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RESEARCH ARTICLE

Determining the Effect of Different Combinations of NSAIDs on Hemato-Immunological Parameters and the Association with Heat Shock Protein-70 in Broilers

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ABSTRACT

The use of NSAIDs in stress conditions, like summer heat stress, winter cold stress, vaccination and drug intake stress may help to prevent or minimize the disease outbreaks in poultry birds. This study was planned to investigate the effects caused by short term and prolonged use of acetylsalicylic acid (ASA), acetaminophen (APAP) and ibuprofen with different combinations in broilers and their association with heat shock protein 70, i.e., to determine the safe level of NSAID's use in poultry. For this purpose, total 200 day-old broiler chicks were procured and performed study having 42 days. The broilers were equally disseminated at 10th day into A-D groups, where group A act as control negative. The B, C and D groups were divided into different subgroups. In current experiment, using completely randomized design three levels in different combinations with different doses of NSAIDs were given orally in drinking water from day 10 to 32 of age of birds. The NSAIDs had no effect on hematological parameters while, immunological parameters showed significant results according to dose manner of NSAIDs especially in treatment groups (APAP @160 mg/L, ASA @1200 mg/L), (APAP @90mg/L, ibuprofen @60 mg/L) and (APAP @160 mg/L, ibuprofen @120 mg/L) as compared to control negative. The gene expression of HSP-70 in treated groups (ASA @600 mg/L, ibuprofen @60 mg/L), (ASA @1200 mg/L, ibuprofen @120 mg/L), (APAP @ 30mg/L, ASA @300 mg/L), (APAP @ 90mg/L, ASA @600 mg/L), (APAP @ 160mg/L, ASA @1200 mg/L) and (APAP @160mg/L, ibuprofen @120mg/L) had significant results as compared to control negative group. The study presented that low and medium doses of NSAIDs could be used as possible substitute for antipyretic, anti-inflammatory and analgesic drugs for poultry. The toxicity due to excess dose of APAP in different combinations was also observed.

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INTRODUCTION

The non-steroidal anti-inflammatory drugs (NSAIDs) are normally used to decrease pain in different diseases and under post-operative conditions. The NSAIDs have three main functions like anti-inflammatory, analgesic and antipyretic. The enzyme COX is responsible for normal physiological turnover of prostaglandin (Bindu et al., 2020). The NSAIDs are broadly used to cure inflammatory disorders, such as (arthritis) by inhibiting COX- enzymes which play an important role in the development of pro-inflammatory prostaglandin (Zihran et al., 2022). Moreover, nonsteroidal anti-inflammatory drugs are also used in birds, (Bauck, 1990) in relieving inflammatory conditions and pain in pet birds and food producing birds like chickens, turkeys, ducks, geese, quails, swans, guinea fowls and ostrich, etc. and also in zoo birds (Mohan, 2010). Although, a large number of nonsteroidal anti-inflammatory drugs are being discovered following the initial innovation of aspirin, relatively in poultry very small amount of NSAIDs are being used unlike in livestock and human beings (Muneeb et al., 2022). In ascites, moderate reduction was reported to ASA treated broiler birds kept in a hypobaric chamber to mimic high altitude (Khajali, 2022).

Acetaminophen (N-acetyl-P aminophenol) is very effective antipyretic and analgesic drug, which is used in large scale in human medicine, having a wide range of dose rate and is also used as growth stimulant (Ayoub, 2021). It metabolizes to a potentially toxic metabolite, N-

acetyl-Parabanzoqinimin, conjugated with glutathione that is excreted out through renal system, but overdose of acetaminophen results in high production of the toxic metabolites which may lead to increased serum transaminases concentrations (liver cell necrosis) and liver dysfunction (Al-Zubaidy, 2021). The APAP breakdown takes place in liver, where it is converted into 60% acetaminophen-glucuronide and about 32% acetaminophen-sulfate, while 9% is oxidized to N-acetyl-p-benzoquinone-imine and 3-hydroxyacetaminophen and 3% is eliminated unaffected (Israr et al., 2021). The APAP by the peroxidase function of COX-2, produces the reactive metabolites which can reduce glutathione, a cofactor enzyme like PGE synthesis (Ayuso et al., 2021).

