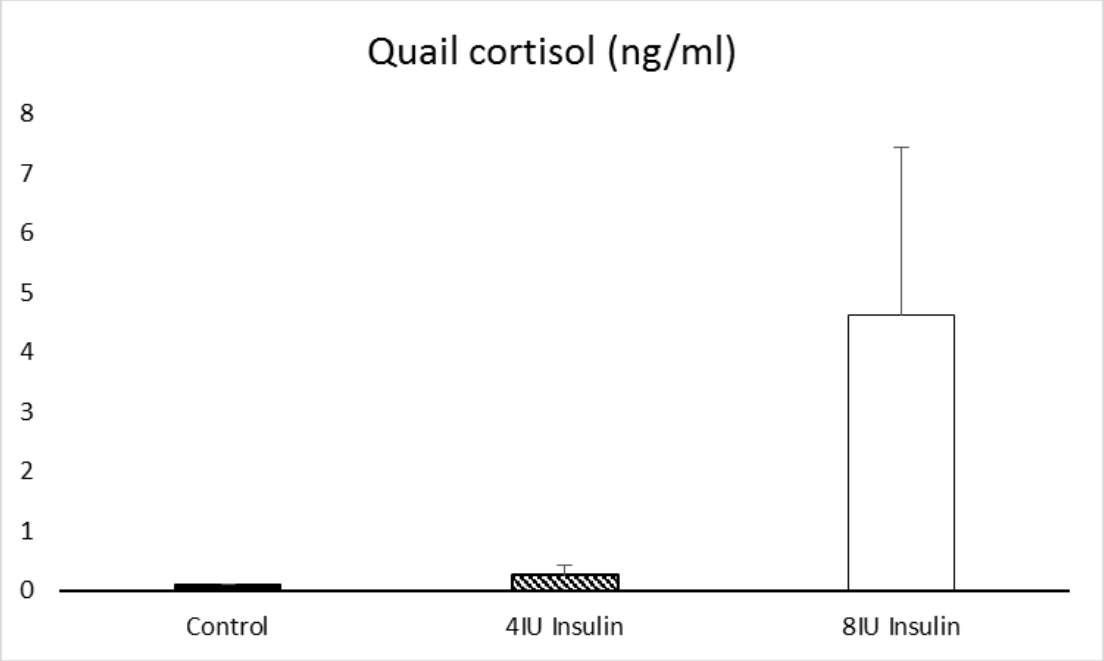

Figure 3: Cortisol level in Quail after insulin administration

4.2 H:L Ratio Levels Post-Insulin Administration

4.2.1 Broiler Chickens

The heterophil:lymphocyte (H:L) ratio in broiler chickens decreased significantly after insulin administration at both dose level (Figure 4).

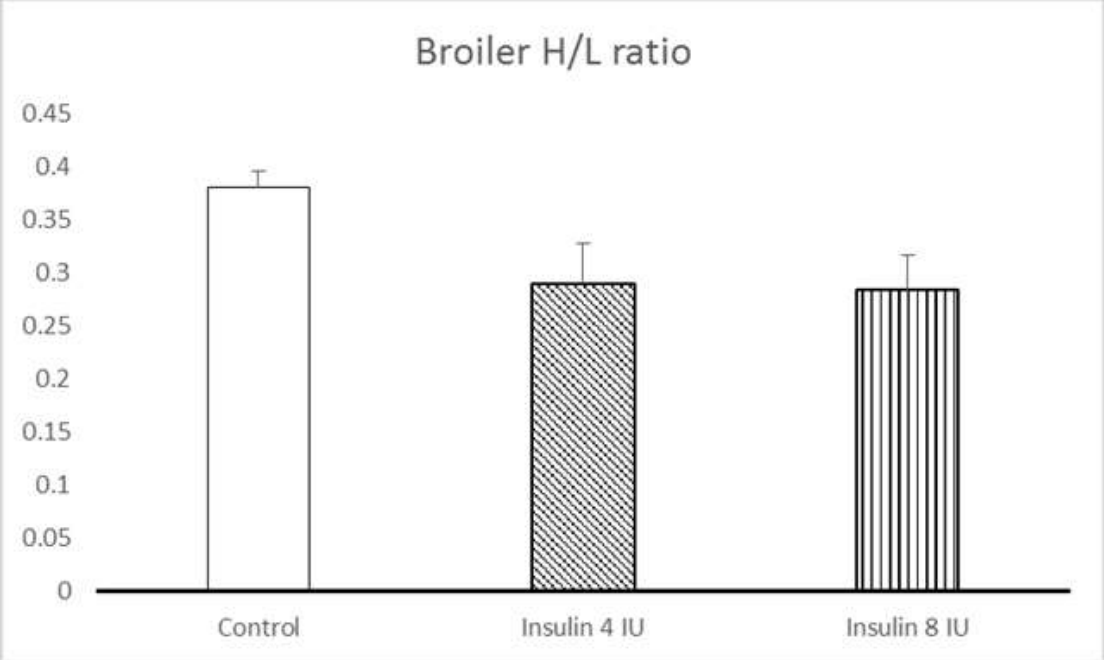

Figure 4: H:L ratio in broiler chicken after insulin administration

4.2.2 Sonali Chickens

The H:L ratio in Sonali chickens decreased significantly at both dose levels of insulin administration (Figure 5).

