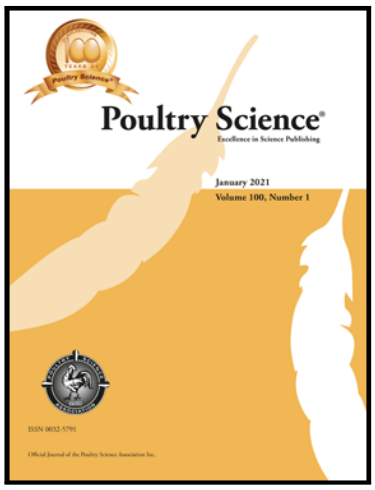

Research Note: Adipogenic differentiation of embryonic fibroblasts of chicken, turkey, duck, and quail in vitro by medium containing chicken serum alone

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RESEARCH NOTE

Research Note: Adipogenic differentiation of embryonic fibroblasts of chicken, turkey, duck, and quail in vitro by medium containing chicken serum alone

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ABSTRACT

The study of adipogenesis is one of the most important areas for not only regulating meat quality, but production efficiency associated with fat accretion in the poultry species. Current *in vitro* models for avian adipogenesis require adipogenic inducers including dexamethasone, 3-isobutyl-1-methylxanthine (**IBMX**), fatty acids, or insulin. However, problems still remain in these models for testing/screening potential nutritional, hormonal, and pharmaceutical factors because of interfering/overriding effects of the inducing factors. Therefore, the purpose of this study was to develop a simple *in vitro* method for avian adipogenesis. In this study, chicken serum (**CS**) and fetal bovine serum (**FBS**) were compared for adipogenic potential using chicken embryonic fibroblasts (**CEF**). Oil-red O staining at 4 days in culture of CEF under CS revealed that lipid droplet formation was increased by CS in a dose-dependent manner (0 to 10%). On the contrary, all concentrations of FBS (0 to 10%) alone did not show lipid droplet formation. In accordance with the morphological data of CEF,

mRNA expression of genes involved in adipocyte differentiation/determination, fatty acid uptake, and triacylglycerol (**TAG**) synthesis, was most significantly up-regulated by 10% CS at day 4 compared to 1 or 5% CS. In addition, embryonic cells isolated from quail (**QEF**) at E5, duck (**DEF**) at E6, and turkey (**TEF**) at E6, were tested for adipogenic differentiation by media containing the same concentrations of CS. Similar to the morphological data from CEF, quantitative data of the Oil-red O staining showed that lipid droplet formation in QEF, DEF, and TEF was increased by CS in a dose-dependent manner (0 to 10 %). The current study demonstrates that CS alone can induce adipogenesis on embryonic fibroblasts of various poultry species. By providing a new simple *in vitro* method of avian adipogenesis, diverse nutritional, hormonal, and pharmaceutical factors can be broadly and easily tested for scientific and industrial purposes.

Keywords: Adipogenesis; Chicken Serum; Poultry; Embryonic cells

INTRODUCTION

Adipose tissue stores excessive energy as triacylglycerol (**TAG**) in adipocytes. Selection of broiler chickens for fast growth is accompanied with increased feed intake and accretion of body fat, negatively affecting feed efficiency. In addition, meats with high fat have been regarded as unhealthy meats by consumers. Therefore, a developmental model for investigating avian adipogenesis is essential to understand regulatory roles of nutritional, hormonal, and environmental factors in adipocyte differentiation, development, and fat accretion. Although *in vivo* avian models are ultimate systems to verify effects of these factors in regulation of adipogenesis, *in vitro* models can be useful and beneficial by saving time, cost, and resources and investigating direct effects of these factors on avian adipogenesis.

