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Effect of *in ovo* betaine supplementation during normal and early embryonic thermal conditioning on the hatchability as well as post-hatch performance in broiler chickens

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ABSTRACT

Two separate experiments carried out to study effect of *in ovo* betaine supplementation during normal as well as elevated incubation condition on post-hatch broiler performance. In experiment 1, fertile eggs (400 nos) incubated under 4 different groups and standard hatchery parameters were noted. *In ovo* administration of betaine was carried out on 18th day at the rate of 20 mg (T_1), 40 mg (T_2), 60 mg (T_3) and 0 mg (T_4) dissolved in 0.1 ml of PBS/egg. In experiment around 400 eggs were injected betaine at (T_1), 50 (T_2), PBS at 0.1 ml (T_3) and µn-injected control (0 mg) (T_4). *In ovo* injection was carried out at 10th day of incubation as per standard protocol. After 48 h following betaine injection (12th day), the setter temperature had increased to 103±0.5°F from for 99±1°F in all the groups for a period 5 days (12 to 17 days of incubation). Chicks were hatched out and transferred to experimental house by maintained them in respective groups and distributed evenly into six replicates in each group. The *in ovo* betaine supplementation did not affect the hatchability percentage, hatch weight but improved the starter phase production performance. However, *in ovo* betaine administration followed by thermal conditioning adversely affected the hatchability percentage. The post hatch performance, i.e. production, immunity and serum biochemical profile were comparable among all the treatment groups. In conclusion, the overall production of broiler chicken during early phase of life can be positively improved by *in ovo* betaine supplementation with normal incubation temperature.

Keywords: Betaine, Broilers, Embryonic stage, *In ovo,* Thermal condition

Myriad of innovative approaches are being tried by researchers to strengthen the early embryo conditioning *via in ovo* nutrition and perinatal heat acclimation for ameliorating heat stress (Saeed 2019). Early exposure of broiler birds to high temperature sensitize the embryo and could increase their thermotolerance adaptability in later stages of life without compromising growth (Zaboli *et al*. 2017). Induction of epigenetic mechanisms will help to regulate heat stress tolerance for long time is physiological basis of this approach (Vinoth *et al*. 2018). Thermal manipulation may decrease abdominal fat in broilers to have better physiological responses to environmental conditions during rearing (Fernandes *et al*. 2016).

The juvenile nutrition and management is considered as more critical period, compounding to production of weak chicks which could not survive the harsh heat stress conditions. These obstacles can be bowled over by feeding the chicks during their embryonic stage itself, which could help the chicks to survive the post-hatch delay in feeding, disease susceptibility and better production performance. As *in ovo* feeding fetches the property of supplying nutrients directly during prenatal period without any dissipation, can aid in supplementation of various additives and other substances during prenatal period. Betaine a trimethyl derivative of the amino acid glycine protects cells during stress (osmoregulation) and also donate methyl group via transmethylation, for the synthesis of critical metabolites like DNA/RNA, protein and choline (Wafaa A Abd El-Ghany and Daryoush Babazadeh 2022). Betaine have osmoregulatory property and it reduce symptoms like panting and even prevent the increase of body temperature. Betaine also improves the hatchability and chick weight; increases breast yield; increases immunity and reduce homocysteine which is responsible for chick mortality (Rokade *et al.* 2020.)

It is hypothesized that *in ovo* supplementation of betaine could establish a potential channel for transfer of nutrients from prenatal stage to developing chick. Also, to evaluate the potential of betaine (*in ovo)* to counter act the manipulated prenatal heat stress in the hatchery set up further studies are required. With this background, comparative study was executed to assess the role of *in ovo*

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betaine supplementation at different incubation conditions to study their effect on hatchability, growth performance, immune response in heat stressed broiler chickens.