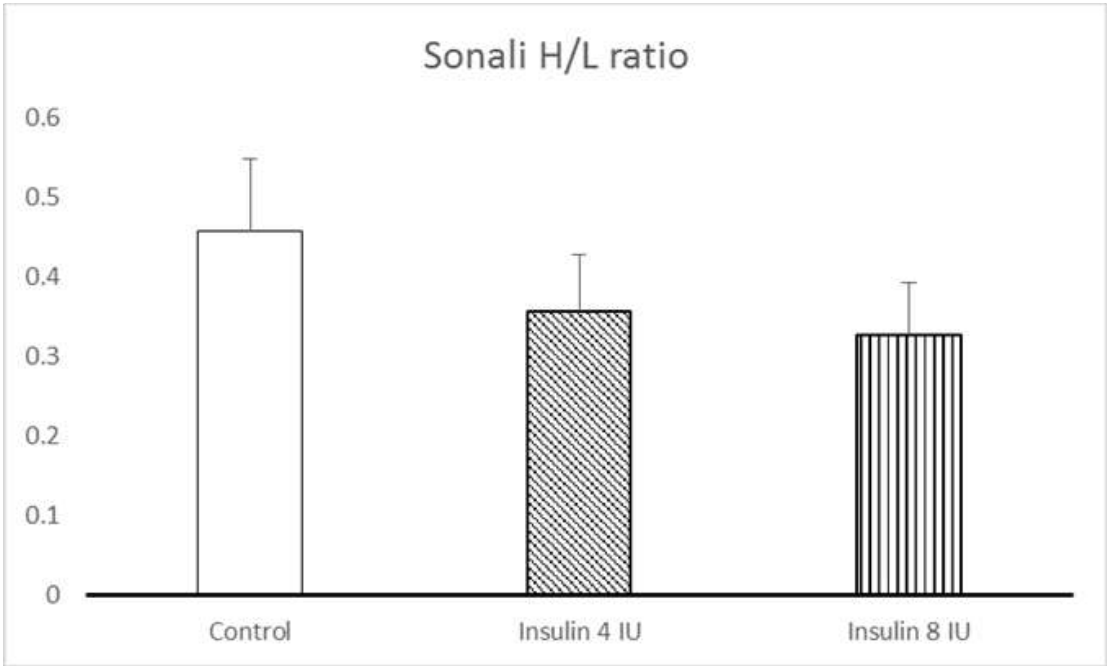

Figure 5: H:L ratio in sonali chicken after insulin administration

4.2.3 Quail

The H:L ratio in quail decreased significantly at both dose levels of insulin administration. (Figure 6).

