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Physiological response of some anti-stressful agents in broilers exposed to dexamethasone

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Supervised by

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By

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بسم الله الرحمن الرحيم

رَبِّ أَوۡزِعۡنِيٓ أَنۡ أَشۡكُرَ نِعۡمَتَكَ ٱلَّتِيٓ أَنۡعَمۡتَ عَلَيَّ وَعَلَىٰ وَٰلِدَيَّ وَأَنۡ أَعۡمَلَ صَٰلِحٗا تَرۡضَىٰهُ وَأَدۡخِلۡنِي بِرَحۡمَتِكَ فِي عِبَادِكَ ٱلصَّٰلِحِينَ ١٩

صدق الله العظيم

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Abstract

The current study aims to reveal the effect of sodium butyrate (SB) and betaine (BET) as anti-stressful agents on some of the physiological parameters in the broiler chickens exposed to dexamethasone (DEX)-induced non-specialized stress in an experimental setting. Ninety-six broiler chicks (rose-type) at one day of age were reared in the animal house/ College of Veterinary Medicine- the University of Mosul from September to November 2021 in the appropriate conditions for this study. After that, they were divided into 4 groups, each one consisting of 24 birds and three replications, and two periods of 21 and 42 days of age. The first group was considered the control group while the second group was the DEX group, the broiler was injected with DEX every 3 days at an experimental dose of 1 mg/kg of body weight (subcutaneous). While the third and fourth groups were injected with DEX and treated with SB and BET at a dose of 1.2 g/kg feed, and 2 g/kg feed, respectively.

The results of this study showed that DEX adversely affected the feed conversion ratio (FCR) and final body weight in both periods. There was a significant decrease in weight gain and an increase in feed consumption at 21 days compared to the control group. A significant rise in liver weight and gizzard weight was observed at both periods and a rise in heart and bursa weight at 21 and 42 days. There was a significant increase in both the packed cells volume (PCV) and the stress index values of blood parameters in the DEX group in the 21 and 42 days of the bird's life, as well as an increase in the levels of liver enzymes (alanine aminotransferase ALT- aspartate aminotransferase AST) compared with the control group. The injection of DEX resulted in a significant decline in glutathione (GSH) levels and a significant rise in malondialdehyde (MDA) levels in serum, liver, spleen, and

bursa tissues. The stress biomarkers also showed a negative impact of the DEX by increasing the heat shock protein 90 (HSP90) and caspase-3 values, these findings were later reflected in the correlation between HSP90 and caspase-3 which increased significantly during the first period.

The addition of SB or BET to the feed of DEX-injected broilers enhanced growth performance by improving FCR values at 42 days of age, while SB lower significantly the liver weight at 21 days compared to the DEX group. At 42 days of age, these feed additives had similar effects by overcoming the negative effect of DEX by reducing liver weight compared to the DEX group. Recent results of this study indicate that the addition of SB or BET to the ration of DEX-injected broilers resulted in a significant decrease in PCV and stress index values in both periods as well as a significant decrease in the liver enzymes (ALT and AST) in the 21 days and at 42 days of age, BET decrease significantly these two enzymes was reduced compared to DEX. The results also showed that the addition of SB or BET in feed for DEX-injected broilers resulted in a significant rise in GSH level and a significant decrease in MDA in serum in both periods compared to the DEX group and in 21 days the MDA values were returned to the control group. And for liver tissue both feed additions caused GSH to increase in both periods and reduced MDA level compared to DEX and return the MDA values in 21 and 42 days to the control group values. The third and fourth groups showed an increase in the level of GSH in spleen tissue in the 42 days compared with DEX and return the values to the control values; for MDA in spleen tissue, there was a significant decrease in third and fourth for both periods compared to DEX and return the values for the control values in the first period. There has also been a significant rise in the level of GSH in the bursa in groups three and four compared to DEX in 21 and 42 days and the values have been returned to control values in the 21 days, while MDA level in the bursa was

significantly decreased in groups three and four compared with DEX group in both periods and return values to control values in the 42 days. The results showed that the addition of both SB or BET in the stressed birds' resulted in a significant decrease in the HSP90 in both periods compared to the DEX group while the addition of SB had the greatest ability to reduce the level of HSP90 by the age of 21 days as well as the significant reduction of caspase-3 activity after one of the two feed additives to stressed birds compared with DEX in the two periods and returned the values for the 21-day control values.

The current study concluded that through both periods of the broilers DEX has a negative impact on growth performance, the PCV, the stress index, liver enzymes, the level of GSH and MDA in the serum, liver, spleen, bursa tissue, and on HSP90, and programmed cell death in the liver. The addition of SB or BET to the stressed broilers has anti-stressful and antioxidant properties by improving and enhancing all the different physiological values that were used in this study, but the results of these additions are better in a 21-day period than in a 42-day period.

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List of abbreviations

Abbreviation	Meaning
Abs	Absorbance
ALT	Alanine aminotransferase
APPs	Acute phase proteins
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
B.W.	Body weight
Bax	Bcl-2 associated x protein
Bcl-2	B-cell lymphoma 2
BET	Betaine
CASP	Caspase
CAT	Catalase
cDNA	Complementary DNA
CORT	Corticosterone
DEX	Dexamethasone
DLC	Differential leukocyte count
DNA	Deoxyribonucleic acid
FCR	Feed conservation ratio
GCs	Glucocorticoids
GPx	Glutathione peroxidase
GR	Glucocorticoids receptor

GSH	Glutathione
H/L	Heterophiles/Lymphocytes ratio
Hb	Hemoglobin
HCT	Hematocrit
HSP90	Heat shock protein 90
HSPs	Heat shock proteins
ICE	Interleukin-1b-converting enzyme
KDa	Kilo Dalton
MDA	Malondialdehyde
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen
P53	Tumor suppressor protein
PCV	Packed cell volume
PKa	Acid dissociation constant
RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RS	Reactive species
S/C	Sub cutaneous
STAT	Signal transducers of activated transcription
SB	Sodium butyrate
SOD	Superoxide dismutase
VLDL	Very-low-density lipoprotein

Chapter one

Introduction

In the year 2020, Billman recast the physiological notion of internal environment equilibrium as a self-process that enables an organism to preserve internal stability and adjust to changing external situations (Billman, 2020). While Tortora and Derrickson (2017) found negative feedback, the challenge was to preserve a stable range of values. When ecological changes occur, animals experience stress, which is coupled with bodily responses to reestablish homeostatic conditions (Scanes, 2016). Stress occurs when homeostasis is disrupted, and it refers to the overall biological reaction to any stimulation that threatens the bird's homeostasis in the manner of behavioral, physiological changes, or both, (Elitok and Bingüler, 2018). The fowl industry as a whole is subjected to a wide range of stressor stimuli, which causes oxidative stress in poultry in general and broilers in particular, resulting in decreased chicken production (Surai *et al*., 2019). When any type of stress occurs, as a response to perceived stress agents, the endocrine system is triggered to release stress hormones, resulting in a variety of physiological and behavioral responses aimed at preserving the balance of essential processes (Collier *et al*., 2017).

Dexamethasone (DEX) is a synthetic corticoid that begins stress-related signal transduction pathways and has the same impact as elevated corticosterone (CORT) levels. It is frequently used to investigate the effects of a number of non-specific stresses on the induction of stress in chickens (Calefi *et al*., 2016), and because it can change avian metabolic activity by inducing oxidative stress via the hypothalamic-pituitary-adrenal axis, it has frequently been used to create a prolonged stress model (chronic) to assess the adverse implications of physiological stress in poultry (Lv *et al*.,2018).

The reason that dexamethasone has been used as a stress moderator, injectable dexamethasone duplicates the deleterious effects of increased corticosterone (Maccari *et al*., 2003).

The HSPs family in general, and HSP90 in particular, which is linked to a rise in corticosterone levels, is another sign of elevated oxidants when subjected to stress. It possesses cytoprotective characteristics, acting as a molecular chaperone to help with protein folding and prevent aggregation when proteins are damaged by stress (Kalmar and Greensmith, 2009). It's important to note that cells that are stressed or apoptotic have a relationship with HSPs that are produced in response to increased free radicals in the cells. As a result, HSPs appear to play a role in apoptosis regulation at critical regulatory points (Kennedy *et al*., 2014).

Apoptosis was defined by Nicholson and Melino. (2013) as a widely known type of programmed cell death (cell suicide) used to eliminate undesirable or even more cells that are distinguished by plenty of morphologic changes caused primarily by the excitation of a group of cysteine proteases identified as caspase. Reactive species (RS) emerge from cellular oxidative participation in programmed cell death because apoptotic cells release large amounts of oxidizing agents (Nunes *et al*., 2003). As a result of increased ROS synthesis in the mitochondria, free radical invasion of lipid membranes might occur, resulting in membrane depolarization and caspase activation (Nazıroğlu, 2012).

An antioxidant, described by Halliwell, (2007) as "any chemical that delays, blocks, or removes oxidation to a biomolecule," to slow the oxidation reaction even at trace levels, and thereby plays a number of physiological roles in the body. So, a common nutritional method for increasing poultry output is to add antioxidants, also known as anti-stressful agents, to feed

supplements to safeguard the animal cells from the damage caused by the high production of oxidants (Panda and Cherian, 2013).

Sodium butyrate (SB) is a short-chain fatty acid that has molecular, biological, and tissue impacts. Whenever butyric acid loses its H^+ ion, it is converted to a butyrate ion as a result. Overall, sodium salt has a benefit over unbound acids in that it is without undesired odor and enables the feed production process to be simpler (Elnesr *et al*., 2020). According to some researchers, SB is capable of reducing lipid peroxidation by enhancing endogenous antioxidant enzymes as well as avoiding weight loss following corticosteroid rushes (Zhang *et al*., 2011).

Betaine (trimethylglycine) is an amino acid present in a range of food products such as wheat, spinach, and sugar beets, and also generated in the body naturally from its precursor, choline, as well as exogenously from the diet (Craig, 2004). And it has the ability to reverse the effects of many types of stress by offering a methyl group and having a zwitterionic reaction, which helps to preserve cell homeostasis in hot weather (Nutautaitė *et al*., 2020). The objectives of the up-to-date study are: -

1-Determining the effect of dexamethasone as nonspecific stress on growth performance, blood parameters, liver biomarkers, and stress biomarkers in broilers at 21 and 42 days of bird age.

2-Determining the physiological response of the body when given sodium butyrate and betaine to alleviate oxidative stress induced by exogenous dexamethasone exposure on growth performance, blood parameters, liver biomarkers, and stress biomarkers in broilers at 21 and 42 days of bird age.

3-Determining the correlation between HSP90 and caspase-3 in broilers at 21 and 42 days of bird age.

Chapter two

Literature Review

2-1 Homeostasis

Homeostasis has risen as physiology's key combining idea and is defined as a self-regulating activity that permits a creature to retain internal permanence while responding to shifting external conditions (Billman, 2020). As pointed out by Cannon, (1963) homeostasis is not a stationary system; rather, it is a dynamic self-adapting system that provides sustainability in the face of altering environmental necessities. Not long ago, Turner, (2017) expressed homeostasis as a steady internal environment that dictates invariable monitoring and modifications to keep up a balance between contradictory dynamisms, admitting to a free and autonomous life. Allostasis represents how homeostatic manners drive at the level of the cell, tissue, and organ, as well as at the level of the intact organism (McEwen and Wingfield, 2010). Therefore, the internal environment of an animal is preserved surrounded by a minute sort of standards by a self-adjusting system, which is a crucial factor of homeostasis and by both feedback and feedforward processes of homeostasis (Billman, 2020). Previously a feedback system is a closed-loop construction in which the sequels of prior events, for instance, alterations that unbalanced the internal environment, are used to direct future action (Forrester, 1976), So there are two categories of feedback systems: negative feedback, which tries to find a goal and responds as an outcome of failure to meet that goal, while positive feedback is the means of boost up or intensifying a consequence by its guidance on the process that triggers it (Tortora and Derrickson, 2017). The keystone for

physiological control is homeostasis, which is the after-effect of the obscure back-and-forth and match between many negative and positive feedback mechanisms, by three prime sections include: a receptor, a control center, and an effector (Modell *et al*., 2015; Tortora and Derrickson, 2017). There are 4 chief styles of homeostatic organizing that take place in the body involve: thermoregulation; is one of the utmost noticeable and dynamic homeostatic systems, to survive all species from giant animals to the smallest kinds must maintain an optimum temperature (Ruuskanen *et al*., 2021), osmoregulation; is the activity through which the body maintains the appropriate sum of water and electrolytes within and outside of cells or the balance of salt and water across cells membrane (Samue *et al*., 2018), chemical regulation to maintain systems sense of balance, animals control other chemical mechanisms as well such as in the instance of blood sugar levels, the pancreas would release either insulin when blood sugar concentrations are elevated or glucagon when blood sugar concentrations are run-down (Roder *et al*., 2016). End with cellular redox homeostasis which is a vital and self-motivation mechanism that upholds the balance of diminishing and oxidizing activities within cells and dominates an extensive range of biological reactions and events (Le Gal *et al*., 2021).

2-2 Stress

The expression "stress" is so recognizable to numerous researchers nevertheless, there is no inclusive definition for stress (Virden and Kidd, 2009). Hans Selye (father of stress) in 1975 saw that stress is the non-specific response of the body to any instance, hence stress overall is a biological response advanced when an animal is aware of a hazard to its homeostasis (Moberg, 2000). likewise, Stull, (1997) explained stress as adversative effects in the controlling or environmental system which inflict

modifications in the animal's behavior or physiologic to evade physiological out of order and upkeep the animal to survive in its environment. The animal's feedback with stressors is determined by the former familiarity of the animal with stressors, the stress severity, the extent of the stressors, the physiological condition of the animal, and proximate environmental limitations (Stull, 1997). So, animals respond to stress through various means including behavioral, metabolic, and physiological adaptations at all points of a vertebrate configuration, from cellular to the whole animal body (Collier and Gebremedhin, 2015). There are 2 phases to the stress response acute and chronic, acute stress reactions can take wherever from a few minutes to days subsequently after the stress initiates (Horowitz, 2001). As fight-or-flight hypothesis, additionally known as the acute stress response, appeals that when animals are endangered, their sympathetic nervous system is triggered, prepping them for fighting or getaway (Fink, 2016). But a chronic response to stress is delimited by the endocrine system (as well as acute stress) and is correlated with a modified receptor population, changing the susceptibility of the tissue to homeostatic signals and consequential in a new physiological state (Collier *et al*., 2017). The hypothalamic-pituitary-adrenal (HPA) axis is actuated by responding to stressor agents, giving rise to a sequence of neuroendocrine and behavioral responses that direct reserve the balance of critical developments (Sheng *et al*., 2021).

In poultry commerce, there are four fundamental classifications of stress: environmental stress, dietary stress, stress due to technology or management, and internal stress, which is associated with health circumstances and difficulties (Surai *et al*., 2019). Oxidative stress in the broilers trade is a hot issue presently for an assortment of intentions, one of which is interrelated with more than a few pathologies that concern bird growth (Fellenberg and Speisky, 2006). And is described as a disproportion between free radical

creation and endogenous antioxidant defense in cells/tissues, which guides lipid peroxidation, protein nitration, DNA damage, and apoptosis (Reyer *et al*., 2015). Free radicals are persistently found in cells consequently of physiological oxygen metabolism at particular amounts, in cooperation, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are signaling molecules concerned with upholding homeostasis (Rehman *et al*., 2018). Furthermore might create by various processes other than the addition of a single electron to non-radical, endogenous resources of free radicals exclude several cell organelles such as mitochondria, peroxisomes, and endoplasmic reticulum, other than some enzyme actions, fatty acid metabolism, and phagocytic cells, whereas X-rays, ultraviolet light, and heavy metals are exogenous sources (Martemucci *et al*., 2022). The chief resource of reactive oxygen species are mitochondria, manufactured by the electron transport chain in aerobic respiration as byproducts (Adwas *et al*., 2019), and excessive synthesis of ROS and RNS, or ineffectual scavenging of these molecules, triggers oxidative stress which simultaneously diminished feed ingestion, inadequate elaboration, immunosuppression, hypoxia, and extreme mortality percentage (Mujahid *et al*., 2007). In critical physiological processes, ROS modulates cell development, programmed cell death, and other signaling processes (Brieger *et al*., 2012). External threats or any stressors might end in pathological overproduction of ROS in the endoplasmic reticulum, peroxisomes, and mitochondria as a consequence of rising protein-folding load, and higher fatty-acid oxidation, as well as an increase in energy consumption (Rosca *et al*., 2012; Sandalio *et al.*, 2013). Even though ROS oxidation potentially results in persistent conformational alterations to proteins and lipids, along with DNA mutations, multiple enzymatic and non-enzymatic systems have been initiated to safeguard cells from ROS build-up (Lü *et al.*, 2010). These oxidants may trigger caspase-dependent apoptosis and inflammasome-driven pyroptosis, and other

caspase-independent programmed cell death mechanisms like necroptosis, ferroptosis, and autophagic cell death (Amri *et al*., 2017; Shen *et al*., 2017). However, chronic or severe oxidative stress somehow doesn't induce enough development of cytoprotective proteins, which could cause cell damage and/or death (Circu and Aw, 2010).

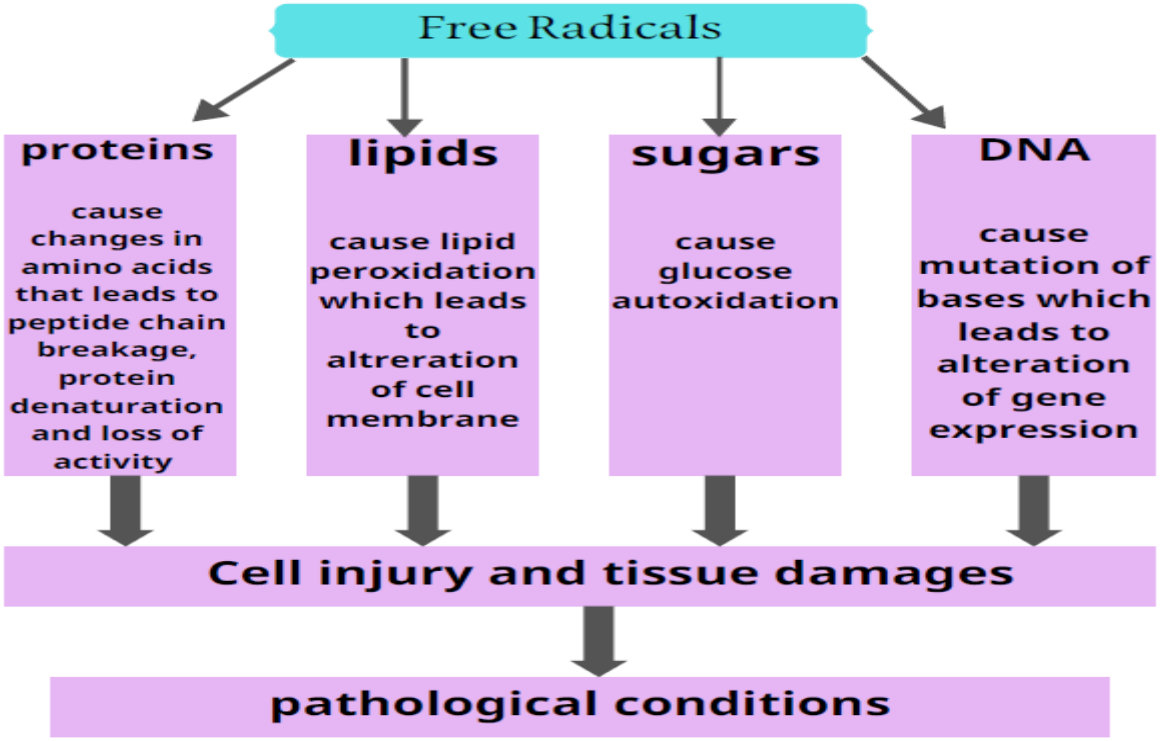

Figure (1): The molecular target of free radicals and oxidative damages. (Martemucci *et al*., 2022)

2-3 Corticosterone and dexamethasone analog

Corticosteroids are steroid hormones are formulated from cholesterol that is either made biologically by vertebrates or synthesized and released into the circulation to set diversity of physiological processes (Kirkgoz and Guran, 2018). The adrenal gland comprises 3 functional areas firstly, the zona glomerulosa which releases mineralocorticoids (mainly aldosterone), secondly, the zona fasciculata which generates Glucocorticoids (cortisol in

man and corticosterone in rodents and poultry). Whereas the third is zona reticularis production of androgens and sex hormones, involving progesterone, estrogens, and testosterone (Williams, 2018). Glucocorticoids (GCs) are the principal steroid hormones produced in the adrenal cortex when reacting to physiological cues and stress (Miller and Auchus, 2011). Although corticosterone, additionally identified as 17-deoxycortisol and 11β,21-dihydroxyprogesterone, is a foremost glucocorticoid in countless genera, counting amphibians, reptiles, rodents, and birds, and it is immersed in the management of energy, immunological reactions, and stress responses (Liebl and Martin, 2012).

