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Effects of *In ovo* Injection of Stimulant Solutions on Growth and Yolk Utilization in Broiler Embryos[1,2]

B.M. McGruder[1], W. Zhai[1], M.M. Keralapurath[1], P.D. Gerard[2] and E.D. Peebles[1]
[1]Department of Poultry Science, Mississippi State University, Mississippi State, MS 39762, USA
[2]Department of Applied Economics and Statistics, Clemson University, Clemson, SC 29634, USA

Abstract: Effects of the automated *in ovo* injection of stimulant solutions (200 μL) on growth and yolk utilization in the broiler embryo was investigated in 3 trials. Treatment solutions were injected into the amnion of embryos on d 16 of incubation. Treatment effects on 0 to 18 d percentage incubational egg weight loss, proportional embryo BW, proportional yolk sac weight and embryo and yolk sac moisture contents on d 18 were determined. In trial 1, 1 mM caffeine decreased proportional embryo BW and increased percentage embryo mortality and moisture content. At 1 x 10^{-3} and 10^{-6} mM concentrations, caffeine decreased proportional embryo BW and at a 1 x 10^{-6} mM concentration, caffeine increased percentage embryo moisture concentration. However, caffeine at concentrations of 1 x 10^{-3}, 10^{-6} and 10^{-9} mM had no detrimental effect on embryo survivability. Theophylline, creatine, or phosphocreatine at a 1 mM concentration in trial 2 and theophylline, creatine, or L-arginine at a 1 mM concentration in combination with 5.5 mM potassium chloride in trial 3 did not affect any of the parameters examined. The current study indicated that except for 1 mM caffeine, the tested stimulant solutions had no detrimental effects on embryogenesis, suggesting their potential use as stimulants in the commercial injection of broiler hatching eggs. However, they may have to be used at different concentrations or at different times of incubation than those used in this study in order to improve embryo growth and yolk nutrient utilization, while having no detrimental effect on embryo survivability.

Key words: Broiler, embryo, *in ovo* injection, stimulant, yolk

INTRODUCTION

Broiler breeder eggs contain an excess of fat and moisture, whereas available protein and carbohydrate stores may be limited (Al-Murrani, 1978; Ohta *et al*., 1999; Uni *et al*., 2005). However, despite the fact that embryogenesis requires increased amounts of energy, particularly late in incubation, not all yolk fat can be efficiently utilized (Zhai *et al*., 2008). On day of hatch, approximately one fourth of initial yolk stores remain unabsorbed by chicks. Glycogen can be synthesized from fat or protein and stored in the liver of embryos as a supply of glucose for hatching; however, the *in ovo* injection of external nutrients such as amino acids (Ohta *et al*., 1999; Kadam *et al*., 2008), carbohydrates (Tako *et al*., 2004) and vitamins (Gore and Qureshi, 1997), has been shown to promote post-hatch growth and BW gain in birds.

Caffeine, the active ingredient in coffee, is the world's best known stimulant. It serves as both a metabolic and central nervous system stimulator (Voet and Voet, 2004). Theophylline is the main active ingredient in tea and also an active metabolic product of caffeine catabolism (Siegel *et al*., 1999). There are certain cell types that respond better to theophylline than caffeine and as such each form may cause a distinctive response in different cells (Devlin, 1997). However, caffeine may provide more sustained stimulation effects than theophylline (Fang *et al*., 1998). Consumption of caffeine may stimulate the mobilization of fatty acids from adipose tissue and increase the level of circulating fatty acids in the blood, which permits these fat stores to be oxidized to produce energy (Graham *et al*., 2008). Furthermore, caffeine is known to increase the utilization of exogenous carbohydrate in humans undergoing exercise by as much as 26% (Yeo *et al*., 2005).