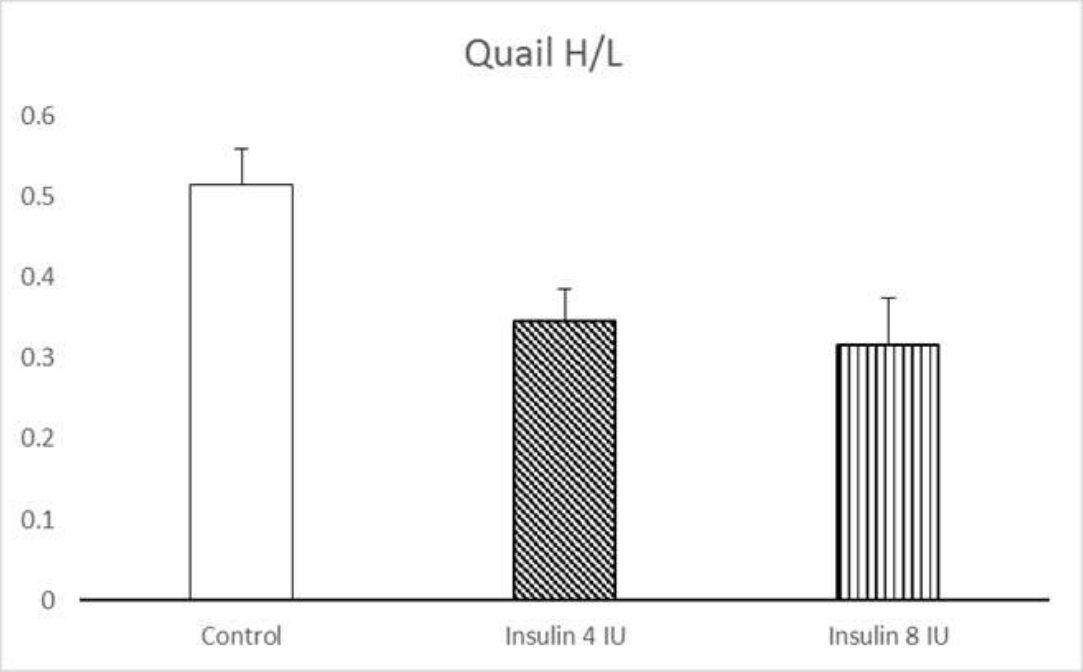

Figure 6: H:L ratio in Quail after insulin administration

4.3 Glucose and Hemoglobin Levels Post-Insulin Administration

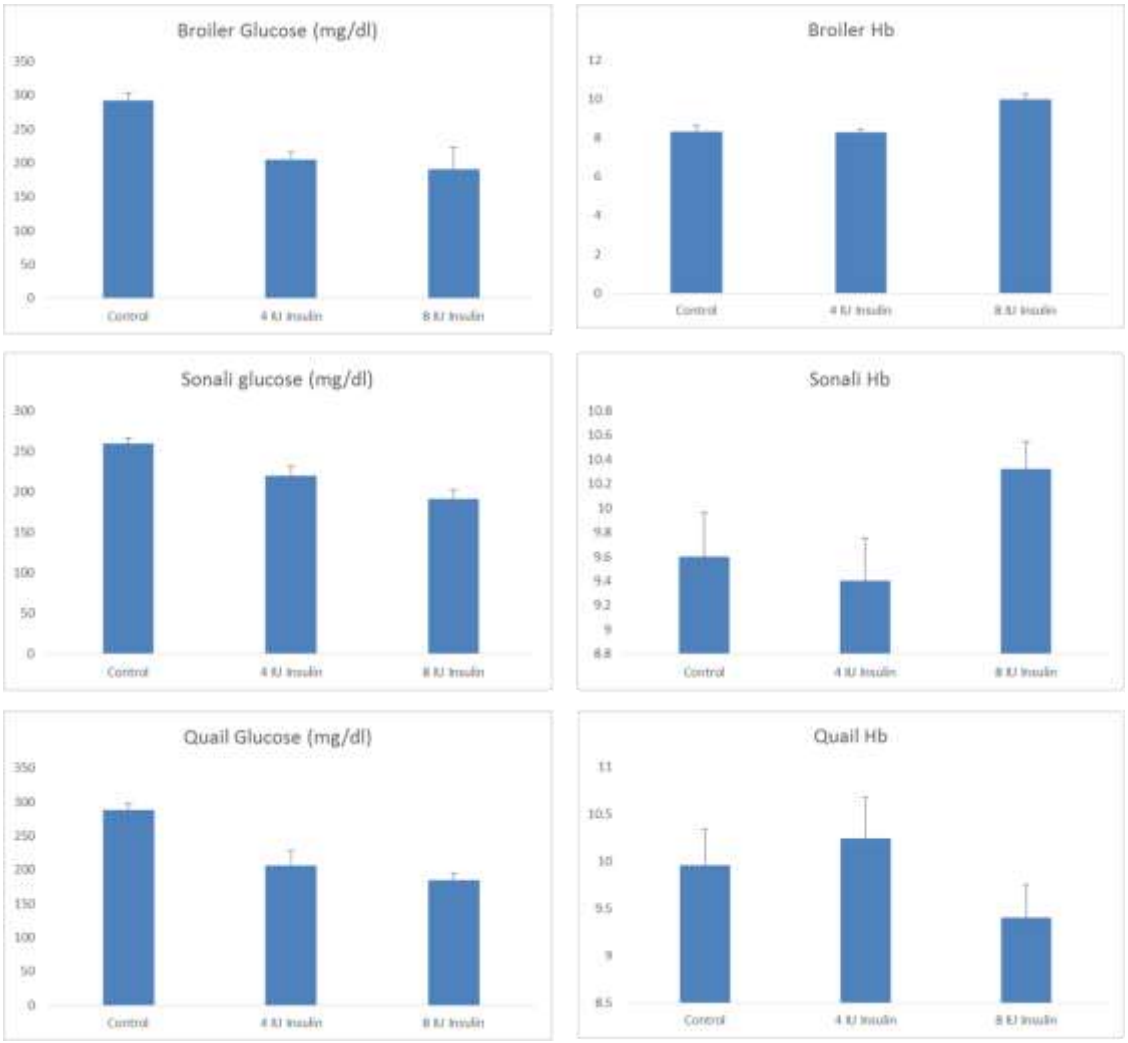

Figure 7: Glucose and hemoglobin level after insulin administration in bird species

4.3.1 Glucose Levels

Serum glucose levels significantly decreased in all species after insulin administration ($p < 0.05$, ANOVA)

4.3.2 Hemoglobin Levels

Changes varied in hemoglobin level according to species after insulin administration

Part 2: Effect of Insulin on the mRNA Expression of Genes Involved in Glucose and Energy Metabolism

4.4 Liver Tissue:

4.4.1 GLUT12 mRNA Expression

Hepatic mRNA expression of GLUT12 increased significantly at 30 minutes post-insulin administration, with the 4 IU insulin group showing a higher increase compared to the control and 8 IU groups (Figure 8).