So far, several *in vitro* models of avian adipogenesis have been developed by supplementing differentiation media containing combinations of several factors including insulin, dexamethasone, and fatty acids to stromal vascular (**SV**) cells isolated from chicken adipose tissues (Ramsay and Rosebrough, 2003; Shang et al., 2014). In recent studies, *in vitro* adipogenic differentiation using chicken embryonic fibroblasts (**CEF**) or SV cells isolated from embryonic and adult chickens has been achieved by supplementation of adipogenic inducers such as fatty acids, insulin, or all-*trans* retinoic acid (Serr et al., 2011; Kim et al., 2020a; b). Although these inducers can promote adipogenic differentiation of CEF and SV cells, these robust inducers might override regulatory effects of potential factors that are needed to be tested in these cell models. Therefore, the objective of the current study is to establish a simple method for adipogenic differentiation in which direct effect of factors of interest can be analyzed, ultimately identifying potential regulatory factors reducing fat accretion in poultry.

MATERIALS AND METHODS

Isolation of Embryonic Cells and Adipogenic Differentiation

Experiments using poultry embryos are exempt from requiring University Institutional Animal Care and Use Committee approval. Fertile eggs of Hy-Line White Leghorn chickens and Nicholas breed turkeys were kindly donated from Hy-Line North America, LLC, and Cooper Hatchery, respectively. Fertile eggs of Japanese quail were obtained from The Ohio State University poultry research farm and eggs of the Khaki Campbell duck were purchased from Fifth Day farm. To isolate and culture embryonic cells, embryos were sampled at embryonic day (**E**) 5 or 6 as following our previous study (Kim et al., 2020b). The next day after seeding, adipogenic differentiation of the cells was induced with Dulbecco's Modified Eagle Medium (**DMEM**, #11965, Gibco, Grand Island, NY, USA) containing different concentrations (0, 0.5, 1, 2, 5, or 10 %) of chicken serum (**CS**, #16110, Gibco) or fetal bovine serum (**FBS**, #F4135, Sigma-Aldrich) for 4 days.

Visualization of Lipid Droplets

After 4 days of inducing adipogenic differentiation, cells were fixed with 10% normal buffered formalin for 1 h. After fixation, cells were washed with distilled water for 3 times and stained with 60% Oil-Red-O (**ORO**) solution (#O0625, Sigma-Aldrich) for 1 h at room temperature. After washing with distilled water for 3 times, stained plates were scanned by a scanner (Epson perfection 4490 Photo, EPSON, Los Alamitos, CA), and lipid droplets were visualized using a microscope (EVOS cell imaging system, Thermo Fisher Scientific, Waltham, MA, USA). To quantify relative OD values from ORO-stained cells, the ORO was extracted with 100% isopropanol and absorbance values were measured at 490 nm by a spectrophotometer (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA, USA).

Analysis of Gene Expression

Total RNA was isolated from chicken embryonic cells at 4 days after induction of adipogenic differentiation using Trizol reagent (#15596026, Life Technologies Inc. Grand Island, NY, USA) according to the manufacturer's instructions. Synthesis of cDNA from RNA and quantitative Real-time PCR (**qPCR**) were performed as following our previous studies (Kim et al., 2020a). For qPCR, three independent experiments were performed and each of the experiments were duplicated. qPCR for each sample was performed in duplicate and all primer sequences with qPCR condition in this study were described in our previous studies (Kim et al., 2020a) except *fatty acid transporter 1* (***Fatp1***) (NCBI reference sequence: NM_001039602.2, F: 5'-TCGTTTGGTGAAGGTGAATGAG, R: 5'-CGAGCTCATCCATCACCAACA, size: 242 bp), *Glycerol-3-phosphate dehydrogenase 1* (***Gpd1***, Ensembl Reference Sequence: ENSGALT00000076926.2, F: 5'-GGCTTTTGCCAAGACTGGGAA, R: 5'-GGTTTGCCCTCATAGCAGATCTG, size: 177bp). *Ribosomal Protein S13* (***Rps13***) was used as a housekeeping gene. All qPCR data were analyzed using the 2-ΔΔCT method (Livak and Schmittgen, 2001).

Statistical Analysis

Data for expression levels of genes were expressed as means ± SEM (n = 3). Multiple means were compared by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism software, version 6.02. *p*-value, $p < 0.05$, was considered statistically significant.

