ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 10 (12): 927-932, 2011
ISSN 1682-8356

Effects of *in ovo* Injection of Theophylline and Electrolyte Solutions on Hatchability and Growth of Broilers from Day 0 to Day 10 Post-Hatch[1,2]

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Abstract: The *in ovo* feeding of supplemental nutrients, electrolytes and stimulants during late embryogenesis has the potential of benefitting subsequent broiler performance. Therefore, the effects of the automated *in ovo* injection of 200 μL of a 1:400 carbohydrate/electrolyte nutrient solution, 5.0 mM tripotassium citrate, or 1.0 mM creatine in combination with 5.5 mM potassium chloride and 1.0 mM theophylline solution on hatchability and post-hatch performance of Ross x Ross 308 broilers were investigated. Non-injected and 117 mM saline-injected controls were included. All 5 treatment groups, each containing 20 eggs, were equally represented on each of 4 replicate tray levels in a single incubator. All eggs were incubated under standard incubation conditions with incubator dry and wet bulb temperature set at 37.6 and 29.0°C, respectively. Treatment solutions were injected into the amnion of embryos on d 18 of incubation. After hatch, chicks were brooded under standard commercial grow-out conditions. Hatchability; BW on d 0 (d of hatch), 3 and 10 post-hatch; d 3 yolk sac weight and yolk sac moisture concentration and post-hatch mortality were not affected by any injection treatments. Plasma refractive index decreased and liver moisture concentration of broiler progeny increased between d 3 and 10 post-hatch; however, no treatment effects on either parameter were observed. The current study indicated that the tested stimulant solutions had no detrimental effects on broiler hatchability or post-hatch performance, suggesting their potential use alone or in combination in the commercial injection of broiler hatching eggs to improve growth and post-hatch broiler performance.

Key words: Electrolyte, hatchability, *in ovo* injection, post-hatch performance, theophylline

INTRODUCTION

In ovo technology has proven to be effective for the commercial vaccination of broilers in the United States. It provides a safer, faster and more uniform means by which to deliver vaccines to developing embryos (Johnston *et al*., 1997; Williams, 2005). This method has not only become a standard procedure for vaccination, but is a potentially effective and pragmatic route by which to introduce external nutrients to embryos that may have limited nutrient reserves. During late embryogenesis, chick embryos grow rapidly in association with an increased metabolic rate and with increased energy requirements (Davison, 1976; Christensen *et al*., 1982; Lourens *et al*., 2011; Pulikanti *et al*., 2010; Zhai *et al*., 2011a). A recent study has also shown that a large number of proteins detected in the pipping muscle are involved in energy producing metabolic pathways and in nucleic acid, carbohydrate, lipid and protein metabolism (Sokale *et al*., 2011). Previous studies have shown that supplementary carbohydrates and amino acids may improve embryo energy status and growth (Uni *et al*., 2005). Despite the fact that embryos need greater amounts of energy during pipping, not all the nutrients stored in the yolk are mobilized and utilized by embryos at hatch (Zhai *et al*., 2008). Injected substances can be actively or passively ingested by the embryo via the amnionic fluid and can be subsequently absorbed into various organs prior to hatch (Jochemsen and Jeurissen, 2002; Uni *et al*., 2005).

Caffeine and theophylline are the two most common exogenous stimulants for consumption, which are naturally found in coffee and tea, respectively. Theophylline is also an active metabolic product of caffeine catabolism (Siegel *et al*., 1999). Theophylline has a similar stimulatory function as does caffeine, but there are certain types of cells that respond better to theophylline than caffeine and these may cause distinctive responses in different cells (Devlin, 1997). Theophylline may stimulate the utilization of available nutrients and increase metabolic rate to facilitate the high energy requirement of embryos during pipping. Therefore, the benefits of the *in ovo* feeding of supplemental nutrients, electrolytes and stimulants during late embryogenesis have the greatest potential of being realized. A previous study has shown that the injection of 200 μL of 1.0 mM caffeine on d 16 of

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incubation decreased embryo BW and increased embryo mortality (McGruder *et al*., 2011b). However, 1.0 mM theophylline alone or in combination with 5.5 mM KCl (potassium chloride) did not cause such negative effects in embryos. Similarly, the injection of 1.0 mM creatine alone or in combination with 5.5 mM KCl was found to not be detrimental to embryo development (McGruder *et al*., 2011b). Creatine occurs naturally in vertebrates and helps to supply energy to cells in the body.