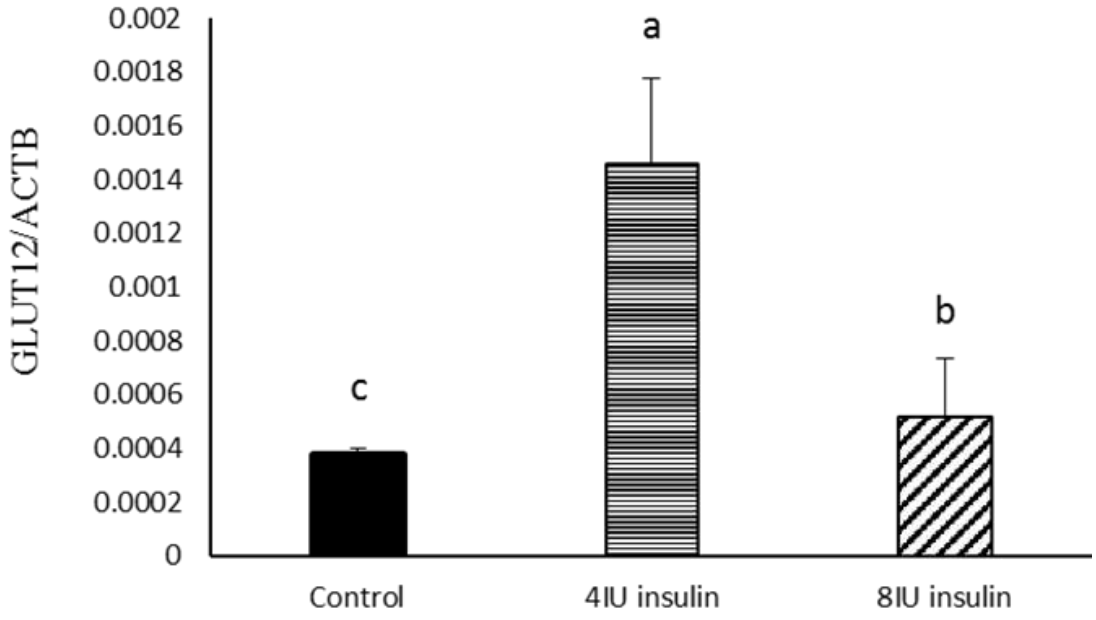

Figure 8: Hepatic mRNA expression of GLUT12 at 30 minutes after insulin treatment in the sonali chicken

4.4.2 IR mRNA Expression

Hepatic mRNA expression of the insulin receptor (IR) also increased significantly at 30 minutes post-insulin administration, with the 4 IU insulin group exhibiting a greater increase compared to the control and 8 IU groups (Figure 9).

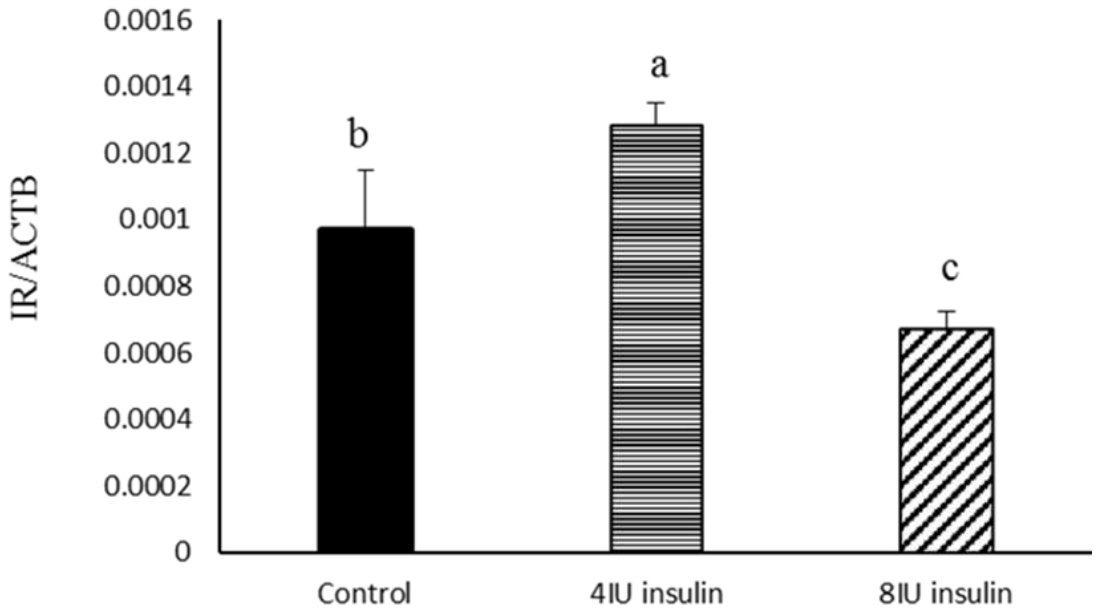

Figure 9: Hepatic mRNA expression of IR at 30 min after insulin treatment in the Sonali chicken

4.5 Pancreas Tissue

4.5.1 GLUT12 mRNA Expression

Pancreatic mRNA expression of GLUT12 increased significantly with insulin treatment, with the 4 IU insulin group showing a higher increase compared to the control and 8 IU groups (Figure 10).

