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Effects of in ovo feeding of creatine pyruvate on the hatchability, growth performance and energy status in embryos and broiler chickens

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The effects of in ovo feeding (IOF) of creatine pyruvate (CrPyr) on the hatchability, growth performance and energy status of embryos and broilers (Arbor Acres) were investigated. Five treatments were arranged as non-injected treatment (Control), 0.6 ml physiological saline (0.75%) injected treatment (Saline), and IOF treatments injected with 0.6 ml physiological saline (0.75%) containing 3, 6 or 12 mg CrPyr (CrPyr$_3$, CrPyr$_6$ or CrPyr$_{12}$) into the amnion per fertile egg on day 17.5 of incubation. After hatching, 80 male chicks from each treatment with similar weight close to the average BW of their pooled group were selected and randomly assigned into eight replicates of 10 chicks each. The results showed that the hatchability was not affected among groups, whereas the hatching weight of broilers in CrPyr$_{12}$ was significantly higher than the control and saline groups (P < 0.05). At 21 day post-hatch, the BWs of broilers in CrPyr$_6$ and CrPyr$_{12}$ were increased relative to the control and saline groups (P < 0.05). Chickens in CrPyr$_6$ and CrPyr$_{12}$ exhibited higher BW gain and feed intake than the control and saline groups during 8 to 21 days post-hatch and the entire experiment period (P < 0.05). Compared with the control and saline groups, the total and relative weight of pectoral muscle of embryos or chickens were greater in CrPyr$_6$ and CrPyr$_{12}$ at 19th day of incubation (19 E), hatch, 3 and 21 days post-hatch (P < 0.05). The concentrations of glucose and glycogen in liver were increased in CrPyr$_6$ and CrPyr$_{12}$ at 19 E and hatch (P < 0.05). Neither glycogen nor glucose concentration in pectoral muscle was altered among treatments (P > 0.05). Irrespective of dosage, the concentrations of creatine and phosphocreatine, and activities of creatine kinase in embryos were enhanced in CrPyr treatments at 19 E when compared with the control and saline groups (P < 0.05). The activities of glucose-6-phosphatase in liver in CrPyr$_6$ and CrPyr$_{12}$ treatments were higher than the control and saline groups at 19 E (P < 0.05). In conclusion, these results indicated that IOF of CrPyr, especially at the level of 12 mg/egg, could improve energy status of embryos and hatchlings, which was useful for enhancing hatching weight, BW and pectoral muscle weight until the end of the experiments at 21 days post-hatch in broilers.

Keywords: in ovo feeding, creatine pyruvate, growth performance, energy status, broilers

Implications

Nowadays, early nutritional regulation (in ovo feeding (IOF) of exogenous nutrients) has been indicated to offer the promise of sustaining progress in production efficiency of commercial poultry. The present study showed that IOF of creatine pyruvate (CrPyr) (which contains pyruvic acid molecularly bound to creatine (Cr) at a concentration ratio of 40 : 60), especially at the level of 12 mg/egg, could improve hatching weight, BW and pectoral muscle weight until 21 days post-hatch in broilers. These findings provide a basis for future work on the use of CrPyr to solve the deficiency of energy reserves during the late embryogenesis.

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Introduction

Unlike mammals, avian species, which do not have a continuous maternal energy supply, possess limited nutrient and energy deposits in the fertile egg to support embryonic and neonatal growth. During pre-hatch period, glucose and glycogen are preferentially utilized as the main energy sources for the nutrition of avian embryos (Shafey *et al.*, 2012). However, the glycogen reserves are significantly depleted in order to meet the high energy demand toward the end of incubation. This may consequently force the embryo to mobilize more muscle protein for gluconeogenesis thereby inhibiting early growth and development (Chen *et al.*, 2009; Noy and Uni, 2010). In addition, under commercial industry practices, chicks are deprived of feed and

1689

water for 24 to 72 h because of variation in hatch time, chick handling, and transportation time (Willemsen *et al.*, 2010). Delaying access to feed and water for 36 to 72 h aggravates the deficiency of energy and leads to irreversible damage to broilers, such as retard of BW, depression of intestinal development and lower pectoral muscle weight (Kornasio *et al.*, 2011; Lamot *et al.*, 2014). Therefore, the few days pre- and post-hatch are crucial for the development of hatchlings, suggesting that the improvement of energy storage during this period may promote subsequent growth performance of chickens.

In ovo feeding is a technique of administrating exogenous nutrients into the amnion of the late-term avian embryos, as the embryos can orally consume the amniotic fluid and then absorb the added nutrients by the intestine before piping (Uni and Ferket, 2004). Several attempts have revealed that IOF of exterior nutrient substances such as carbohydrate, amino acids or protein could increase hatching weight, liver glycogen reserves, marketing weight and breast muscle yield (Uni and Ferket, 2004; Uni *et al.*, 2005; Foye *et al.*, 2006a; Tangara *et al.*, 2010). Therefore, the IOF, an inspiring insight for perinatal nutrition of poultry embryos, may be beneficial to overcome the restriction of finite energy in late-term bird embryos.