Dexamethasone (DEX) (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) is a synthetic GCs that are counted in the World Health Organization's model list of needed medications (WHO Model Lists of Essential Medicines 2021). although DEX has practically no mineralocorticoid action, the addition of a fluorine atom in the DEX molecule renders it about 7 times more effective than prednisolone and about 35 times extra powerful than cortisone (Czock *et al*., 2005). Therefore, the DEX effect lasts 36–72 hours, much longer than cortisone and hydrocortisone 5–12 hours and prednisone 12–30 hours (Villanueva *et al*., 2017). DEX is a lipophilic compound that can be liquified in organic solvents such as acetone, ethanol, or chloroform, but also have one more propriety, it has a solubility in water as sodium phosphate form which is transformed into active DEX within the body, whereas it has a littler lipid membrane permeability but it is devoted as a more soluble shape of the active pharmaceutical ingredient in intravenous medications (Zhang *et al*., 2011). DEX has anti-inflammatory, anti-allergic, immunosuppressive, anti-shock, and anti-toxicity possessions that are analogous to those of natural glucocorticoids (Coutinho and Chapman, 2011). Its action mechanisms are

able to be achieved together with genomic and non-genomic means, the genomic mechanism: Lipophilic DEX quickly accesses cell membranes and binds to intracellular cytoplasmic glucocorticoid (GC) receptors found in virtually all organs and tissues then the resultant receptor-GC complex binds to a specific DNA spot and initiates gene transcription (Zen *et al*., 2011). Accordingly, mRNA is manufactured and assists as a scaffold for the creation of precise proteins, which modify cell functions, whereas the non-genomic outcomes seem approximate taking from seconds to a few minutes next to the receptor-GC complex is shaped (Buttgereit *et al*., 2002). Briefly, the non-genomic effects of DEX involve (i) cell membrane steadying, comprising mast cell membranes and organelle membranes such as lysosomes; (ii) inhibition of mononuclear phagocyte action; (iii) restraint of leukocyte movement to the inflammation site; and (iv) lessening in the activity of endothelial cells, monocytes, macrophages, neutrophils, and fibroblasts (Hardy *et al*., 2020). The injection of dexamethasone duplicates the negative after-effects of raised corticosterone therefore dexamethasone (dosages between 0.2 and 4.0 mg/kg) has been applied as an immune suppressant (Giles *et al*., 2018), as a prenatal stress mediator (Maccari *et al*., 2003), and to create oxidative stress in laying hens (El-Habbak *et al*., 2005) as well as cockerels (Eid *et al*., 2006). Aengwanich, (2007) realized that administering dexamethasone, a synthetic glucocorticoid, at levels of up to 6mg/kg had numerous consequences on broilers that were parallel to internal glucocorticoids. Though giving corticosterone or analogs of corticosterone may be a potential approach in the study of broiler chicken stress adaption (Post *et al*., 2003). Longstanding corticosteroid usage in general produces mitochondrial accumulation and expansion, in addition to mitochondrial dysfunction, which has been linked to DNA damage affected by oxidative stress (Oshima *et al*., 2004).

2-4 Heat Shock Protein 90 (HSP90)

Heat shock proteins (HSPs) are a family of greatly conserved proteins that were identified in 1962 in the chromosomes of Drosophila (Ritossa, 1962). It was far along exposed that most HSPs have high cytoprotective properties and represent molecular chaperones for further cellular proteins (Kalmar and Greensmith, 2009). Molecular chaperones are proteins that cooperate translationally with the nascent polypeptide chain or folding intermediates to steady or assist a non-native protein in obtaining its native conformation, inhibiting premature misfolding and aggregation (Hartl and Hayer-Hartl, 2009; Vabulas *et al*., 2010). They are sorted based on the molecular weight stated in the chaperone's name, HSP40, HSP60, HSP70, HSP90, and HSP100, although tiny HSPs have a molecular weight between 15 and 40 kDa, and some chaperones encourage folding (foldases), while others retain solubility (holdases), and the third type of chaperone is responsible for protein aggregate disaggregation (disaggregases) (Gregersen *et al*., 2006). HSPs are created in comeback to a varied assortment of cellular stressors, counting heat stress, oxidative stress, hypoxia, ischemia, hypothermia, virus infections, and numerous toxicants, such as mycotoxins, it's worth noting that the rise of HSP invention is believed as an endogenous adaptive system that subsequently upgraded tolerance to numerous stresses (Velichko *et al*., 2013). It's mentioned that cell stress and cell death are related, and HSPs start to act at key regulatory locations in the apoptotic controller when created in response to stress. (Kennedy *et al*., 2014).

HSP90 (heat shock protein 90) a complementary DNA clone for the 90kDa heat-shock protein was separated by the direct immunological screening of a chicken smooth muscle cDNA expression library, the protein was discovered to be an elongated dimer with a molecular weight of 160,000, and a friction coefficient ratio of 1.6 (Catelli *et al*., 1985). The amino acid

sequence of the chick HSP90 homologous to that of the HSP90 from yeast to human demonstrated 64-96 percent identity, with this sequence uncovering a "DNA-like" structure: potential site of reaction with steroid receptors with two hydrophilic regions A and B (Surai, 2015). The C-terminal half of HSP90 comprises a region that is critical for the protein's cytoplasmic organization, and the cytoplasmic anchoring signal is situated between amino acids 333 and 664 (Passinen *et al*., 2001), but N-terminal is ATP binding domain (Bouhouche-Chatelier *et al*., 2001). HSP90 has two locations where it can bind ATP; one is in its N-terminal domain, where it contains an unconventional nucleotide-binding site, and the second is in its C-terminal region of the protein (Garnier *et al*., 2002). After being exposed to higher temperatures for two hours, broilers' hearts, livers, and kidneys expressed more HSP90 (Lei *et al*., 2009).

Under physiological grade, the utmost impacts of GCs are mediated through heat shock protein (HSP)-bound cytoplasmic GC receptors (GR), so in the nonappearance of glucocorticoids, GR is linked with two HSP90 molecules, HSP70, HSP40, and a peptide named p23 (Sionov *et al*., 2006). Upon GC-GR binding, the HSP chaperone molecules are announced and the hormone-receptor complex passes into the nucleus where it binds to specific DNA sequences named glucocorticoid responsive elements (GREs), with follow-on inhibition or development of the relevant genes (Sionov *et al*., 2006). In reality, HSP90 is thought to be a key participant in upholding cellular homeostasis and adaptive response to stress (Jackson, 2012). It has been shown that the chaperoning function of HSP90 under oxidative stress may be disturbed by protein cleavage caused by hydrogen peroxide or other ROS (Panopoulos *et al*., 2005). So under oxidative stress, aggregation of essential proteins and DNA when dysfunctional oxidized proteins accumulate, inflammatory pathways turn out to be activated and the apoptotic cascade is

also activated, in this circumstance the function of HSPs turn up to repair the dysfunctional proteins a lot of methods such as protein sorting and directing for degradation, and refolding damaged proteins (Kalmar and Greensmith, 2009). It has been demonstrated that downregulating or impairing HSP90 is sufficient to make a cell susceptible to apoptosis, indicating that endogenous amounts of those chaperones appear to be high enough to restrict apoptosis, it's known that these chaperones could even interact with important proteins of the apoptotic signaling pathways (Lanneau *et al*., 2007). So, overexpression of HSP90 inhibits programmed cell death that is generated by a myriad of stressors, including oxidative stress and hyperthermia (Parcellier *et al*., 2003). In eukaryotes, the HSP90 chaperones, which are generally intracellular molecules, assist in the maturation of a number of receptors and kinases mature to exist in four different forms, two of which are cytosolic isoforms and the other two are detected in the endoplasmic reticulum and mitochondrion (Zininga *et al*., 2018). Extracellular HSP90 is hypothesized to promote receptor folding, which in turn promotes the activity of these receptors in immune cells like T lymphocytes and natural killer cells (Graner, 2016). It is believed that HSP90-mediated phagocytosis of the wound debris is how topical application of HSP90 enhances wound healing (Li *et al*., 2012). As well as HSP90 which has been exposed to the surface acts as a signal for molecular patterns linked to injury or harm, triggering the innate immune system and eventually the adaptive immune system (Multhoff *et al*., 2012). Therefore, HSP90 is vital in the presentation of antigens both intracellularly and extracellularly; the intracellular type of HSP90 attaches to antigenic proteins to speed up the alteration of those proteins into antigenic peptides (Graner, 2016). While extracellular HSP90 functions as an adjuvant by binding to substrate peptide antigens and binding with cell surface receptors to promote endocytosis of the HSP90-antigen complex (Oura *et al*., 2011).

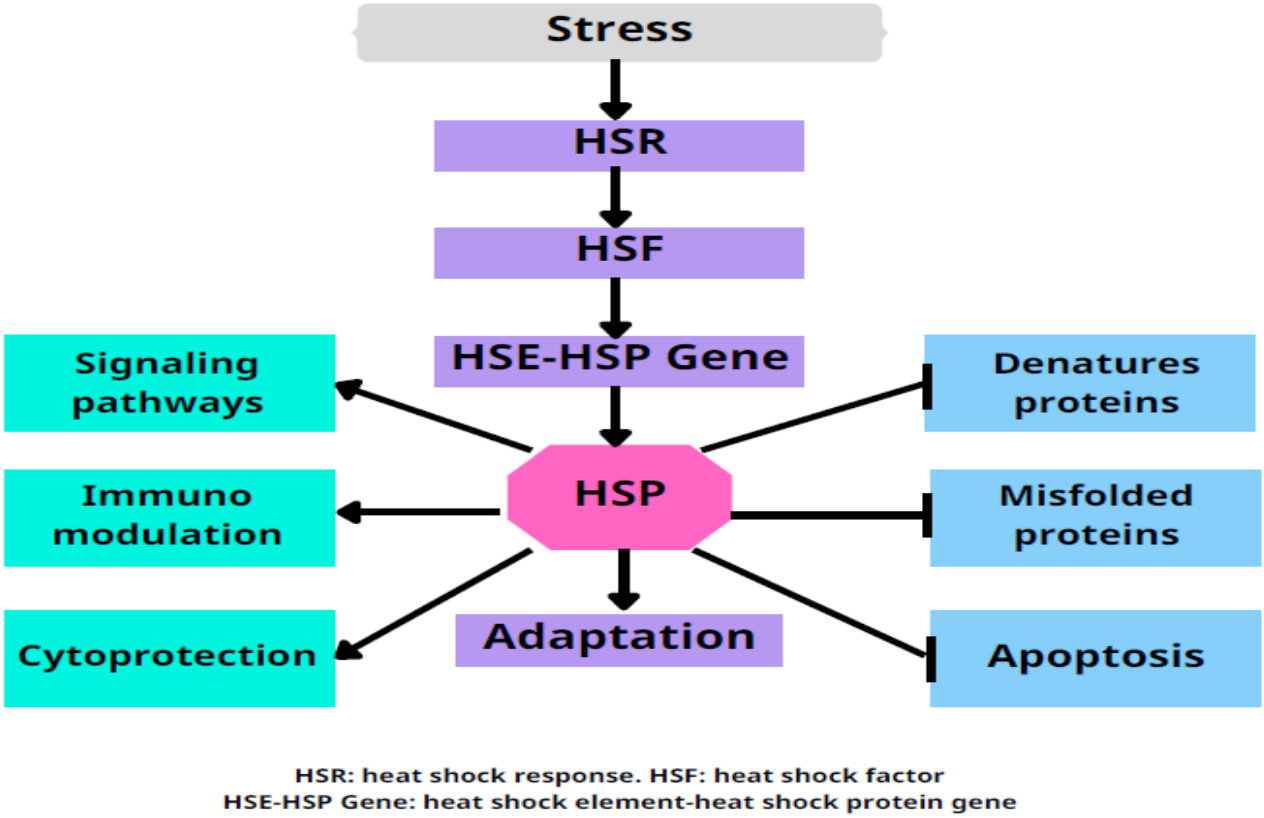

Figure (2): Functions of HSP under physiological and stress status. (Surai, 2015)

2-5 Caspase-3

Kerr, Wyllie, and Currie set up the word "apoptosis" in 1972 to illustrate a sort of cell death-related to genomic DNA fragmentation, it's distinguished morphologically by cytoplasmic condensation, nuclear pyknosis, chromatin condensation, cell rounding, membrane blebbing, cytoskeletal collapse, and the development of membrane-bound apoptotic cells that are swiftly phagocytosed and consumed by macrophages or nearest cells without triggering an immune response (Saraste and Pulkki, 2000). Currently, apoptosis identifies as a familiar style of cell death employed to get rid of undesirable or extra cells and is characterized by a slew of morphological and biochemical alterations caused typically by the stimulation of a family of cysteine proteases called caspase (Nicholson and Melino, 2013). So that caspases are a group of protease enzymes that have a vital role in

programmed cell death (Teng and Marie Hardwick, 2015). Caspase-1 was the initial member of the caspase family uncovered firstly as an interleukin-1b-converting enzyme (ICE), this enzyme is critical for interleukin-1b maturation, and after years an assortment of caspases from countless mammalian and non-mammalian species have been spotted (Lamkanfi *et al*., 2002). Twelve caspases have been characterized in humans, four in chickens, four in zebrafish, and ten in mice (Lamkanfi *et al*., 2002). Caspases are made up of three structural domains: a pro domain, which has a great subunit, and a minor subunit that acts a fundamental role in the control of the caspases that are activated enzymatically; the active enzyme includes the large and small subunits together (Nicholson and Melino, 2013). The caspase gene family is organized into three groupings. Group 1 enzymes (caspases 1, 4,5, and 12) are concerned with the control of inflammation whereas group 2 (caspases 2, 3, and 7) and Group 3 (caspases 6, 8, 9, and 10) are apoptosis-regulating caspases (Widmann, 2007). Caspases occupied in apoptosis have been sub-divided into initiator caspases, such as caspase-9 in mammals, and effector or executioner caspases, such as caspases-3 and -7 in mammals and chickens (McIlwain *et al*., 2013). They are made as dormant zymogens (pro-caspases) that are merely stimulated in response to a definite stimulus therefore at this level of post-translational the control permits the enzyme's quick and precise regulation (Shi, 2004). Existence 2 caspase-dependent apoptotic pathways: intrinsic pathway or mitochondrial pathway and extrinsic pathway which are caspase-activated by extracellular ligands via cell surface death receptors (Danial and Hockenbery, 2018). Caspase-3 is a caspase protein that cooperates with caspases 8 and 9 and the CASP3 gene is in charge of its expression it has been found in a diversity of mammals for whom whole genomic data are existing well as birds and lizards all have unique orthologs of caspase-3 too (Choudhary *et al.*, 2015).

Reactive oxygen species and the follow-on oxidative stress have been implicated in both apoptosis and necrosis, it has been recommended because apoptotic cells contain significant quantities of oxidants or stress oxidative indicators (Nunes *et al*., 2003). besides elevated quantities of ROS in the mitochondria might cause a free radical attack on membrane phospholipids, which brings about depolarization of the mitochondrial membrane (Nazıroğlu *et al*., 2004). Then the depolarization of mitochondria, which is an irreversible stage in the programmed cell death process, can set off a cascade of caspases (Nazıroğlu *et al*., 2012). When the bird is under oxidative stress and a lot of -OH accumulation occurs, which initiates DNA damage and brings about a rise in the level of tumor suppressor protein "P53" which can order gene expression of B-cell lymphoma 2 "Bcl-2" gene family and subsequently a pro-apoptotic member Bcl-2 gene family called Bcl-2 associated X protein "Bax" (Eisenberg-Lerner *et al*., 2009). So, when these proteins increased considerably, they activated caspase-9 upregulation and finally triggered caspase-3, and this pathway of apoptosis is called the intrinsic pathway (Nazarabadi *et al*., 2019).

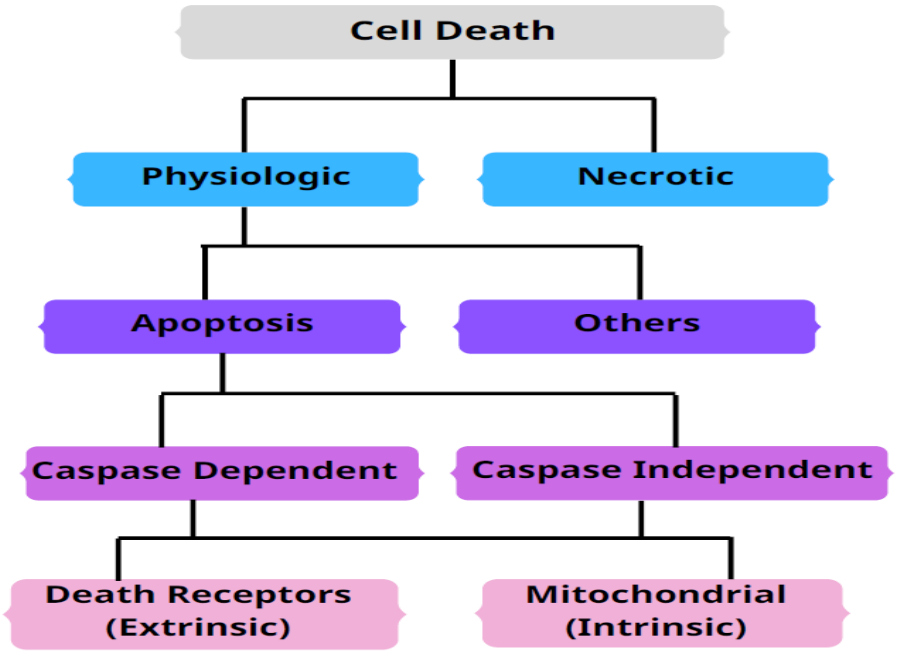

Figure (3): Classification of cell death pathways. (Danial and Hockenbery, 2018)

2-6 Antioxidants

Chemically as Lü *et al.* (2010) established that antioxidants are agents that could squelch free radicals by providing or receiving electrons to eradicate the radical's unpaired status. Formerly, the expression antioxidant meant an element that avoided the intake of oxygen (Lobo *et al*., 2010). Although early findings on the position of antioxidants in biology directed on their capacity to inhibit the oxidation of unsaturated lipids, which produces rancidity (Lobo *et al*., 2010). Also, when antioxidants react with free radicals affecting alters into a form that is converted to less active, and less risky (Lü *et al*., 2010). Briefly, antioxidants motivate biological systems through a diversity of manners, including electron donation or taking, metal ion chelation, co-antioxidants, and gene expression modulation (Lobo *et al*., 2010). The antioxidants acting in the defense systems act at 3 focal stages which are: "the first line of defense" is the preventive antioxidants by suppressing the creation of free radicals which include superoxide dismutase (Sindhi *et al*., 2013), "second line" is antioxidants that hunt active radicals to stop chain initiation and/or disrupt chain proliferation reactions such as glutathione, while "the third defense line" is antioxidants which include a multiplex collection of enzymes for restoring damaged proteins, damaged DNA, oxidized lipids and peroxides examples: Lipase, protease (Sindhi *et al*., 2013).