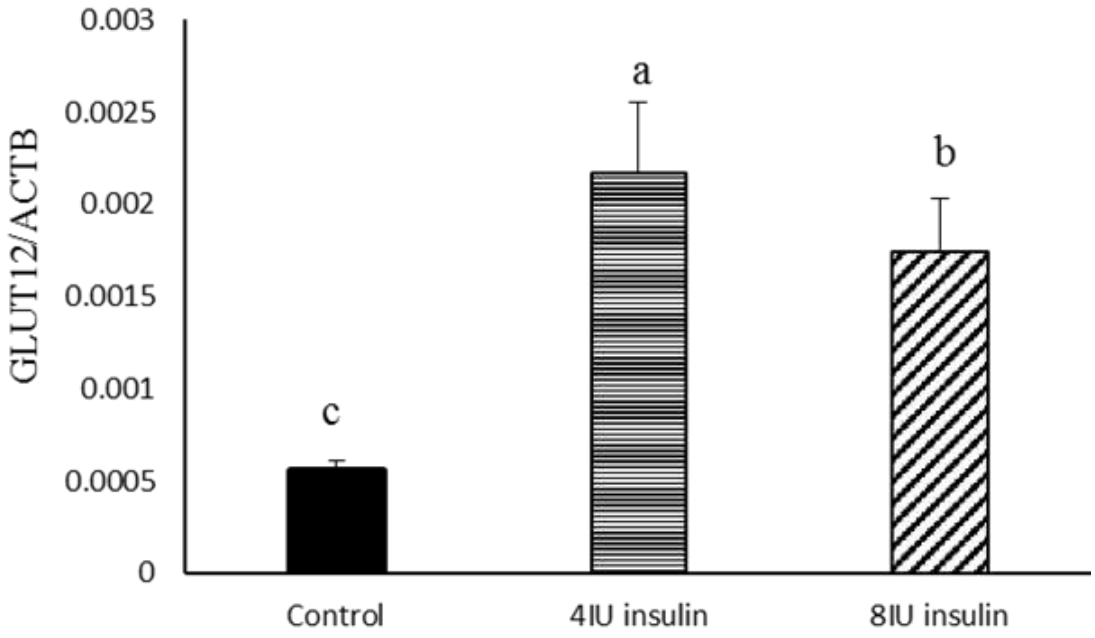

Figure 10: Pancreatic mRNA expression of GLUT12 at 30 min after insulin treatment in the Sonali chicken

4.5.2 Pancreatic mRNA Expression

Pancreatic mRNA expression of insulin increased significantly post-insulin administration, with the 4 IU insulin group exhibiting the highest increase compared to the control and 8 IU groups (Figure 11).

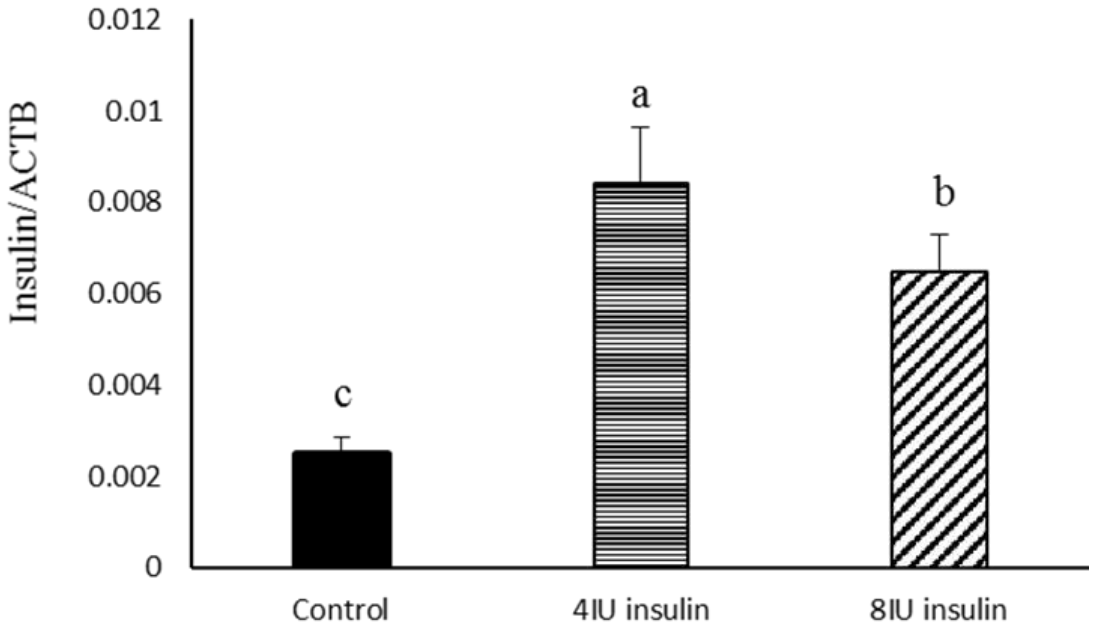

Figure 11: Pancreatic mRNA expression of insulin at 30 min after insulin treatment in the Sonali chicken

4.6 Cortisol Concentration Post-Insulin Administration in Sonali Chickens

Cortisol concentration in Sonali chickens decreased significantly at 30 minutes post-insulin administration. 4IU insulin group decreased Cortisol concentration than 0(control) group but not less than 8 IU insulin group.