Ibuprofen is an NSAID, which stops the biosynthesis of prostaglandins with immunosuppressive and pro-inflammatory properties. Therefore, in the broiler chickens, ibuprofen projected as an applicant molecule for the cure of coccidiosis (Pal et al., 2024). It is a potential drug, that inhibits both COX-1 and COX-2 pathway. It appears that its analgesic, antipyretic and anti-inflammatory activities are achieved principally through COX-2 inhibition, whereas COX-1 inhibition is responsible for its unwanted effects on the gastrointestinal mucosa (Sohail et al., 2023). Relatively, paracetamol has slight anti-inflammatory activity, unlike other common analgesics such as NSAIDs including ibuprofen. However, APAP, paracetamol and ibuprofen have similar effects in the treatment of headache. The APAP can relieve pain in mild arthritis; however, it has no effect on the underlying redness inflammation, and swelling of joints (Freo et al., 2021).

The nonsteroidal anti-inflammatory drugs have been shown to enhance the heat shock response and a reaction to hyperthermia and other toxic situations in addition to their anti-inflammatory response which is characterized by the induction of HSPs (Batulan et al., 2005). It is probable that acetylsalicylic acid (NSAIDs) deliberates the protection of heart by another mechanism, e.g., upregulation of expression of many different HSPs protein family members, including HSP-27 (Ebert et al., 2005), in many other species (Sandoval-Montiel et al., 2013). Heat shock proteins are widely supposed to protect cell against any damage caused by several stress-factors. It is reasonable to consider that, NSAIDs may protect by modulating HSP-expression against myocardial injury from stress. Previous research; however, has indicated that acetylsalicylic acid may decrease stress in injury, that it is closely correlated to the HSR (Ghavami and Hardy, 2002).

Keeping in view of above situation, this study was planned to investigate the effect of NSAIDs on hematological and immunological parameters in different combinations and check the gene expression of HSP-70.

MATERIALS AND METHODS

Ethical approval: The proposed study design was approved by ethical committee of University of Agriculture Faisalabad with ref # (D.No.3676/ORIC, 30/08/2021).

Experimental layout: The 200 broiler chicks were purchased from local hatchery. After 10 days of acclimatization under suitable environmental conditions, birds were distributed into 10 different groups (A-D), having 9 subgroups. The water and feed was provided at *ad-libitum*. Group A acted as control -ve group. The various combination of NSAIDs at different doses via drinking water were given to B, C and D subgroups. 5 birds from each group were slaughtered on 14th and 42nd day of trial. The total duration of trial was 42 days. The treated birds with different doses of the individual NSAIDs were mentioned in Table 1.

Table 1: Experimental Design.

Groups	No.	of Birds	Treatment
A		20	Control negative group
	B1	20	Acetylsalicylic acid (tab) + Ibuprofen (syrup) @ 300 mg/L + @ 20 mg/L
B	B2	20	Acetylsalicylic acid (tab) + Ibuprofen (syrup) @ 600 mg/L+ @ 60 mg/L
	B3	20	Acetylsalicylic acid (tab) + Ibuprofen (syrup) @ 1200 mg/L+ @ 120 mg/L
	C1	20	Acetaminophen (syrup) + Acetylsalicylic acid (tab) @ 30 mg/L + @ 300 mg/L
C	C2	20	Acetaminophen (syrup) + Acetylsalicylic acid (tab) @ 90 mg/L + @ 600 mg/L
	C3	20	Acetaminophen (syrup) + Acetylsalicylic acid (tab) @ 160 mg/L+ @ 1200 mg/L
	D1	20	Acetaminophen (syrup) + Ibuprofen (syrup) @ 30 mg/L + @ 20 mg/L
D	D2	20	Acetaminophen (syrup) + Ibuprofen (syrup) @ 90 mg /L+ @ 60 mg/L
	D3	20	Acetaminophen (syrup) + Ibuprofen (syrup) @ 160 mg/L+ @ 120 mg/L

Parameters studied:

Hematology: 5 birds from each group were euthanized humanely on 14th and 42nd day of trial. Blood samples with anti-coagulant were used for hematology. The TLC and TEC was done by using hemocytometer as previously described by Kansal *et al.* (2017). The Hb- concentration was determined by using Sahli's haemoglobinometer as previously described Muneer *et al.* (2021).