The antioxidant system of the birds can be generated endogenously or taken exogenously through diet or nutritional extras (Munteanu and Apetrei, 2021). Endogenously have 2 chief sub-categories: enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants are made up of three valuable enzymes that inhibit or neutralize the development of free radicals (Carocho and Ferreira, 2013). Rahman. (2007) mentions that Glutathione peroxidase, which gives two electrons to the reduction of peroxides in

addition to the eradication of peroxides; catalase, which exchanges hydrogen peroxide into water and molecular oxygen; and superoxide dismutase, which converts superoxide anions into hydrogen peroxide as a subtract for catalase. Non-enzymatic antioxidants are molecules that can hastily deactivate radicals and oxidants (Mirończuk-Chodakowska *et al*., 2018). Besides, the fundamental intrinsic non-enzymatic antioxidants take account of fat-soluble antioxidants (vitamins A & E, ubiquinone, carotenoids), and water-soluble antioxidants (vitamin C, glutathione, taurine, uric acid) (Halliwall and Gutteridge, 2015).

The feeding factor for broilers should fulfill physiological necessities as much as possible, which further will get on its efficacy, so when the birds suffered from variable external stressors such as temperature, light effects, noise, and chemical impacts, medication, feed modification, transporting, and many more thus modern technological and biological techniques should be available for rearing poultry (Pashtetsky *et al*., 2019). The acclimatization of broilers under oxidative stress is due to their use of organic acids, betaine, and other non-enzymatic antioxidants, hence the chance of offering extra poultry supplies with a set of critical nutrients can be regarded among the key elements for satisfactorily fighting stressors (Fisinin and Kavtarashvili, 2015).

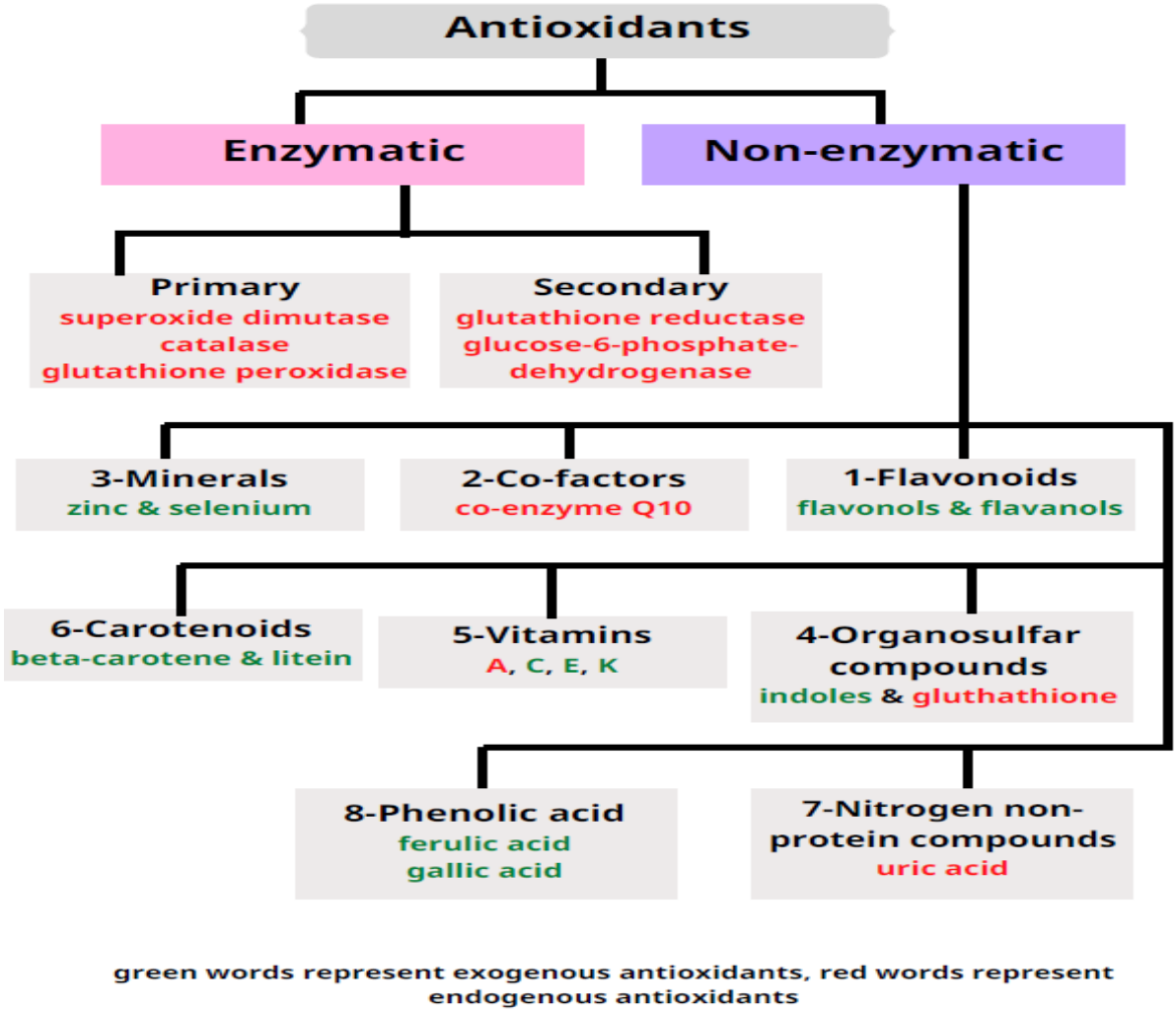

Figure (4): Natural antioxidants separated in classes. (Carocho and Ferreira, 2013)

2-7 Sodium butyrate

Sodium butyrate (SB) [C4H7NaO2] is a short-chain fatty acid with molecular, cellular, and tissue impacts, While Butyric acid (BA) [CH3CH2CH2COOH] is converted to butyrate ion when it misses its H ion [CH3CH2CH2COO] (Elnesr *et al*., 2020). It's the sodium salt of butyric acid with a sodium atom replacing the H in the -OH group and because SB is compact, steady, and much less odor-fumed, it was put to use as a supply of

BA, which is prominent for its positive outcomes on the gut (Jiang *et al.*, 2015). In general, salt has a benefit over free acids in that it is odorless and assists make the feed manufacturing process trouble-free to hold due to its hardness and less volatility nature (Elnesr *et al*., 2020). More than a few clinical sorts of investigations have just revealed that butyrate or its salts like sodium also intermediated the immune reaction and antioxidant capability of both *Vivo* and *Vitro* (Vanhoutvin *et al*., 2009). Also, certain reports expose that butyrate can use as a safe alternative to antibiotics (Lan *et al.*, 2020). It has been made known to be a crucial fuel source for gastrointestinal epithelial cells. Besides having antibacterial, anti-inflammatory, and antioxidant benefits (Song *et al*., 2017).

Certain findings realized that broilers treated with butyric acid glycerides had higher live weights at slaughter and better feed conversion rates, and it suggested that these compounds be taken into consideration as an effective supplementation to broilers' diet plans and, in particular, as a potential replacement for antimicrobial medications that have been outlawed in Europe (Antongiovanni *et al*., 2007). SB intake in any phase of Japanese quail induces stimuli growth performance, blood biochemistry, hematology, and histomorphometry of immunological organs (Elnesr *et al*., 2019). But not only could sodium butyrate (SB) restore the body weight (BW) gain and high serum level CORT in hens, but also it could be improved serum superoxide dismutase (SOD) activity and drop the levels of malondialdehyde (MDA) in chickens that were raised normally (Zhang *et al.*, 2011). While Elnesr *et al*. (2020) described that consuming broiler with 0.1% sodium butyrate as dietary supplementation at 21 days of age raised the serum SOD and CAT levels and drop off the levels serum malondialdehyde (MDA), also SB lowers albumin to globulin ratio and rises serum globulin concentration (Deepa *et al*., 2018).

Lan *et al*. (2020) exposed that SB can be utilized as a beneficial dietary addition to boosting the productive efficiency, liver function, and meat quality of broilers that have undergone oxidative stress due to hot weather, however, several investigations concluded that 4 g SB/kg dietary supplementation recovered the immunoglobulin indicators and oxidative damage of breast meat in broiler birds (Ogwuegbu *et al*., 2022). These beneficial results of sodium butyrate administration in broilers imply that it possesses free radical scavenging capability along with lowering oxidative harm inflicted by these radicals to cells or tissues (Qaisrani *et al*., 2015). Several studies documented the immunological benefits of SB on cellular and humoral immunity including the morphology of viscera, such as the thymus, spleen, and bursa of Fabricius in broiler chickens, and they supposed SB as an immune enhancer (Ahsan *et al*., 2016). Additionally, an immunostimulatory BA in chicken by generating host defense peptides has been emphasized (Sunkara *et al*., 2011). Moreover, in broiler chickens, SB impacted the lymphoid organs and enhanced growth performance and immunity (Sikandar *et al*., 2017). Besides that, SB considerably raised the body mass of meat-type chicken, bursa of Fabricius relative weights, and antibody titers versus Newcastle vaccination (Eshak *et al*., 2016). Butyrate has a significant influence on the biotransformation of xenobiotics and medications and also on steroid metabolism, and it contributes to the oxidative metabolism of hepatocytes, and may also have an impact on hepatic metabolic activities (Beauvieux *et al*., 2001). Csikó *et al*. (2014) expected that Butyrate has an effect on medication metabolism in hepatocytes, such as metabolic changes that alter pharmacological potency, and toxicity as well as the time of the withdrawal period in food-producing livestock. Gümüş *et al*. (2021) concluded that a mixture of coated calcium butyrate and natural antioxidants could be optimized to enhance carcass characteristics and meat antioxidant capability in broilers. While Zhao *et al*.

(2020) mentioned that SB tends to keep body weight, insulin sensitivity, and energy balance, as well as somewhat inhibit fat accumulation by increasing fatty acid oxidation, even though a few investigations have indicated that adding butyrate derivatives to the diet of broiler chickens lowered their ratio of abdominal fat and changed the expression of some hepatic genes (Yin *et al*., 2016; Bedford *et al*., 2017). By reducing glycolysis, rising beta-oxidation, and boosting oxidative phosphorylation, SB regulates energy metabolism and mitochondrial function, it also increased catalase, superoxide dismutase, glutathione peroxidase, and other antioxidant enzymes in the cell, moderates cell death, and the build-up of reactive oxygen species, and recovered a number of antioxidant parameters (Xing *et al*., 2016). And by enhancing antioxidative enzyme activity, SB reduces oxidative damage, it also inhibits caspase-3, caspase-9, and Bax protein levels and thus participates in limiting apoptosis (Li *et al*., 2019). In reproduction (Alhaj *et al*., 2018) hypothesized that SB would encourage testicular maturation by boosting testosterone hormone output and antioxidant capability in mature roosters. Supplementing SB to chicken diets assist in reducing the pH of the glandular stomach, fast-moving the transformation of pepsinogen to pepsin, which enhances the nutritional absorption of peptides, minerals, and proteins (Youn *et al*., 2005). The low pH also executes dangerous bacteria by depleting metabolic energy and reducing cell membrane metabolism, an outflow of cellular fluids, and nutrient utilization, as a consequence, the crucial purpose of administering SB with diet is to preserve the health of the fowl intestine by reducing the counting of pathogenic microorganisms (Zhang *et al*., 2011). Giving dietary SB significantly improves the shape of all intestinal parts from their length and weight in broilers at 21 and 42 days (Wu *et al*., 2018).

2-8 Betaine

Betaine (BET), likewise identified as trimethylglycine and glycine betaine, is a naturally occurring chemical found in microorganisms, invertebrate marine animals, vertebrates, and plants such as sugar beet, and lucerne meal (Hassan *et al.*, 2005). Sugar beet comprises a great amount of betaine, which mounts up in condensed molasses soluble, a byproduct of sugar beet handling (Eklund *et al*., 2005). Besides its existence as a refined feed chemical addition. It can be produced endogenously from choline, which is considered its metabolic precursor (Craig, 2004). Animals can quickly absorb betaine by the duodenum, and it may be readily filtered in the kidney and absorbed into the circulation, thus it's expelled largely in sweat rather than urine and is largely distributed to the kidneys, liver, and brain, and it's used in most tissues (Zhao *et al*., 2018). BET as a chemical compound is a neutral methyl derivative of the amino acid glycine with a charging trimethylammonium group positively and a charging carboxyl group negatively, a sort of zwitterion that regulates osmoregulation and methylation (Rao *et al*., 2011). It has two vital metabolic properties; works as a donor of methyl group and as a proper osmolyte, which assistances to sustain cellular osmolarity, that's why using betaine as upgrading poultry homeostasis during heat stress (Norouzian *et al*., 2018). Amongst organic osmolytes like “proline, glycine, glutamine, and taurine”, betaine is accepted as the best effectual osmoprotectant as an osmolyte (Yancey, 2001). Compatible osmolytes are chiefly momentous in instances of cellular dehydration since they aid in limiting water loss in contrast to the prevailing osmotic gradient (Lipiński *et al*., 2012). However, BET as a methyl donor has a broad range of actions, including DNA methylation and boosting methionine remethylation from homocysteine (Shakeri *et al.*, 2018). DNA methylation acts a tremendously noteworthy function in keeping up cell’s

normal function in animals, gene expression control, genetic imprinting, embryonic maturity, and an upward number of papers about the poultry industry recommended that DNA methylation played a momentous role in adipogenesis (Zhang *et al*., 2020). It depends upon the obtainability of methyl groups from *S*-adenosylmethionine, which originated from methionine, the transmethylation metabolic paths intimately interrelate with choline, betaine, methionine, and tetrahydrofolate (Niculescu and Zeisel, 2002).

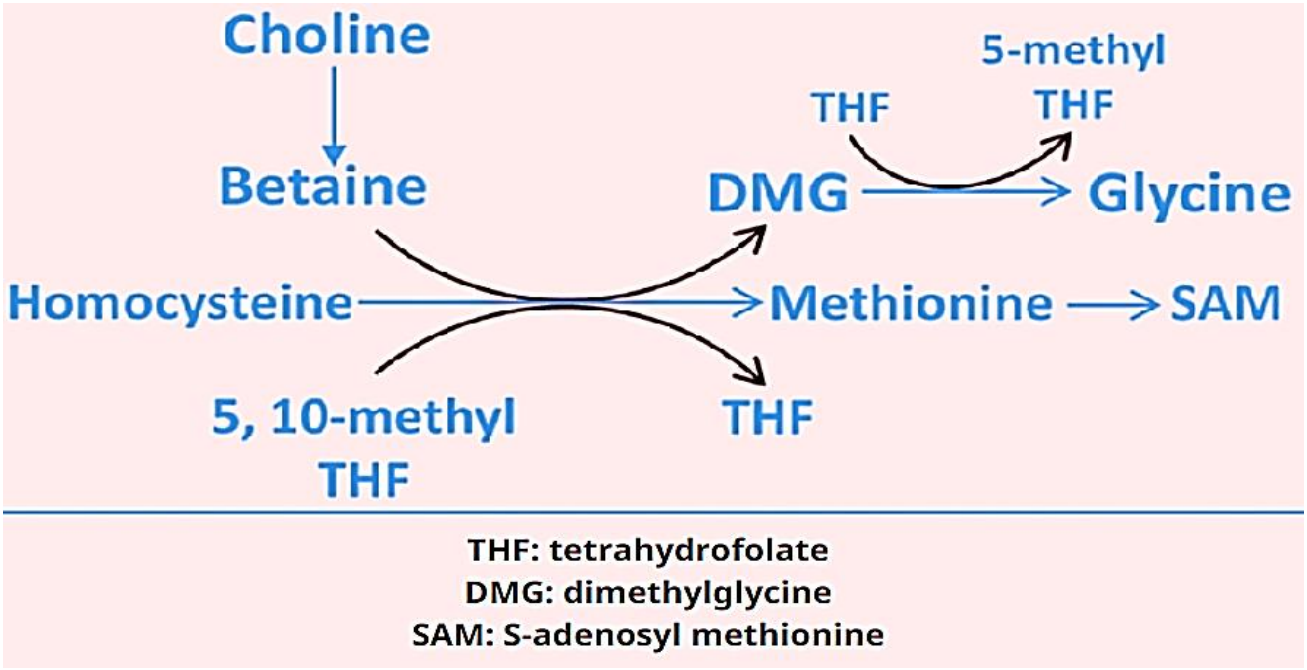

Figure (5): BET as a major methyl donor from diet. (Xu *et al*., 2018)

BET has a significant role in maintaining the physiological parameters and recovering immunological indices of chickens undergone oxidative stress in hot circumstances (Singh *et al*., 2015). And in numerous animal kinds, betaine supplementation has been shown off to moderate oxidative stress and its outcomes (Ahn *et al*., 2014). It has also been described to avert mitochondrial cell death and apoptosis (Ji and Kaplowitz, 2003). BET might inhibit caspase family proteins, it is reported that BET can reduce caspase-3

action in hepatocytes, likewise its considerably decrease caspase-8, caspase-9, and caspase-3/7 in corneal epithelial cells under hyperosmotic stress (Zhao *et al*., 2018). And also, there is a study told that BET reduced apoptosis rates of cardiomyocytes by diminishing the numbers of apoptotic cells and by converse the biochemical changes related to heart apoptosis (Yang *et al*., 2020). Betaine's antioxidant action might also be recognized for its competence to slow the initiation of mitochondrial lipid peroxidation (Ganesan *et al*., 2007). As well as, it employs its antioxidant impact by regulating HSPs (Dangi *et al*., 2016). Supplemental BET to 2 years old laying hens has been demonstrated to lessen the heterophil % while increasing the lymphocyte %, bringing about a lower heterophil/lymphocyte ratio (Gudev *et al*., 2011). Furthermore, giving dietary betaine raises the counts of monocytes, erythrocytes, hemoglobin, and hematocrit levels (Park and Kim, 2017).

