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Short communication: *In ovo* injection of all-*trans* retinoic acid causes adipocyte hypertrophy in embryos but lost its effect in posthatch chickens

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ABSTRACT

The regulation of adipose deposition in broiler chickens is an important factor for production efficiency to poultry producers and health concerns to customers. Although vitamin A and its metabolite [all-*trans* retinoic acid (**atRA**)] have been used for studies on adipogenesis in mammals and avian, effects of embryonic atRA on adipose development in embryonic (**E**) and posthatch (**D**) ages in broiler chickens have not been studied yet. Different concentrations of atRA (0 M–2 μM) were injected in broiler eggs at E10, and adipose tissues were sampled at E16. Percentages of adipose tissues in chicken embryos were significantly increased in the group injected with 500 nM of atRA compared to the 0 M group ($P < 0.05$). In addition, the adipocyte cross-sectional area (**CSA**) was significantly greater by *in ovo* injection of 500 nM atRA compared to the injection of 0 M ($P < 0.01$). Moreover, *in ovo* atRA-injected embryos were hatched and BWs were measured at D0, D7, and D14. BWs were not different from those of the 0 M group. Percentages of adipose tissues and CSA of the *in ovo* atRA-injected group (500 nM) were not different from those of the 0 M group at D14. Taken together, the current study clearly showed that *in ovo* injection of atRA promoted adipose deposition with hypertrophy during embryonic development, but its effects were not maintained in early posthatch age in broiler chickens, implying that embryonic atRA has an important role in the regulation of adipose development in chicken embryos.

Implications

In poultry industry, fast-growing performance for meat production accompanies with excessive adipose deposition. To satisfy poultry producers and consumers in aspects of production efficiency and health concerns, respectively, regulating adipose development has been considered. This study demonstrated that, by injecting all-*trans* retinoic acid (an active form of retinol) into broiler eggs, adipose deposition was promoted with adipocyte hypertrophy during embryonic development, however, its effects were not maintained in early posthatch age, implying that embryonic atRA has an important role in the regulation of adipose development in chicken embryos.

Introduction

With the fast-growing performance in broiler chickens, excessive adipose deposition is one of the concerns to producers in aspects of reduced feed efficiency and production of carcass yield, and customers in aspects of health concerns and meat flavor. The development of adipose tissues is processed by two steps: the adipocyte hyperplasia which is increasing numbers of adipocytes, and the adipocyte hypertrophy which is increasing sizes of adipocytes. In mammals, the molecular and cellular mechanisms in adipose accumulation during development have been well studied. However, understanding adipose development in chickens is still a paucity of knowledge.

Vitamin A (retinol) is an essential nutrition which is involved in adipose development. As an activated metabolite form of retinol, all-*trans* retinoic acid (**atRA**) has been used to study adipose development. Most *in vivo* studies have been performed with dietary challenges or injection of retinoids in growing or adult animals

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having a large population of mature adipocytes. In mice, fat mass was reduced by injection of atRA (Felipe et al., 2005; Mercader et al., 2006) but, in cattle, fat ratio and marbling score were increased by the injection of retinol (Harris et al., 2018). Due to potential difficulties in direct delivery of retinoids into the fetus in mammals, the effects of atRA on fetal adipose development have not been studied in mammals. However, the capable injection of atRA into quail eggs allowed us to study its effect in embryonic adipose development (Kim et al., 2021), resulting in adipose hypertrophy in quail embryos. However, the roles of atRA in adipose development of chicken embryos and its effect on posthatch chicks have not been reported.

For higher meat production of broiler chickens, controlling adipose deposition is an important factor for higher feed efficiency and better poultry meat with low fat. So, it is important to evaluate the effects of *in ovo* injection of atRA in adipose deposition of broiler chickens. Therefore, the present study aims to investigate the cellular effects of *in ovo* injection of atRA on adipose growth of chickens in embryonic and posthatch periods.

Material and methods

Animal care

Commercially available broiler chickens used in this study were maintained at The Ohio State University Poultry Facilities in Columbus, OH, USA. All animal care protocols and procedures were approved by the Institutional Care and Use Committee at The Ohio State University (Protocol 2020A00000046). All chickens were raised in the same circumstances such as incubation time and temperature to hatch eggs, room temperature, the size of brooder cages and a same kind of feed (Table 1), free access to food and water after hatch.