Immunology:

Antibody response against SRBCs: Serum samples were collected to check antibody titers against injected 3% washed SRBCs at 21st, 28th, 35th day of experimental birds and antibody response was assessed by Regnier *et al.* (1980). Serum was separated and kept at -20°C. The stored serum samples were tested to check antibody titers against SRBCs by using titration method. Serum samples were heat inactivated by using water bath at 56ºC for 30 min. 50µl of phosphate buffer saline was poured in every well of titration plate. Then, 50µL of inactivated sample of serum was added in first well and incubated at 37°C for 30 min. After incubation, a two-fold serial dilution was done for each sample. Then, 50µl of 3% SRBCs was poured in every well and incubated at 37°C for 30 min and titers were recorded.

Lymphoproliferative response to avian tuberculin: The cutaneous lymphoproliferative response to avian tuberculin was measured in-vivo at 28th day. 3 birds were selected to study lympho-blastogenic response. 0.1ml of avian tuberculin was injected in inter-digital space between 3rd and 4th digit of right foot in each bird. The responses were evaluated by measuring thickness of intra-digital skin at 24, 48 and 72hrs, described by Corrier (1990).

Mononuclear phagocytic system function assay: Black Indian ink was centrifuged at 3000rpm for 30 min and supernatant was collected. Three glass tubes having 4ml of 1% sodium citrate solution were labelled as 0, 3 and 15min for each bird. 0.2ml blood was collected from wing vein and transferred in 0 min glass tube. Supernatant of ink was injected with 1ml/kg B.W of bird in wing vein of right side. Blood was collected from wing vein of left side and added in 3 and 15min glass tubes. After centrifugation, remaining particles in supernatant was measured at 640 nm by spectrophotometer based by Sarker *et al.* (2000).

Detection of gene expression of HSP-70 by real-time PCR:
RNA isolation: Tissue samples of liver were kept at -70°C in liquid nitrogen. Sample was triturated in liquid nitrogen. 100µl trizol reagent was added in sample. After homogenization, 200µl chloroform was added. 3 layers were formed after 15min. Took supernatant and added 200µl ISO-propanol and mixed it. Sample was preserved at -20°C for 24 hrs. After centrifugation, pellet was observed. Supernatant was discarded and centrifuged it for 2min by adding 70% ethanol. The previous step was repeated three time. RNA pellet was preserved in RNA's free water.

Primers: To check gene expression of HSP-70, oligo primers were used with the following sequence. 5'-AGCGTAACACCACCATTCC-3' (forward primer) and 5'-TGGCTCCCACCCTATCTC-3' (reverse primer) (Yu and Bao, 2008).

cDNA synthesis*:* After RNA extraction, synthesis of cDNA was done by kit method. The thermos scientific RevertAid First Strand cDNA Synthesis kit was used having catalog # K1622.

Relative quantification by real-time PCR: SYBER Green I-based one-step real-time quantitative amplification was done by using (iCycler iQ) Real-Time PCR detection system. In RT-PCR conditions, the denaturation, annealing and extension temperature was 94°C, 58°C and 72°C for 30 secs, respectively.

Statistical analysis: The experimental data was collected and analyzed by using analysis of variance technique (P≤0.05) and means were analyzed by Tukey, s test by using the SAS statistical software version 9.2 (SAS, 2007).

RESULTS

Hematological analysis: At 14th and 42nd day of the experiment, the erythrocyte count (TEC), leukocytes count (TLC), Hb-concentration of all treatment groups with different combination of NSAIDs were non-significantly (P≥0.05) different as compared to the control negative group (Fig. 1-3).

Immunological analysis
Antibody response against sheep RBCs: On the 21st day of the primary injection, the total antibody titers of groups B3 (ASA + Ibuprofen), C2 (APAP+ASA), C3 (APAP+ASA), D1 (APAP+Ibuprofen), D2 (APAP+Ibuprofen) were significantly (P≤0.05) lower than control negative group. The IgG and IgM titers were non-significantly different of treatment groups as compared to the control negative group. On 28th day of the secondary injection, the total antibody titers, IgG and IgM titers in different combination of NSAIDs treated groups were non-significantly different than the control negative group. On 35th day of the tertiary injection, the group B3 (ASA+Ibuprofen) had significantly (P≤0.05) lower antibody titers as compared to the control negative group. While the IgG and IgM titers in different combination of NSAIDs in all treated groups were non-significantly different as compared to the control negative group (Fig. 4-6).