Creatine and phosphocreatine are important compounds in the normal energy metabolism of muscle and the creatine/phosphocreatine system works as a buffer when adenosine triphosphate concentrations become elevated (Clark, 1997). Phosphocreatine is a high energy storage compound found in many different cells (Devlin, 1997; Voet and Voet, 2004) and may serve as a phosphate reservoir, thereby reducing the need for adenosine triphosphate as a source by which to load creatine with phosphate. The consumption of creatine can improve intramuscular phosphocreatine concentrations and increased phosphocreatine concentrations increase muscle cell volume, which subsequently triggers additional protein and glycogen synthesis in association with a reduction in protein

Corresponding Author: E.D. Peebles, Department of Poultry Science, Mississippi State University, Mississippi State, MS 39762

breakdown (Haussinger, 1996; Ziegenfuss *et al*., 2002). Dietary creatine supplementation has been shown to improve feed efficiency in chickens (Stahl *et al*., 2003). However, because phosphocreatine itself is an energy source, it may be more useful than creatine in stimulating yolk utilization during incubation and subsequently reducing yolk wastage.

Although glucose is important in electrolyte solutions as an electrolyte balancer and as a means by which to increase water absorption (Snyder, 1991; Rehrer, 2001), amino acids have proven useful in the place of glucose in some tested electrolyte solutions (Lima *et al*., 2002). L-arginine acts as an inducer of lipolytic hormones (Kalkhoff *et al*., 1973) and is also a known glycolytic amino acid (Devlin, 1997; Voet and Voet, 2004). L-arginine has been further evaluated for its potential as a glycolytic amino acid and has proven effective as such in turkeys (Foye *et al*., 2006).

It is proposed that the *in ovo* injection of the above stimulants may increase metabolic activity by increasing nutrient availability in the egg and subsequently promote the growth of embryos. The objective of this study was to determine which of these stimulants and their combinations used for *in ovo* injection might stimulate embryo metabolism and promote embryo yolk nutrient utilization and subsequent growth.

MATERIALS AND METHODS

Incubation: The current experimental protocol was approved by the Institutional Animal Care and Use Committee of Mississippi State University. Broiler hatching eggs (Ross x Ross 308) were obtained from flocks from a commercial source that were between 35 and 54 wk of age. However, all the eggs used within a trial were collected from a common flock at the same age. All eggs were held for 3 to 4 d under standard storage conditions prior to setting. Eggs were weighed individually and were arranged randomly in each of 4 incubator trays in trial 1 and in 2 incubator trays in trials 2 and 3, in a Jamesway AVN single stage incubator. All treatment groups were represented on each tray and the total number of eggs set in each treatment group among all trays in trials 1, 2 and 3, were 20, 10 and 20, respectively. Incubator dry and wet bulb temperatures were set at 37.6±0.1°C and 29.0±1.0°C, respectively.

Treatment solutions: All injected solutions were prepared within 2 to 5 d prior to injection using deionized water as a diluent. Unless specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). In all trials, a 200 µL 117 mM saline solution injection control treatment and a non-injected control treatment were included and all treatment solutions were prepared to achieve a specific molarity. In trial 1, an initial caffeine concentration of 1 mM and 1000-fold serial dilutions were performed to achieve lower concentrations at 1 x 10^{-3}, 1 x 10^{-6} and 1 x 10^{-9} mM. In trial 2, 1 mM solutions of theophylline, creatine and phosphocreatine were tested. In trial 3, 1.0 mM concentrations of theophylline, creatine and L-arginine were used in conjunction with 5.5 mM KCl (Fisher Scientific, San Jose, CA).

Injection procedure: Injections were performed using an IntelliLab single egg injector (AviTech, LLC, Salisbury, MD). Eggs were injected through the air cell with a blunt tip injector needle [18.4-cm length and 1.27-mm bore width (o.d.)] to target the amnion. The needle provided an approximate 2.49 cm injection depth from the top of the large end of the egg (Keralapurath *et al*., 2010). Injection volumes of 200 µL were used in all 3 trials. The standard error for injection volume was 0.1 %. To ensure that the material was being delivered into the amnion, a validation test was performed using a water soluble dye that was injected on d 16 of incubation. The test confirmed that the solution was being correctly injected into the amnion. On d 16 of incubation in trials 1, 2 and 3, eggs were weighed and randomly assigned to an injection treatment group prior to injection. In order to avoid subsequent contamination and treatment solution crossover, the injector was equipped with an automated cleaning cycle after the injection of each individual egg and on each tray, all eggs belonging to a particular treatment were injected with their corresponding solution before switching to another treatment. The eggs were placed back into the incubator on their corresponding tray after eggs in all treatment groups were injected. All eggs were held outside the incubator less than 5 min while injecting.