Creatine pyruvate is an organic compound, which contains pyruvic acid molecularly bound to Cr at a concentration ratio of $40:60$ (Chen *et al.*, 2012). Pyruvate, which works as an intermediate product of carbohydrate, protein and lipid, can modulate energy metabolism through the glycolytic/gluconeogenesis pathway and the Krebs cycle; while Cr, a nitrogen containing compound, can be phosphorylated as phosphocreatine (PCr), which are directly involved in the muscle energy buffering system by transferring a phosphate group to ADP to replenish ATP (Chen *et al.*, 2011; Allen, 2012). Meanwhile, previous study in our lab demonstrated that combined IOF of creatine monohydrate and glucose during the last stage of incubation had synergistic effects on elevating the glycogen reserves in liver and increasing the concentrations of Cr and PCr in muscle of embryos and hatchlings (Zhang *et al.*, 2016). Thus, we hypothesize that IOF of CrPyr would enhance energy reserves and support the growth of avian embryos and neonates. Therefore, the objectives of the present study were to evaluate the effects of IOF of CrPyr on hatchability, growth performance and energy status of embryos and broilers from 19 days pre-hatch until 21 days post-hatch.

Material and methods

Egg incubation
All procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. A total of 1400 fertile broiler eggs (Arbor Acres) from a laying flock at 34 weeks of age were pre-weighed and selected from a commercial hatchery with an average weight of 62.17 ± 1.63 g (range = 60 to 65 g). Eggs were then randomly assigned in a microcomputer automatic incubator (ZCA-A; Zhicheng Incubation Equipment Co., Ltd., Dezhou, China) under routine conditions ($37.8 \pm 0.1°C$ of temperature and 60% of relative humidity) and turned through $270°$ every 1.5 h until 19th day of incubation (19 E). On embryonic day 6, eggs were candled and unfertilized eggs were removed from the incubator.

In ovo feeding procedure
All injected solutions were freshly prepared on the day of injection. The CrPyr (Ju sheng Technology Co., Ltd., Wuhan, China) was dissolved in physiological saline (0.75%) to achieve concentrations of 5, 10 or 20 mg CrPyr/ml, respectively. Then solutions were sterilized by filtration through a 0.22 µm membrane filter and then subsequently kept in the incubator at 37.8°C. At the end of embryonic day 16, all eggs were illuminated again and non-viable eggs were removed. Of the remaining eggs, 1200 available eggs with similar weight within $\pm 1\%$ of the mean weight (56.64 ± 0.51 g) were randomly divided into five treatment groups with eight replicates of 30 eggs each. In total, 40 incubator trays were used, and each tray was taken as one replicate. Treatment 1 was non-injected group (Control), treatment 2 was 0.6 ml physiological saline (0.75%) injected group (Saline), treatments 3 to 5 were injected with 0.6 ml physiological saline (0.75%) solution containing 3, 6 or 12 mg CrPyr/egg (CrPyr$_3$, CrPyr$_6$ or CrPyr$_{12}$), respectively. On embryonic day 17.5, the operation procedures were performed as described in detail by Uni *et al.* (2005) and Zhai *et al.* (2011b). The location of the amnion was identified by candling and the injection place was disinfected with 75% ethyl alcohol at the surface of the large end of the egg. A hole was then punched using a needle and the IOF solution was injected into the amnion using a 21-gauge needle (the syringe was used in a disposable way) to a depth of about 2.49 cm. Immediately after the injection experiment, the injected holes on the eggs were sealed with petroleum wax, and transferred to hatching trays. All eggs were exposed outside the incubator for <30 s to complete the IOF procedure. Until hatch, all eggs were incubated according to the routine procedure. The remaining eggs served as the non-injected control were subjected to the same handling procedures as the IOF groups.

Animal husbandry
Upon hatch, the number of hatchlings within each treatment was recorded. Hatchability was calculated as (%) = (number of hatchlings/number of fertile eggs) $\times 100$. All male hatched chicks from one treatment were pooled and weighted. In all, 80 male chicks with similar weight close to the average BW of their pooled group were selected and randomly assigned into eight replicates of 10 chicks within each treatment. In total, 40 pens were provided for the five treatment chicks, with each replicate allocated for a pen ($110 \times 60 \times 50$ cm). The chickens were allowed free access to feed and water in three-layer cages in a temperature-controlled room and the temperature was set at 32°C to 34°C for the first 3 days and then reduced by 2°C to 3°C per week. All birds were reared

Table 1 *The composition and calculated nutrient levels of the basal diets*

Items	Value
Ingredients (%)	
Corn	57.61
Soybean meal	31.00
Corn gluten meal	3.29
Soybean oil	3.11
Limestone	1.20
Dicalcium phosphate	2.00
L-lysine	0.34
DL-methionine	0.15
Salt	0.30
Premix[1]	1.00
Calculated nutrient levels	
ME (MJ/kg)	12.56
CP (%)	21.10
Ca (%)	1.00
Available phosphorus (%)	0.46
Lysine (%)	1.20
Methionine (%)	0.50
Methionine + cysteine (%)	0.85

ME = metabolizable energy.