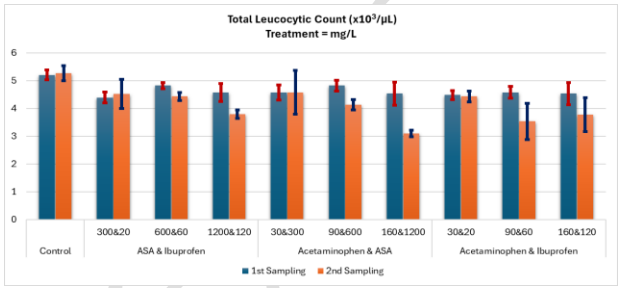

Fig. 1: Hematological values (TLC) of broilers treated with different combination of NSAIDs.

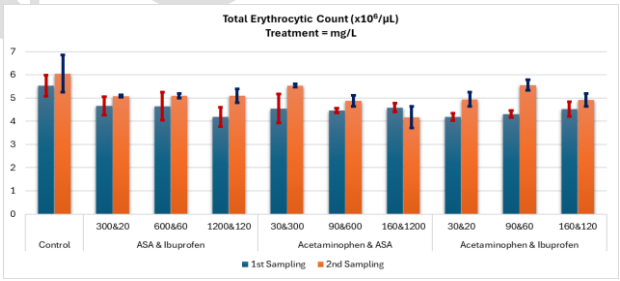

Fig. 2: Hematological values (TEC) of broilers treated with different combination of NSAIDs .

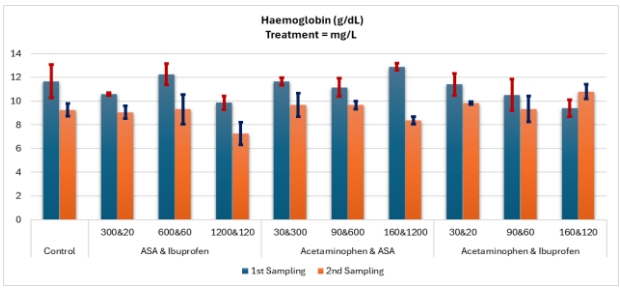

Fig. 3: Hematological values (Hb) of broilers treated with different combination of NSAIDs.

Lymphoproliferative response to avian tuberculin: The avian tuberculin revealed that after 24h of injection, the different combination of NSAIDs treated groups were non-significantly different in thickness at interdigital space as compared to the control negative group. The response after 48h of injection to avian tuberculin showed that, the groups C3 (APAP+ASA), D1 (APAP+ Ibuprofen), D2 (APAP+Ibuprofen) and D3 (APAP+ Ibuprofen) significantly (P≤0.05) increased thickness, while all other treated groups were non-significantly different from control negative group. After 72h of

injection, all treatment groups with different combination of NSAIDs were non-significantly different to lymphoproliferative response than control negative group (Fig. 7).

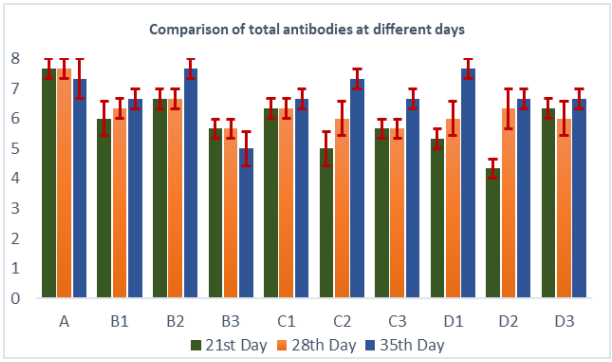

Fig. 4: Antibody titers (total Abs) against S-RBCs of broilers treated with different combination of NSAIDs.
(Group A= Control negative; Group B1= ASA + Ibuprofen, 300mg/L + 20mg/L; Group B2= ASA + Ibuprofen, 600mg/L + 60mg/L; Group B3= ASA + Ibuprofen, 1200mg/L + 120mg/L; Group C1= APAP + ASA, 30mg/L + 300mg/L; Group C2= APAP + ASA, 90mg/L + 600mg/L; Group C3= APAP + ASA, 160mg/L + 1200mg/L; Group D1= APAP + Ibuprofen, 30mg/L + 20mg/L; Group D2= APAP + Ibuprofen, 90mg/L + 60mg/L; Group D3= APAP + Ibuprofen, 160mg/L + 120mg/L).