Broilers, turkeys, and ducks of meat types' performance, physical appearance, and physiological features are influenced positively by the administration of betaine from 0.05 percent to 0.20 percent at high ambient temperature and presented raising in the body weight gain to 23.3% (Park and Kim, 2017). Another exploration of meat types poultry has been conducted after betaine administration up to a 9.8% increase in feed intake has been noted (Al-Shukri *et al*., 2012). Even though the feed conversion was boosted by up to 24.2% due to betaine supplementation (Chand *et al*., 2017). Under environmental stress, dietary supplementation of 0.05%-0.20% betaine enhances feed intake by up to 14.1% in breeders, laying hens, ducks, and quails, which is accompanied by a 23.6% raising in egg production (Awad *et al*., 2014). While Attia *et al.* (2016) have seen an elevation in ovary and oviduct weight, besides oviduct length, in consort with an increase in big follicle weight in laying hens due to 0.10 percent betaine supplementation.

Whereas Ratriyanto *et al*. (2017) found an increase in the protein and/or energy efficacy ratio in quails, reflective to get better protein and energy consumption for egg production. It is extensively presumed that intestinal cells and bacteria are endangered to osmotic alterations as they are portable through the digestive tract because the content of the intestinal lumen is hyperosmotic in comparison to blood plasma (Eklund *et al*., 2005). Furthermore, after all, intestinal cells accelerate the exchange of water, and with little solutes like "ions, nutrients, and macromolecules" between plasma and intestinal fluid, osmolyte-defending mechanisms are necessary (Ratriyanto *et al*., 2009).

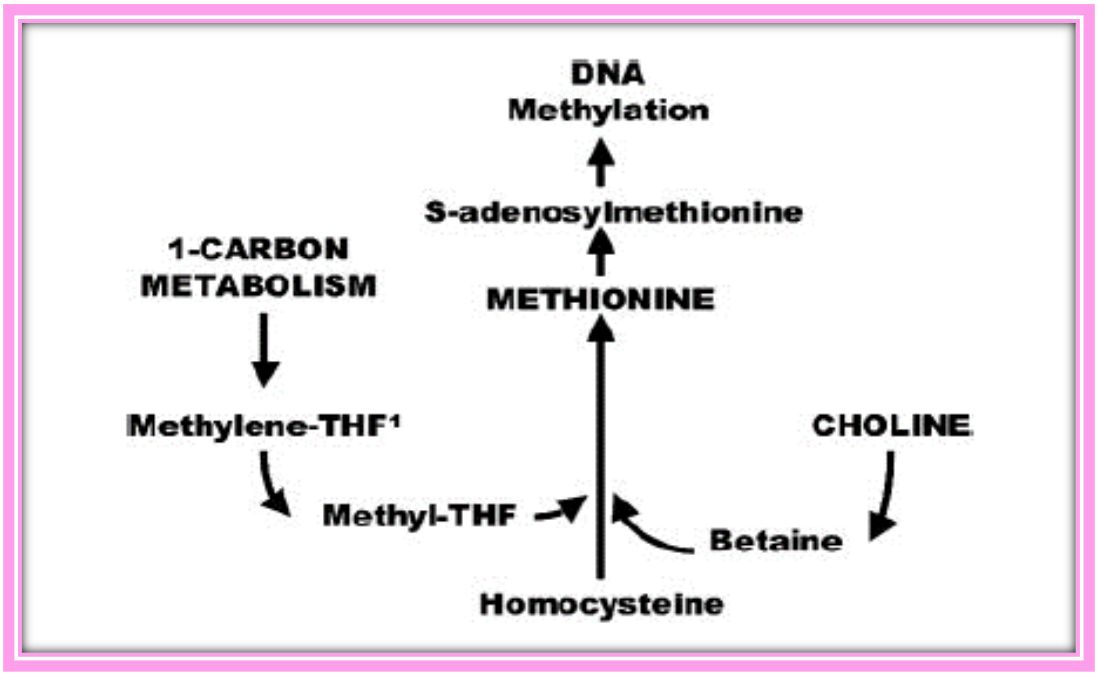

Figure (6): DNA methylation. (Niculescu and Zeisel, 2002)

Chapter Three

Materials and Methods

3-1: Devices and equipment and chemicals used in the experiments:

3-1-1 Devices:

Table (1): The devices and their origin

Number	Devices	The company and its origin
1	Centrifuge	Gima, Italy
2	Digital scale	China
3	EDTA tubes	BD vacutainer, England
4	Gel tubes	EZMDLAB, China
5	Glass microscope slides	Sail Brand, China
6	Haemometer	Superior, Germany
7	Incubator	JRAD, Syria
8	Microcentrifuge	TGL-12B, China
9	Microscope	Leitz Wetilar, Germany
10	Microplate reader	BioTek instruments, USA
11	Microplate washer	BioTek instruments, USA
12	Na-heparinized microhematocrit tubes	Vitrex medical A\S, Denmark
13	Pocket scale	M-H series pocket scale, China
14	Sartorius electrical balance	ADAM, Turkey
15	Spectrophotometer	Lovibond, England
16	Visible spectrophotometer	Shanghai Yoke Instrument Co., Ltd. China
17	Water bath	Memmert, Germany

3-1-2 Equipment:

Table (2): The equipment and its origin

Number	Equipment/Kits	The company and its origin
1	ALT & AST kits	BioLabo, France
2	Caspase-3 kit	Solarbio, China
3	Chicken Heat Shock Protein 90	Sunlong Biotech Co., Ltd, China

3-1-3 Chemicals:

Table (3): The chemicals and their origin

Number	Chemicals	The company and its origin
1	Absolute methyl alcohol	Laboratory reagent, India
2	Betaine	Biopoint company, Poland
3	Dexamethasone	Pioneer company, Slemani
4	DTNB 5,5-Dithiobis (2-nitrobenzoic acid)	HIMEDIA, India
5	Ethylene diamine tetraacetic acid-EDTA	Fluka AG, Switzerland
6	Hydrochloric acid (HCL)	GCC, Uk
7	KH2PO4	Fluka AG, Switzerland
8	L-Glutathione	MERCK, Germany
9	Na2HPO4	Thomas Baker, India
10	Normal saline- 0.9% sodium chloride	Polifarma, Turkey
11	Powdered wright stain	Scharlau, Spain
12	Propylene glycol	Laboratory reagent, India

13	Sodium arsenate	RIEDEL-DEHAEN AG SEELZE-HA NNOVER, Germany
14	Sodium azide	B.D.H chemical LTd poole, England
15	Sodium butyrate	Biopoint company, Poland
16	Sodium hydroxide- NaOH	B.D.H, England
17	Sulfosalicylic acid	LAB tech chemical, Canada
18	The Hydrogen peroxide	Laboratory reagent, India
19	Thiobarbituric acid (TBA)	HIMEDIA, India
20	Tri-chloroacetic acid (TCA)	Nottengham, England
21	Tris (Hydroxymethyl) aminomethane	Fluka AG, Switzerland
22	Trisodium-citrate	Sdfime limited, India

3-2 Solutions

3-2-1 Wright stain:

Wright stain preparation:

1- In a mortar put 0.1 g of powdered wright stain.

2- Add 60 ml of acetic acid-free absolute methyl alcohol, a few milliliters at a time, while grinding the stain with a pestle.

3- After all of the alcohol has been applied, pulverize the stain for several minutes.

4- Place the stain in a tightly sealed brown bottle and keep it in the dark for 2-4 weeks.

5- Filter the stain before use. (Coles, 1986)

3-2-2 Solutions for estimation of malondialdehyde (MDA) in serum:

*Material:

Tri-chloroacetic acid (TCA), Thiobarbituric acid (TBA), Hydrochloric acid (HCL)

*Solutions:

1- Tri-chloroacetic acid (TCA) solution 15%:

It's made by melting 15 g of TCA in 100 ml of distilled water (D.W) and kept in the refrigerator.

2- HCl solution (0.25 N):

Prepared by diluting 0.2 ml of concentrated HCL in 9.8 ml of distilled water and refrigerated.

3- Thiobarbituric acid (TBA) solution:

It's prepared by dissolving 0.0375 g of TBA in 10 ml of HCL solution. It's prepared at once. (Buege and Aust, 1978)

3-2-3 Solutions for estimation of glutathione (GSH) in serum:

*Materials:

Sulfosalicylic acid, DTNB 5,5-dithiobis (2-nitrobenzoic acid), Na_2HPO_4, KH_2PO_4

*Solutions:

1- Sulfosalicylic acid 4% solution:

It's prepared by thawing 4 g of sulfosalicylic acid in 100 ml of distilled water and refrigerating.

2- Phosphate buffer solution (KH_2PO_4 0.6 M and Na_2HPO_4 0.08 M):

It's made by putting 8.16 g of KH_2PO_4 with 2.8 g of Na_2HPO_4 and liquifying in 100 ml of distilled water.

3- Ellman's reagent (0.1 mM):

It's prepared by melting 0.00396 g of DTNB in 100 ml of a phosphate buffer solution with a setting pH within (8) and refrigerated. (Burtis and Ashwood, 1999)

3-2-4 Solutions for estimation of malondialdehyde in tissues:

*Materials:

Tris (Hydroxymethyl) aminomethane, Ethylene diamine tetraacetic acid/EDTA, Sodium azide, Normal saline/0.9% sodium chloride, Hydrogen peroxide, Sodium arsenate, Thiobarbituric acid/TBA, Tri- chloroacetic acid/TCA, Sodium hydroxide/NaOH.

*Solutions:

1- Tris buffer solution:

The solution was prepared by dissolving 6.057 g of Tris in 900 ml of distilled water, then adding 0.0292 g of EDTA and bringing the volume to 1 liter with distilled water, and adjusting the pH of this solution to 7.6 and refrigerating.

2- Peroxidizing azide solution:

It was formulated by thawing 130.02 mg of sodium azide in 1 liter of normal saline with 0.9% sodium chloride and refrigerating.

3- Peroxidizing solution:

It was prepared by adding 10 µL of 30% hydrogen peroxide to 9.09 ml of peroxidizing azide solution, it prepares at once.

4- Tri-chloroacetic acid (TCA)/ sodium arsenite solution:

It was made by dissolving 1.2991 g of sodium arsenite in 90 ml of D.W, then adding 28 g of TCA (28%) and bringing the volume to 100 ml of distilled water and refrigerating.

5- Sodium hydroxide solution:

It is prepared by dissolving 2 g of NaOH into 1 liter of distilled water.

6- Thiobarbituric acid (TBA) with sodium hydroxide solution:

It was prepared by liquifying 1 gm of TBA (1%) in 100 ml of NaOH solution that was prepared previously. The solution needs a little heating to better dissolve the TBA and the preparation is instant. (Gilbert *et al.*, 1984).

3-2-5 Solutions for estimation of glutathione in tissues:

*Materials:

Tri- chloroacetic acid/TCA, Na_2HPO_4, DTNB, Trisodium-citrate, L-Glutathione

*Solutions:

1- Tri-chloroacetic acid (TCA) solution 6%:

It was prepared by melting 6 g of TCA in 100 ml of distilled water and refrigerating.

2- Na_2HPO_4 buffer solution (0.3 M):

The solution is prepared by dissolving 42.588 g of Na_2HPO_4 in 1 liter of distilled water and refrigerating.

3- Trisodium-citrate buffer solution (10%):

It was made by dissolving 10 g of trisodium-citrate in 100 ml of distilled water (refrigerated).

4- DTNB solution (0.04%):

The solution was prepared by dissolving 0.04 g of DTNB in 100 ml of a trisodium-citrate buffer solution prepared previously. It is prepared instantaneously.

5- L-Glutathione standard solutions:

A standard concentration of glutathione was prepared (0.003125- 0.00625- 0.025- 0.05- 0.10 mg/ 0.5 ml distilled water), to obtain the final concentration of glutathione in the sample. (James *et al*., 1982)

3-3 Birds

Ninety-six Rose-type broiler chicks unsexed were brought from Nebras hatchability to conduct this study, then raised under standard conditions in the animal's house/College of Veterinary Medicine/University of Mosul for a period from September to November 2021. Then they were separated into 4 groups, every group containing 24 birds with 3 replications. Sawdust was used as a mattress for the floor and the hall was equipped with heaters, and ventilating fans to maintain the temperature (the temperature at hatchability 31-33 c˚, then diminished 1-degree c˚ after 4th day of age, subsequently continue lowering 1-degree c˚ every 3 days of bird age) and adequate ventilation. The birds were exposed to a period of natural lighting and completed with artificial lighting (60-watt lamps) during the first 7th day of age, then it was gradually reduced to 2 h/ week until it became 12 hours/day at the end of the rearing. Each cage was provided with feed at a fixed timing, while the water was provided freely *ad libitum* throughout the experiment.

3-4 Poultry feed:

The chicks were fed broiler feed obtained from the Erbil Feed mill; the chicks were fed starter feed from the 1st to 15th days of age, then grower feed from the 16th to 28th days of age, and finally finisher feed from the 29th to 42nd days of age (NRC, 1994); the components and details of the feed are shown in Table (4), and the chemical analysis of the three types of forage was included in Table (5).

Table (4): The percentage of feed items that were employed in the research

Ingredients	Starter%	Grower%	Finisher%
Premix 2.5%	2.5	2.5	2.5
Soybean meal	27.9	22.00	16.5
wheat	30.80	33.86	35.6
Wheat bran	5.8	3.78	3.6
Wheat floor	10	15	17
maize	20	20	21
oil	1	1	2
limestone	2	1.86	1.8

Table (5): The nutritional value calculated for the ration used in the study

Composition	Starter	Grower	Finisher
Crud protein	22.11%	20.20%	18.8%
Energy	3000 Kcal/Kg	3100 Kcal/Kg	3175 Kcal/Kg
Dry matter	92.31%	92.20%	92%
Fat	2.63%	2.63%	5%
Fiber	4.55%	4.11%	2.5%
Ash	6.32%	5.5%	4.7%

3-5 Dose determination and preparation of dexamethasone:

Initial experiments were conducted to calculate the suitable stressing dose of dexamethasone after it was scheduled to be 5 mg/kg B.W (Li *et al*., 2009), and because of the high mortality among chicks therefor the dose was adjusted based on preliminary experiences to be 1 mg/kg B.W in 3 days interval to induced oxidative stress (Eid *et al*.,2006; Ademu *et al*., 2018)

The dose was prepared by using dexamethasone as a raw material obtained from Pioneer company and dissolved with propylene glycol as follows every 1 mg of dexamethasone dissolved in 1 ml of propylene glycol. (Al-Zubaidy, 2021)

Propylene glycol is 1,2- dihydroxy propane or 1,2- propanediol, pure, viscid, colorless, almost odorless liquid with a molecular weight of 76.095. It is soluble in water, acetone, and chloroform and is extensively used as an excipient in several medications, as well as permitted in food and cosmetic items. Furthermore, it has a variety of other practical products, such as antifreeze, as an ingredient in latex paints and coatings to advance freeze-thaw capabilities (European Medicines Agency, 2013).

3-6 Experimental design

This methodology includes 96 chicks which were randomly divided into four groups after three days of acclimation. There are 24 broiler chicks for each group, with tri-replicates and two age periods of 21 and 42 days.

1- Control group: It was given the water and usual ration with subcutaneously (S/C) injected normal saline at 3 days interval.

2- Dexamethasone group (DEX): It was given the water and usual ration and the birds were injected with dexamethasone 1mg/kg B.W subcutaneously in the abdomen region at 3 days intervals.

3- Dexamethasone + sodium butyrate group (DEX+SB): It was treated with sodium butyrate 1.2 g/kg feed (Lan *et al*., 2020), with subcutaneously injected of dexamethasone 1 mg/kg B.W in the abdomen at 3 days interval.

4- Dexamethasone + betaine group (DEX+BET): It was treated with betaine 2 g/kg ration (Liu *et al*., 2019), with subcutaneously injected of dexamethasone 1 mg/kg B.W in the abdomen at 3 days interval.

3-7 Blood sampling

A day before the date of slaying, blood samples were gathered from the jugular veins of the birds in the aforementioned groups, and a portion of it was placed in plastic tubes containing EDTA for hematological tests. The remaining blood sample was placed in gel tubes and centrifuged at a speed of 3000 rounds/min. for 15 minutes, after which the serum was separated and distributed into small volumes in Eppendorf tubes and kept in the freezer at - 20 C˚ until the measurement is done.

3-8 Growth performance parameters

Within two periods of age (21 and 42 days of age) data on initial body weight, final body weight, total feed intake/bird, and food conversion ratio (FCR) were collected and introduced into the equation below (Matty and Hassan, 2020): -

3-8-1 Mean body weight

The average of the weight was calculated by weighing the birds in each group and dividing the result by the total number of birds in the group

The sum of the weight of the birds(g)/total number of birds

3-8-2 Mean weight gain

Mean body weight = final body weight(g) - initial body weight(g)

3-8-3 Feed consumption

Feed consumed (FC) = weight of feed consumed during 21,42 days of age (g) - the weight of feed remaining at the end of 21 and 42 days of age (g)

The result is divided by the number of birds present to extract the feed consumption rate per bird (g/bird).

3-8-4 Feed conservation ratio (FCR)

FCR = amount of feed consumed by birds/weight gain rate (for each period 21 and 42 days of age)

FCR = gram feed/ gram weight gain.

3-8-5 Internal organs weight

The carcass was opened using scalpel and forceps from the bottom of the abdomen, then continues to the chest from two sides. The visceral organs including (the heart, gizzard, spleen, liver, and bursa of Fabricius) were weighed up and calculated as organ weight/100 g body weight with an electronic scale. Then the organs (liver, spleen, and bursa of Fabricius) were taken and kept at - 20 C˚ until their measurements were made.

3-9 Blood parameters

Blood samples kept in tubes containing EDTA were used for the assays of packed cell volume, hemoglobin estimation, and differential leukocyte count (DLC).

3-9-1 Packed cell volume (PCV) estimation:

It was discovered in 1891 by Swedish physiologist Magnus Blix as hematocrit.

The PCV of a blood sample can be calculated fast (Barger and MacNeill, 2015).

-Equipment needed: 2 microhematocrit tubes precoated with EDTA or heparin, micro hematocrit centrifuge, clay, and PCV scale.

-Procedure:

1-Two microhematocrit tubes are filled with whole blood. To prevent blood leakage, each microhematocrit tube puts a small bit of clay at one end.

2- Clay-facing rotor and tubes are inserted into a microhematocrit centrifuge. lasting 5 minutes long, the samples are centrifuged at 12,000 rounds/min.

3-The centrifuged tube is placed on a PCV scale so that the edge between the clay and the blood sample is at 0% and the top of the sample (at the line between the plasma and the air in the tube) is at 100%.

4-To determine the proportion of cells in the blood sample, the line that spans the border between the packed cells and the plasma is traced back to the PCV scale.

5-The stripe that marks the border between the packed RBCs and the buffy coat is traced back to the PCV scale to read the percentage of RBCs in the blood sample to more precisely estimate hematocrit.

3-9-2 Hemoglobin estimation:

It was discovered in 1894 by swiss internist Hermann Sahli.

1-Draw five drops of N/10 hydrochloric acid in a measuring tube and draw fresh blood or anticoagulant-added blood to the 20 marks in a pipette.

2-Wipe away any excess blood with your fingertip, just as you would for a total erythrocyte count. Place the pipette in the tube containing HCl and transfer the blood into the acid to convert to acid hematine.

3-Suck the acid and rinse the contents into the tube. After 5 minutes, add distilled water drop by drop with a dropper and mix with a stirring rod to match the color of the standard.