[1]Premix provided per kilogram of diet: retinyl acetate for vitamin A, 12 000 IU; cholecalciferol for vitamin D_3, 2500 IU; DL-α-tocopheryl acetate for vitamin E, 20 IU; menadione sodium bisulfate, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8.0 mg; nicotinamide, 40 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyridoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamin), 0.013 mg; Fe (from ferrous sulfate), 80 mg; Cu (from copper sulfate), 8.0 mg; Mn (from manganese sulfate), 110 mg; Zn (from zinc sulfate), 60 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg.

under incandescent white light with a light schedule of 23 h light and 1 h dark according to Zhang *et al.* (2014). The diets were formulated to meet the nutrient requirements of Arbor Acres broiler chickens (Table 1). At 7 and 21 days, birds were weighed after feed deprivation for 12 h and feed intake was recorded by replicate to calculate BW gain, feed intake and feed/gain ratio.

Tissue sampling
The entire embryos were removed and cleaned of yolk sac and membrane after the eggs were opened from air chamber at 19 E. They were then euthanized with sodium pento-barbital (20 mg/kg of BW; Beijing Chemical Co., Beijing, China). One male embryo was randomly selected by obser-ving the morphology of the gonads (embryo with two tubular shaped gonads of about equal length was identified as male) from each replicate (eight per treatment) according to the method of Burke (1994). Then the yolk-free body and entire pectoral muscle were weighed and recorded. The samples of liver and pectoral muscle were collected and frozen imme-diately in liquid nitrogen for further analysis.

On the age of hatch, 3, 7 and 21 days post-hatch, one bird (eight birds per treatment) with a BW close to the average BW of the replicate was selected and weighed, then killed by cervical dislocation. The entire pectoral muscle was obtained and weighed. Moreover, samples of liver and pectoral muscle were obtained and frozen in liquid nitrogen until analysis.

Liver and muscle glycogen and glucose analysis
The concentrations of glycogen in liver and pectoral muscle were estimated with a 1200 UV spectrophotometer (Mapada Instruments Co. Ltd., Shanghai, China) according to the directions of commercially available liver glycogen/muscle glycogen detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The concentration of glucose was measured using commercial glucose oxidase kits (Shanghai RongSheng Biotech Co. Ltd., Shanghai, China).

Concentrations of creatine and phosphocreatine in pectoral muscle
The concentrations of Cr and PCr in pectoral muscle were determined by reverse-phase-HPLC according to Li *et al.* (2016). Briefly, each 200 mg frozen muscle sample was homogenized in 2 ml ice-cold 5% $HClO_4$ for 1 min and cen-trifuged at 10 000 × **g** at 4°C for 10 min after being lixiviated in an ice bath for 15 min. The supernatant was then mixed with 900 μl of 0.8 M K_2CO_3. The mixture was centrifuged for 10 min at 10 000 × **g** at 4°C again after being neutralized in an ice bath for 10 min. Next, the supernatant was filtered through a 0.45 μm filtration membrane and injected into the Waters-2695 Alliance HPLC system (Waters, Milford, MA, USA) equipped with an integrated auto-sampler. The analy-tical column used in the experiments was Waters SunFire C18 (250 × 4.6 mm, 5 μm; Waters) with a column temperature of 25°C. The mobile phase consists of 2% methyl cyanides and 98% KH_2PO_4 buffer (29.4 mM) and the flow rate was kept at 1.0 ml/min. Other chromatographic conditions were set as follows: UV detection wavelength, 210 nm; and the injection volume, 20 μl. The standard curves were established according to the method reported by Zhang *et al.* (2010).

Determination of creatine kinase activity in pectoral muscle
For each bird, 200 mg frozen muscle sample was weighed and homogenized in a centrifuge tube with 1.8 ml of 0.75% saline, and then centrifuged at 3500 × **g** for 10 min at 4°C. The supernatant was used for assaying the activity of creatine kinase with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The result was normalized against total protein concentration in each sample. The concentrations of protein in tissue extracts were estimated according to the manufacturer's protocol of total protein quantitative assay kits (Nanjing Jiancheng Bioengi-neering Institute, Nanjing, China).

Determination of glucose-6-phosphatase activity in liver
The activity of glucose-6-phosphatase in liver was deter-mined using modified procedures described by Donaldson and Christensen (1991). Each 150 mg liver sample was homogenized in a 0.25 M sucrose solution (1 g liver/10 ml) and centrifuged at 14 000 × **g** at 4°C for 10 min. The super-natant was diluted 1 : 4 with 0.25 M sucrose solution. Three tubes were prepared for each experimental sample contain-ing the following: 0.3 ml of 0.1% histidine solution, 0.1 ml 0.25 M sucrose solution, and 0.1 ml of diluted sample. At 15 s intervals each tube was placed into a 37°C water bath

1691

Table 2 *The effects of in ovo feeding (IOF) of creatine pyruvate (CrPyr) on embryo characteristics on 19th day of incubation (19 E), hatchability, hatching weight and BW at 3, 7 and 21 days post-hatch of broilers*