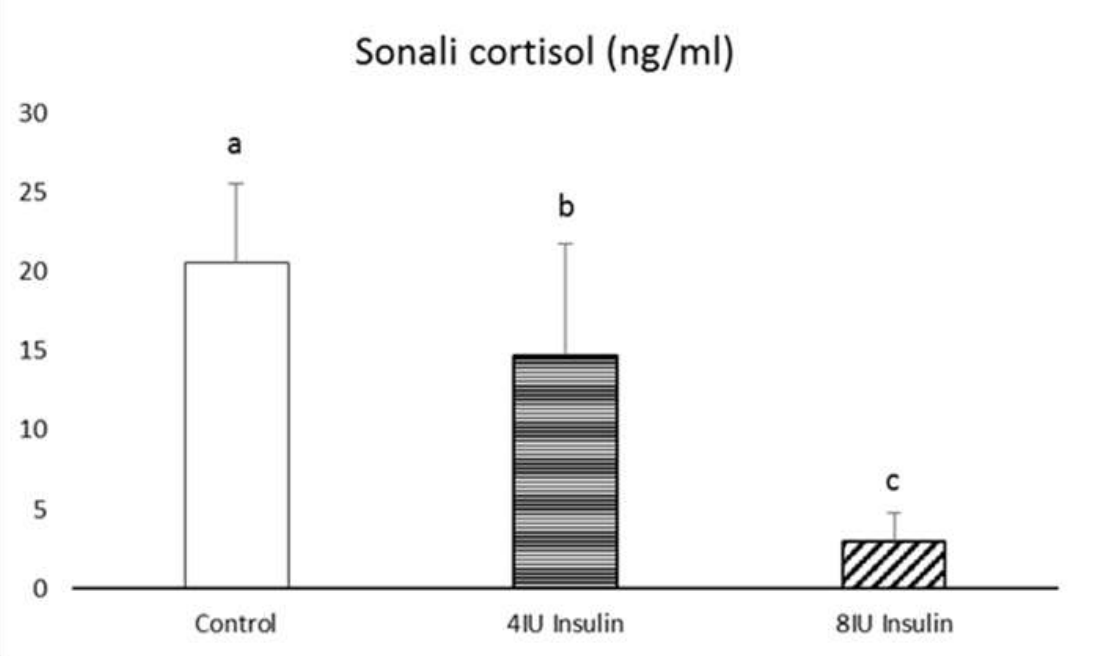

Figure 12: Cortisol concentration at 30 minutes after insulin treatment in the Sonali chicken

4.7 Summary of Key Findings

1. Insulin administration significantly alters cortisol levels in birds during slaughter, with species-specific responses.
2. Insulin reduces stress (as indicated by cortisol levels and H:L ratio) in Sonali chickens, potentially improving welfare during slaughter.
3. Insulin significantly upregulated GLUT12 and IR mRNA expression in the liver, and GLUT12 and insulin mRNA in the pancreas of Sonali chickens.
4. The 4 IU insulin dose showed the most pronounced effects on both hormonal and genetic parameters.

These findings provide insights into the potential role of insulin in modulating stress responses and metabolic regulation in birds, with implications for improving animal welfare and productivity in the poultry industry.

Chapter 5: Discussion

Part 1: Interplay Between Insulin and Cortisol in Birds

In the first part of the study, we found there has been a good interplay between insulin and cortisol in Sonali chickens, a popular breed known for their high productivity and adaptability, is often subjected to stressors that can negatively affect its health and performance. Managing stress, particularly through the regulation of cortisol, is essential for maintaining the well-being of these birds. Our findings showed that cortisol levels significantly decreased in Sonali chickens after insulin administration (both 4 IU and 8 IU doses), suggesting that insulin has a stress-mitigating effect in this breed. This decrease in cortisol supports previous studies suggesting that insulin, beyond its role in glucose regulation, may also modulate the stress response by influencing cortisol levels (Sohail et al., 2010).

Insulin is predominantly recognized for its role in glucose homeostasis and as a regulator of carbohydrate and lipid metabolism. Studies conducted on rodents have shown that insulin administration can reduce stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, which is responsible for regulating cortisol production. By dampening the HPA axis response, insulin may help mitigate the release of cortisol in response to stressors (Janssen, 2022).This aligns with our finding of reduced cortisol levels in Sonali chickens following insulin administration.

Cortisol, often referred to as the "stress hormone," is released in response to various stressors. Chronically elevated cortisol levels can have detrimental effects on the health and performance of Sonali chickens. Insulin has been found to interact with cortisol through multiple pathways, suggesting a potential regulatory role. Insulin administration has been shown to decrease cortisol production by influencing the activity of key enzymes involved in cortisol synthesis and metabolism. Additionally, insulin may indirectly affect cortisol levels by promoting glucose uptake in tissues, thus preventing excessive glucose availability, which can stimulate cortisol secretion (Vijayan et al., 1997). However, in broiler chickens and quail, cortisol levels increased significantly following insulin administration, indicating species-specific differences in how insulin interacts with the HPA axis and modulates stress responses.

Beside that, heterophil:lymphocyte ratio (H:L) was decreased significantly at all the studied bird species which is quite interesting. The H:L is a commonly used indicator of stress and immune function in poultry. Insulin, primarily recognized for its role in glucose regulation, has been found to exert modulatory effects on the immune system. It plays a vital role in maintaining immune homeostasis and promoting immune cell function. Insulin receptors are present on immune cells, including heterophils and lymphocytes, indicating that insulin may directly influence their activity. Our results showed that insulin administration decreased the H:L ratio significantly across all species, suggesting a stress-reducing effect.Insulin administration has been shown to enhance the proliferation and activity of lymphocytes while reducing the activation of heterophils, thereby potentially balancing the H:L (Wasti et al., 2020).