Saline (NaCl) has been used as a traditional carrier for injected nutrients (Jochemsen and Jeurissen, 2002; Tako *et al*., 2004; Uni *et al*., 2005; Foye *et al*., 2006; Zhai *et al*., 2008). However, a previous study has shown that 5.5 mM KCl may actually serve as an electrolyte that is superior to that of NaCl for the *in ovo* injection of solutions. Electrolytes are necessary to regulate the physiological function of birds subjected to heat stress (Brake *et al*., 1994). Potassium chloride is a common physiological salt. However, heat stress may increase potassium excretion and thereby decrease plasma potassium in birds (Ait-Boulahsen *et al*., 1989; Naseem *et al*., 2005). Therefore, KCl supplementation may be beneficial to embryos during the last few days of incubation, since their level of heat production increases dramatically during pipping.

The World Health Organization has utilized CEN (carbohydrate-electrolyte nutrient solution) in catch-up feeding programs for malnourished children. The carbohydrate content in CEN may benefit embryos by providing them with a source to supplement their limited carbohydrate stores. Supplemental electrolytes may also help to maintain electrolyte balance and improve endurance. Tripotassium citrate ($C_6H_5K_3O_7$) is a good source of potassium and citric acid facilitates the metabolism of basic nutrients including protein, fat and carbohydrate (Lehninger *et al*., 2005). It has been shown that $C_6H_5K_3O_7$ has the potential for use as an electrolyte for the commercial injection of broiler hatching embryos (McGruder *et al*., 2011a). Because of the potential physiological benefits of CEN, $C_6H_5K_3O_7$, KCl, theophylline, theophylline/KCl and creatine/KCl on embryonic development when administered individually or in combination (McGruder *et al*., 2011a,b), CEN, $C_6H_5K_3O_7$, or creatine was injected in combination with KCl and theophylline in the current study to test for the effects of these solutions on the hatchability and post-hatch performance of broilers.

MATERIALS AND METHODS

Incubation: Broiler hatching eggs were obtained from a commercial source from a common flock (Ross x Ross 308). All eggs were held for approximately 4 d under standard storage conditions prior to setting. A total of 400 eggs were weighed individually and were arranged randomly in each of 4 incubator trays in a Jamesway AVN single stage incubator. Each tray represented a replicate unit. Eggs were randomly allocated to 3 experimental treatment groups, as well as non-injected and saline-injected control groups. All 5 treatment groups, each containing 20 eggs, were equally represented on each of the 4 replicate trays. Incubator dry and wet bulb temperatures were set at 37.6±0.1°C and 29.0±1.0°C, respectively.

Injection solutions and procedures: Solutions were prepared in autoclaved water and then were filtered through a single use, gamma sterilized syringe filter (0.22 μm) the day before injection. Unless specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Three treatment solutions were tested. Firstly, a CEN solution (carbohydrate/electrolyte nutrient solution), used by the World Health Organization for catch-up feeding of malnourished children (Table 1), was provided at a relative concentration of 1:400. In addition, 5.0 mM tripotassium citrate and 1.0 mM creatine solutions were also tested. Each of these 3 solutions were separately used in combination with solutions containing both 5.5 mM KCl and 1.0 mM theophylline. A 117 mM saline solution was prepared to serve as a control for comparative purposes. The concentration, osmolality and pH of the tested solutions are listed in Table 2. On d 18 of incubation, eggs were injected with

Table 1: Constitutive elements concentrations in the 1:400 carbohydrate/electrolyte solution

Elements	Concentration (x 10^{-2} mM)
Potassium chloride[1]	5.5
Tripotassium citrate[2]	0.25
Magnesium chloride[1]	0.37
Zinc acetate[2]	0.07
Copper sulfate[2]	0.00875
Sodium citrate[2]	2.46
Sodium chloride[2]	14.97
Glucose[1]	27.75
Sucrose[2]	18.25

[1]Fisher scientific.
[2]Sigma-aldrich

Table 2: Concentration, osmolality and pH of tested solutions: NaCl, CEN, $C_6H_5K_3O_7$ or creatine in combination with KCl and theophlline

	Concentration[1] (mM)	Osmolality[1] (mOsm)	pH[2]
Control-NaCl	117	222.3	8.32
CEN/KCl/Theophylline	0.18/5.5/1.0	12.3	8.12
$C_6H_5K_3O_7$/KCl/Theophylline	5.0/5.5/1.0	30.4	8.38
Creatine/KCl/Theophylline	1.0/5.5/1.0	12.4	7.85