Items	Treatments[1]					SEM	*P*-value
	Control	Saline	CrPyr$_3$	CrPyr$_6$	CrPyr$_{12}$		
EW (g)	56.9	56.6	58.0	58.8	58.4	0.5	0.499
YBW (g)	42.7^b	43.1^b	45.2^a	45.5^a	45.1^a	0.3	0.002
YSW (g)	9.3^b	9.5^b	10.6^a	10.9^a	10.7^a	0.2	0.001
HF (%)	90.0	89.4	89.3	89.4	89.7	0.3	0.962
HW (g)	44.2^b	44.1^b	44.3^b	44.5^b	45.2^a	0.1	0.007
BW at 3 days (g)	60.9^b	60.7^b	60.3^b	61.0^b	63.4^a	0.4	0.047
BW at 7 days (g)	139.7^b	139.0^b	138.9^b	138.6^b	143.7^a	0.6	0.020
BW at 21 days (g)	775.5^b	780.8^b	794.0^b	816.9^a	832.6^a	4.8	<0.001

EW = egg weight on 19 E; YBW = weight of embryos with the yolk sac on 19 E; YSW = yolk sac weight on 19 E; HF = hatchability of fertilized eggs (both male and female); HW = hatching weight of male chicks.
The results are presented by mean values and the SEM.
^{a,b}Means within a row with different superscript letters are different at *P* < 0.05.
[1]Control is the non-injected treatment. Saline is 0.6 ml physiological saline (0.75%) injected treatment. CrPyr$_3$, CrPyr$_6$ and CrPyr$_{12}$ are IOF treatments injected with 0.6 ml physiological saline (0.75%) containing 3, 6 or 12 mg CrPyr per egg.

for a total incubation time of 10 min. A volume of 0.1 ml of 0.1 M glucose-6-phosphate solution was added to two tubes of the triplicate set before incubation, and the remaining tube of the triplicate served as a sample blank. After 10 min of incubation, 1 ml of 10% $C_2HCl_3O_2$ was added to each tube. For the sample blanks, 0.1 ml of 0.1 M glucose-6-phosphate solution was added. Subsequently, the inorganic phosphate levels were measured using phosphate assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer. The activity of glucose-6-phosphatase was calculated and expressed in micromoles of substrate hydrolyzed per minute per milligram of protein in the tissues (μmol/min per mg of protein).

Statistical analysis
Data analysis was performed by one-way ANOVA using SAS statistical software (version 8.02, SAS Institute Inc., Cary, NC, USA). In animal incubation and husbandry trials, the incubator trays and raising cages per treatment served as the experimental unit ($n = 8$). Therefore, the data on total and relative weight of pectoral muscle, activities of creatine kinase and glucose-6-phosphatase and the concentrations of glycogen, glucose, Cr, PCr were analyzed using the individual embryo or broiler as the experimental unit ($n = 8$). Differences among treatments were examined using Duncan's multiple range tests. The means and pooled standard error of means were presented and differences were considered to be significant at *P* < 0.05.

Results

Embryo characteristics, hatchability, hatching weight and body weight
Injection treatment had no significant effect on the egg weight at 19 E (*P* > 0.05, Table 2). However, compared with

the control and saline groups, all IOF of CrPyr groups increased the weight of embryos with the yolk sac, as well as the yolk sac weight at 19 E (*P* < 0.05). No difference on hatchability was observed among treatments (*P* > 0.05). The hatching weight and BW of birds in CrPyr$_{12}$ were significantly elevated compared with the control and saline groups at 3 and 7 days post-hatch (*P* < 0.05). On 21 days post-hatch, the BW was 5.34% and 7.37% greater in CrPyr$_6$ and CrPyr$_{12}$ than the control group (*P* < 0.05), respectively.

Growth performance
Birds showed similar growth performance among treatments during 1 to 7 days post-hatch (*P* > 0.05, Table 3). However, the chickens in CrPyr$_6$ and CrPyr$_{12}$ exhibited higher BW gain and feed intake than the control and saline groups during 8 to 21 days post-hatch and the entire experiment period (*P* < 0.05). All treatments had similar feed/gain ratio irrespective of the growth period (*P* > 0.05).

Pectoral muscle weight and relative pectoral muscle weight
As shown in Table 4, significant increases in total and relative weight of pectoral muscle were observed in CrPyr$_6$ and CrPyr$_{12}$ treatments compared with the control and saline groups at 19 E, hatch, 3 and 21 days of age (*P* < 0.05). In addition, the birds in CrPyr$_{12}$ gained greater total and relative weight of pectoral muscle than the control and saline groups at 7 days post-hatch (*P* < 0.05).

Concentrations of glycogen and glucose in liver and pectoral muscle
The concentrations of glycogen were increased in CrPyr$_6$ and CrPyr$_{12}$ in comparison with the control, saline and CrPyr$_3$ groups in liver at 19 E (*P* < 0.05, Figure 1A). All groups showed a pattern of reduction in liver glycogen reserves as the embryo approached hatch, whereas the glycogen concentrations of hatchlings in CrPyr$_6$ and CrPyr$_{12}$ were 1.79-fold and

1692

Table 3 *The effects of in ovo feeding (IOF) of creatine pyruvate (CrPyr) on the growth performance of broilers*