MATERIALS AND METHODS

Experimental design and interventions: Two separate experiments were carried out to explore the thermal manipulation and *in ovo* betaine injection under different incubation temperatures. In experiment 1, fertile eggs (400 nos) of CARIBRO-VISHAL broilers were collected, fumigated, weighed and incubated under four different groups, each having 100 eggs. The eggs were set in a force draft incubator at temperature 99±0.5°F and relative humidity of 60±2%. *In ovo* administration of betaine was carried out on 18th day of incubation by using a 24 gauge 25 mm needle at the broad end of egg (Bhanja *et al*. 2004). First three groups were injected with betaine at the rate of 20 (T_1), 40 (T_2) and 60 mg (T_3) dissolved in 0.1 ml of PBS/ egg. The fourth group (T_4) served as control (un-injected). Overall incubation (setting and hatching) procedures were carried out at standard temperature and relative humidity. After *in ovo* injection the eggs were sealed with wax and transferred to a hatcher and kept in pedigree hatching boxes.

In experiment 2, total of 400 fertile eggs of Bro-Vishal were divided into four different groups. The first three treatment groups were injected with betaine at 25 mg (T_1), 50 mg (T_2), 0.1 ml - PBS (T_3) and T_4 considered as control (un-injected control). The PBS will not have nutrients but that group undergo injection to stimulate the stress so will act as second control. The betaine levels here were decided based on observations of post-natal broiler performance in the first experiment and given by dissolving in 0.1 ml of PBS/egg. In order to get early embryo conditioning by *in ovo* nutrition and perinatal heat acclimation the *in ovo* injection was carried out at 10th day of incubation followed by thermal manipulation of incubator temperature. Forty-eight hours following betaine injection (12th day), the setter temperature had increased to 103±0.5°F from 99±1°F in all the groups for a period five days (12 to 17 days of incubation). On 19th day of incubation, the eggs were shifted to a hatcher and kept in pedigree hatching boxes to differentiate between the treatment groups. The chicks were hatched out and transferred to the experimental house, maintained them in their respective groups and uniformly reared for the entire experimental duration. The chicks were distributed evenly into six replicates in each group.

Micro-climatic observations: The present study was carried out at ICAR-Central Avian Research Institute, Bareilly during the peak summer months of May to July. Immediately after hatching the chicks were transported to the open sided deep litter experimental shed. During the initial 14 days, the chicks were provided with artificial heat source according to the recommended temperature and relative humidity. From 15 days of age, the chicks exposed to outside environmental conditions. The daily shed minimum and maximum temperature (°C) and relative humidity (%) was recorded and the average temperature and humidity index (THI) values were determined (Moraes *et al.* 2008). The THI during experiment was 85 which is consider as heat stress conditions.

$$THI=0.8DBT+ RH \times (DBT-14.4) + 46.4$$

Where, DBT, dry bulb temperature (°C); RH, Relative humidity.

Hatching performance: The effects of higher incubation temperature and *in ovo* administration of betaine on the hatchability, embryonic mortality, break out analysis were studied once the complete hatch was out by using following formulas.

$$\text{Hatchability of set eggs (HR\%)} = \left(\frac{\text{Number of hatched chicks}}{\text{Number of set eggs}}\right) \times 100$$

$$\text{Rate of early embryonic mortality (EEM, \%)} = \left(\frac{\text{Number of early embryonic mortalities}}{\text{Total number of fertile eggs}}\right) \times 100$$

Experimental diet and management: The birds of both the trails were fed with broiler diet as per the nutrient recommendation of ICAR, 2013. The diets were offered in mash form and two phases feeding, starter (0-21 days) and finisher (22-42 days) was followed in both experiments (Supplementary Table 1). The birds were reared at open shed housing with THI around 82-84.

Growth performance: Body weight changes were recorded weekly to ascertain the weekly and overall body weight gain. A weighed quantity of diet was offered *ad lib* daily to each group in the morning and the residue was weighed next day on daily basis in order to arrive at overall feed intake. Based on the data pertaining to the FI and BWG, the weekly and period wise FCR of birds was determined by the method described by Fritz *et al.* (1969) using following equation.

$$\text{Feed conversion ratio} = \frac{\text{Total feed intake (g)}}{\text{(final body weight – initial body weight (g)}}$$

Immune response: Immune response of the experimental birds as affected by *in ovo* betaine injected, embryonic thermal exposure and heat stress was evaluated in terms of humoral (HA/HI) and cell mediated immune response (foot pad index).