Insulin has been found to decrease the neutrophil:lymphocyte ratio in various animal models, suggesting a potential similar effect in Sonali chickens. Stressors can disrupt the delicate balance of the immune system, leading to alterations in the H:L. Chronic stress can elevate the H:L, indicating increased stress and compromised immune function. Insulin has been shown to modulate the stress response by reducing the release of stress hormones, such as cortisol, and dampening the activation of the hypothalamic-pituitary-adrenal (HPA) axis. By mitigating the stress response, insulin may indirectly contribute to maintaining a balanced H:L in Sonali chickens (Müller et al., 2011).

Part 2: Effect of Insulin on mRNA Expression of Genes Involved in Glucose and Energy Metabolism

The impact of insulin on the mRNA expression of the GLUT12 gene in the liver and insulin receptor gene in the pancreas of chickens is not well-studied, and thus, specific information on this topic is limited. However, based on our current understanding of insulin signaling and glucose metabolism in other vertebrates, we can propose potential mechanisms by which insulin may affect the expression of these genes in chickens

Insulin is known to regulate glucose metabolism in various tissues, including the liver in mammals, insulin promotes glucose uptake by upregulating the expression of glucose transporter genes, such as GLUT2 and GLUT4, in insulin-responsive tissues (Chadt and Al- Hasani 2020)

It is plausible that insulin may exert a similar effect on GLUT 12 gene expression in the liver of chickens Insulin signaling pathways, such as the PI3K/Akt pathway, may be involved in the regulation of GI UT12 gene expression by insulin (Rosa et al. 2011) Further investigations are needed to explore the direct impact of insulin on the transcriptional regulation of GLUT 12 in the chicken liver.

Exogenous insulin interacts with insulin receptors on the surface of liver cells, initiating a cascade of intracellular signaling events. This leads to the activation of downstream signaling molecules, such as insulin receptor substrates (IRS), phosphoinositide 3-kinase (PI3K), and protein kinase B (PKB/Akt).

The activated signaling pathways result in the activation of transcription factors, such as forkhead box protein O1 (FoxO1) and sterol regulatory element-binding protein Ic (SREBP- lc) (Deng et al, 2012). These transcription factors bind to specific regions of the insulin receptor gene (INSR) promoter, enhancing its transcription and resulting in increased insulin receptor mRNA expression. In our study, insulin administration significantly increased the expression of insulin receptor mRNA in the liver, with the highest expression observed in the 4 IU insulin group.

Insulin signaling and increased insulin receptor expression in liver cells can also trigger feedback mechanisms. These include the activation of negative feedback loops, such as the phosphorylation and inhibition of IRS proteins, to regulate the intensity and duration of the insulin signaling response.

Exogenous insulin administration can activate insulin receptors in the pancreatic beta cells. This activation triggers intracellular signaling cascades that lead to the activation of transcription factors, including pancreatic and duodenal homeobox ! (PDX-1) and MafA. These transcription factors bind to specific regions of the insulin gene (INS) promoter promoting its transcription and resulting in increased insulin mRNA expression (Wang et al. 2001). Our results confirmed that insulin administration significantly increased the expression of both GLUT12 and insulin mRNA in the pancreas, with the 4 IU group exhibiting the highest levels of mRNA expression.

Insulin itself acts as a key regulator of beta cell function. Exogenous insulin administration can provide additional insulin to pancreatic beta cells, which can improve their functionality and enhance insulin production. This enhanced beta cell function can contribute to increased insulin mRNA expression.

Exogenous insulin administration helps regulate blood glucose levels When glucose levels are controlled, it reduces the inhibitory effect of high blood glucose on insulin gene expression in the pancreas. This allows for the upregulation of insulin mRNA expression in response to exogenous insulin administration.

Increased insulin levels resulting from exogenous insulin administration can trigger negative feedback loops that regulate insulin synthesis and secretion. These feedback loops involve the activation of various signaling pathways that can modulate the expression of insulin mRNA in the pancreatic beta cells (Miller and O'Callaghan, 2002)

The HPA axis is involved in the regulation of cortisol production. Insulin has been shown to inhibit the HPA axis by suppressing the release of corticotropin-releasing hormone (CRH) from the hypothalamus and adrenocorticotropic hormone (ACTH)

from the pituitary gland. This inhibition subsequently reduces the stimulation of cortisol synthesis and secretion by the adrenal glands (Papadimitriou & Priftis, 2009)

High blood glucose levels can activate the HPA axis and stimulate cortisol production (Aronoff et al., 2004). By maintaining glucose homeostasis, exogenous insulin reduces the need for the HPA axis to respond to glucose dysregulation, leading to decreased cortisol concentration. In this study, cortisol concentrations in Sonali chickens decreased significantly following insulin administration, indicating insulin's role in modulating cortisol production and maintaining stress homeostasis.

Insulin exhibits anti-inflammatory properties, and chronic inflammation can contribute to cortisol release. By reducing inflammation, exogenous insulin may indirectly lower cortisol levels. Insulin and cortisol have complex interactions within the body. Insulin can inhibit cortisol release by interfering with the enzymatic processes involved in cortisol synthesis or by affecting the sensitivity of adrenal gland receptors to ACTH stimulation.