[1]Calculated values.
[2]Measured values using a pH meter

200 µL of either 117 mM saline or one of the 3 test solutions using an IntelliLab single egg injector (AviTech, LLC, Salisbury, MD), as described in studies conducted by Keralapurath *et al.* (2010a,b) and McGruder *et al.* (2011a,b). The standard error for injection volume was 0.2 µL. Eggs were injected through the air cell with a blunt tip injector needle which provided an approximate 2.49 cm injection depth and a 1.27-mm bore width to target the amnion. A validation test was performed before injecting and confirmed that the solution was being appropriately injected into the amnion. The injector was equipped with an automated cleaning cycle after the injection of each individual egg to prevent contamination. In order to avoid treatment solution crossover, all eggs belonging to a particular treatment were injected before switching to another treatment. After injection, the eggs were placed back into the incubator on their corresponding tray levels. All eggs were held outside the incubator less than 5 min while injecting.

Chick brooding: The current experimental protocol was approved by the Institutional Animal Care and Use Committee of Mississippi State University. On d 21 of incubation (i.e. d 0 post-hatch), all hatched chicks were marked with indelible colored ink and labeled with coded tags representing each of the replicate trays of the incubator and were assigned to a corresponding floor pen. Therefore, there were a total of 4 pens. Each pen contained approximately 15 chicks from each of the 5 treatment groups, so that the chicks intermingled from each treatment group provided a total of 75 chicks in each pen. The concrete pen floors were covered with pine shavings and were pre-warmed before bird placement. Brooding heat lamps were used to maintain standard commercial growout temperatures in each pen. The temperature of all 4 pens were recorded twice daily throughout the growout period. Chicks were provided ad libitum access to feed and water and diets were formulated to meet or exceed NRC (1994) recommendations throughout the growout period.

Data collection: Set Egg Weight (SEW), hatchability of fertilized eggs, hatching chick BW and chick BW on d 3 and 10 post-hatch were recorded. On d 3 and 10 post-hatch, a minimum of 5 chicks were randomly selected from each treatment within each replicate pen and weighed. Therefore, a total of at least 40 chicks were selected from each treatment across replicate pens and both time periods. The chicks were then euthanized by cervical dislocation. On d 3 post-hatch, yolk sacs from 16 chicks euthanized in each treatment group across the 4 replicate pens (4 chicks per treatment per pen) were collected for determination of their weight as a proportion of SEW and as a proportion of total BW (PYSW). Yolk sacs were then dried for calculation of their moisture concentration (YSMOI). On d 3 and 10 post-hatch, fresh liver samples were collected from 16 chicks in each treatment for determination of their weight in proportion to BW (PLW) and for calculation of their moisture concentration (LMOI). Blood samples were also collected from 4 chicks in each treatment for the determination of Plasma Refractive Index (PRI), total glucose (PGLUC, mg/dL) and triglycerides (PTGL, mg/dL) concentrations.

Blood samples were collected in capillary tubes and then centrifuged for plasma extraction. An Ektachem-Vitros system DT 6011 (Eastman Kodak Co., Rochester, NY) was used to determine PGLUC and PTGL according to the procedure of Peebles *et al.* (2008). The PRI (a sensitive method for determining plasma protein concentration and which indicates tissue dehydration status), was determined using a Model 10406 TS plasma refractometer (American Optical Co., Scientific Instrument Division, Buffalo, NY) according to the procedure described by Morgan *et al.* (1975) and Peebles *et al.* (2005).

Statistical analysis: Individual egg or chick within each treatment was considered as a replicate unit for SEW, BW, PYSW, YSMOI, PLW and LMO; whereas, individual tray was considered as a replicate unit for hatchability, post-hatch mortality, PRI, PTGL and PGLUC. A split-plot analysis was used to test for the main effects of age and treatment on blood plasma and liver parameters and their interaction with treatment and day designated as fixed effects and tray as a random effect. A spatial power correlation structure was used to model dependence of BW measurements made on the same chick over time. A one-way ANOVA was used to test for the effects of treatment with treatment designated as a fixed effect and tray as a random effect for the analysis of SEW, hatchability, d 3 PYSW, YSMOI and post-hatch mortality. The MIXED procedure of SAS software (SAS Institute, 2003) was used in all data analysis. Fisher's protected LSD (least significant difference) test was used to compare means (Steel and Torrie, 1980). Comparisons between means were made when there were significant global effects, with all differences considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

In previous studies, several types of solutions have been used to track amniotic fluid uptake by the embryo. The uptake of injected materials is more expedient and dispersive in d 18 rather than d 16 embryos. For all solutions tested, it was determined that all solution types were easily assimilated when injected on d 18 of incubation (Jochemsen and Jeurissen, 2002). The poultry industry also performs *in ovo* vaccinations on d 18 or 19 of incubation (depending on incubator type) when the eggs are transferred from incubator to hatcher (Zhai *et al.*, 2011b). In order to simulate commercial operations which use the same brand of incubator as was used in this study, eggs were injected on d 18 of incubation.