Humoral immune response: Humoral immunity was evaluated as hemagglutination (HA) and hemagglutination inhibition (HI) antibody titre against newcastle disease virus (NDV) at 7th and 14th day of post vaccination. The microtitre HA and HI procedure by Siegal *et al.* (1980) was followed to measure total HA and HI antibody titre in chickens.

Cell mediated immune response (CMI): Using Cheng and Lamont (1988) method CMI response to PHA-P mitogen was estimated. In eight birds from each treatment group 0.1 ml PHA-P (1mg/ml of PBS) was injected intra-dermally in the right foot web. Also in left foot web of the same birds 0.1 ml sterile PBS was injected and thus served as control. By using micrometer before and 24 h

after injection of mitogen thickness of foot webs (right and left) of injected birds was measured. By subtracting the skin thickness at 0 h from that of 24 h of injection of both feet, the difference in the foot web was calculated. Foot web index (FWI) was calculated by subtracting the difference in thickness at 0 h and 24 h of PBS injected (control) foot web from the difference in thickness of mitogen injected foot web at 0 and 24 h. FWI formula is presented here.

Foot web index (mm) = [(Web thickness post PHA-P injection) – (Web thickness pre PHA-P injection)] – [(Web thickness post PBS injection) – (Web thickness pre-PBS injection)]

Serum biochemical parameters: Blood samples were collected randomly from six birds per treatment group into sterile glass test tubes without addition of anticoagulant. Serum was separated by centrifugation at 3000 rpm for 10 min. and serum was decanted into plastic vials, and then stored at -20°C till further processing. The serum glucose, serum total cholesterol was estimated by CHOD/PAP method and total protein was estimated by biuret method using diagnostic biochemical kits obtained from Coral Clinical Systems Ltd., Goa, India.

Statistical analysis: The data obtained from both the experiments were subjected to analysis of variance – single factor using SPSS version 16.0. The values obtained in percentage were subjected to arsine transformation before statistical analysis. The means compared for significance using Tukey's range test and P-values less than 0.05 were considered as significant.

RESULTS AND DISCUSSION

Hatchery performance: The effects of *in ovo* betaine injection on egg weight, day old chick weight and hatchability parameters were presented in Table 1. In Experiment 1, the *in ovo* injection of betaine did not affect (P>0.05) the egg weight, hatchability of fertile egg and chick weight. In consistent to present results, Uni and Ferket (2005 revealed that the *in ovo* betaine injection did not affect hatchability and chick weight in broilers. These results indicate that the *in ovo* intervention neither have beneficial nor adverse effect on hatchability and hatch weight in broiler chickens. However, in experiment 2, the *in Ovo* betaine administration on 10th day accompanied by thermal exposure (12-17th day of incubation) had no effect on hatch weight but the hatchability was negatively affected (P<0.05). This adverse effect on hatchability might be due to early age embryonic interventions, which were quiet evident with reduction in hatchability in all the *in ovo* injected groups including betaine free PBS solution compare to control.

The higher thermal exposure at approximately 4°C increment during mid-embryonic stage (12-17 days) did not affect the hatchability in control groups which were comparable to that of control group in experiment 1. Therefore, to find out the root cause for hatchability reduction, break out analysis study were carried out. The break out analysis of hatching of eggs in experiment 2 revealed higher incidence of early embryonic mortality (23 to 39%) in all the treatment groups compare to control (0%), which might be due to early (10th day) *in ovo* intervention (Table 1). However, no other studies have reported a negative effect of *in ovo* betaine administration in chicken embryo. Due to very poor hatchability, the post-hatch performance, immunity and serum biochemical parameters study could not be performed in the experiment 2.