	Treatments[1]						
Items	Control	Saline	$CrPyr_3$	$CrPyr_6$	$CrPyr_{12}$	SEM	*P*-value
BWG (g/bird)							
1 to 7 days	97.0	96.1	96.1	95.9	98.8	0.6	0.441
8 to 21 days	635.8^b	641.8^b	655.1^b	678.3^a	689.0^a	4.7	<0.001
1 to 21 days	732.8^c	738.0^c	751.1^{bc}	774.2^{ab}	787.8^a	4.8	<0.001
FI (g/bird)							
1 to 7 days	108.8	107.4	109.0	108.3	110.9	0.7	0.643
8 to 21 days	925.1^b	928.9^b	925.9^b	963.7^a	971.6^a	5.8	0.009
1 to 21 days	1033.8^b	1036.3^b	1034.9^b	1072.1^a	1082.5^a	6.0	0.008
F : G (g : g)							
1 to 7 days	1.12	1.12	1.14	1.13	1.12	0.01	0.956
8 to 21 days	1.46	1.45	1.41	1.42	1.41	0.01	0.417
1 to 21 days	1.41	1.40	1.38	1.39	1.37	0.01	0.506

BWG = BW gain; FI = feed intake; F : G = feed : gain
The results are presented by mean values and the SEM.
[a,b,c]Means within a row with different superscript letters are different at $P < 0.05$.
[1]Control is the non-injected treatment. Saline is 0.6 ml physiological saline (0.75%) injected treatment. $CrPyr_3$, $CrPyr_6$ and $CrPyr_{12}$ are IOF treatments injected with 0.6 ml physiological saline (0.75%) containing 3, 6 or 12 mg CrPyr per egg.

Table 4 *The effects of in ovo feeding (IOF) of creatine pyruvate (CrPyr) on the pectoral muscle weight and relative pectoral muscle weight of embryos and broilers on 19th day of incubation (19 E), the day of hatch, and 3, 7 and 21 days post-hatch*

	Treatments[1]						
Items	Control	Saline	$CrPyr_3$	$CrPyr_6$	$CrPyr_{12}$	SEM	*P*-value
Pectoral muscle weight (g)							
19E	0.74^b	0.76^b	0.78^b	0.89^a	0.92^a	0.02	<0.001
Hatch	0.60^b	0.63^b	0.65^b	0.84^a	0.88^a	0.02	<0.001
3 days	1.05^c	1.10^c	1.15^{bc}	1.24^{ab}	1.31^a	0.02	<0.001
7 days	7.21^b	7.07^b	7.28^b	7.30^b	8.02^a	0.10	0.027
21 days	113.60^c	112.69^c	117.19^c	133.49^b	141.42^a	2.08	<0.001
Relative pectoral muscle weight (%)							
19E	2.20^b	2.23^b	2.27^b	2.59^a	2.70^a	0.05	<0.001
Hatch	1.40^b	1.45^b	1.49^b	1.88^a	1.96^a	0.04	<0.001
3 days	1.92^b	1.96^b	2.07^{ab}	2.19^a	2.26^a	0.03	0.002
7 days	5.91^b	5.92^b	6.18^{ab}	6.19^{ab}	6.25^a	0.05	0.049
21 days	15.42^c	15.25^c	15.21^c	16.16^b	16.77^a	0.13	<0.001

Relative pectoral muscle weight = pectoral muscle weight as a percentage of BW.
The results are presented by mean values and the SEM.
[a,b,c]Means within a row with different superscript letters are different at $P < 0.05$.
[1]Control is the non-injected treatment. Saline is 0.6 ml physiological saline (0.75%) injected treatment. $CrPyr_3$, $CrPyr_6$ and $CrPyr_{12}$ are IOF treatments injected with 0.6 ml physiological saline (0.75%) containing 3, 6 or 12 mg CrPyr per egg.

2.13-fold of the control at hatch ($P < 0.05$). Meanwhile, these two groups had higher concentration of glucose than other samples at 19 E and hatch ($P < 0.05$, Figure 1C). At 3 days post-hatch, birds in $CrPyr_6$ and $CrPyr_{12}$ had higher concentration of glycogen in liver than other groups, whereas this result maintained at 7 days post-hatch in $CrPyr_{12}$ ($P < 0.05$). Neither glycogen nor glucose concentration in pectoral muscle was altered among treatments at any of the time points measured ($P > 0.05$, Figure 1B and D).

Concentrations of creatine and phosphocreatine in pectoral muscle
There were significant positive effects on concentration of Cr in pectoral muscle compared with the control and saline groups in all IOF of CrPyr groups at 19 E and hatch ($P < 0.05$), and the results were also found in $CrPyr_{12}$ group on 3 days post-hatch ($P < 0.05$, Figure 2A). In addition, the concentrations of PCr were higher in all CrPyr-treated groups than the control and saline groups at 19 E ($P < 0.05$, Figure 2B).