RESULTS AND DISCUSSION

Lipid metabolism and its regulation in avian species have become an interesting research field because, unlike mammals, avian species are oviparous which means they use only egg

yolk as an energy source during embryonic development. Primary cell cultures isolated from adipose tissues and embryonic cells have been used for research on avian adipogenesis by supplementing diverse adipogenic inducers (Ramsay and Rosebrough, 2003; Serr et al., 2011; Shang et al., 2014; Kim et al., 2020a; b). Although previous methods including various adipogenic inducers have been actively used in the avian adipogenesis studies, these inducers may too strongly influence adipogenesis to discern direct effects of potential nutritional, hormonal, and pharmaceutical factors that will be tested or screened in the future.

In this study, to establish a simple *in vitro* model, adipogenic differentiation of CEF isolated from E6 chicken embryos were induced by different concentrations of CS or FBS for 4 days without supplementations of adipogenic inducers such as fatty acids and insulin (Figure 1). Morphological examination revealed that lipid droplet formation in CEF was increased with increasing percentages of CS; whereas lipid accumulation of CEF was not changed at any concentrations of FBS that were tested. (Figure 1A). In agreement with the data showing a dose-dependent increase in intensities of Oil-red O staining in multi-well plates for cultures of CEF (Figure 1A), relative amounts of ORO measured by spectrophotometry were increased by CS (Figure 1B). To further investigate developmental processes of adipogenesis that might be affected by CS, expression levels of genes involved in adipogenic determination/differentiation were analyzed by qPCR (Figure 1C). A well-known marker of adipogenic determination/differentiation, *Zinc finger protein 423* (***Znf423***) was significantly up-regulated in the 10% CS group at D4 compared to 1 and 5% CS groups at D4 and all groups at D2. Adipocyte differentiation markers, *CCAAT/enhancer-binding protein beta* (***C/ebpβ***), *peroxisome proliferator-activated receptor γ* (***Pparγ***), and *fatty acid binding protein 4* (***Fabp4***), were also dose-dependently increased with CS concentrations. Especially, the highest expression levels of those genes were observed in the 10% CS at D4 ($p < 0.05$, Figure 1C).

Compared with mammals, sources of TAG accumulation in avian adipocytes are lipids from egg yolk during embryonic adipose development and from serum after hatching, because *de novo* lipogenesis occurs exclusively in avian liver, not in adipose tissues (Goodridge and Ball, 1967; Leveille et al., 1975). Therefore, in this study, expression levels of genes involved in fatty acid uptake, *fatty acid transporter 1* (***Fatp1***), and *acyl-CoA synthetase long-chain family 1* (***Acsl1***), were analyzed (Figure 1C). Similar with the expression patterns of adipogenic factors above, *Fatp1* and *Acsl1* were dose-dependently up-regulated with CS concentrations. Especially, the 10% CS group at D4 resulted in the highest expression of these genes ($p < 0.05$, Figure 1D). In addition, expression levels of genes involved in TAG synthesis, *Gpd1*, *acylglycerolphosphate acyltransferase 1* (***Agpat1***), and *diacylglycerol O-acyltransferase homology 2* (***Dgat2***), were significantly increased at D4 in the dose-dependent manner ($p < 0.05$, Figure 1E). Taken together, increased formation of lipid droplets by CS in a dose-dependent manner and up-regulation of adipogenic and lipogenic markers by 10% CS indicate that chicken serum itself is sufficient to induce differentiation of CEF into adipocytes *in vitro*.

Previous studies reported that lipoproteins such as high-density lipoprotein (**HDL**), low-density lipoprotein (**LDL**), and very low-density lipoprotein (**VLDL**), promote induction of adipogenic differentiation in 3T3-L1 cells and human preadipocytes *in vitro* (Stanton et al., 1997; Chiba et al., 2003). Also, it was reported that CS contains 6-fold more of triglyceride, 4-fold more of VLDL, and 2-fold more of cholesterol and HDL compared to FBS (Khaki et al., 2012). In addition, components in FBS compared to CS might not be fully compatible with the chicken cell system. For these reasons, adipogenic differentiation of CEF can be induced with CS alone, but not with FBS alone.