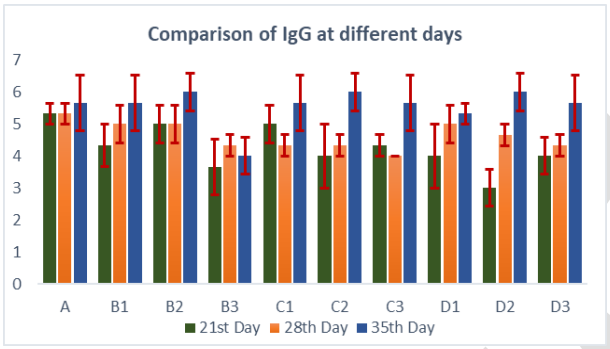

Fig. 5: Antibody titers (IgG) against S-RBCs of broilers treated with different combination of NSAIDs.
(Group A= Control negative; Group B1= ASA + Ibuprofen, 300mg/L + 20mg/L; Group B2= ASA + Ibuprofen, 600mg/L + 60mg/L; Group B3= ASA + Ibuprofen, 1200mg/L + 120mg/L; Group C1= APAP + ASA, 30mg/L + 300mg/L; Group C2= APAP + ASA, 90mg/L + 600mg/L; Group C3= APAP + ASA, 160mg/L + 1200mg/L; Group D1= APAP + Ibuprofen, 30mg/L + 20mg/L; Group D2= APAP + Ibuprofen, 90mg/L + 60mg/L; Group D3= APAP + Ibuprofen, 160mg/L + 120mg/L).

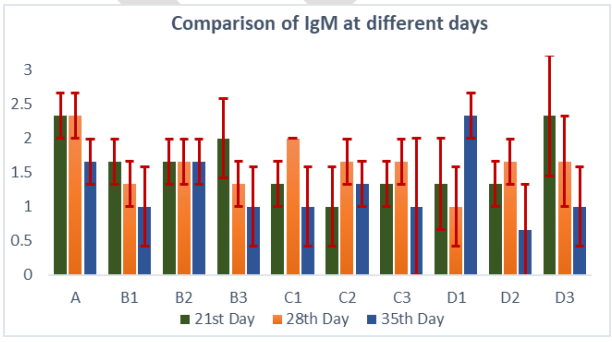

Fig. 6: Antibody titers (IgM) against S-RBCs of broilers treated with different combination of NSAIDs.
(Group A= Control negative; Group B1= ASA + Ibuprofen, 300mg/L + 20mg/L; Group B2= ASA + Ibuprofen, 600mg/L + 60mg/L; Group B3= ASA + Ibuprofen, 1200mg/L + 120mg/L; Group C1= APAP + ASA, 30mg/L + 300mg/L; Group C2= APAP + ASA, 90mg/L + 600mg/L; Group C3= APAP + ASA, 160mg/L + 1200mg/L; Group D1= APAP + Ibuprofen, 30mg/L + 20mg/L; Group D2= APAP + Ibuprofen, 90mg/L + 60mg/L; Group D3= APAP + Ibuprofen, 160mg/L + 120mg/L).