Production performance: The effect of supplemental *in ovo* betaine on production performance in experiment 1 was presented in Table 2. During starter period (0-3 week), highest feed intake was observed in the T_1 group (20 mg betaine injected group) followed by T_3 (60 mg betaine injected group) compared to control (P<0.05). Whereas, in finisher and overall period, no significant

Table 1. Effect of *in ovo* betaine injection on day old chick weight hatching parameters

Trial	Group	Egg weight (g)	Chick weight (g)	Hatchability (%)
Exp. 1	T1 (20mg)	59.80 ± 1.52	47.20 ± 3.24	85.47 ± 1.54
	T2 (40 mg)	55.80 ± 0.86	47.80 ± 2.49	87.13 ± 0.67
	T3 (60 mg)	57.80 ± 2.26	45.20 ± 1.77	85.24 ± 1.10
	T4 (Control)	58.05 ± 2.35	48.40 ± 1.20	87.19 ± 0.51
	Significance	NS	NS	NS
Exp. 2	T1 (25mg)	62.60 ± 0.22	42.40 ± 1.16	5.71 ± 3.84^{b}
	T2 (50 mg)	62.34 ± 0.23	47.00 ± 1.00	2.85 ± 1.88^{b}
	T3 (0.1ml PBS)	57.57 ± 0.23	47.20 ± 0.95	14.28 ± 3.95^{b}
	T4 (Control)	62.30 ± 0.01	46.57 ± 0.56	88.67 ± 2.84^{a}
	Significance	NS	NS	0.001
*Break out analysis of unhatched eggs** (Experiment 2)*				
Group	Dead after pipping	Early mortality	Mid mortality	Late mortality
T_1 (25 mg)	0.52%	29.47%	2.1%	-
T_2 (50 mg)	-	31.57%	1.05%	1.05%
T_3 (PBS)	1.05%	23.68%	3.68%	3.68%
T_4 (Control)	1.05%	-	-	-

** Due to very poor hatchability observed in experiment 2, break out analysis done to study in detail.

Table 2. Effect of *in ovo* supplementation of betaine on phase wise and overall production performance (Experiment 1).

Attribute	Treatment	0-3 weeks	4-6 weeks	0-6weeks
Feed intake /bird (g)	T_1 (20 mg)	797.34 ± 13.90[a]	2203.34 ± 9.90	3000.69 ± 23.20
	T_2 (40 mg)	725.25 ± 11.10[b]	2168.13 ± 9.60	2893.38 ± 10.20
	T_3 (60 mg)	734.69 ± 20.00[ab]	2188.75 ± 9.90	2923.44 ± 11.10
	T_4 (Control)	698.69 ± 16.50[b]	2040.06 ± 31.60	2738.75 ± 40.50
	P-value	0.005	0.336	0.175
Body weight gain /bird (g)	T_1 (20 mg)	433.30 ± 13.30[ab]	936.14 ± 43.90	1369.44 ± 43.90
	T_2 (40 mg)	417.07 ± 13.40[b]	964.02 ± 47.50	1381.09 ± 59.40
	T_3 (60 mg)	475.97 ± 17.00[a]	987.40 ± 15.70	1463.37 ± 18.20
	T_4 (Control)	407.16 ± 1.70[b]	999.44 ± 51.10	1406.60 ± 50.30
	P-value	0.012	0.727	0.497
Feed conversion ratio	T_1 (20 mg)	1.84 ± 0.05[a]	2.37 ± 0.10	2.20 ± 0.06
	T_2 (40 mg)	1.74 ± 0.06[ab]	2.27 ± 0.18	2.11 ± 0.14
	T_3 (60 mg)	1.55 ± 0.06[b]	2.21 ± 0.06	2.00 ± 0.06
	T_4 (Control)	1.72 ± 0.04[ab]	2.06 ± 0.12	1.95 ± 0.08
	P-value	0.018	0.387	0.255

[ab]Means within column bearing different superscript differ significantly (P<0.05).

effect of in ovo betaine injection on feed intake were recorded. Current results are comparable with findings of Anderson *et al.* (2012). During the starter phase (0-3 days), a significant increase (P<0.01) in body weight gain was observed in treatment group T_3 (60 mg betaine) followed by 20 mg betaine supplemented group (T_1). The improved body weight gain and feed intake in first three weeks can be attributed to well-known ability of betaine to improve the performance of broilers under heat stress (Sayed and Downing 2011) and due to its ability to spare other methyl donors like methionine and choline. Thus, it can be inferred that, similar mechanism might have played an instrumental role for improved weight gain in treatment birds in their early life. The body weight gains and feed intake of birds during 4 to 6 weeks of age as well as for overall trial period was comparable among all the groups.