To further investigate adipogenic effect of CS in other poultry species, adipogenic differentiation of embryonic cells isolated from quail (**QEF**) at E5, and duck (**DEF**) and

turkey (**TEF**) at E6, were tested using different concentrations of CS for 4 days. Similar to the morphological data from the CEF in Figure 1A, lipid droplet formation in QEF, DEF, and TEF was increased dose-dependently by CS (Figure 2A and 2B). In agreement with the data from intensities of Oil-red O staining in multi-well plates for cultures of QEF, DEF, and TEF (Figure 2A and 2B), relative amounts of ORO measured by spectrophotometry were dose-dependently increased by CS (Figure 2C). These data further demonstrated the adipogenic ability of CS in avian embryonic fibroblasts and thus, providing QEF, DEF, and TEF as *in vitro* models for adipogenesis study.

Our previous study showed that supplementation of fatty acids and insulin in 10% CS medium induced adipogenic differentiation of CEF at day 2, but not in 10% CS alone (Kim et al., 2020a). However, in the prolonged supplementation of CS alone, genes involved in adipogenesis were significantly over-expressed at day 4 after inducing adipogenic differentiation (Figure 1C-E). Using the current method, adipogenesis of various avian embryonic cells can be easily obtained and cultured with a low chance of experimental contamination due to the germ-free condition of the eggs. Therefore, this new *in vitro* method for avian adipogenesis will be widely and easily used in research for investigating genetic, developmental, nutritional, phytochemical, and environmental factors in most poultry species.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Effect of various concentrations of FBS or CS on the lipid accumulation in CEF. Oil-Red-O (**ORO**) staining (A). Chicken embryonic fibroblasts (**CEF**) were harvested at embryonic day (**E**) 6 and adipogenic differentiation of CEF were induced by different concentrations of chicken serum (**CS**) or fetal bovine serum (**FBS**) for 4 days. ORO stained cells were visualized under a scanner and microscope. 1: 0% CS; 2: 0.5% CS; 3: 1% CS; 4: 2% CS; 5: 5% CS; 6: 10% CS; 7: 0% FBS; 8: 0.5% FBS; 9: 1% FBS;10: 2% FBS; 11: 5% FBS; 12: 10% FBS. Scale bar: 100 μm. O.D. values (B). ORO was quantified using a spectrophotometer at 490 nm (n=4). Expression levels of genes involved in adipogenesis *Znf423*, *C/ebpβ*, *Pparγ*, and *Fabp4*, (C), fatty acid uptake, *Fatp1* and *Acsl1*, (D) and triglyceride synthesis, *Gpd1*, *Agpat1*, and *Dgat2* (E) were analyzed by qPCR at D0, D2 and D4 after inducing adipogenic differentiation. *Rps13* was used as a housekeeping gene. All data for expression levels of the genes were shown as mean ± SEM (n=3). ND: non-detected. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis by the GraphPad PRISM 6.02 program and statements of significance noted by a, b, ab, or c were based on testing at $p < 0.05$.