In ovo all-trans retinoic acid injection and tissue sampling

atRA (#R2625, Sigma-Aldrich, St. Louis, MO, USA) was injected at embryonic day (**E**) 10 as following our previous study (Kim et al., 2021). Different concentrations of atRA (final concentrations: 0 M, 100 nM, 500 nM, 1 μM, or 2 μM) dissolved in 15 μl of DMSO were directly injected into egg white through the shell membrane. The injected eggs were incubated at 37.5 °C with turning through 90-arc rotation every 2 hours until to be sampled or hatched. At E16 and posthatch days (**D**) 0, 7, and 14, BW were measured.

Table 1
Ingredient and calculated nutrient composition from DM of diets fed to broilers from 1 to 14 d of age.

Item	Value
Corn (kg 100 kg^{-1})	41.9
Soybean meal – 48% (kg 100 kg^{-1})	44.4
Meat and bone meal, 55% (pork) (kg 100 kg^{-1})	5
Blended fat (kg 100 kg^{-1})	2.9
D,L-Methionine (kg 100 kg^{-1})	0.25
L-Lysine (kg 100 kg^{-1})	0.15
Salt (kg 100 kg^{-1})	0.4
Limestone (kg 100 kg^{-1})	0.7
Dicalcium phosphate 18.5% (kg 100 kg^{-1})	2.85
Copper sulfate, fine 25.2% (kg 100 kg^{-1})	0.05
Amprolium 2.5% (kg 100 kg^{-1})	1
Selenium, 90.8 mg/lb (kg 100 kg^{-1})	1
Choline Chloride (kg 100 kg^{-1})	0.15
L-lysine (kg 100 kg^{-1})	0.15
Vitamin A (IU/kg^{-1})	13 200

Weights of inguinal fat pads were measured at E16 and D14. Exact numbers of animals were indicated in the figure legends.

Histological processing and measurement of fat cell size

To measure fat cell size, inguinal fat pads were sampled at E16 and D14, and fixed in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin and then cut into 5-mm slices. After staining with hematoxylin and eosin (**HE**), the samples were visualized under a microscope (EVOS cell imaging system, ThermoFisher Scientific, Waltham, MA, United States). To measure fat cell size, ImageJ software (NIH ImageJ 1.52 s) was used and the average of the adipocyte cross-sectional area (**CSA**) was calculated according to our previous study (Kim et al., 2021) by measuring randomly selected areas. At least 400 cells per animal were evaluated.

Statistical analysis

Data of BW at embryonic day and proportion of inguinal fat in BW at various concentrations of atRA (0–2 μM) were analyzed using the general linear model (**GLM**) of SAS (version 9.4). Preplanned polynominal contrasts were made for linear, quadratic, cubic, and quartic effects where the procedure of Interactive matrix Language (**IML**; SAS, version 9.4) was used to obtain orthogonal coefficients for unequal spaced levels of atRA concentration. Data of HE sain of inguinal fat tissue in Figs. 1C and 2C were analyzed by *t*-test using the GraphPad Prism software, version 6.02. To compare the differences of BW in Fig. 2A, BW was measured at D0, D7 and D14, respectively, and *t*-tests were used for statistical analysis to compare between the two groups (0 M or 500 nM) at the each of posthatch days. All data were expressed as means ± SEM. The results with $P < 0.05$ were considered to be significant.

Results and discussion

Adipogenesis occurs during both embryonic and postnatal development. Due to the fact that avian species have confined energy sources in their eggs during embryonic development, the chicken is a great model to track the effect of nutritional factor from embryonic to posthatch ages. In line with our previous findings demonstrating that atRA promotes adipocyte accumulation in quail *in vitro* and *in vivo* (Kim et al., 2021; 2022), and chicken *in vitro* (Kim et al., 2020; 2022), the current study was performed to investigate whether *in ovo* injection of atRA affects both embryonic and posthatch adipose growth and development in chickens.