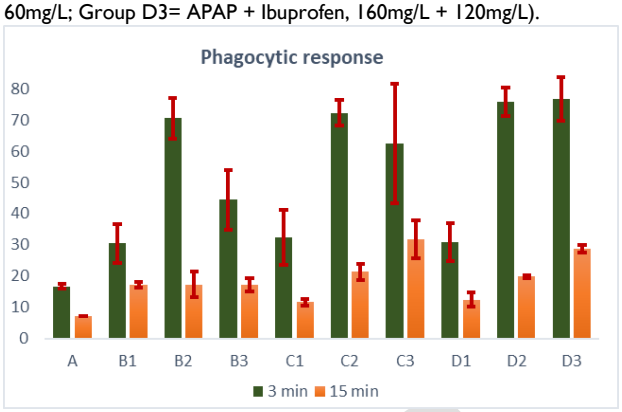

Fig. 8: In-vivo phagocytic response of mononuclear cells to carbon particles of broilers treated with different combination of NSAIDs.
(Group A= Control negative; Group B1= ASA + Ibuprofen, 300mg/L + 20mg/L; Group B2= ASA+ Ibuprofen, 600mg/L + 60mg/L; Group B3= ASA+ Ibuprofen, 1200mg/L + 120mg/L; Group C1= APAP + ASA, 30mg/L + 300mg/L; Group C2= APAP + ASA, 90mg/L + 600mg/L; Group C3= APAP + ASA, 160mg/L + 1200mg/L; Group D1= APAP + Ibuprofen, 30mg/L + 20mg/L; Group D2= APAP + Ibuprofen, 90mg/L + 60mg/L; Group D3= APAP + Ibuprofen, 160mg/L + 120mg/L).

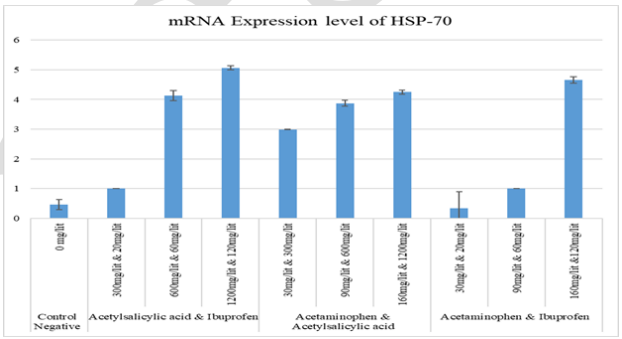

Fig. 9: The mRNA expression level of HSP-70 of broilers treated with different combination of NSAIDs.
(Group A= Control negative; Group B1= ASA + Ibuprofen, 300mg/L + 20mg/L; Group B2*= ASA+ Ibuprofen, 600mg/L + 60mg/L; Group B3*= ASA+ Ibuprofen, 1200mg/L + 120mg/L; Group C1*= APAP + ASA, 30mg/L + 300mg/L; Group C2*= APAP + ASA, 90mg/L + 600mg/L; Group C3*= APAP + ASA, 160mg/L + 1200mg/L; Group D1= APAP + Ibuprofen, 30mg/L + 20mg/L; Group D2= APAP + Ibuprofen, 90mg/L + 60mg/L; Group D3*= APAP + Ibuprofen, 160mg/L + 120mg/L).

Mononuclear phagocytic system function assay: The phagocytic response to carbon particles was observed with different combination of NSAIDs treatment groups. At three (3) minutes, maximum in-vivo phagocytic potential was observed in groups D2 (APAP+Ibuprofen) and D3 (APAP+Ibuprofen), while all other treatment groups with different combination of NSAIDs showed non-significant difference from the control negative group. At 15 minutes, the absorbance percentage was significantly ($P \leq 0.05$) greater in groups C3 (APAP+ASA) and D3 (APAP+Ibuprofen), while all other treatment groups with different combination of NSAIDs showed non-significant difference as compared to the control negative group (Fig. 8).

Detection of gene expression of hsp-70 mRNA by real-time PCR: The results demonstrated that, the mRNA expression level of hsp-70 in different combination of NSAIDs treatment groups B2 (ASA+Ibuprofen), B3 (ASA+Ibuprofen), C1 (APAP+ASA), C2 (APAP+ASA), C3 (APAP+ASA) and D3 (APAP+Ibuprofen) were

significantly (P≤0.05) higher than the control negative group. While the levels of hsp-70 mRNA of other treatment groups was non-significantly different as compared to the control negative group (Fig. 9).

DISCUSSION

Poultry meat demand has been increased in Pakistan during the last few years. The NSAIDs are used in poultry for treatment of different conditions such as locomotor disorders and stress (cold, hot, fever and etc.), by the poultry farmers without prescription. It is also widely used for the treatment of respiratory and digestive problems (Baert, 2003). This was the basis of the research was conducted; to illustrate the effects of NSAIDS, either in different combinations in broilers. No side effects of NSAIDs have been reported by Pozniak *et al.* (2010).

In the current study, the different combination of NSAIDs (ASA, APAP and ibuprofen) showed parallel values of TLC, TEC and Hb at 1st and 2nd slaughtering than control negative group A, similar to the results described by Derakhshanfar *et al.* (2013). The erythrocyte indices are directly related to the values of TEC and Hb. Almost similar pattern of rise and decline in hematological parameters in treatment groups has been reported by Majid *et al.* (2015) and Baki *et al.* (2019).