Figure 1

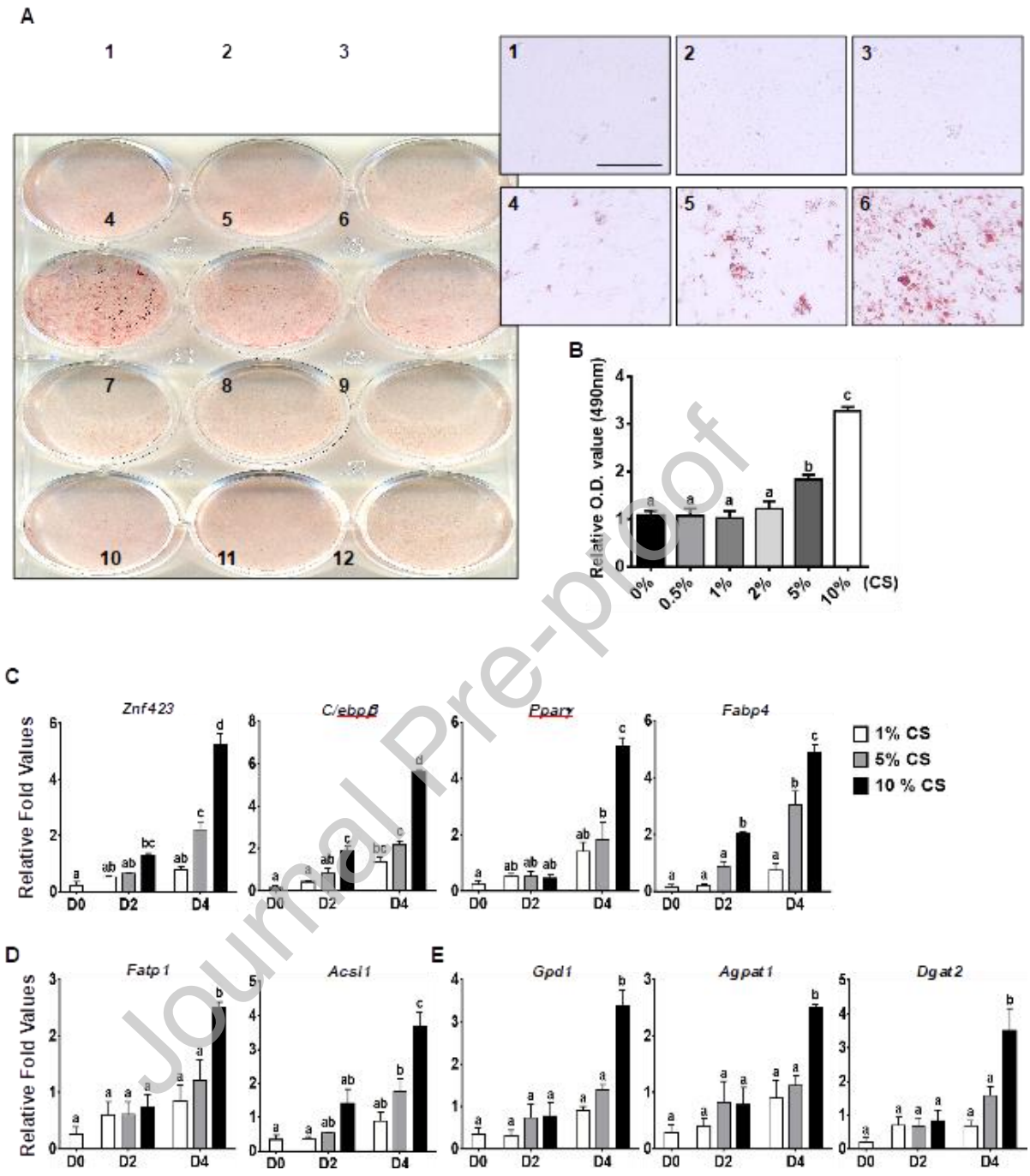

Figure 2. ORO staining on embryonic cells of quail, duck, and turkey. Quail embryonic fibroblasts (**QEF**), duck embryonic fibroblasts (**DEF**), turkey embryonic fibroblasts (**TEF**) were harvested at E 5, 6, or 6, respectively, and adipogenic differentiation was induced by different concentrations of CS for 4 days and stained by ORO at D4. ORO stained cells were visualized under a scanner (A) and a microscope (B). Scale bar: 100 μm. O.D. values (C).

ORO was quantified using a spectrophotometer at 490 nm (n=4). 1: 0% CS; 2: 0.5% CS; 3: 1% CS; 4: 2% CS; 5: 5% CS; 6: 10% CS.

Figure 2