Our previous study demonstrated that atRA have both pro- and anti-adipogenic effects by supplementing different concentrations in mouse preadipocytes (Kim et al., 2019). In addition, *in ovo* injection of atRA was the most effective at E7 in quail when embryonic adipose tissues started to appear (Kim et al., 2021). Because adipose tissues can be visible around E10 in chicken embryos (Chen et al., 2014), to determine to what extent concentrations of atRA on adipose accumulate in chicken embryos, five different dosages (0–2 μM) of atRA were directly injected *in ovo* at E10 and sampled at E16 in the current study. Although there was no difference in BW among all groups by a statistical analysis using the GLM of SAS (Fig. 1A), the 500 nM injected chicken embryos tend to increase percentages of weights of inguinal fat pads compared with the 0 M group (500 nM vs 0 M: 0.68 vs 0.60%, $P = 0.08$, Fig. 1B). The relationship between percentages of inguinal fat tissues and the different concentrations of atRA (0–2 μM) was showed by linear, quadratic, cubic and quartic regressions, and significant trends were detected by the linear and cubic ($P = 0.0345$ and 0.0119, respectively, Fig. 1B). To determine whether the increased

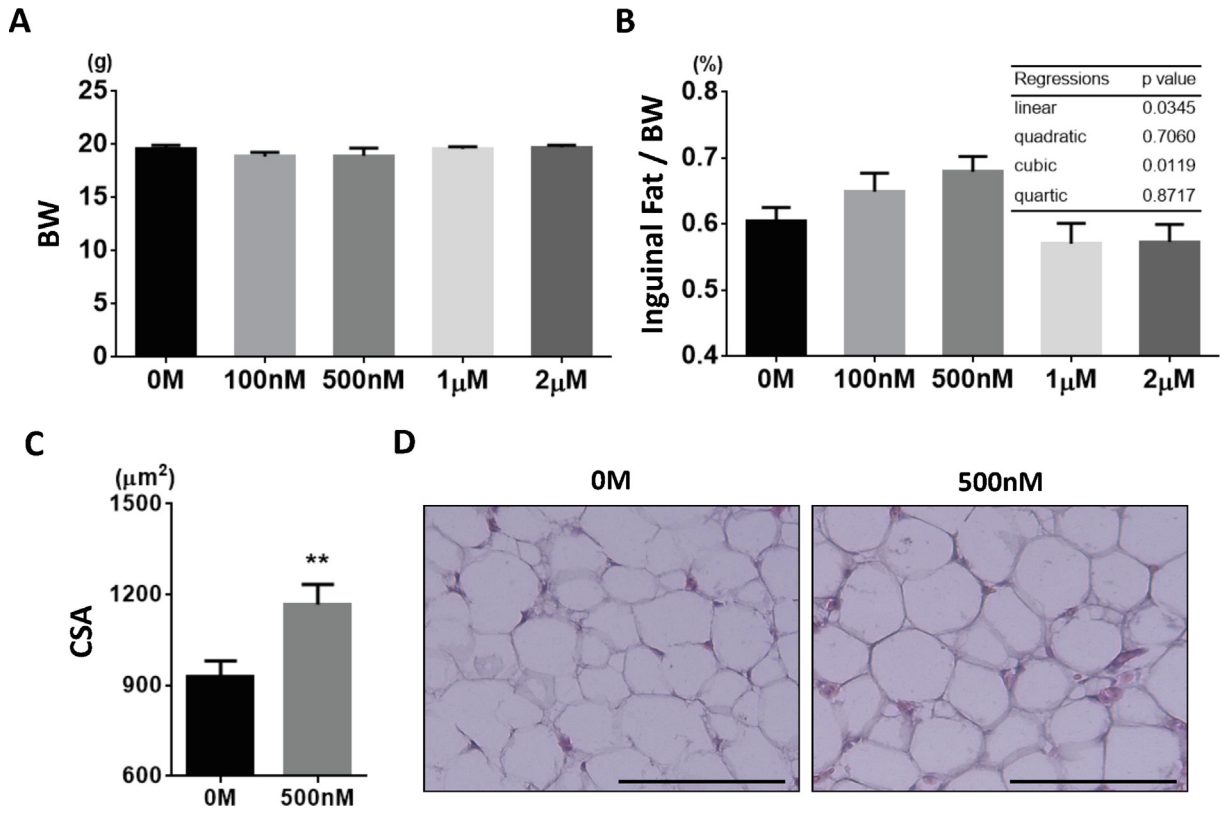

Fig. 1. Hypertrophic fat cells by *in ovo* injection of *all-trans* retinoic acid (atRA) at embryonic day of broiler chickens. (A) BW at embryonic day (E) 16. Various concentrations of atRA (0–2 μM) were injected *in ovo* at E10 and sampled at E16. (B) Percentages of inguinal fat weights in BW. For (A) and (B), n = 9 for 0 M, 8 for 100 nM, 7 for 500 nM, 12 for 1 μM, and 12 for 2 μM. The data were analyzed using the general linear model (GLM) of SAS. (C and D) hematoxylin and eosin (HE) stain of inguinal fat tissue. $^{**}P < 0.01$. n = 9 for 0 M, and 7 for 500 nM. Results are given as mean ± SEM of five randomly selected areas per individual embryo. *t*-test was used for the statistical analysis between 0 M and 500 nM. Scale bar: 100 μm. CSA: cross-sectional area.

percentages of fat pads by atRA at E16 can be attributed to increasing cell size, histological examination was performed using inguinal fat tissues. In chicken embryos that were injected with 500 nM of atRA at E10 and sampled at E16, CSA of inguinal fat cells was significantly increased compared with the 0 M (0 M vs 500 nM: 929.3 ± 51.3 μm^2 vs 1 167 ± 65.8 μm^2, $P < 0.01$, Fig. 1C and D). Our previous studies demonstrated that supplementation of atRA can promote adipogenesis *in vitro* in murine preadipocytes (Kim et al., 2019) and chicken preadipocytes (Kim et al., 2020; Lee et al., 2021). Moreover, *in ovo* injection of atRA resulted in hypertrophic fat accretion in quail embryos (Kim et al., 2021). In the current study, *in ovo* injection of atRA in chicken embryos resulted in increased fat pad weight with adipocyte hypertrophy during embryonic development, suggesting a consistent pro-adipogenic effect of atRA in avian embryos.

To investigate whether the hypertrophic effects of atRA on embryonic adipose tissues can affect adiposity in posthatch chickens, adipose deposition after hatch were further investigated. There was no difference in BW at D0, D7, and D14 between the control group and atRA group that were injected with 0 and 500 nM of atRA into E10 embryos, respectively. In addition, percentages of inguinal fat pads were calculated, and CSA was measured at D14. Although the percentages of fat pad to BW and CSA of adipocytes were significantly increased in chicken embryos by atRA (Fig. 1), these effects were not found in adipose tissues at D14 (Fig. 2B); no difference in percentages of fat pad to BW (Fig. 2B) and fat cell sizes at D14 (0 M vs 500 nM: 896.0 ± 70.3 μm^2 vs 965.3 ± 67.9 μm^2, Fig. 2C and D). As avian embryos are developing in confined nutritional environments in eggs, *in ovo* injected atRA might continuously affect adipose development during embryonic periods. However, posthatch feeding for the first two weeks might result in no difference in fat contents between the two groups. In addition, dynamic changes in adipose tissues accompanied with fast growth at early posthatch ages may diminish the effects of *in ovo* atRA injection.

The current study clearly showed that *in ovo* injection of atRA could increase adipose deposition with hypertrophy during embryonic development, but these effects were not maintained in posthatch broiler chickens. A previous study reported that dietary supplementation of RA resulted in an increased fat accretion rate in early broiler chickens (Savaris et al., 2020). Therefore, it will be interesting to further investigate whether modulation of atRA levels for combinations of embryonic and/or posthatch periods can differentially affect fat deposition in market-age chickens.

Ethics approval

All animal care protocols and procedures were approved by the Institutional Care and Use Committee at The Ohio State University (Protocol 2020A00000046).

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request.