1693
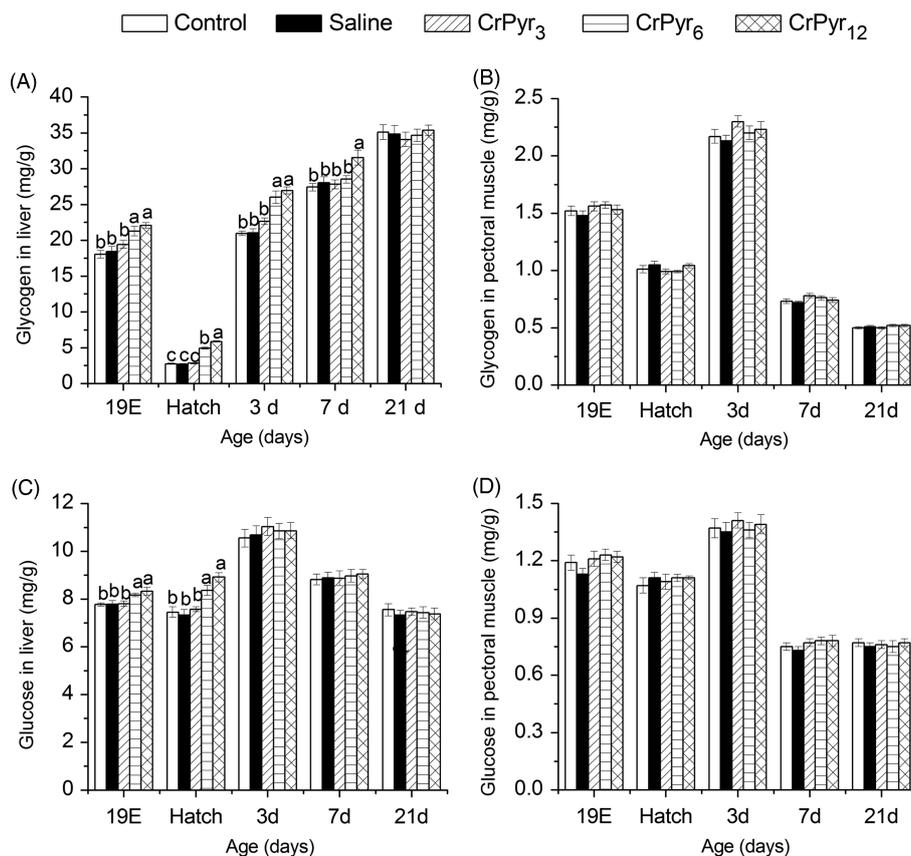
Figure 1 The effects of in ovo feeding (IOF) of creatine pyruvate (CrPyr) on the concentrations of glycogen and glucose in liver ((A) and (C)) and pectoral muscle ((B) and (D)) of embryos and broilers on 19th day of incubation (19E), the day of hatch, 3, 7 and 21 days post-hatch. Control is the non-injected treatment. Saline is 0.6 ml physiological saline (0.75%) injected treatment. $CrPyr_3$, $CrPyr_6$ and $CrPyr_{12}$ are IOF treatments injected with 0.6 ml physiological saline (0.75%) containing 3, 6 or 12 mg CrPyr per egg. All data are represented as the mean value ± SE of eight sample embryos or birds per treatment. [a,b,c]Different letters within the same time points indicate significant differences between the five treatments ($P < 0.05$).

*Activities of creatine kinase and glucose-6-phosphatase*
A notable increase in activity of creatine kinase in pectoral muscle was observed in all IOF of CrPyr groups at 19 E when compared with the control and saline groups ($P < 0.05$, Table 5). Simultaneously, the activities of glucose-6-phosphatase in liver in $CrPyr_6$ and $CrPyr_{12}$ groups were higher than other treatments ($P < 0.05$).

Discussion

The hatchability of poultry, as one of the major determinants of profitability in a hatchery enterprise, is influenced by many factors such as genetics, breeder hen age, egg size and incubation conditions (Kadam *et al.*, 2013). As reported in previous study, IOF of 0.5 ml/egg of carbohydrate (maltose, sucrose and dextrin mixture in a proportion of 1 : 1 : 8) varied from 50 to 250 mg/egg had no significant effect on hatchability of broilers (Shafey *et al.*, 2012). On the contrary, Dong *et al.* (2013) asserted that injection of 0.2 ml/egg of 4.5% maltose +4.5% sucrose into amnion of pigeon eggs reduced hatchability and concluded that the concentration of injection solution should be limited in order to prevent excessive energy metabolism of the embryos. Another research

claimed that hatchability was negatively related to injection volume of carbohydrate solution (Zhai *et al.*, 2011b). These results suggested that the effects of IOF on hatchability might be attributed to other profound factors including the solution formulation, concentration and appropriate injection volume. No significant difference in hatchability was observed among treatments, suggesting that the injection dose in the present study was safe.