Data collection: Egg weights at set (SEW) and at d 16 of incubation (time of injection) were recorded and were further used to correct for the weight of the injected solution in order to accurately determine 0 to 18 d percentage incubational Egg Weight (EW) Loss (PEWL) [(d 0 EW - d 18 EW) / d 0 EW x 100]. On d 18, eggs were weighed and broken out. Embryos were subsequently removed and yolk sacs and embryos were weighed separately. Embryos and yolk sacs were placed in a drying oven at 70°C until no further weight loss was observed. Later, samples were kept overnight at room temperature before their dry weights were determined. Tissue moisture content was calculated as a percentage of fresh tissue sample weight (Peebles *et al*., 1998; 1999). The following parameters were evaluated in all 3 trials: SEW, PEWL, d 18 proportional embryo BW (PEMBW; d 18 embryo BW/d 18 EW x 100), d 18 proportional yolk sac weight (PYSW; d18 yolk sac weight/d 18 EW x 100), d 18 Percent Yolk Sac Moisture (PYSMOI) [(d 18 fresh yolk sac weight - d 18 dry yolk sac weight)/d 18 fresh yolk sac weight x 100] and d 18 Percent Embryo Moisture (PEMBMOI) [(d 18 fresh BW - d 18 dry BW)/d 18 fresh BW x 100]. Eggs containing early dead embryos were recorded for calculation of post-injection mortality percentage in trial 1.

Statistical analysis: Individual egg within each treatment was considered as a replicate unit for BW, SEW, PEWL, PEMBW, PYSW, PYSMOI and PEMBMOI. Individual tray was considered as a replicate unit, with the number of replicate units sufficient for embryonic mortality data analysis in trial 1. 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. 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 all the trials of this study, there were no differences in SEW among all treatment groups, which would eliminate SEW as a possible confounding factor on the parameters investigated. In trial 1, caffeine at a 1 x 10^{-3}, 1 x 10^{-6}, or 1 x 10^{-9} mM concentration had no detrimental effect on embryo mortality. There were no significant differences between the control treatments and the 1 x 10^{-3}, 1 x 10^{-6}, or 1 x 10^{-9} mM caffeine injected treatments. However, embryonic mortality in the 1 mM caffeine injection group was higher ($p \leq 0.01$) than that in the non-injected control and the 117 mM saline injection control groups. Mean mortality in the non-injected control, 117 mM saline and 1, 1 x 10^{-3}, 1 x 10^{-6} and 1 x 10^{-9} mM caffeine treatments were 0, 0, 25.0, 5.0, 15.0 and 11.3%, respectively (SEM = 4.44). It has been shown in a previous study by Lee *et al.* (1982) that caffeine caused neural tube defects and inhibited morphogenesis of nearly all primordial organs in early chick embryos. The effects of caffeine have further been shown to be concentration dependent (Lee *et al.*, 1982). This study has confirmed that the effects of caffeine in selected commercial broiler embryos is also concentration dependent and more specially that survivability is reduced when caffeine is administrated at a 1 mM concentration on d 16 of incubation. Caffeine has been shown to be associated with growth inhibition in several species (Muther, 1988; Nolm, 1988; Golding, 1995).

In the current study, even though the lower concentrations of caffeine that were injected (1 x 10^{-3}, 1 x 10^{-6}, or 1 x 10^{-9} mM) did not cause embryonic death, they retarded growth. In trial 1, the *in ovo* injection of caffeine at 1, 1 x 10^{-3} and 1 x 10^{-6} mM concentrations decreased PEMBW and caffeine at 1 and 1 x 10^{-6} mM concentrations increased PEMBMOI (Table 1). During late embryogenesis, lipid concentrations increase in the bodies of broiler embryos (Peebles *et al.*, 1999). The most extensive catabolism of lipids begins on d 15 of incubation and contributes approximately 7.5% of the total water content of the egg in the form of metabolic water (Romanoff and Romanoff, 1967; Simkiss, 1980; Berg *et al.*, 2002). Caffeine stimulates fatty acid oxidation, which produces water as a metabolic by-product (Acheson *et al.*, 1980). The increase in PEMBMOI in response to the injection of 1 and 1 x 10^{-6} mM caffeine, therefore, may be a result of its stimulation of lipid hydrolysis. However, a similar PYSW among all treatments would also indicate that the injection of caffeine did not accelerate yolk absorption or yolk utilization in the embryos. Therefore, it is suggested that the increase in PEMBMOI was a result of an increase in the oxidation of body fat rather than yolk fat. A similar PYSMOI among all treatments would also suggest that the water in the embryonic tissues produced as a metabolic by-product was not subsequently transferred to the yolk sac. This is supported by the finding of Simkiss (1980) that water movement towards the embryo occurs at a rate that is 4 to 5 times higher than that of the reverse flux.