It is important to note that the proposed mechanisms are speculative and require experimental validation in the context of chicken physiology Future research employing molecular techniques, such as gene expression analysis, promoter studies, and mechanistic studies of insulin signaling pathways in chickens, would provide a more comprehensive understanding of the specific impact of insulin on the mRNA expression of GLUT12 and the insulin receptor gene in the liver and GLUT12 and insulin in pancreas, respectively.

Chapter 6: Conclusion

This thesis investigated the interplay between cortisol and insulin during the slaughter process in birds and the effect of insulin on the mRNA expression of genes involved in glucose and energy metabolism. The findings revealed significant species-specific responses to insulin administration, with Sonali chickens showing a marked decrease in cortisol levels and heterophil to lymphocyte (H;L) ratios, indicating insulin's stress-reducing potential in this breed. Additionally, the upregulation of key genes, such as GLUT12 and insulin receptor (IR) in the liver and GLUT12 and insulin in the pancreas, further underscores insulin's role in enhancing glucose uptake and utilization in Sonali chickens. These results highlight insulin's dual role in both stress mitigation and metabolic regulation, which could improve animal welfare and production efficiency.

The study also found that a 4 IU dose of insulin was more effective than an 8 IU dose in modulating both hormonal and genetic parameters. This suggests that moderate insulin doses are optimal for achieving the desired physiological effects without triggering negative feedback mechanisms. This provides a foundation for developing insulin-based interventions aimed at reducing stress and enhancing metabolic health in poultry, particularly during the high-stress slaughter process. Moving forward, future research should investigate the long-term impacts of insulin administration across various poultry species and refine dosing strategies to optimize outcomes, ultimately contributing to more ethical and effective poultry management practices.

Chapter 7: Recommendations

Based on the findings of this thesis, several recommendations can be made to enhance poultry management practices, improve animal welfare, and optimize productivity:

1. Optimizing Insulin Dosage for Stress Reduction:

The results of this study suggest that moderate insulin doses (4 IU) are more effective in reducing stress and enhancing metabolic gene expression than higher doses (8 IU). It is recommended that future research focuses on optimizing insulin dosing regimens for different poultry species to maximize the stress-reducing effects without triggering negative feedback mechanisms. This could improve animal welfare during stressful periods, such as slaughter, and contribute to better productivity.

2. Species-Specific Management Practices:

The study demonstrated species-specific responses to insulin administration, indicating that a one-size-fits-all approach may not be effective. Future research should conduct species-specific trials to determine the most effective insulin dosage and administration protocols for their flocks. This approach ensures that the benefits of insulin administration are maximized for each species.

3. Implementation of Insulin Administration in Poultry Management:

Given the significant reduction in cortisol levels and the H:L ratio in Sonali chickens, insulin administration could be considered as a welfare-improving intervention in poultry management, particularly during slaughter. It is recommended that poultry producers and researchers collaborate to investigate the practical application of insulin treatments in commercial settings, ensuring that protocols are safe, cost-effective, and scalable.

4. Integration with Other Stress-Reduction Strategies:

While insulin administration has shown promise in reducing stress, it should be integrated with other stress-reduction strategies for a holistic approach. These strategies can include environmental enrichment, proper handling techniques, and nutritional interventions. Combining multiple approaches can lead to synergistic effects and further enhance animal welfare.

5. Long-Term Impact Studies:

Further research is needed to investigate the long-term impacts of insulin administration on poultry. Studies should focus on growth rates, feed efficiency, reproductive performance, and overall health. Understanding the long-term effects will help in developing sustainable and effective management practices.

6. Exploration of Alternative Insulin Analogues:

Research should explore the use of alternative insulin analogs that may offer better efficacy or longer-lasting effects. These analogs could potentially provide more sustained stress reduction and metabolic benefits, reducing the need for frequent administration.

Broader Application Beyond Sonali Chickens: While Sonali chickens were the primary focus of this study, the differing responses in broiler chickens and quail suggest that insulin's effects may vary across species. Therefore, it is recommended that future research investigate the effects of insulin in a wider range of poultry breeds to assess its potential as a universal intervention for reducing stress and enhancing metabolic efficiency.

By following these recommendations, poultry farms can leverage the findings of this thesis to enhance animal welfare, optimize productivity, and adopt more sustainable and ethical farming practices. Future research and continued collaboration with industry stakeholders will be crucial in refining these practices and ensuring their long-term success.

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Brief Biography

M. A. Rahman Rahim, a veterinarian from Chattogram, Bangladesh, completed his Secondary School Certificate in 2014 and Higher Secondary Certificate in 2016. He earned his Doctor of Veterinary Medicine (DVM) degree from Chattogram Veterinary and Animal Sciences University (CVASU) in December 2022 and is currently pursuing a Master of Science (MS) in Physiology at CVASU. His thesis research focuses on the effects of exogenous insulin on stress response and the expression of carbohydrate metabolic genes during slaughter in poultry. Dr. Rahim's research interests include hormonal regulation and environmental factors influencing gene expression to improve animal welfare and productivity. He aims to pursue a PhD to explore how these factors can be utilized to reduce disease risks and enhance the welfare of animal and human health. In addition to his academic pursuits, Dr. Rahim is currently serving as a veterinarian at the Veterinary Specialized Hospital and Diagnostic Center in Uttara, Dhaka, applying his veterinary expertise in a clinical setting to further his commitment to animal health and welfare.