4- When the colors match, read the scale on the tube to get the hemoglobin gram per 100 ml of blood. (Chanhan and Agarwal, 2008)

3-9-3 Differential leukocyte count:

A differential blood count estimated the relative percentage of every sort of white blood cell and serves in the recognition of deviant white blood cell populations (Voigt and Swist, 2011).

*Procedure:

1- Smear a blood drop from the specimen on a clean glass slide to diffuse the blood around. The blood smear is then dyed with a wright stain.

-the method for preparation and staining the smear is done by modified wright's method (Coles, 1986):

a- For one to two minutes, place 4-5 drops of Wright's stain on the blood smear on the slide.

b- Dilute the stain with the same amount of neutral distilled water.

c- Drain and flood the slide with neutral D.W. When the smear turns a touch pink, replace the water.

d- Place the slide in a Coplin jar with 100% absolute methyl alcohol for a few minutes. Rather than putting the slide in a Coplin jar with the methyl alcohol, a little amount of the methyl alcohol can be poured into the smear.

e- Using neutral distilled water, remove the absolute methyl alcohol. Replace with more distilled water and leave on the discolored smear until a rosy tint emerges. Get rid of any unwanted smears and examine them.

2- Place the prepared slide in the oil immersion of the microscope and move downwards in a chain-like fashion until 100 cells are observed.

3-After counting the different types of WBCs, apply the following equation to estimate the heterophil-to-lymphocyte ratio:

Stress index = Heterophile/ Lymphocyte

3-10 Liver enzymes

3-10-1 Alanine aminotransferase (ALT) estimation kit:

Method for estimation ALT by spectrophotometer. The reaction scheme is:

$$\text{L- Alanine + 2-Oxoglutarate} \xrightleftharpoons{\text{ALT}} \text{Pyruvate + L- Glutamate}$$

$$\text{Pyruvate + NADH + } H^{+} \xrightarrow{\text{LDH}} \text{L- Lactate + } NAD^{+}$$

At 340 nm, the reduction in absorbance owing to NADH alternating into NAD+ and proportionate to ALT activity in the specimen is determined.

*Manual procedure:

1- To remove the aluminum cap, use a non-sharp device. Add the amount of demineralized water specified on the label to the contents of the vial as soon as possible.

2- Blend softly and wait for the finish dissolution before using the reagent (roughly 2 minutes).

3- Take a cuvette for a sample and put 1 ml from the reagent and 100 µl from the serum and mix. While for blank take a cuvette and put only 1 ml of distilled water.

4- Set up a timer, and note the 1st absorbance after 1 minute at 340 nm.

5-Write the absorbance changes per minute for 3 minutes, and evaluate absorbance changes per minute (Δ Abs/min).

6-The final equation for calculating the ALT in IU/L is as follows:

$$\text{IU/L} = (\Delta \text{ Abs/min}) \times 1746$$

3-10-2 Aspartate amino transferase (AST) estimation kit:

Method for estimation AST by spectrophotometer. The reaction scheme is as follows:

$$\text{L- Aspartate + 2-Oxoglutarate} \xrightleftharpoons{\text{AST}} \text{Oxaloacetate + L-Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^{+} \xrightleftharpoons{\text{MDH}} \text{L-Malate} + NAD^{+}$$

At 340 nm, the drop in absorbance proportional to AST activity in the specimen is measured.

*Manual procedure:

The procedure is the same steps with the same equation for estimation ALT as mentioned previously.

3-11 Glutathione and Malondialdehyde estimation

3-11-1 Estimation of glutathione in serum:

The level of GSH in serum was estimated using the modified method that used (Burtis and Ashwood, 1999), where this method depends on using Ellman's reagent containing (DTNB) as it reacts with GSH and is reduced by (Glutathione-SH group) which makes a colored product whose absorption is measured at (412 nm wavelength).

*Procedure:

1-Put 150 µ of serum in a dry test tube.

2-Add 150 µ of 4% sulfosalicylic acid solution to it and shake well.

3-The solution is split by centrifuge at speed of 3000 rounds/min for 15 minutes.

4-150 µ of supernatant was drawn and placed in a test tube, 4.5 ml of Ellman's reagent was added to it, shaken well, and left for 5 minutes.

5-The absorbance of the solution was calculated by spectrophotometer at a wavelength of 412 nm versus blank, which contains all solutions except for serum.

6-The equation for the calculation of GSH in serum is:

$$\text{GSH concentration (µ Mol/L)} = \frac{\textbf{(A test – A blank)}}{\textbf{Eo} \times \textbf{L}} \times 10^{6}$$

Eo (Exinction coefficient) = 13600 M^{-1}, cm^{-1}

L (Length path) = 1 cm

3-11-2 Estimation of malondialdehyde in serum:

MDA, which is one of the principal consequences of lipid peroxidation activity, was estimated using the modified (Thiobarbituric acid reaction substance-TBARS), a method used by (Buege and Aust, 1978). The method is based on the reaction between lipid peroxides, especially MDA and TBA. As it flows in an acidic medium, forming a pink-colored product, the intensity of its absorption is measured at a wavelength of 532 nm by a spectrophotometer.

*Procedure:

1-50 µ of serum is placed in test tubes, 25 µ of TBA solution and 1 ml of TCA 15% solution are added to it and shaken well.

2-The test tubes are arranged in a water bath with a temperature of 100 C, with their nozzles closed with glass balls, for 15 minutes.

3-Samples are cooled and the supernatant is separated using a centrifuge of 2000 rounds/min for 10 minutes.

4-The absorbance of the formed supernatant is measured at wavelength 532 nm.

5-Blank is treated with the same steps mentioned above, but using 50 µ distilled water instead of serum.

6-The level of MDA is calculated by the following equation:

$$\text{MDA concentration (n Mol/ml)} = \frac{\textbf{(A test – A blank)}}{\textbf{Eo} \times \textbf{L}} \times 10^6$$

Eo (Exticion coefficient) = 1.56 × 105 M^{-1}, cm^{-1}

L (Light path) = 1 cm.

3-11-3 Estimation of malondialdehyde in tissues:

The (Gilbert *et al.*, 1984) method was used to measure MDA, which is a marker of the lipid peroxidation process in different tissues, as this analysis depends on the interaction of MDA with TBA and this interaction depends on pH (7.4).

*Procedure:

1-One g of wet cold tissue (spleen, liver, bursa of Fabricius) was weighed using a sensitive scale, and the tissue was placed in a bowl covered with a block of crushed ice. Then 10 ml of tris buffer solution was added and the tissue was crushed manually until a homogeneous solution was obtained.

2-Take 0.5 ml of the homogenous solution in a test tube and add to it 0.5 ml of the cold peroxidizing solution to start the reaction with good shaking, then put it in a water bath at a temperature of 37 C for 30 minutes.

3-Stop the reaction by putting 0.5 ml of TCA/sodium arsenate solution and mixing well.

4-Separation of supernatant by using centrifuge 3000 rounds/min for 5 minutes.

5-One ml of clear supernatant was drawn into a clean test tube and 0.25 of D.W and 0.5 ml of TBA/hydroxide solution were added to it and mixed well.

6-The tubes, after sealing their nozzles with glass balls, were placed in a water bath at boiling point for 15 minutes.

7-The tubes were cooled to room temperature, then the absorbance of each sample was read by spectrophotometer at a wavelength of 532 nm and then read again at another wavelength of 453 nm.

-To prepare the blank put in a test tube 0.5 ml D.W with 0.5 ml of peroxidizing solution and 0.5 ml of TBA/sodium hydroxide solution.

8-To estimate the MDA applied the following equation:

$$\text{MDA (n Mol/g)} = \frac{\textbf{(test 532 – blank 532) – 20\% (test 453 – blank 453)}}{\textbf{1.56 × 10}^{\textbf{5}}}$$

1.56×10^5 = EO (Extinction coefficient).

3-11-4 Estimation of glutathione in tissues:

The concentration of GSH in tissues (liver- spleen- bursa of Fabricius) was measured by the modified Ellman method (James *et al*., 1982), where it depends on the principle of interaction between Ellman's reagent with GSH in the sample, which made a complex with a yellow- golden color and the intensity of this color depends on the concentration of GSH and its intensity is measured by using a spectrophotometer.

*Procedure:

1- 0.5 g of cooled wet tissue was weighed and placed in a bowl covered with crushed ice. Then 2.5 ml of 6% TCA solution was added and crushed manually until a homogeneous solution was obtained.

2- The homogeneous samples were transferred to test tubes to be placed in a centrifuge at a speed of 3000 rounds/ min for 20 minutes and then 0.5 ml of the supernatant was taken from each sample.

3- Two ml of Na_2HPo_4 buffer solution were added and the tubes were shaken well, then 0.5 ml of DTNB was added to the tubes and left for 5 minutes.

4- The optical absorbance of the samples was measured against blank by spectrophotometer at a wavelength of 412 nm.

-Blank: it was prepared by adding 0.5 D. W with 0.5 ml Na_2HPo_4 buffer solution and 0.5 ml of DTNB solution.

5-To calculate the final concentration of GSH in the organs, different standard concentrations of glutathione (0.003125, 0.00625, 0.025, 0.05, 0.10 powder glutathione/ 0.5 ml distilled water) were prepared and the absorption of these solutions was read after treatment.

6-According to the concentration of GSH in the treated samples using the standard curve, the simple linear regression equation for standard concentrations was also to find the unknown GSH concentration in the sample, as the final result is µ Mol/L.

7-The equation for estimation of the GSH in tissue is:

$Y = a + b\ x.$

Which:

Y: the absorbance of the sample at a wavelength of 412 nm.

a: point of intersection of the vertical axis of standard concentration (Interception).

b: the slope of the regression line sample for standard concentration.

x: the unknown GSH concentration in the sample.

3-12 Stress biomarkers

3-12-1 Estimation of heat shock protein 90 (HSP90)

Sandwich-ELISA is the technique used in this ELISA kit (Chicken Heat Shock Protein 90, Sunlong Biotech Co., Ltd, China). The micro ELISA strip plate contained in this kit has been pre-coated with an antibody specific to HSP90. Micro ELISA strip plate bore holes were coupled with the identified antibody. Formerly, in every micro ELISA strip plate well, a Horseradish peroxidase (HRP)- conjugated antibody identified for HSP90 is put in and incubated. The superfluous parts are rinsed away. Every borehole obtains the TMB substrate solution. solitary the wells containing the HSP90 antibody will be looked at as blue colored and then back yellow next to the addition of the stop solution. At 450 nm, the optical density is calculated spectrophotometrically. The optical density is related to HSP90 concentration. By evaluating the optical density of the specimens to the standard curve, we computed the concentration of HSP90 in the samples.

*Procedure:

1-Sample formation: let the blood sample coagulate next to collecting the whole blood throughout leaving it at room temperature. This in general lasts between 10 and 20 minutes. Centrifuge the clot for 20 minutes at 2,000-3,000 rpm. If precipitates form during the reservation process, the sample must centrifuge again.

2-Standard dilution: dilute the standard in small tubes and then pipette 50 µl from each tube to a microplate well, every tube using two holes for a total of ten wells.

Table (6): shows standard solutions for the HSP90 ELISA kit

36 ng/ml	Standard 1	150 µl standard diluents + 300 µl original standard
24 ng/ml	Standard 2	150 µl standard diluents + 300 µl standard 1
12 ng/ml	Standard 3	150 µl standard diluents + 150 µl standard 2
6 ng/ml	Standard 4	150 µl standard diluents + 150 µl standard 3
3 ng/ml	Standard 5	150 µl standard diluents + 150 µl standard 4

3- Control as blank, let a well vacant in the Microelisa strip plate. 40 µl of sample dilution buffer and 10 µl of material are sited in sample wells (dilution factor is 5). Samples must be put onto the bottom without touching the well wall. Shake lightly to intermix.

4-Incubation: once sealed by closure plate membrane, put in incubation for 30 minutes at 37°C.

5-Dilution: use D.W to dilute the concentrated washing buffer (30 times for 96T and 20 times for 48T).

6-Washing: eliminate the closure plate membrane gently, aspirate, and fill up with the wash solution. Once 30 seconds of relaxing finish, discard the wash solution. Repeat the 5 more times of washing process.

7-Excluding for the blank control well, add 50 l HRP-Conjugate reagent to every hole.

8-Incubate according to Step 3.

9-Washing in the same manner as the 5^{th} step.

10-Coloring: in every hole, add 50 µl Chromogen Solution A and 50 µl Chromogen Solution B, shake carefully, and incubate at 37°C for 15 minutes.

11-Termination: To halt the reaction, add 50 µl stop solution to each well. The well's hue should transition from blue to yellow.

12-Using a Microtiter Plate Reader, determine the absorbance O.D. at 450 nm. The blank control hole's OD value is set up to zero. Once the stop solution is added, the assay must complete within 15 minutes.

3-12-2 Estimation of caspase-3

The caspase-3 colorimetric assay kit is based on caspase-3 hydrolyzing the peptide substrate DEVD-PNA (Asp-Glu-Val-Asp-p-nitroanilide) and releasing the p-nitroaniline (PNA) molecule. At 405 nm, p-Nitroaniline shows a high absorption. Caspase activity can be estimated by detecting PNA. This kit is intended for use with mammalian tissue and cells.

*Procedure:

1-Sample preparation: according to the tissue mass (g): reagent ratio 1:5-10 volume (mL) (it is recommended to weigh about 0.1 g of tissue and add 1 mL of Reagent), crush it in an ice bath or thoroughly cut it, set it on ice for 15 minutes, then centrifuge it at 4. Take the supernatant and place it on ice for testing after 10-15 minutes.

2-Preheat the spectrophotometer/microplate reader for 30 minutes, then set the wavelength to 405 nm and set the distilled water to zero.

3-Before use, standard solution diluent is used to dilute 5 μmol/L PNA standard solution to 200, 100, 50, 25, 12.5, and 0 mol/L standard solutions.

4-Sample determination (in a 96 well plate / EP tube, add the following reagents in the following order):

Table (7): shows the standard solutions of the caspase-3 assay kit

Reagent name (μL)	Test tube (AT)	Blank tube (AB)	Standard tube (AS)
Reagent I	40	40	
sample	50		
Reagent II		50	
Reagent III	10	10	
standard solution			100

5-Mix thoroughly, then cover the 96-well plate tightly with sealing film. Incubate for 60-120 minutes at 37°C. When the color shift is visible, the absorbance at 405 nm can be calculated. If the color change is not visible, the incubation period can be increased, even overnight. The blank tube only needs to be done 1-2 times.

6-Calculate ΔAT (μmol/L) =AT-AB.

3-13 Statistical analysis:

Duncan's multiple ranges A one-way analysis of variance (ANOVA) test was used to examine data at the probability level ($P < 0.05$) between groups to find the significance in growth performance and relative organ weight, but a two-way analysis of variance (ANOVA) was used to see if there was a significant difference at the probability level ($P < 0.05$) between groups and between the two periods of bird age of the resting parameters. Person correlation was used to detect the value of the correlation factor between caspase-3 and HSP90 at a probability of ($P < 0.01$). SigmaPlot (14.0), a statistical analysis application, was used to evaluate the data. (Steel *et al*., 1997).

Chapter Four

Results

4-1 Growth performance:

In table (8), From starting the experiment, the chicks were divided randomly into 4 groups, then the initial body weight was taken and there wasn't an obvious statistical difference ($P > 0.05$) between them, but groups injected with DEX and third and fourth treated groups caused a significant decrease in both final BW and weight gain ($P < 0.05$) compared with control, while group DEX revealed the lowest statistical value relative to other groups. Feed intake of all treated groups DEX, DEX+SB, and DEX+BET created a significant decrease compared with the control. However, the lowering in significance was graduated from DEX+BET, DEX, and DEX+SB. The group given DEX shows the highest statistical value in FCR, but supplementary groups including DEX+BET and DEX+SB caused a decrease in the significance when compared with the DEX group although not reached the value of FCR as in the control group, and these groups caused a significant raising in FCR compared with control ($P < 0.05$), but group DEX created the highest one among them.

At 42 d of age, the final body weight of the first period was the same initial body weight of the second period. The final BW at end of 42 d showed a decrease significantly in all three treated groups relative to the control, whereas the weight gain revealed no variance between groups. when calculating the feed intake, found that group DEX had no significance compared with control ($P > 0.05$) and broilers feed DEX+SB and DEX+BET caused a significant decrease related to control ($P< 0.05$). Finally, the FCR of all treated groups clarified no statistical difference except group DEX recorded an improvement in FCR compared with control and rest groups.

Table (8): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on the growth performance

Treatment		Initial body weight (g)	Final body weight (g)	Weight gain (g)	Feed intake (g)	Feed conversion ratio
21 days of age	Control	47.56 ± 0.62 a	1005.6 ± 17.48 a	957.9 ± 18.03 a	1928.1 ± 15.07 a	1.98 ± 0.01 c
	DEX (1mg/Kg B.W)	46.56 ± 0.81 a	372.1 ± 10.33 c	325.7 ± 9.84 c	1235.3 ± 44.51 c	3.80 ± 0.22 a
	DEX+SB (1.2 g/kg diet)	46.20 ± 0.85 a	467.9 ± 10.11 b	421.7 ± 10.62 b	1356.5 ± 23.91 b	2.91 ± 0.05 b
	DEX+BET (2 g/kg diet)	48.03 ± 0.57 a	430.2 ± 17.58 b	381.8 ± 17.91 b	1051.5 ± 27.89 d	2.68 ± 0.12 b
42 days of age	Control	1005.6 ± 17.48 a	2379.6 ± 55.38 a	965.5 ± 33.58 a	2083.0 ± 63.57 a	1.99 ± 0.08 b
	DEX (1mg/Kg B.W)	372.1 ± 10.33 c	1326.0 ± 63.10 b	738.6 ± 77.17 a	2058.0 ± 43.29 a	2.83 ± 0.26 a
	DEX+SB (1.2 g/kg diet)	467.9 ± 10.11 b	1555.0 ± 157.24 b	950.8 ± 51.60 a	1650.5 ± 52.71 b	1.84 ± 0.10 b
	DEX+BET (2 g/kg diet)	430.2 ± 17.58 b	1473.6 ± 106.59 b	968.2 ± 66.19 a	1830.0 ± 71.88 b	1.90 ± 0.14 b

Analysis one-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($P < 0.05$). / 96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-2 Organ weights

The data during the first period of bird growth logged in the table (9), there were no significant variations ($P > 0.05$) between groups in spleen and bursa weight, but gizzard and heart weight increased significantly in three treated groups related to control. The liver weight in a bird subjected to DEX has a highly significant value compared with control and broilers treated with DEX+SB, while the liver weight in the group injected with DEX didn't significantly differ from the group administered with DEX+BET whereas the DEX+BET group also didn't variable significantly to DEX+SB birds.

The organ weight relative to body weight is also listed in the same table for the second period, there wasn't a difference significantly in the weight of the spleen and heart between the experimental total. However, bursa and gizzard weight have a high significant value in all treated birds compared with non-treated. When the liver is weighted in the DEX group there was an increase significantly relative to the control and resting groups.