Because 1 mM caffeine caused an elevated rate of mortality in trial 1, the effects of caffeine were not tested again in trial 2. Instead, 1 mM theophylline, a metabolite of caffeine, was used in trial 2. In a previous study conducted in our laboratory (McGruder *et al.*, 2011), it was shown that KCl was superior to NaCl for use as an electrolyte in solutions prepared for *in ovo* injection. In trial 3, theophylline, creatine, or L-arginine were tested

Table 1: Set Egg Weight (SEW), Percentage Egg Weight Loss (PEWL), days 18 Proportional Embryo Body Weight (PEMBW), Proportional Yolk Sac Weight (PYSW), Percentage Yolk Sac Moisture (PYSMOI) and Percentage Embryo Moisture (PEMBMOI) in non-injected control, injected with 117 mM NaCl, injected with 1, 1 x 10^{-3}, 1 x 10^{-6}, or 1 x 10^{-9} mM caffeine treatments in trial 1

Solution	Concentration (mM)	SEW (g)	PEWL (%)	PEMBW (%)	PYSW (%)	PYSMOI (%)	PEMBMOI (%)
Non-injected control		63.80	10.50	54.40^{a}	25.00	52.10	81.30^{b}
Control-NaCl	117	64.90	10.50	52.10^{ab}	25.80	51.90	82.30^{ab}
Caffeine	1	65.50	10.50	48.30^{c}	25.10	52.20	83.00^{a}
Caffeine	1 x 10^{-3}	64.00	10.80	49.80^{bc}	28.80	53.90	82.70^{ab}
Caffeine	1 x 10^{-6}	64.70	9.90	50.00^{bc}	26.70	51.80	83.10^{a}
Caffeine	1 x 10^{-9}	64.70	10.90	50.60^{abc}	25.90	51.10	82.30^{ab}
SEM		1.20	0.36	0.82	0.88	0.66	0.32
p-value		0.91	0.48	0.003	0.08	0.14	0.02

^{a,b}Means among treatments with no common superscript differ significantly ($p \leq 0.05$). n = 20 (where individual embryonated egg served as a replicate unit)

Table 2: Set Egg Weight (SEW), Percentage Egg Weight Loss (PEWL), days 18 Proportional Embryo Body Weight (PEMBW), Proportional Yolk Sac Weight (PYSW), Percentage Yolk Sac Moisture (PYSMOI) and Percentage Embryo Moisture (PEMBMOI) in non-injected control, injected with 117 mM NaCl, injected with 1.0 mM theophylline, creatine, or phosphocreatine treatments in trial 2

Solution	SEW (g)	PEWL (%)	PEMBW (%)	PYSW (%)	PYSMOI (%)	PEMBMOI (%)
Non-injected control	66.90	10.30	53.80	26.40	51.30	82.90
Control-117 mM NaCl	68.10	10.70	51.00	24.50	50.60	83.00
1.0 mM Theophylline	66.10	12.00	49.30	28.00	51.90	82.20
1.0 mM Creatine	66.50	10.40	50.60	27.20	53.40	83.00
1.0 mM Phosphocreatine	68.00	10.40	50.70	27.60	54.90	83.00
SEM	1.36	0.64	1.43	1.08	1.80	0.38
p-value	0.77	0.53	0.39	0.31	0.50	0.68

n = 10 (where individual embryonated egg served as a replicate unit)