This study indicated that IOF of 12 mg/egg CrPyr improved chick hatching weight and this advantage was sustained up to 21 days of age at least, which agreed the results observed in turkeys, ducks and domestic pigeons (Foye *et al.*, 2006b; Chen *et al.*, 2009; Dong *et al.*, 2013). In fact, hatching weight is a vital indicator of marketing weight in poultry, whereas this correlation may differ among strains (Sklan *et al.*, 2003; Willemsen *et al.*, 2008). Wen *et al.* (2014) reported that 6.6 g of increase in hatching weight of Arbor Acres broiler chickens led to 252 g of increase in BW at 42 days post-hatch. However, our research showed that a 0.98 g difference in BW at hatch due to IOF of 12 mg/egg CrPyr resulted in 57.12 g of increase in BW at 21 days post-hatch. In addition, Kornasio *et al.* (2011) reported that IOF of carbohydrates and β-hydroxy-β-methylbutyrate increased the pectoral muscle weight of broilers on 35 days post-hatch. The broilers in 6
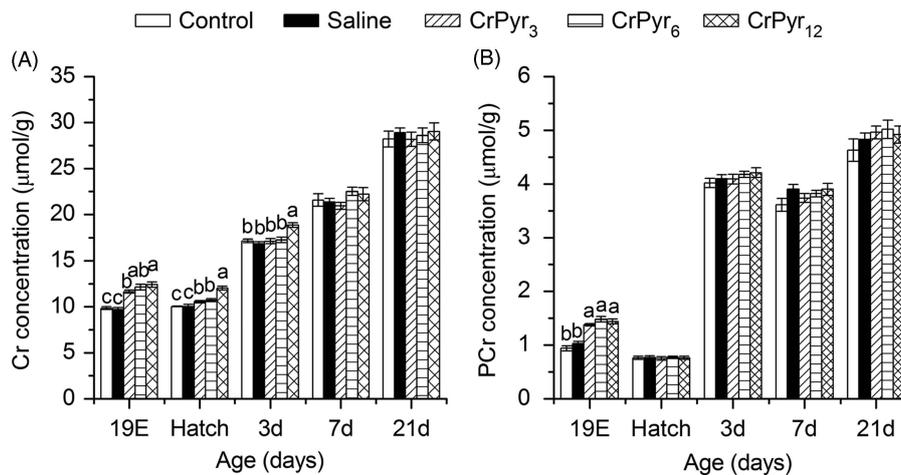
Figure 2 The effects of in ovo feeding (IOF) of creatine pyruvate (CrPyr) on the concentrations of creatine (Cr) and phosphocreatine (PCr) in pectoral muscle ((A) and (B)) of embryos and broilers on 19th day of incubation (19E), the day of hatch, 3, 7 and 21 days post-hatch. Control is the non-injected treatment. Saline is 0.6 ml physiological saline (0.75%) injected treatment. $CrPyr_3$, $CrPyr_6$ and $CrPyr_{12}$ are IOF treatments injected with 0.6 ml physiological saline (0.75%) containing 3, 6 or 12 mg CrPyr per egg. All data are represented as the mean value ± SE of eight sample embryos or birds per treatment. [a,b,c]Different letters within the same time points indicate significant differences between the five treatments ($P < 0.05$).

Table 5 The effects of in ovo feeding (IOF) of creatine pyruvate (CrPyr) on the activities of creatine kinase in pectoral muscle and glucose-6-phosphatase in liver of embryos and broilers on 19th day of incubation (19 E), the day of hatch, and 3, 7 and 21 days post-hatch

Items	Treatments[1]					SEM	P-value
	Control	Saline	$CrPyr_3$	$CrPyr_6$	$CrPyr_{12}$		
Activity of creatine kinase (U/mg of protein)							
19E	4.41[c]	4.50[c]	4.98[b]	5.29[ab]	5.57[a]	0.09	<0.001
Hatch	5.66	5.85	5.57	5.94	6.03	0.07	0.252
3 days	6.58	6.45	6.56	6.91	6.76	0.07	0.308
7 days	9.88	9.85	9.99	9.88	9.92	0.11	0.996
21 days	3.97	3.89	4.07	4.03	3.98	0.05	0.785
Activity of glucose-6-phosphatase (μmol/min per mg of protein)							
19E	0.121[b]	0.125[b]	0.129[b]	0.152[a]	0.161[a]	0.003	<0.001
Hatch	0.114	0.110	0.121	0.119	0.120	0.002	0.235
3 days	0.068	0.065	0.067	0.070	0.068	0.001	0.584
7 days	0.061	0.059	0.062	0.061	0.063	0.001	0.724
21 days	0.068	0.066	0.070	0.069	0.072	0.001	0.460

The results are presented by mean values and the SEM.
[a,b,c]Means within a row with different superscript letters are different at $P < 0.05$.
[1]Control is the non-injected treatment. Saline is 0.6 ml physiological saline (0.75%) injected treatment. CrPyr3, CrPyr6 and CrPyr12 are IOF treatments injected with 0.6 ml physiological saline (0.75%) containing 3, 6 or 12 mg CrPyr per egg.

and 12 mg/egg CrPyr group showed a significant increase in the pectoral muscle weight and relative pectoral muscle weight on 21 days post-hatch in the present study. Based on the current results, if the benefits persist to slaughter age, it is suggested that IOF of appropriate nutrients might be an effective technique to stimulate avian embryo development, increase the market weight and pectoral muscle weight of growing chickens.