Table 3: Hatch rate, BW on hatch day, d 3 BW, Proportional Yolk Sac Weight (PYSW), Yolk Sac Moisture (YSMOI), d 10 BW and post-hatch mortality in non-injected control, injected with 117 mM NaCl, injected with 1:400 CEN, 5.0 mM $C_6H_5K_3O_7$ or 1.0 mM creatine in combination with 5.5 mM KCl and 1.0 mM theophlline treatments

	Hatch rate[1] (%)	D 0 BW[2] (g)	D 3 BW[3] (g)	D 3 PYSW[4] (%)	D 3 YSMOI[4] (%)	D 10 BW[3] (g)	Post-hatch mortality[1] (%)
Non-injected control	82.5	44.7	55.9	1.76	58.2	98.6	0.50
Control-NaCl	76.3	45.6	57.4	1.80	55.7	97.4	0.75
CEN/KCl/Theophylline	73.8	46.1	59.1	1.38	58.7	93.0	0.50
$C_6H_5K_3O_7$/KCl/Theophylline	73.8	46.0	57.6	1.69	56.5	99.0	0.25
Creatine/KCl/Theophylline	73.8	43.8	54.3	1.77	57.3	93.1	1.50
SEM	3.03	1.37	1.74	0.226	1.24	1.89	0.329
p-value	0.25	0.76	0.41	0.68	0.46	0.08	0.09

[1]n = 4 (where individual tray or pen served as a replicate unit). [2]n = 80 (where individual chick served as a replicate unit).
[3]n = 40 (where individual chick served as a replicate unit). [4]n = 16 (where individual chick served as a replicate unit).

Table 4: Plasma Refractive Index (PRI), Plasma Triglycerides (PTGL) and Glucose (PGLUC), Proportional Liver Weight (PLW) and Liver Moisture (LMOI) in non-injected control, injected with 117 mM NaCl, injected with 1:400 CEN, 5.0 mM $C_6H_5K_3O_7$ or 1.0 mM creatine in combination with 5.5 mM KCl and 1.0 mM theophlline treatments

Treatment	Age (d)	PRI[1]	PTGL[1] (mg/dL)	PGLUC[1] (mg/dL)	PLW[2] (%)	LMOI[2] (%)
Non-injected control		76.9	75.6	239.0	3.68	72.9
Control-NaCl		76.5	80.8	240.5	3.60	72.8
CEN/KCl/Theophylline		76.0	77.1	232.9	3.63	71.9
$C_6H_5K_3O_7$/KCl/Theophylline		74.8	84.4	239.3	3.60	73.3
Creatine/KCl/Theophylline		76.9	70.3	233.1	3.66	72.2
SEM		2.35	4.44	6.14	0.075	0.39
	3	80.5[a]	73.1	234.2	3.62	71.5[b]
	10	72.0[b]	82.2	239.7	3.64	73.7[a]
	SEM	1.26	2.50	7.04	0.051	0.25
Non-injected control	3	81.8	74.8	240.5	3.77	71.5
Control-NaCl	3	80.8	71.5	232.0	3.59	71.7
CEN/KCl/Theophylline	3	79.5	80.3	228.8	3.64	71.1
$C_6H_5K_3O_7$/KCl/Theophylline	3	77.5	75.5	236.8	3.49	72.3
Creatine/KCl/Theophylline	3	82.8	63.5	233.0	3.63	70.8
Non-injected control	10	72.0	76.5	237.5	3.58	74.3
Control-NaCl	10	72.3	90.0	249.0	3.60	73.9
CEN/KCl/Theophylline	10	72.5	74.0	237.0	3.62	72.6
$C_6H_5K_3O_7$/KCl/Theophylline	10	72.0	93.3	241.8	3.70	74.3
Creatine/KCl/Theophylline	10	71.0	77.0	233.3	3.70	73.6
SEM		2.83	5.58	8.39	0.105	0.55
p-value	Treatment	0.96	0.28	0.52	0.89	0.14
	Day	0.009	0.06	0.62	0.80	0.007
	Day x Treatment	0.67	0.09	0.28	0.40	0.75