Table 3: Set Egg Weight (SEW), Percentage Egg Weight Loss (PEWL), days 18 Proportional Embryo Body Weight (PEMBW), Proportional Yolk Sac Weight (PYSW), Percentage Yolk Sac Moisture (PYSMOI) and Percentage Embryo Moisture (PEMBMOI) in non-injected control, injected with 117 mM NaCl, injected with 1.0 mM theophylline, creatine, or phosphocreatine in combination with 5.5 mM KCl treatments in trial 3

Solution	SEW (g)	PEWL (%)	PEMBW (%)	PYSW (%)	PYSMOI (%)	PEMBMOI (%)
Non-injected control	62.20	11.00	54.80	24.30	51.50	81.90
Control-117 mM NaCl	60.10	10.10	51.10	25.80	53.10	83.10
1.0 mM Theophylline/5.5 mM KCl	61.50	11.60	54.20	23.70	52.00	83.20
1.0 mM Creatine/5.5 mM KCl	61.80	11.80	56.40	24.60	52.00	82.40
1.0 mM L-arginine/5.5 mM KCl	60.10	10.70	52.50	26.10	53.30	82.60
SEM	1.19	0.70	1.09	0.92	1.28	0.31
p-value	0.74	0.40	0.13	0.41	0.61	0.15

n = 20 (where individual embryonated egg served as a replicate unit)

in combination with 5.5 mM KCl. Creatine rather than phosphocreatine was used in trial 3, because creatine was more available and was less expensive than phosphocreatine and the effects of creatine in trial 2 were not different from those of phosphocreatine. However, no evidence was found that all stimulants that were tested individually in trial 2 or in combination with KCl in trial 3 had detrimental effects. All of these stimulants did not affect any parameters examined. To some degree, embryos have the ability to regulate their development based on the availability of nutrients under various conditions (Simkiss, 1980). The creatine/ phosphocreatine system activates glycogenolysis and glycolysis by liberating phosphate. This in turn integrates phosphocreatine and carbohydrate degradation for the maintenance of ATP levels necessary for skeletal muscle development. Conversely, high levels of phosphocreatine can reduce ADP-stimulated mitochondrial respiration (Greenhaff, 2001; Walsh *et al*., 2001). Carbohydrates are preferentially used instead of fat to meet energy demands, particularly when excess carbohydrates are available. However, in order to satisfy this demand, fat utilization may be accelerated when carbohydrates are unavailable. Exogenous stimulants can accelerate carbohydrate and fat oxidation for the production of energy; however, via negative feed-back inhibition, the accumulation of adenosine triphosphate may inhibit the further mobilization of fat and carbohydrates for energy production (Guyton and Hall, 2000; Berg *et al*., 2002). Theophylline, creatine, phosphocreatine and L-arginine at the concentrations tested in this study, apparently did not cause a sufficient change in the energy metabolism of the embryos so as to affect their development. Although the aforementioned parameters were not significantly affected by treatment, the treatment means for the parameters investigated in trials 2 and 3 are presented in Tables 2 and 3, respectively, for observation.

The results of the current study suggest that except for 1 mM caffeine, at the concentrations used for amniotic injection, the stimulants tested were compatible to embryogenesis. However, it is apparent that embryonic metabolic changes, such as the observed decrease in PEMBW and the increase in PEMBMOI elicited by the lower concentration of caffeine (1×10^{-6} mM), can occur without negatively affecting embryo survivability. Further research should be conducted to determine how the fat, protein and carbohydrate concentration profiles of embryonic and post-hatch chick body tissues might be affected by the injection of stimulants. Moreover, because the embryo may use external nutrients to meet its energy needs, thereby preserving its own nutrients for growth, different developmental responses may result when external nutrients are injected into growing embryos together with these stimulants. In conclusion, the combined results of these current aforementioned trials confirm that the tested stimulants, within certain concentration ranges, may be safely used individually or in combination with electrolyte solutions for the *in ovo* injection of broiler hatching eggs. However, they may

have to be used at different concentrations or at different times of incubation than those used in this study in order to achieve significant improvements in embryo growth, yolk utilization, hatchability and subsequent post-hatch performance. Nevertheless, the *in ovo* injection of 200 μL of caffeine at a 1 mM concentration should be avoided.

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