In current study, the combination of NSAIDs treated groups B3, C2, C3, D1 and D2 showed significantly (P<0.05) lower values of antibody titers against SRBCs, while IgG and IgM has shown parallel values at 21st day of experiment after primary injection of than control negative group. On 28th day of the experiment, the antibody titers (total Ig, IgG, IgM) against SRBCs showed parallel values to the control negative group in different combinations. At 35th day of the experiment, the group B3 had signed (P<0.05) lower value of total Ig, while other groups showed parallel values of antibody titers (total Ig, IgG, IgM) against SRBCs treated with different combinations than control negative group. Almost similar results about the antibody production have been documented by Saleh *et al.* (2016). The variation in antibody response against SRBCs according to dose manner of NSAIDs. The NSAIDs helps to inhibit the activity of COX enzymes by affecting the production of PGF2 which regulates the immune responses at low and medium doses due to reduced stress.

The lymphoproliferative response presented significantly (P<0.05) higher values in combination of treatment groups C3, D1, D2 and D3 after 48 hrs. of avian tuberculin injection than control negative group. At 24, 48 and 72 hours, the other groups treated with different combination of NSAIDs (ASA, APAP, ibuprofen) showed parallel values to the control negative group. By the use of avian tuberculin, another cellular immune response was accessed. The NSAIDs not only interferes the development of antibody response, but also helps to improve the immune tolerance in dendritic and various immune cells by (Huemer, 2015; Raaijmakers *et al.* 2022). The cell mediated response showed significantly (P<0.05) higher values in combination of NSAIDs treated groups D2 and D3 at 3 minute than control negative group. At 15 minutes, the phagocytic activity showed significantly (P<0.05) higher values in groups C3 and D3 than control negative group. The NSAIDs have anti-inflammatory properties and may also have complex immunological results on the proliferation of cells, cytokine and production of antibodies. The cell-mediated immune response may involve in obliteration of infected cells or I/C pathogens by cytotoxic T-cells and macrophages.

In present study, the combination of NSAIDs treated groups B2, B3, C1, C2, C3 and D3 were showed significantly (P<0.05) increased values of mRNA expression of Hsp-70 than control negative group, almost similar findings are also observed by (Yu and Bao, 2008; Dervisevik *et al.* 2022). The increased expression of Hsp-70 showed that, heat shock proteins might have been produced as a stress of these drugs and thus may also help to improve the survival of cell by protecting proteins from the degradation and facilitating the re-folding of proteins. Similar results were seen by Hartl, (1996). The NSAIDs have been shown to enhance the heat shock response in different situations in addition to their anti-inflammatory response which is characterized by the induction of HSPs.

Conclusions: In conclusion, the hematological and immunological findings indicate that, the NSAIDs had no adverse effects on production level of broilers. The different combination of NSAIDs at different doses persuades an mRNA expression and increase the levels of HSP-70 protein in broilers. These drugs can be used to control infections (i.e. coccidiosis) and in stressed conditions. So, NSAIDs could be a possible substitute as antipyretic, anti-inflammatory and analgesic drugs for poultry use.

Authors contribution: IH, MTJ and MKS contributed in research planning, data analysis and manuscript writing. MKS and MMG helped in proof reading. IH, MTJ, MKS and MMG executed research plan, laboratory analysis. MTJ, MMG and MKS participated in manuscript preparation and data interpretation.

Abbreviations

APAP	n-acetyl-para-aminophenol / acetaminophen
ASA	acetylsalicylic acid
BW	body weight
cDNA	complementary DNA
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
Hb	hemoglobin
h	hour
HSP-70	heat shock protein-70
IgG	immunoglobin G
IgM	immunoglobin M
kg	kilogram
L	liter
min	minute
ml	milliliter
mRNA	ribonucleic acid
nm	nanometer
NSAIDs	non-steroidal anti-inflammatory drugs
PGE	prostaglandin

RNA	ribonucleic acid
rpm	revolutions per minute
sec	second
SRBC	sheep red blood cells
tab	tablet
TEC	total erythrocyte count
TLC	total leukocyte count
µl	microliter

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