The glucose, mainly stored as glycogen in the liver and glycolytic muscles of embryos, is preferentially utilized as energy source over lipid and protein because of the limitation of oxygen availability especially during late incubation

(Moran, 2007). Nevertheless, it is well known that the demand for glucose is high and the primary mechanism of glucose production depends on hepatic gluconeogenesis using the substrates of lactate (Kobayashi et al., 1989) and glucogenic amino acid from the amnion and muscles in the avian embryos and neonates (Edwards et al., 1997). In the current study, the data in non-injected control group proved that the depletion of energy reserves might occur in late-term chick embryos, as illustrated by exhausting up to 15% of liver glycogen and 66% of pectoral glycogen concentrations from 19 E to hatch. In addition, our results indicated that IOF of 6 and 12 mg/egg CrPyr raised liver glucose and glycogen

1695

accumulation at 19 E and hatch, which were consistent with the findings of Foye *et al.* (2006b) and Chen *et al.* (2010). These results implied that IOF of CrPyr might have a substrate-mediated effect on the concentrations of glucose and glycogen in liver, due to CrPyr providing pyruvate as the possible substrates for hepatic gluconeogenesis. As expected, the activity of liver glucose-6-phosphatase enzyme, one of the key enzymes in the gluconeogenesis pathway, was also increased in embryos IOF of CrPyr solutions at 19 E. Another similar study reported that there was a high positive correlation between BW and the concentration of glycogen in liver (Tangara *et al.*, 2010), suggesting that the improvement of the BW of broilers in this study could be partially attributed to the higher concentration of glycogen in liver.

In addition, the Cr–PCr system has been reported to maintain energy homeostasis by buffering ADP and ATP ratios via a freely reversible reaction catalyzed by creatine kinase in muscles (Allen, 2012). The creatine monohydrate supplementation in diet has been recently reported to elevate the concentration of Cr, activity of creatine kinase (Li *et al.*, 2016) and the level of PCr (Young *et al.*, 2007) in muscle of pigs. Wang *et al.* (2015) also showed that dietary 1200 mg/kg creatine monohydrate supplementation increased the concentrations of Cr and PCr in pectoral muscle of 3 h transported broilers in comparison to a 45 min transported control. Similarly, the present study proved that IOF of CrPyr increased the concentrations of Cr and PCr at 19 E, which could provide more ATP to avoid energy imbalance when energy demand increased at hatch. Therefore, this additional energy sources resulting from Cr–PCr system probably, at least in part, could improve the energy status of embryos and hatchlings, which was useful for development of BW. Uni *et al.* (2005) suggested that IOF of carbohydrates and β-hydroxy-β-methylbutyrate in late-term embryos could improve liver glycogen by two to five fold and elevate relative breast muscle size by 6% to 8% on the day of hatch. In the present study, compared with non-injected birds, the total and relative weight of pectoral muscle of broilers in 6 and 12 mg/egg CrPyr-injected groups were increased by 19.89 g (4.80%) and 27.82 g (8.75%) at 21 days of age, respectively. It is reasonable to assume that the higher energy reserves including glycogen and PCr probably reduce the need for glucose synthesis via gluconeogenesis from muscle proteins, resulting in higher pectoral muscle weight of embryos and broilers. Moreover, the yolk sac weight was increased in CrPyr groups in comparison with the control and saline groups at 19 E, which is consistent with the results of Zhai *et al.* (2011b) and Zhang *et al.* (2016). These findings suggested that the embryos in late embryonic stage could utilize the exogenous energy nutrients thereby sparing the yolk sac nutrient utilization. The higher residual yolks may be beneficial to the maintenance and growth of hatched broilers (Zhai *et al.*, 2011a).

In contrast to glycogen dynamics in liver, there was no significant change in glycogen reserves of pectoral muscle in this study. Similarly, Foye *et al.* (2006a) and Tangara *et al.*

(2010) maintained that IOF of protein or arginine alone also had no influence on muscle glycogen levels. Conversely, another study claimed that IOF of carbohydrates (simpler sugars) into amnion of pigeon eggs increased the glycogen reserves in pectoral muscle (Dong *et al.*, 2013). This discrepancy may be explained by the lack of glucose-6-phosphatase enzyme needed for gluconeogenesis and the requirement of insulin for uptake of glucose from the blood in skeletal muscle (Foye *et al.*, 2006a). It has been noted that IOF of carbohydrates could accelerate the uptake and storage of glucose in the form of glycogen in the muscles mainly through the release of insulin (Foye *et al.*, 2006b). Hence, IOF of a part of non-carbohydrate nutrients, such as protein and arginine, may not stimulate the secretion of insulin, thus, the glucose produced from hepatic gluconeogenesis is mainly stored as glycogen in liver rather than pectoral muscle (Foye *et al.*, 2006a), which corroborate with the effects of CrPyr in this study. In addition, the glycogen concentration in liver showed an increasing trend during 3 to 21 days post-hatch, whereas the glycogen concentration in muscle decreased from 3 to 7 days and kept nearly constant to 21 days post-hatch. The reason may be attributed to the different physiological functions of glycogen in liver and muscle. The liver glycogen mainly regulates the stability of blood glucose to provide energy for multiple organs. However, the muscle glycogen is primary used to generate ATP for muscle protein synthesis (very high in the breast muscle of 1- to 3-week-old chickens) and muscular contractions under the oxidative or glycolytic pathways.

In conclusion, the present study demonstrated that IOF of CrPyr on 17.5 days of incubation was beneficial to increase the concentrations of glucose and glycogen in liver, as well as the concentrations of Cr and PCr in pectoral muscle, which may contribute to the improvement of energy status of embryos and hatchlings, and subsequently improved the BW and pectoral muscle weight until the end of the experiments at 21 days post-hatch in broilers. The appropriate injection level of CrPyr was recommended at 12 mg/egg in the present study.

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