Table (9): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on the different organ weights

Treatment		Spleen g/100 g BW	Liver g/100 g BW	Bursa g/100 g BW	Heart g/100 g BW	Gizzard g/100 g BW
21 Days of age	Control	0.06 ± 0.007 a	2.26 ± 0.10 c	0.18 ± 0.02 a	0.52 ± 0.07 b	1.60 ± 0.19 b
	DEX (1mg/Kg B.W)	0.13 ± 0.02 a	5.02 ± 0.40 a	0.11 ± 0.03 a	1.00 ± 0.07 a	2.92 ± 0.25 a
	DEX + SB (1.2 g/kg diet)	0.12 ± 0.02 a	4.02 ± 037 b	0.08 ± 0.01 a	1.28 ± 0.19 a	2.82 ± 0.20 a
	DEX + BET (2 g/kg diet)	0.08 ± 0.01 a	4.32 ± 0.19 ab	0.09 ± 0.03 a	1.08 ± 0.12 a	2.94 ± 0.27 a
42 Days of age	Control	0.06 ± 0.01 a	1.96 ± 0.22b	0.04 ± 0.009 b	0.40 ± 0.07 a	0.82 ± 0.19 b
	DEX (1mg/Kg B.W)	0.08 ± 0.007 a	3.32 ± 0.20 a	0.08 ± 0.008 a	0.68 ± 0.07 a	1.96 ± 0.12 a
	DEX + SB (1.2 g/kg diet)	0.09 ± 0.004 a	2.22 ± 0.05 b	0.08 ± 0.006 a	0.56 ± 0.10 a	1.70 ± 0.11 a
	DEX + BET (2 g/kg diet)	0.08 ± 0.008 a	2.52 ± 0.18 b	0.08 ± 0.005 a	0.48 ± 0.08 a	1.70 ± 0.13 a

Analysis one-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($P < 0.05$). / 96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-3 Blood parameters

4-3-1 Hemoglobin and packed cell volume

At 21 and 42 days of age, the results revealed there was no significant difference ($P > 0.05$) in Hb value among the 4 groups. DEX injection induced a significant elevation ($P < 0.05$) in the percentage of PCV related to control and resting groups, but DEX+SB and DEX+BET groups had the same significance to control, as well as Hb and PCV in the two times of the bird's age. (Table 10).

Table (10): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on the blood parameters

Treatment	21 days of age		42 days of age	
	Hb g/dl	PCV %	Hb g/dl	PCV %
Control	14.0 ± 1.34 aA	31.8 ± 2.31 bA	13.2 ± 0.58 aA	33.0 ± 1.54 bA
DEX (1mg/Kg B.W)	13.1 ± 0.87 aA	38.8 ± 3.30 aA	13.0 ± 0.44 aA	41.2 ± 2.35 aA
DEX+SB (1.2 g/kg diet)	15.4 ± 1.28 aA	31.8 ± 2.47 bA	15.0 ± 0.70 aA	29.0 ± 1.18 bA
DEX+BET (2 g/kg diet)	14.4 ± 0.98 aA	32.0 ± 1.78 bA	14.2 ± 0.86 aA	29.6 ± 1.43 bA

Analysis two-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($P < 0.05$). / (A, B) different letters in rows refer to significant variance between data groups ($P < 0.05$). /96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-3-2 Differential leukocytes count

The number of lymphocytes and monocytes in the DEX and DEX+SB groups didn't differ significantly ($P > 0.05$) from DEX+BET and nontreated birds after examining and counting WBCs during the first period of age, but there was a statistically significant difference between them. When completing the statistical analysis in the DEX group compared to resting groups, the heterophils ($P < 0.05$) raised significantly, whereas groups treated with DEX+SB and DEX+BET from nontreated groups exhibited no significant ($P > 0.05$) difference. The basophil and eosinophil counts were unaffected statistically ($P > 0.05$) by any of the treatment groups and within two periods of age. The outcome is displayed in the same table (11). The stress biomarker when calculated shows in the DEX group, there were a lot of significant variances ($P < 0.05$) compared to the other groups, while DEX+SB and DEX+BET didn't differ ($P > 0.05$) between them, and with control.

The percentage of cells when DLC was performed was caused by a significantly decreased ($P < 0.05$) lymphocyte and an increasing ($P < 0.05$) heterophils percentage and stress index in broilers when injecting with DEX compared to resting groups. While supplementary groups did not show variation ($P > 0.05$) with each other or even with the control group in lymphocytes, the heterophils% in the DEX+SB group revealed no statistically significant ($P > 0.05$) difference from the control with a significant decrease ($P < 0.05$) from the DEX+BET group. The counting of monocytes revealed a significant reduction ($P < 0.05$) in the DEX group related to the control, whereas the DEX+SB and DEX+BET groups revealed no significant ($P > 0.05$) variable value between them and the resting group, as well as adding feed additive improved the stress index significantly ($P < 0.05$) relative to the DEX group.

DLC and stress index values are not affected ($P > 0.05$) by the two-period time of broiler production.

Table (11): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on DLC and stress index

Treatment	21 days of age						42 days of age					
	Lymphocyte %	Heterophile %	Monocyte%	Basophile %	Eosinophile%	Stress index	Lymphocyte %	Heterophile %	Monocyte%	Basophile %	Eosinophile%	Stress index
Control	36.20 ± 1.53 abA	37.20 ± 1.35 bcA	24.60 ± 1.50 abA	1.00 ± 0.31 aA	1.00 ± 0.31 aA	1.02 ± 0.06 bcA	35.80 ± 1.06 aA	35.20 ± 1.88 cA	27.60 ± 1.91 aA	1.00 ± 0.44 aA	0.40 ± 0.24 aA	0.97 ± 0.07 bcA
DEX (1mg/Kg B.W)	31.20 ± 1.62 bA	47.00 ± 2.02 aA	20.60 ± 0.81 bA	0.60 ± 0.40 aA	0.60 ± 0.40 aA	1.55 ± 0.14 aA	27.00 ± 1.41 bA	49.80 ± 1.39 aA	21.60 ± 0.92 bA	0.80 ± 0.37 aA	0.80 ± 0.37 aA	1.73 ± 0.05 aA
DEX+SB (1.2 g/kg diet)	40.20 ± 2.03 aA	33.00 ± 1.22 cA	25.80 ± 1.02 aA	0.60 ± 0.40 aA	0.40 ± 0.24 aA	0.82 ± 0.06 cA	38.60 ± 2.29 aA	35.40 ± 1.63 cA	24.80 ± 1.59 abA	0.60 ± 0.40 aA	0.60 ± 0.40 aA	0.83 ± 0.07 cA
DEX+BET (2 g/kg diet)	35.40 ± 2.56 abA	40.00 ± 1.37 bA	22.80 ± 1.39 abA	0.80 ± 0.20 aA	1.00 ± 0.31 aA	1.17 ± 0.14 bA	35.20 ± 0.91 aA	40.00 ± 0.70 bA	23.60 ± 1.07 abA	0.40 ± 0.24 aA	0.80 ± 0.37 aA	1.13 ± 0.04 bA

Analysis two-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($P < 0.05$). / (A, B) different letters in rows refer to significant variance between data groups ($P < 0.05$). /96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-4 Liver enzymes

At the first period of broiler age (21 days) a remarkable increase ($P < 0.05$) in liver enzymes, including ALT and AST in the DEX group compared with control and the remaining groups, while when broilers were supplemented with SB and BET after being exposed to DEX, a significant decrease ($P < 0.05$) in ALT and AST compared to the group treated just with DEX group and at the same time, didn't differ significantly ($P > 0.05$) from the control group.

At 42 days of age broilers injected with DEX had significantly ($P < 0.05$) greater ALT and AST levels than controls, but groups given with DEX+SB did not significantly ($P > 0.05$) vary from treated and non-treated groups, although adding BET to broiler feed caused a significant ($P < 0.05$) decrease in ALT relative to DEX group whereas that was equivalent to the value of control groups. but when analyzing the data of AST in broiler supplement with BET didn't differ ($P > 0.05$) from other groups.

All groups in the 2 experiments when compared with each other for both periods didn't show any significant difference ($P > 0.05$) (Table 12).

Table (12): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on the liver enzymes

Treatment	21 days of age		42 days of age	
	ALT IU/L	AST IU/L	ALT IU/L	AST IU/L
Control	35.91 ± 2.67 bA	202.1 ± 27.01 bA	36.39 ± 2.97 bA	208.6 ± 25.12 bA
DEX (1mg/Kg B.W)	50.15 ± 3.63 aA	321.7 ± 33.74 aA	52.33 ± 4.08 aA	291.6 ± 28.68 aA
DEX+SB (1.2 g/kg diet)	36.94 ± 2.18 bA	225.1 ± 17.16 bA	44.17 ± 1.33 abA	248.0 ± 14.85 abA
DEX+BET (2 g/kg diet)	36.35 ± 3.25 bA	228.7 ± 18.85 bA	42.01 ± 1.52 bA	241.8 ± 19.53 abA

Analysis two-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($P < 0.05$). / (A, B) different letters in rows refer to significant variance between data groups ($P < 0.05$). /96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-5 Glutathione and Malondialdehyde estimation

4-5-1 GSH and MDA in serum

At 21 days of age in the table (13), groups treated with DEX reduced GSH levels in serum to the lowest significant value compared to control and treatment birds ($P < 0.05$), when broilers given SB after being injected with DEX their serum GSH level increase significantly ($P < 0.05$) as compared with group DEX and the value didn't return to the control value. Giving BET to boilers injected with DEX led to a significant rise in the level of GSH and a return to the control value. The injection of DEX into broilers resulted in a significant increase in MDA levels when compared to the control. Administration of both SB and BET to broilers injected with DEX led to a significant decline ($P < 0.05$) in the level of MDA compared with the group injected with DEX alone, but the value did not return to the control value.

At the age of 42 days, the present results showed a significant decrease ($P < 0.05$) in the level of GSH in the DEX group compared to the control, while giving SB with DEX caused a significant increase ($P < 0.05$) in the level of GSH compared with DEX group, and back to control value. The addition of BET to the diet of broilers injected with DEX caused a significant increase ($P < 0.05$) in the level of GSH at the same age compared to the DEX group. The results of the current study showed a significant increase ($p < 0.05$) in the level of MDA in the DEX group compared with the control, while the addition of each of both SB and BET in the diet of broilers injected DEX led to a significant decrease ($P < 0.05$) in MDA compared with DEX group and the values return to the control.

All groups related to each other within the 2 periods. GSH test resulted in the nontreated group, DEX, and DEX+SB groups achieving the highest significant value within 42 days of age relative to 21 d, while group DEX+BET showed no significant change during either period. Analysis of data MDA, except for the control, all the treated groups compared to each other within 2 periods pointed the highest values within 21 days of age compared with 42 days of age ($P < 0.05$).

Table (13): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on the GSH and MDA in serum

Treatment	21 days of age		42 days of age	
	GSH µMol/L	MDA nMol/ml	GSH µMol/L	MDA nMol/ml
Control	4.58 ± 0.37 aB	2.90 ± 0.64 cA	7.78 ± 0.56 aA	3.84 ± 0.51 bA
DEX (1mg/Kg B.W)	2.28 ± 0.38 cB	14.22 ± 0.67 aA	4.74 ± 0.33 bA	7.40 ± 0.59 aB
DEX+SB (1.2 g/kg diet)	3.90 ± 0.40 bB	9.08 ± 0.66 bA	7.50 ± 0.67 aA	5.06 ± 0.46 bB
DEX+BET (2 g/kg diet)	4.52 ± 0.55 aA	10.04 ± 0.51 bA	5.26 ± 0.39 bA	5.02 ± 0.53 bB

Analysis two-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($P < 0.05$). / (A, B) different letters in rows refer to significant variance between data groups ($P < 0.05$). /96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-5-2 GSH and MDA in liver tissue

The following table (14) shows that the group DEX had a significantly ($P < 0.05$) lowering level of GSH in liver tissue than the control, at 21 and 42 days of age. The addition of SB and BET to the diet of broiler chickens injected with DEX led to a significant increase ($P < 0.05$) in the level of GSH in liver tissue compared with group DEX and didn't return to the normal control value. Furthermore, no significant change ($P > 0.05$) in the level of GSH in the liver tissue at both periods of age except in the group DEX+BET which showed a significant decrease in the first period compared with the second one.

At 42 days of age when broilers were injected with DEX, the level of MDA in liver tissue increased significantly as compared with the control. The results observed that administration of SB and BET to broilers injected with DEX caused a significant decline ($P < 0.05$) in the level of MDA in the liver tissue compared with the DEX group and return to the control values at both periods. The results of this study didn't record a significant difference in the

liver MDA between the two treatment periods except for the DEX group and control, which recorded a greater level at 21 days of age than at 42 days of age.

Table (14): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on the GSH and MDA in liver

Treatment	21 days of age		42 days of age	
	GSH µMol/L	MDA nMol/g	GSH µMol/L	MDA nMol/g
Control	0.41 ± 0.04 aA	2.30 ± 0.24 bA	0.50 ± 0.04 aA	1.00 ± 0.31 bB
DEX (1mg/Kg B.W)	0.17 ± 0.02 cA	7.36 ± 0.67 aA	0.25 ± 0.01 cA	2.76 ± 0.37 aB
DEX+SB (1.2 g/kg diet)	0.31 ± 0.01 bA	2.26 ± 0.27 bA	0.35 ± 0.03 bA	1.32 ± 0.28 bA
DEX+BET (2 g/kg diet)	0.24 ± 0.02 bcB	2.00 ± 0.24 bA	0.34 ± 0.02 bcA	1.16 ± 0.18 bA

Analysis two-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($p < 0.05$). / (A, B) different letters in rows refer to significant variance between data groups ($p < 0.05$). /96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-5-3 GSH and MDA in spleen tissue

The statistical table's data (15) At 21 days of age, all treated groups had a significant decrease in GSH levels in the spleen tissue compared to the control ($P < 0.05$). However, MDA estimation revealed that the DEX+SB and DEX+BET groups had values that were approximately the same as non-treated birds ($P > 0.05$), whereas the DEX group produced an increase ($P < 0.05$) in MDA levels when compared to the control and supplementation groups.

The GSH level at 42 days of bird age, when the groups were given DEX+SB and DEX+BET, was shown to be similar to the control ($P > 0.05$) and different from the group injection with DEX into broilers, causing significance to decrease when compared to the non-treated group. During the

same period, MDA levels were elevated ($P < 0.05$) in the DEX group as compared to the control. However, groups DEX+SB and DEX+BET was also significantly ($P > 0.05$) similarly related to the control and DEX groups.

When statistically analyzed between both periods, the GSH test showed that within 42 days of age, the treated and nontreated groups had the highest significant values ($P < 0.05$) from 21 d. In the MDA test, in the control and DEX+BET groups, which had the lowest significance value ($P < 0.05$) at 21 days of age vs 42 d, no groups within either period showed significance ($P > 0.05$).

Table (15): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on the GSH and MDA in the spleen

Treatment	21 days of age		42 days of age	
	GSH µMol/L	MDA nMol/g	GSH µMol/L	MDA nMol/g
Control	0.33 ± 0.02 aB	6.50 ± 0.41 bB	0.58 ± 0.02 aA	7.86 ± 0.46 bA
DEX (1mg/Kg B.W)	0.17 ± 0.008 bB	10.34 ± 0.54 aA	0.34 ± 0.02 bA	9.38 ± 0.46 aA
DEX+SB (1.2 g/kg diet)	0.20 ± 0.01 bB	6.96 ± 0.58 bA	0.53 ± 0.02 aA	8.22 ± 0.45 abA
DEX+BET (2 g/kg diet)	0.19 ± 0.008 bB	6.62 ± 0.37 bB	0.56 ± 0.02 aA	8.32 ± 0.30 abA

Analysis two-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($P < 0.05$). / (A, B) different letters in rows refer to significant variance between data groups ($P < 0.05$). /96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-5-4 GSH and MDA in bursa tissue

The level of GSH in the bursa tissue found in the table (16) at 21 days of age, there was no significant ($P > 0.05$) difference between DEX+SB and DEX+BET especially in comparison to control, whereas the birds subjected to DEX had a decline in significance ($P < 0.05$) value compared to both the control and treated groups. However, MDA levels in the bursa increased significantly ($P < 0.05$) in all three treated groups related to the nontreated group, even though adding BET to the feed of broilers injected DEX caused a marked decline ($P < 0.05$) in MDA levels compared to the DEX and DEX+SB groups but in both lasted mention groups still raised significantly ($P < 0.05$) relative to the control group.

The GSH value remained the lowest ($P < 0.05$) in the DEX group at 42 d compared to the resting group in the experiments, whereas nutritional supplements BET and SB to boilers injected with DEX caused higher ($P < 0.05$) GSH levels than the DEX group but were still significantly ($P < 0.05$) different from the control group. On the other hand, estimation of MDA in the same tissue and similar period showed a significant height ($P < 0.05$) in birds injected with DEX compared to the remnant groups, and the remnant groups showed similar significant ($P > 0.05$) values between them and the control group.

There's no significant difference ($P < 0.05$) in GSH content in the bursa tissue among groups in either period, except for the control in the first period being significantly lower ($P < 0.05$) than in the second period. Furthermore, the MDA estimation revealed reduced in significance ($P < 0.05$) at 21 days of age in contrast to the second period of age, except for the DEX+SB group, which didn’t t produce statistical significance ($P > 0.05$) between both periods.

Table (16): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on GSH and MDA in the bursa

Treatment	21 days of age		42 days of age	
	GSH µMol/L	MDA nMol/g	GSH µMol/L	MDA nMol/g
Control	0.27 ± 0.02 aB	0.32 ± 0.08 cB	0.32 ± 0.02 aA	1.94 ± 0.13 bA
DEX (1mg/Kg B.W)	0.14 ± 0.007 bA	1.64 ± 0.15 aB	0.17 ± 0.01 cA	3.10 ± 0.20 aA
DEX+SB (1.2 g/kg diet)	0.25 ± 0.008 aA	1.28 ± 0.16 abA	0.25 ± 0.01 bA	1.66 ± 0.19 bA
DEX+BET (2 g/kg diet)	0.23 ± 0.008 aA	0.96 ± 0.14 bB	0.25 ± 0.01 bA	2.04 ± 0.21 bA

Analysis two-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($P < 0.05$). / (A, B) different letters in rows refer to significant variance between data groups ($P < 0.05$). /96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-6 HSP90 and caspase-3

In table (17) broilers were given DEX+SB, their HSP90 levels didn't change significantly ($P > 0.05$) compared to the control, but when DEX was given to the broiler responsible for the significant ($P < 0.05$) elevation HSP90 levels, it was higher than the control, while the values of group-administered DEX+BET similar to that found in control and DEX at 21 days of age. After subjecting broilers to DEX at 42 days of age, there was an increase in HSP90 ($P < 0.05$) among the other groups, followed by a significant ($P < 0.05$) reduction in the DEX+SB, DEX+BET, and control groups, respectively. The effect of both periods on HSP90 levels in the control and DEX+BET groups increased in significance ($P < 0.05$) in the first period compared with the second period. But the DEX group had the exact opposite in significance between both dates while the DEX+SB group had no statistical variance between the two periods.

The experimental data of this investigation was conducted at 21 and 42 days of age, indicating a highly significant value ($P < 0.05$) of caspase-3 in birds treated with DEX, especially in contrast to resting groups, although no significant difference ($P > 0.05$) was noticed between DEX+SB and DEX+BET and even with the control group at the first period, while DEX+SB and DEX+BET didn't differ ($P > 0.05$) between them but varies significantly with the control group that shows a lower level of caspase-3 at the second period. When the statistical analysis of caspase-3 was performed to check if there were any significant changes between the two periods (21 and 42 days), it was revealed no significant variance in groups control, DEX+SB, and DEX+BET between 2 periods except for DEX group at 21 days, which indicates a significant ($P < 0.05$) decline when contrasted to the DEX group at 42 days.