The observations revealed that the FCR was significantly different (P<0.05) with superior performance in T_3 group (1.55) followed by control group (1.72). During 4- 6 weeks of age as well as in overall, the *in ovo* supplementation of betaine did not cause significant improvement in FCR. The FCR in first three weeks has shown an improvement with best FCR obtained in 60 mg betaine supplemented group (T_3). The ability of betaine to improve the performance of broilers under heat stress as reported earlier by Sayed and Downing (2011) as well as the sparing action for other methyl donors like methionine and choline, might be responsible for improve FCR in early life of the birds supplemented with *in ovo* betaine in varying doses. Study conducted by Rao *et al.* (2011), have also indicated that betaine can cause improved growth, feed conversion efficiency and breast yield when supplemented in a methionine deficient diet.

Immune response: Table 3 depicts the humoral immune responses (log_2 HI antibody titre against NDV at 7th and 14th day post-hatch) and cell-mediated immune responses (foot pad index at 28th day post-hatch) which did not result in any significant differences in response to *in ovo* betaine injection compared to control (T_4).

Table 3. Effect of *in ovo* supplementation of betaine on immune response of broiler chickens (Experiment 1)

Trial 1	HI antibody titre *(log_2)*		CMI (Foot pad index)
	7d	14d	
T_1 (20mg)	1.05±0.06	1.28±0.10	0.50±0.06
T_2 (40 mg)	0.98±0.05	1.28±0.05	0.55±0.08
T_3 (60 mg)	1.13±0.05	1.43±0.05	0.38±0.06
T_4 (Control)	1.05±0.06	1.36±0.06	0.54±0.09
P-value	0.283	0.321	0.403

HI: Humoral immunity; CMI: Cell mediated immunity.

Serum Biochemistry: In ovo betaine supplementation at any level did not bring any significant variation in serum biochemical profile of broiler chickens (Table 4). The serum glucose and total protein values were quantitatively higher in control birds (T_4) than rest of the treatment groups (Rokade *et al*. 2020). On the other hand, serum cholesterol levels were higher in the birds supplemented with 40 mg of betaine *in ovo* (T_2). Lowest dose of betaine, i.e. 20 mg (T_1) yielded less glucose, total protein and cholesterol among all groups.

In conclusion, the production of broiler chicken during 0-3 week is positively improved by *in ovo* betaine supplementation with normal hatchery temperature. Though, *in ovo* betaine supplementation did not cause

Table 4. Effect of *in ovo* supplementation of betaine on serum biochemical of broiler chickens (Experiment 1)

Group	Glucose (mg/dl)	Total Protein (g/dl)	Total cholesterol (mg/dl)
T_1 (20 mg)	121.23±15.2	1.30±0.26	89.71±16.3
T_2 (40 mg)	155.64±10.9	1.94±0.73	112.95±10.8
T_3 (60 mg)	153.59±23.8	1.85±0.31	92.17±22.2
T_4 (Control)	168.88±7.5	3.44±0.48	106.55±31.2
P-value	0.307	0.120	0.843

significant change in growth performance (after 3rd week), immune status and serum biochemical profile. The higher dose, i.e. 60 mg of *in ovo* betaine injection have produced better zootechnical performance only during juvenile life of broiler birds.

The significantly negative results were seen in hatchery parameters like hatchability, breakout analysis etc. This early embryonic intervention leading to negative effect on hatchability could be attributed due to higher exposure to temperature for 5 days. Further experimentation in this aspect under different climatic as well as management practices would surely help in clear understanding the effects of interaction between the *in ovo* intervention and thermal manipulation at the prenatal days and ultimately to arrive at more precise conclusion.

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