[a,b]Means among treatments within a column with no common superscript differ significantly ($p \leq 0.05$).
[1]n = 4 (where individual pen served as a replicate unit with 4 subsamples/replicate unit).
[2]n = 16 (where individual chick served as a replicate unit)

There were no differences in SEW among all treatment groups, which would eliminate the effects of SEW variance on the parameters investigated. Differences in osmolality (from 12.3-222.3) and pH (7.85-8.38) among all the treatment solutions tested did not affect any of the parameters examined in this study. These results were similar with those of a previous study which has shown that the embryos were very tolerant to osmolality challenges on d 18.5 of incubation. Hatchability was not affected when the concentration of the injection solution varied from 0 to 2,320 mOsm at a volume of 1.2 mL (Zhai *et al*., 2011a).

In a previous study (McGruder *et al*., 2011a), it was shown that 5.0 mM $C_6H_5K_3O_7$ can be used in combination with 5.5 mM KCl as a safe and effective carrier for *in ovo* injection. Also, a CEN solution has been used to maintain the hydration status of humans and animals (Islam *et al*., 2004) and an *in ovo* injection solution containing a combination of 1:400 CEN and 5.5 mM KCl has proved to be safe for chicks during the 0 to 10 d post-hatch period (McGruder *et al*., 2011a). Also, when injected *in ovo* on d 16 of incubation, 5.0 mM $C_6H_5K_3O_7$, 1.0 mM theophylline, or 1.0 mM creatine in combination of 5.5 mM KCl were found to have no deleterious effect on broiler hatchability or BW at hatch (McGruder *et al*., 2011a,b). In the current study, effects of the *in ovo* injection of 1:400 CEN, 5.0 mM $C_6H_5K_3O_7$ or 1.0 mM creatine in combination with 5.5 mM KCl and

1.0 mM theophlline on the post-hatch performance of chicks were tested. None of the injections showed detrimental effects on hatchability, BW at hatch, or on BW at 3 and 10 d post-hatch. This is supported by the observed similarities in hatch rate, post-hatch mortality, chick BW at hatch and BW at 3 and 10 d post-hatch among all treatment groups (Table 3). In addition, a higher plasma refractive index and a lower liver moisture content of chicks on d 3 in comparison to those on d 10 were observed. Because, plasma refractive index is a sensitive method for determining plasma protein concentration and when elevated, is an accurate indicator of tissue dehydration (Morgan *et al*., 1975; Peebles *et al*., 2005), this would infer that the birds may have experienced some degree of dehydration on d 3 (Table 4). However, plasma refractive index and liver moisture were only affected by bird age and not by injection treatment. The results suggest that the tested injection solutions apparently did not cause any significant changes in late embryogenesis that the embryos were not able to physiologically compensate for, so that no observable effects on hatchability or post-hatch performance were found.

Currently, the poultry industry and researchers are looking to expand the use of *in ovo* injection technology in poultry management. Not only are the number of vaccines applied by *in ovo* injection growing, but also the list of nutrients injected *in ovo* is expanding. Many types of nutrients have been injected *in ovo* and tested, such as carbohydrates, amino acids and vitamins (Gore and Qureshi, 1997; Johnston *et al*., 1997; Henry and Burke, 1999; Kocamis *et al*., 1999, 2000; Ohta *et al*., 1999; Jochemsen and Jeurissen, 2002; Tako *et al*., 2004; Uni *et al*., 2005; Foye *et al*., 2006; Kadam *et al*., 2008; Keralapurath *et al*., 2010a,b; Zhai *et al*., 2011a,b). Although the solutions tested in the current study did not significantly affect BW gain or yolk nutrient mobilization and utilization, the results showed that none of the solutions were detrimental to hatchability or post-hatch performance. Therefore, because the tested solutions were shown to be safe to the embryos and chicks, it is suggested that they have potential for use in combination with other electrolytes, nutrients and stimulants for the commercial injection of broiler hatching embryos for the improvement of embryo and early post-hatch chick development and growth. However, effects of the current solutions in combination with other nutrients on broiler feed utilization and processing meat yield need further investigation.

ACKNOWLEDGMENTS

We express our appreciation for the expert technical assistance of Sharon K. Womack of the Mississippi State University Poultry Science Department.

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[1]This is journal No. J-11893 from the Mississippi Agricultural and Forestry Experiment Station supported by MIS-322210.

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