Table (17): Impact of adding sodium butyrate and betaine to the diet of broilers injected with dexamethasone on the growth performance of HSP90 & Caspase-3

Treatment	21 days of age		42 days of age	
	HSP90 ng/ml	Caspase-3 µMol/L	HSP90 ng/ml	Caspase-3 µMol/L
Control	10.78 ± 0.24 bA	1.16 ± 0.10 bA	6.40 ± 0.78 dB	1.01 ± 0.07 cA
DEX (1mg/Kg B.W)	12.85 ± 0.39 aB	1.68 ± 0.05 aB	14.63 ± 0.47 aA	1.98 ± 0.08 aA
DEX+SB (1.2 g/kg diet)	11.58 ± 0.36 bA	1.33 ± 0.08 bA	11.49 ± 0.68 bA	1.29 ± 0.08 bA
DEX+BET (2 g/kg diet)	10.80 ± 0.35 abA	1.23 ± 0.04 bA	9.24 ± 0.45 cB	1.35 ± 0.07 bA

Analysis two-way ANOVA, mean ± SE / (a, b, c, & d) different letters in columns refer to significant variance between data within groups ($p < 0.05$). / (A, B) different letters in rows refer to significant variance between data groups ($p < 0.05$). /96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

4-6-1 Correlation between HSP90 and Caspase-3

At 21 days of age in the table (18), the control, DEX, and SB groups all had a positive significant correlation between HSP90 and Caspase-3 ($r = 0.989^*$, $P < 0.01$), ($r = 0.980^*$, $P < 0.01$), and ($r = 0.988^*$, $P < 0.01$), respectively. The positive significant correlation develops at 42 days in the SB and BET groups, with values of ($r = 0.961^*$, $P < 0.01$) and ($r = 0.993^*$, $P < 0.01$), respectively. In both periods, the rating groups demonstrate a negative correlation between HSP90 and Caspase-3.

Table (18): Correlation between HSP90 and caspase-3 on broilers

Caspase-3 µMol/L								parameters	
42 days of age				21 days of age				Treatment		
DEX+BET (2 g/kg diet)	DEX+SB (1.2 g/kg diet)	DEX (1mg/Kg B.W)	Control	DEX+BET (2 g/kg diet)	DEX+SB (1.2 g/kg diet)	DEX (1mg/Kg B.W)	Control			
							0.98*	Control	21 days of age	HSP90 ng/ml
						0.98*		DEX (1mg/Kg B.W)		
					0.98*			DEX+SB (1.2 g/kg diet)		
				0.91				DEX+BET (2 g/kg diet)		
			0.90					Control	42 days of age	
		0.93						DEX (1mg/Kg B.W)		
	0.96*							DEX+SB (1.2 g/kg diet)		
0.99*								DEX+BET (2 g/kg diet)		

Analysis person correlation/ (*) means there are significant correlation between HSP90 and Caspase-3 ($p < 0.01$). /96 Rose-type broiler chicks, four groups, each group containing 24 birds with 3 replications.

Chapter five

Discussion

Growth performance considers one of the important parameters in most poultry research since the world demand for poultry production is in an increasable manner. The findings of growth performance in the group injected DEX were a decrease in initial body weight (at 42 d), final body weight (both periods), weight gain and feed intake (at 21 d), and an increase in FCR for both periods which approved by Lv *et al*. (2018) who reported a lowering in body weight gain and feed intake and raising in FCR of broilers suffered from oxidative stress induced by DEX given orally by water (20 mg/L water/daily/ 19-41 days of age) and suggested that the cause goes to the inhibitory effects of GCs on growth rate that summarized by increased energy expenditure, protein oxidation, and decreased small intestine absorption. Others have observed that GORT can increase feed intake by suppressing leptin-induced satiety and boosting hypothalamus levels of neuropeptide Y (Cusin *et al*., 2001). Wang *et al*. in 2015 hypothesized that the observed inhibited growth could be the result of highly wasting of energy rather than reducing in feed consumption while, (Virden *et al*., 2007) supposed that glucocorticoids exert a diversion of energy to exercising muscle by mobilizing stored energy, limiting subsequent energy storage, and increasing gluconeogenesis. Catabolism of structural protein is increased by CORT-induced gluconeogenesis, releasing free amino acids for use as gluconeogenic substrates, putting meat-producing animals at a disadvantage (Virden and Kidd, 2009).

After the broilers were injected with DEX and given a dietary SB, the results showed an alleviated effect of DEX on growth performance by an elevation in initial body weight (at 42 d), final body weight, weight gain, and feed intake (all of them at 21 d), and improvement in FCR for both periods, these results approved by Chamba *et al*. (2014) reported that using of partly coated sodium butyrate in broilers diet may be enhanced significantly the growth performance at grower and finisher periods but not at starter period when compared with +ve and -ve controls, and proposed that the explanation to the beneficial impacts of butyric acid on gut functions such as promoting intestinal epithelium cell development and modulating bacterial growth in the intestine by raising the beneficial bacteria and reducing pathogenic bacteria growth. Panda *et al*. (2009) indicated that may be the effectiveness of butyrate depends on its concentration and the form that used in the research, and found 0.2 % of butyric acid in the diet insufficient to improve bird performance, while 0.4% was enough for optimum weight gain and FCR and 0.6% possibly didn't have a detrimental effect on feed intake. Dibner and Buttin (2002) noticed that using organic acids like butyric acid as a dietary supplementation may promote protein and energy digestibility and this effect is done in many ways such as; declining the competition of pathogenic microbes on the host's nutrients, lowering the endogenous nitrogen losses, decrease the production of ammonia and by these effects, they supposed probably the butyrate could improve feed utilization and bird's growth performance (Dibner and Buttin, 2002), or maybe because of the benefits of butyric acid that stimulates the pancreatic exocrine by increasing the digestive enzymes secretions such as lipase and amylase and thus improving nutrient digestion and absorption (Ahsan *et al*., 2016). While some studies contrary to our findings of sodium butyrate, Mahdavi and Torki (2009) reported that supplementation-protected butyric acid glycerides in diet didn't affect positively weight gain and FCR in boilers. And the cause

may be going to that uncoated sodium butyrate when reaches the small intestine and is converted to butyrate, is dissociated because of its low pKa compared with the pH of the small intestine so the enterocytes can't be used the massive quantities of undissociated butyric acid because of low concentrations which may not result in longest villi length, thus the absorption of nutrient lowered which leads to reducing in weight gain and impermeant in FCR (Ahsan *et al*., 2016).

According to this study broilers given betaine with their diets, the results showed an increase in final body weight, weight gain (at 21 d), initial body weight (at 42 d), feed intake, and improvement in FCR (at both periods) compared with the group given DEX, these results were compatible with Rao *et al*. (2011), which revealed that supplementation of (800 mg/ kg diet) of betaine to broilers (cobb 400, male chicks) increase the weight gain at 21 d. and FCR at 42 days of age and suggested that the improvement of growth performance goes to betaine effects as methyl donating property, which could spare the methionine from methyl donor function and the availability of methionine is very important for vital functions such as protein synthesis and immune modulation. Shakeri *et al*. (2018) mentioned more benefits that affected positively growth performance such as; reducing the negative impacts on the intestine and tissues suffering from osmotic stress, strengthening the anti-oxidant status, and helping in the excretion of some toxic metabolites, Hamidi *et al*. (2010) mentioned 2 points about how betaine could improve growth performance of birds, firstly; said betaine might affect positively FCR by increasing the digestibility of nutrients like carotenoids, fats, lysine, and proteins and enhanced the ability of the digestive tract for absorption these nutrients, secondly; betaine when act as an osmolyte, this will assist Na^{+}- K^{-} ATPase pumps of the cell membrane and so on sparing good amounts of energy and so that could contribute for improvement FCR.

However, some studies didn't agree with the results and indicated that betaine didn't have benefits on growth performance in broilers (Esteve-Garcia and Mack, 2000; Xing *et al*., 2011), also the study by Chen *et al*. (2018) disagreed with our study and showed giving betaine to partridge shank broilers with diet at different progressively doses 250, 500 and, 1000 mg/ kg diet and observed that application of betaine didn't effect on growth performance during the starter period but, could improve body weight gain and feed gain ratio during grower period at doses of 500 or 1000 mg/kg diet and thought that betaine showed different responses in the study due to many factors such as; the breeds of chicken, the levels of methionine in diets, the environmental conditions for rearing.

The relative organs weights of this trial found that the group injected with DEX caused an increase in the liver and gizzard weight (at both periods), in the bursa (in 2nd period), in heart weight (in 1st period) compared with the control these results are in line with Puvadolpirod and Thaxton (2000) which caused physiological stress to broilers (6 weeks of age) by given a CORT (8 IU/kg BW/daily- for 7 days) and found an increase in liver weight and the clarification the cause to that elevated levels of endogenous glucocorticoids could lead to an increase the level of free fatty acids in the circulation which then accumulated in the liver and cause the increase the weight of it. Malheiros *et al*. (2003) showed an increase in liver, and gizzard while retardation in spleen and bursa weights in male broiler chickens (aged 21 d) exposed to CORT supplemented at doses (0, 30, 45 mg/ kg diet) and they believed that increase in the proportional weights of the gizzard is not directly affected by exposure to CORT, rather than a side effect of CORT-induced growth retardation, the stomachs (proventriculus and gizzard) are early maturing glands, which means that both organs are primarily developing early in life and lose weight concerning BW as they get older, As

a result, as CORT reduces BW, increase the stomachs retain a higher weight about BW. While the retardation of the spleen and bursa is due to the sensitivity of lymphoid organs to CORT which causes regression of the lymphoid organs (Post *et al*., 2003), our results about bursa relative weight didn't agree with that and we supposed that it's a matter of proportion and proportion, means that sharp decrease in body weight made the bursa higher weight as a compared with whole-body weight even the bursa had suffered from retardation due to CORT.

The results of supplementation of SB and BET on relative organ weights show some of the liver weight diminishing when treated with SB at 21 days compared with DEX, while at 42d, SB and BET could overcome the adverse effect of DEX on liver weight and retain in a normal, whereas the remaining organs didn't differ from the DEX group. According to some researchers, Aghazadeh and Taha-Yazdi (2012), giving a 2.5 g butyrate/kg diet to 42 days of age Ross broilers improves the liver weight and suggested that liver size mainly depends on its activity since many liver functions depend on GIT activities so that when butyrate improves the modulation of digestion processes this leads to an elevation in liver secretions. While Sikandar *et al*. (2017) found that broiler chickens given SB (0.5 & 1 g/kg diet) at 21 days of age noticed there is an increase in the bursa weight (0.5 & 1 g/kg), and thymus weight (1 g/kg), whereas at 35 days showed raising in weight of spleen (1 g/kg), and thymus (0.5 & 1 g/kg), the increase in the immune organs weights have reflected the improvement of the immune system in the body so that when SB offered broilers showed an elevation in immune activities thus thymus, bursa and spleen which consider the essential players of immune system raise in their weights, or maybe the reason for an increase in their weight goes to histological causes that revealed that these organs observed an increased on the thickness of parenchymal areas. In contrast,

Makled *et al*. (2019) found that supplementation of 0.6 g SB/ kg diet to non-stressed SASSO broiler chickens from 1 to 21 days of age didn't affect the liver, spleen, and bursa relative weights and think that SB had no direct effects on the immune organs.

Similar results to our study about betaine Xing and Jiang. (2012) detected that supplementation of betaine (0.04, 0.06, & 0.08%) separately to laying hens at 180 old days reduced the liver weight. Betaine as a methyl donor offers the one-carbon unit which might afford the extra amount of dietary methionine and choline so that when methyl exists in enough quantity in the diet it can diminish the liver fat significantly (Xu *et al*., 2001). Uzunoğlu and Yalcin (2019) found that 0.15 % of betaine in the diet for healthy Ross 308 male broilers (6 weeks of age) didn't affect on relative weights of the gizzard, heart, spleen, and bursa. And El-Shinnawy (2015) said that supplementation of betaine at graded levels (1.0, 1.5, 2.0, and 2.5 g/kg diet) didn't show a significance on the gizzard, and giblets weights of broilers, also (Gudev *et al*., 2011) who disagreed our results and noticed that 2 years old laying hens feed betaine at 1 g/ kg with their diet didn't make any improvement in relative lymphoid organs weights. Şahin *et al*. (2020) revealed that using a 0.3, 0.5, and 0.8 g/kg diet at 42 days of age Ross-308 broilers and exegesis caused to the doses that used in studies, which means that higher doses probably show effectiveness on relative organs weights.

The blood parameters that were estimated for this study didn't show any significance in hemoglobin but an increase in the PCV-only within-group suffered from oxidative stress at both periods, and this result was the same results as Lv *et al*. (2018) who found that exposure to DEX to broilers at 20 mg/L water/ daily/ 19-41 days of age triggered elevation in hematocrit, this is because when a bird is stressed, its compensatory ability of blood to carry a lot of oxygen increases, increasing blood viscosity and red blood cell

fragility, later added one more possible reason for the rise in PCV and discovered that most of the birds that showed an elevation in red blood cell count, Hb, and HCT levels also at the same time suffered from ascites for an unknown reason. In contrast, Hafez *et al*. (2022) noticed a decrease in Hb, PCV, and RBCs count in 2 weeks old Cobb 500 broilers exposed to oxidative stress by inducing high stocking density circumstances and suspected due to impairment of whole bird health conditions associated with diminishing feed intake ratio and reduction in growth rate. Supplementation of SB didn't show any significance in Hb but PCV decrease and retained normal values, (Makled *et al*., 2019) who observed no effect of SB (0.6 g/ kg diet) on Hb, RBCs count in non-stressed SASSO broilers (21 days of age). El-Sawy *et al*. (2015) revealed an increase in PCV, Hb, and RBCs count at 42 days of age Cobb broilers were given SB in different doses and thought that because SB inclusion induced an increase in the count of F-reticulocytes, erythroid progenitors, and F-programmed progenitors the blood parameters also raised.

Given broilers, betaine lessens the elevation in the DEX group in PCV, similar to our results Egbuniwe *et al*. (2021) who showed normal PCV and Hb when given betaine (2 g/kg diet) to female Japanese quails during the season dryness, while Attia *at al*. (2019) revealed improvement in Hb, PCV, RBCs count of broilers suffered from heat stress and had dietary betaine (1000 mg/kg diet) compared with negative control and they said that betaine helped to sustain most of the metabolic functions and by that the birds under heat stress after administration of betaine noticed partial recovery in most of the parameter including blood parameters.

Stress indicators, such as the stress index, indicated statistically significant alterations in dexamethasone-treated broilers due to a drop in lymphocytes and an elevation in heterophil proportion in a recent study. The investigators

Vicuña *et al*. (2015) made sure to quantify the proportion of H/L to a stress evaluation in poultry regularly, and they showed that adding CORT or dexamethasone to the mix causes huge spikes in the H/L ratio with an increase in the lymphoid organs relative weights, and the results they got that feeding dexamethasone to birds as a glucocorticoid may be an effective means of glucocorticoid delivery as well as it creates stress and generates bowel irritation and alterations in mucosal permeability in the intestinal mucosa. The intestinal permeation is implicated in the pathogenesis translocation in the portal or circulatory system, creating greater pathogenic bacterial infections and, subsequently, a transition in the stress index (Ilan, 2012).

SB significantly mark dropping in stress index for both periods compared with birds suffering from oxidative stress, this is supported by Saki *et al*. (2018) who found that hydroxy-methyl Butyrate was a significant increase in WBCs count, including lymphocytes, and also a dramatic drop in Heterophil and H/L rates at day 21 when related to the untreated group, denoting a progression in the immunogenicity in broiler chicks' butyrate consumed diets. Mahdavi and Torki. (2009) not in line with our results they gave butyric acid (0-2-3 g/kg diet) to unsexed Arbor-acres broilers and didn’t affect counts of lymphocytes, heterophile, monocytes, basophils, and eosinophils at 21, 42, 49 days of age, as well as Makled *et al*. (2019) which used SB at dose 0.6 g/kg diet and given to healthy SASSO broilers (21 days of age) and revealed no statistic difference in stress index and WBCs counts compared with control

The effectiveness of betaine on stress index was the same as SB which is a line with Chand *et al*. (2017) who exhibited betaine significantly decline heterophile percentage but the lymphocytes percentage was raised, whereas the H/L ratio significantly decreased. Gudev *et al*. (2011) also reported

significant elevation in lymphocytes and falling heterophile counts when given BET 1.5 g/kg diet to 2 years old laying hens. And because of the betaine's ability to overcome the oxidants, there is a positive impact of betaine when replenishment (0.1%) during the heating challenging task lessens the negative effect on productivity and overall growth with a lower stress index throughout the first 18 days of broiler age (Al-Sagan *et al*., 2021).

In the current study, the levels of ALT and AST for both periods were raised in group-injected DEX compared with non-injectable groups, this agreement with Taha and Tikriti. (2021) oxidative stress in 45 days of age, Japanese male quails are mostly caused by raising in ROS, which leads to a loss of cell membrane selective permeability, where they find an elevation in AST and ALT levels, this may indicate that these enzymes are leaching out of the cells. Sultana *et al*. (2020) said that DEX changes enzymes in the liver at 14 days of age in broilers circulation, which could be due to necrosis of hepatocytes or disrupted liver cell permeation, resulting in a gradual fall in total hepatic ALT. because the ALT molecule's size is (114 kDa), causing raising in hepatic enzymes which indicated the DEX is hepatotoxic.

Our results showed that SB caused a diminishing in ALT and AST at 21 days of age like values in the control group, but marked the half value between control and group DEX within 42 days of age, which agreed with Lan *et al*. (2020) alleged that SB treatment of various ages of broilers (1-35 days) improved hepatic function amid suffering, as a result, the higher anti-oxidant of the birds in the SB administered groups could be the cause, showing a favorable impact induced by butyrate or its salts through diminished H_2O_2-induced DNA damage. But El-Sheikh *et al*. (2019) detected no significant elevation in ALT and AST in 19 days of age unsexed healthy Cobb broilers who drink SB 2 ml/ L water.

Betaine administration in broilers in this study affects ALT and AST activities at both periods except in AST for 42 days of age showed a halving in significance value between control and group DEX, these results were approved by Aboelfadl *et al*. (2020) who marked a decrease in ALT and AST activity compared with control after treatment with 0.1% and 0.2% of commercial betaine to unsexed Alexandria weaned rabbits at 4 weeks of age, whereas (Wen *et al*., 2020) who also agreed with our results seen a decline in the activities of ALT but not AST in 21-day-old male broilers delivered betaine 1000mg/kg diet agonized from oxidative stress from an elevation of temperature. BET could alleviate hepatic injuries from heat stress and maybe the cause goes to that BET affected upholding the structural and functional integrity of hepatocyte cell membrane (Ganesan *et al*., 2010). in contrast Uzunoğlu and Yalcin. (2019) gave 0.15 % betaine to Ross 308 male broilers and noticed no effectiveness of betaine on liver enzymes.