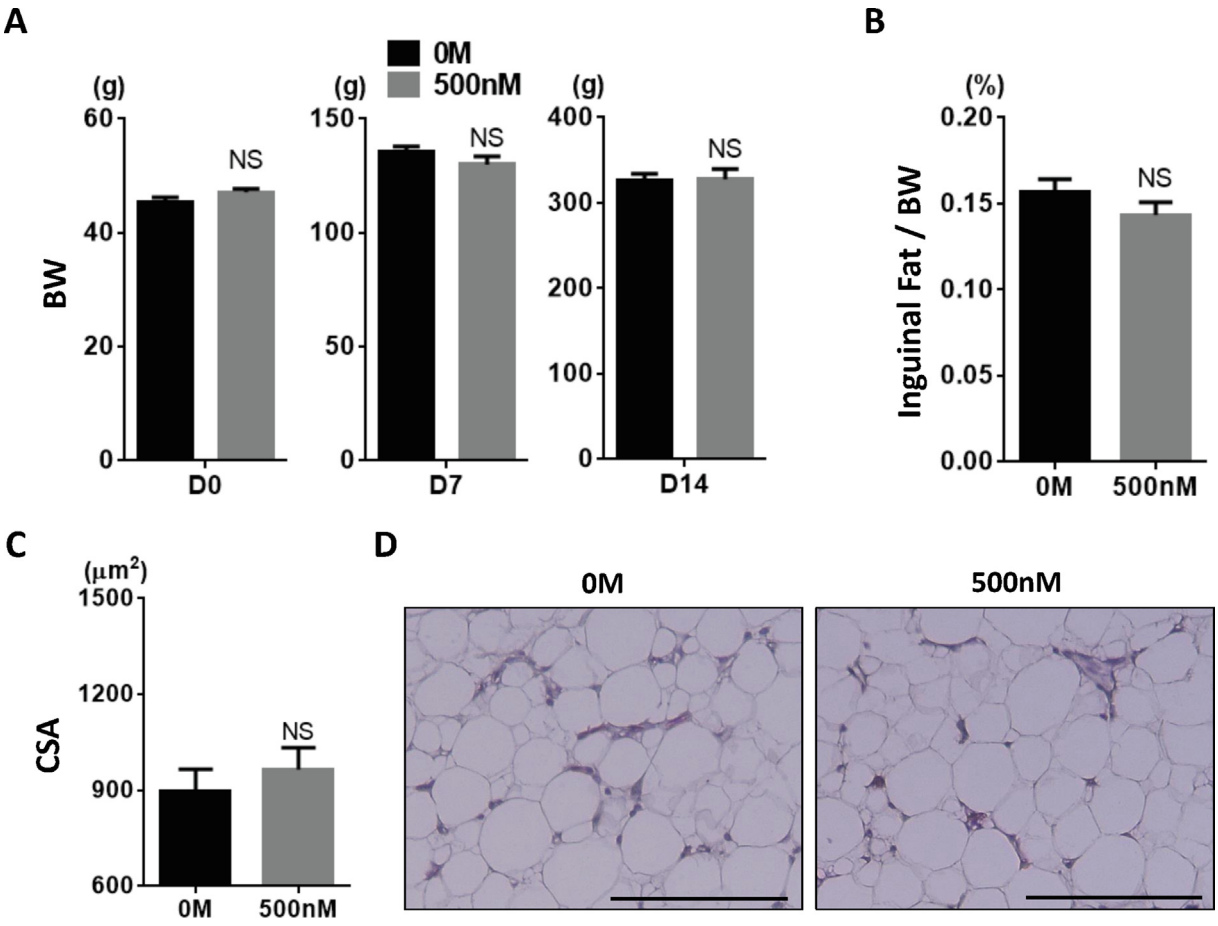

Fig. 2. Effects of atRA-injected *in ovo* at posthatch days of broiler chickens. (A) BW at posthatch days (D) 0, 7 and 14. atRA (0 M or 500 nM) were injected *in ovo* at E10 and measured BW at D0, D7 and D14. *t*-tests were used for statistical analysis to compare between the two groups (0 M or 500 nM) at D0, D7 and D14, respectively. (B) Percentages of inguinal fat weights in BW at D14. (C and D) hematoxylin and eosin (HE) stain of inguinal fat tissue. After injection of 0 M (n = 17) and 500 nM (n = 17) of atRA into eggs at E10, they were sampled at D14. For (B) and (C), *t*-tests were used for statistical analysis to compare between the two groups (0 M or 500 nM). NS: no significant difference. Histological results are given as mean ± SEM of five randomly selected areas per individual chicken. Scale bar: 100 μm. CSA: cross-sectional area.

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D.-H.K.: writing – original draft.
D.-H.K. & K.L.: writing – review and editing.
K.L.: funding acquisition, supervision.
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Declaration of interest

None.

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