The biomarkers can be estimated quantitatively and qualitatively to detect the level of stress, such as thermal stress indicators like heat shock proteins (HSPs), innate immune markers such as Acute Phase Proteins (APPs), chemical secretions in saliva and urine, and oxidative stress indexes such as evaluation of the body's potent anti-oxidant system and values of lipid peroxidation are all possible stress markers (Dhama *et al*., 2019). According to this research, the exposure broilers experienced oxidative stress after receiving DEX, as evidenced by the rise in MDA levels and a decline in GSH levels in serum, liver, spleen, and bursa tissues at both times, these results are supported by Lv *et al*. (2018) It was also shown that after broilers were exposed to DEX orally with water at a level of 20 mg/L for 19 to 41 days, there was an upsurge in lipid peroxidation and a drop in the activity of the enzymatic oxidant system in hepatic tissue, whereas Eid *et al* . (2008) found

the same effects in plasma and hepatic tissues after exposing laying hens to DEX at a dose of 4 mg/hen/day for 7 days at 36 weeks of age. The body's metabolism changes during DEX therapy, causing oxidative stress by reducing mitochondrial antioxidant enzymes system and glutathione levels, producing H_2O_2 radicals, and boosting lipid peroxidation (Ghaisas *et al*., 2009).

In a present study, the supplementation of SB improved the GSH levels in serum, liver, bursa, and spleen (at 42 d), and improved the MDA values in serum, liver, spleen (at 21 d), and bursa (at 42 d) compared with DEX group, which approved the study Jiang *et al*. (2014) after exposure to dietary corticosterone (30 mg/kg of diet) in male broilers from day 7 to 21 days of age, dietary SB significantly enhanced the activity of antioxidant enzymes in duodenal and jejunal mucosa and lessened the MDA levels in the duodenal mucosa, indicating that SB can ameliorate negative outcomes associated with corticosterone-induced oxidative stress. In addition, some researchers observed that SB supplementation also leads to a rise in activation of superoxide dismutase SOD, glutathione peroxidase GPx, and catalase CAT and declined the content of MDA in serum, liver, and breast muscle of broilers suffering from heat stress (Lan *et al*., 2020). Zhao *et al*. (2022) found that SB can enhance antioxidant balance and determined that by measuring of concentration of MDA in the mucous membrane of the small intestine of broilers at 21-day-old.

Broilers treated with supplementary betaine noticed an improvement in GSH level in serum, liver, bursa, and spleen (at 42 d) and MDA values in serum, liver, and spleen (at 21 d), and bursa (at 42 d) when related with group DEX. Our results are in agreement with Alirezaei *et al*. (2012) who suggested the cause of its role in cell membrane stabilization and homocysteine remethylation, dietary betaine can promote the main antioxidant enzyme

concentration such as GPx, CAT, and SOD, lowering MDA levels in breast muscles and supporting the idea that betaine is associated with antioxidant and methyl donor properties. While Wen *et al.* (2019) hypothesized that betaine could have antioxidant capabilities versus oxidative damage by replacing "S-adenosyl methionine", which enhances the availability of substrate for GSH formation, which gives protection from ROS, furthermore betaine-raised glutathione and glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) efficiency. As well as improved hepatic cytoprotection over oxidants (Veskovic *et al*., 2019). Moreover, betaine suppresses both oxidative stress and pathological apoptosis by stimulating the production of "S-adenosyl methionine" and "methionine," which are crucial for the production/secretion of very-low-density lipoproteins VLDL and hepatic beta-oxidation (Zhang *et al*., 2016).

HSP90 is an ATP-dependent molecular engine that is abundant in the cytosol, so it is drawn to client proteins that demand -resource rearrangements and helps them by undergoing nucleotide-induced conformational changes (Kaziales *et al*., 2020). The results of this research marked elevation in HSP90 during injectable with DEX, this is thought to be the cause of the caspase activation and subsequent death that occurs when cells are threatened by extremely high amounts of ROS, which is thought to be a necessary component for activating the cytoplasmic chaperone HSP90 (Park *et al*., 2017). Because HSP90 functions are required by ATP, oxidative stress restricts glycolysis, resulting in a drop in ATP, as a result, ATP defines a new technique to suppress HSP90 when the body is under stress (Beck *et al*., 2009).

Regarding the effect of sodium butyrate on HSP90, we found that it was reduced, Zhang *et al*. (2011) did find that nutritional SB in broilers injected with DEX was beneficial for stressed chickens because of its capability to

lower catabolism and oxidative damage in the body, so according to Zhang supplementation of SB lowers HSP90 levels related to broilers under oxidative stress, so we suspect that SB can positively impact HSP90 by ameliorating oxidative stress.

Recent evidence indicates that dietary betaine can reduce cleavage HSP90, and we recommend that betaine functions similarly to molecular chaperones (i.e., attenuating stress-induced protein denaturation) by sustaining intracellular protein structure mostly through elevated hydrogen bonding between hydrophilic proteins in the folded state (Street *et al*., 2010). According to certain research, betaine additionally assists in cytoprotection, protein stability, and transcription factor modulation in the cell by protecting cellular proteins from heat-induced denaturation and lowering and/or decreasing heat shock protein induction (Uyanga *et al*., 2022). Betaine boosted the rate of HSPs refolding proteins by 30% to 50% and activated protein disaggregation by 2.5-fold, improving the final product and chaperone configuration (Diamant *et al*., 2001).

According to this study, dexamethasone produces cellular injury in the hepatocellular, signaling that apoptosis is provoked; evidence of elevated caspase-3 in the DEX-injected group (non-specific stress) agrees with Tonomura *et al*. (2003), which is accepted because of the various intracellular signaling pathways, glucocorticoids or analogs (DEX) are believed to induce apoptosis and restrict cell growth by elevating oxidative stress, and the formation of mitochondrial cytochrome C which ultimately leads to the inducing apoptosis, as a consequence, apoptotic circuits are disturbed, and unnecessary, faulty, or diseased cells cannot be eliminated. The influence of glucocorticoids (GCs) affects physiology by reducing cytokine expression at the signaling pathway and inducing apoptotic events like caspase activation and mitochondrial dysfunction (Almawi *et al*., 2004).

Recently our findings noticed that SB declined in the level of caspase-3 for both periods compared with the group that suffered from oxidative stress, and since the caspase-3 is a protease enzyme involved in apoptosis, Luciana *et al*. (2002) observed that in the absence of butyrate as an essential nutrient for Guinea pigs caused rapid, massive apoptosis of colonocytes in the colonic mucosa, whereas Jiang *et al*. (2015) discovered that supplementation of SB in the diet significantly suppressed apoptosis and upregulated B-cell lymphoma 2 (Bcl-2) expression in the duodenal mucosa of 21 days of age broiler males (Arbor Acres). Bcl-2 consider a key regulator gene that has been linked to anti-apoptotic activity in enormous cellular systems (Chen *et al*., 2005).

Compared with the group treated with DEX, betaine dropped the caspase-3 level, and this result was approved by Yang *et al*. (2020) who noticed that betaine relieved cardiomyocyte apoptosis rates in geese get excess methionine-induced hyper-homocysteinemia and said that betaine reduced the number of apoptotic cells in the heart by suppressing the caspase-dependent apoptotic pathway which relies on active mitochondrial control. Li *et al*. (2019) mention that betaine had a protective effect when decreased heat-associated apoptosis of bovine mammary epithelial cells by discouraging the mitochondrial apoptotic pathway and inhibiting the caspase-3 activity and reducing the Bcl-2/Bax ratio. The Bcl-2/Bax ratio determines cell survival or programmed cell death following an apoptotic signal (Hardwick and Soane, 2013). Rasineni *et al*. (2020) observed that betaine might alleviate fulminant liver failure caused by lipopolysaccharide/galactosamine in mice by suppressing caspase-3 activation and apoptosis.

A recent study shows a positive correlation between HSP90 and Caspase-3 in control, DEX, and SB groups at 21d while in 42d in supplements groups

(SB and BET), this result is in line with Nollen and Morimoto (2002) which shows during normal condition the HSPs are a group of biologically related chaperone proteins that are normally expressed but remained are activated in response to a wide range of chemical, ecological, and physiological stressors due to the ability to disaggregate, rearrange folding, and compensate for the apoptotic effect is thought to be linked to their ability to attenuate apoptosis and stress effects in diverse ways. HSP90 belongs to the vitagene group and is one of the most important features of the free radical scavenging system because it is the driving force in a cell's or body's (normal status) adjustment to various stress conditions (stress status), and a result, most cell membrane proteins decrease in response to stress, whereas heat shock protein expression typically increases (Surai, 2015). Some research results, such as my recent study, indicate a clear correlation between HSP and caspase families, wherein betaine conquers the hurtful effects of heat stress by observing a decrement in the expression of HSPs when betaine was administered to animals exposed to 42 degrees Celsius (Dangi *et al*., 2016). The addition of several nutrients to the diet of chickens, such as vitamin E, vitamin C, and betaine, frequently resulted in a reduction in oxidative stress consequently as a result, stress proteins' "HSP" expression would also frequently drop, which serves as a mirror for a reduction in apoptotic cells (Kalmar and Greensmith, 2009).

Butyrate therapy lowers STAT1 (single transducer of activated transcription 1) levels in leukemia cells via lowering caspase-3 and 6 cleavages, as well as p53 (Licht *et al*., 2014). So, the relationship between the two families of HSP and caspase is explained when the expression of HSF2 by colitis in the mice results in the altering or expression of Bax, caspase-3,9 through the mitochondrial circuit (Wang *et al*., 2020).

Conclusions

1. Supplementation of sodium butyrate or betaine to the diet of broilers outperforms in terms of growth performance at 42 days.

2. Addition of sodium butyrate to the ration of broilers improved liver weight more than betaine after 21days.

3. Sodium butyrate or betaine reduces the harmful effects of DEX's influence on hematocrit at two stages of bird age.

4. The two supplementary groups (SB or BET), at day 21, counteract the detrimental effects of dexamethasone on liver enzymes, with a more beneficial effect of betaine on ALT at 42 d than sodium butyrate.

5. The GSH and MDA levels in serum, liver, spleen, and bursa are demonstrated by relative improvement in GSH and MDA after two periods of adding sodium butyrate and betaine to the poultry diet to combat the free radicals generated by dexamethasone.

6. The liver cells exhibited cytoprotecting from suicide at 21 d and relatively at 42 d after the broiler was supplemented with sodium butyrate or betaine, whereas sodium butyrate reveals stress protection by the ability to drop stress protein at 21 d.

7. Treatment with sodium butyrate provided greater protection at two periods than betaine when the positive correlation represented by decreased caspase-3 and heat shock protein 90 was observed after exposure to stress by dexamethasone.

8. The two feed additives can reverse the adverse influence of dexamethasone by improving the stress index at both times.

Recommendations

1. A comparison study of the effects of corticosterone and dexamethasone on stress generation.

2. Provoking acute stress with dexamethasone.

3. Demonstrating the impact of dexamethasone as a stress factor on different types of poultry.

4. Use sodium butyrate and betaine at a higher dosage when exposed to chronic stress induced by dexamethasone.

5. Histopathological study to determine the apoptotic effect of dexamethasone and/or sodium butyrate and betaine.

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الخلاصة

الهدف من الدراسة الحالية هو إظهار تأثير بيوتاريت الصويوم والبيتاين كعوامل مضادة للإجهاد على بعض المتغيرات الفسلجية في أفراخ فروج اللحم المعرض للإجهاد الغير متخصص المحدث تجريبا باستعمال ديسكاميثازون تم استعمال 96 من فروج اللحم نوع روز بعمر يوم واحد وذلك بتربيتها في بيت الحيوان\ كلية الطب البيطري\ جامعة الموصل، في الفترة ما بين شهر أيلول الى تشرين الثاني 2021 في الظروف المناسبة لهذه الدراسة وتم تقسيمهم الى 4 مجاميع، كل منها يتكون من 24 فرخ وبثلاث مكررات، وفترتين عمريتين 21 و42 يوم واعتبرت المجموعة الأولى مجموعة السيطرة بينما المجموعة الثانية هي مجموعة الديكساميثازون وتم حقن فروج اللحم بالديسكاميثازون كل 3 أيام بجرعة محددة تجريبيا عند 1 ملغم\كغم من وزن الجسم تحت (تحت الجلد). في حين تم حقن المجموعتين الثالثة والرابعة بالديكساميثازون وعولجتا ببيوتاريت الصوديوم والبيتاين بجرعة 1.2 غم\كغم علف، 2 غم\كغم علف على التوالي.

أظهرت نتائج الدراسة بأن الديكساميثازون أثر سلبيا على معامل التحويل الغذائي ووزن الجسم النهائي في كلتا الفترتين وكان هناك انخفاض معنوي في الزيادة الوزنية واستهلاك العلف عند 21 يوم مقارنة مع مجموعة السيطرة، ولوحظ ارتفاع معنوي في وزن الكبد والقانصة في كلا الفترتين، وارتفاع في وزن القلب في يوم 21 ووزن جراب فابريشيا في 42 يوم. كان هناك ارتفاع معنوي في كل من حجم الخلايا المرصوصة وقيم المؤشر الإجهاد لمعايير الدم في مجموعة الديكساميثازون في الفترتين 21 و42 يوم من عمر الطائر، فضلا عن زيادة في مستويات إنزيمات الكبد (ناقلة أمين الألانين وناقلة أمين الأسبارتيت) مقارنة مع مجموعة السيطرة. أدى الحقن بالديكساميثازون الى حدوث انخفاض معنوي في مستوى كلوتاثيون وارتفاع معنوي في مستوى مالنولديالديهايد في مصل الدم ونسيج الكبد والطحال وجراب فابريشيا وأظهرت أيضا مؤشرات الإجهاد تأثيرا سلبيا ومعنويا لمجموعة الديكساميثازون وذلك بزيادة قيم كاسبيز-3 وبروتين الصدمة الحرارية 90 في الفترتين مما أدى الى انعكاس هذه النتائج لاحقا في العلاقة ما بين كاسبيز-3 وبروتين الصدمة الحرارية 90 بصورة معنوية طردية بسبب الإجهاد التأكسدي في الفترة الاولى.

أدى إضافة كل من بيوتاريت الصوديوم أو البيتاين الى عليقة فروج اللحم المحقونة بالديكساميثازون الى تعزيز إداء النمو من خلال تحسين قيم معامل التحويل الغذائي بعمر 42 يوم، بينما خفض معنويا بيوتاريت الصوديوم من وزن الكبد عند 21 يوم مقارنة مع مجموعة الديكساميثازون، وفي عمر 42 يوما كان لهذه الإضافات العلفية تأثيرات مماثلة بالتغلب على التأثير السلبي للديكساميثازون عن طريق خفض وزن الكبد مقارنة مع مجموعة الديكساميثازون. تشير النتائج الى إن إضافة بيوتاريت

الصوديوم أو البيتاين الى عليقة فروج اللحم المحقون بالديكساميثازون أدى الى حدوث انخفاض معنوي في حجم الخلايا المرصوصة وقيم المؤشر الإجهاد في كلا الفترتين بالإضافة الى حدوث انخفاض معنوي انزيمي الكبد ناقلة الأمين الألانين وناقلة الأمين الأسبارتيت في الفترة 21 يوم، بينما في عمر 42 يوم فقد خفض معنويا البيتاين من هذين الأنزيمين مقارنة مع مجموعة الديكساميثازون. أظهرت أيضا النتائج ان إضافة بوتاريت الصوديوم أو البيتايين في العلف فروج اللحم المعرض للديكساميثازون أدى الى أرتفاع معنوي في مستوى كلوتاثيون وانخفاض معنوي في مالنولديالديهايد لمصل الدم في كلا الفترتين مقارنة مع مجموعة ديكساميثازون وفي الفترة 21 يوم رجعت قيم مالنولديالديهايد لما هو عليه في مجموعة السيطرة، أما نسيج الكبد فقد أحدثت كلتا الاضافتين العلفية الى رفع مستوى كلوتاثيون في كلا الفترتين وخفض في مستوى مالنولديالديهايد مقارنة مع مجموعة الديكساميثازون ورجعت قيم مالنولديالديهايد في 21 و42 يوم لما هو عليه في مجموعة السيطرة. وكما أظهرت المجموعة الثالثة والرابعة ارتفاع معنوي في مستوى كلوتاثيون في نسيج الطحال في الفترة 42 يوم مقارنة مع مجموعة الديكساميثازون ورجعت القيم لما هو عليه في مجموعة السيطرة، اما بالنسبة لمستوى مالنولديالديهايد في نسيج الطحال فكان هناك انخفاض معنوي بمجموعتين الثالثة والرابعة لكلا الفترتين مقارنة مع مجموعة الديكساميثازون ورجعت القيم لما هو عليه في مجموعة السيطرة في الفترة العمرية الأولى، كما ولوحظ هناك ارتفاع معنوي في مستوى الكلوتاثيون في جراب فابريشيا في المجموعتين الثالثة والرابعة مقارنة مع مجموعة الديكساميثازون في 21 و42 يوم وقد رجعت القيم لما هو عليه في مجموعة السيطرة في الفترة 21 يوم, بينما مستوى مالنولديالديهايد في جراب فابريشيا فكان هناك انخفاض معنوي في المجموعتين الثالثة والرابعة مقارنة مع مجموعة الديكساميثازون في كلتا الفترتين ورجعت القيم لما هو عليه في مجموعة السيطرة في الفترة 42 يوم. بينت النتائج ان الإضافة كل من بيوتاريت الصوديوم أو البيتاين في عليقة الطيور المجهدة أدى الى انخفاض معنوي في بروتين الصدمة الحرارية 90 في كلا الفترتين مقارنة مع مجموعة الديكساميثازون في حين كانت لإضافة بيوتاريت الصوديوم القدرة الأكبر على تقليل من مستوى الصدمة الحرارية 90 بعمر 21 يوم، كما قل نشاط الكاسبيز-3 معنويا بعد وضع احدى الاضافتين العلفية للطيور المجهدة مقارنة مع مجموعة الديكساميثازون في الفترتين العمرتين ورجعت القيم لما هو عليه في مجموعة السيطرة في الفترة 21 يوم.

نستنتج من الدراسة الحالية أنه من خلال الفترتين العمرية لفروج اللحم بأن للديكساميثازون تأثير سلبي على إداء النمو وحجم الخلايا المرصوصة ومؤشر الاجهاد وإنزيمات الكبد ومستوى كل من كلوتاثيون و مالنولديالديهايد في مصل الدم ونسيج الكبد والطحال وجراب فابريشيا وعلى بروتينات

الصدمة الحرارية 90 وموت الخلايا المبرمج في الكبد وأنعكس ذلك من خلال الارتباط الإيجابي المعنوي بين الكاسبيز-3 وبروتين الصدمة الحرارية 90، وإن إضافة كل من بيوتاريت الصوديوم أو البيتاين الى عليقة فروج اللحم المجهدة له خصائص مضادة للإجهاد والأكسدة عن طريق تحسين وتعزيز كل المقاييس الفسلجية المختلفة التي استخدمت في هذه الدراسة، لكن النتائج لهاتين الاضافتين أفضل في فترة 21 يوم من فترة 42 يوم.

الاستجابة الفسلجية لبعض العوامل المضادة للإجهاد في فروج اللحم المعرض للدكساميثازون

رسالة تقدمت بها

هالة أسامة عدنان الشرهان

إلى
مجلس كلية الطب البيطري في جامعة الموصل
وهي جزء من متطلبات شهادة الماجستير
في اختصاص الطب البيطري / الفسلجة البيطرية

بإشراف
الاستاذ المساعد
الدكتورة هيام نذير متي

1444هجري **2022 ميلادي**

جامعة الموصل

كلية الطب البيطري

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رسالة ماجستير

الطب البيطري/ الفسلجة البيطرية

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