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Effects of Calcium Lactate on the Development of Chicken Embryos in a Shell-less Culture System up to Day Seventeen of Incubation

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This study examined the effects of calcium lactate on the development of chicken embryos in a shell-less culture system (cSLCS) up to the seventeenth day of incubation. In the presence of calcium lactate, a significant reduction in embryo viability was observed during the first week of incubation in cSLCS. On day 17 of embryo development, no significant difference was observed in the blood plasma calcium concentration or tibia bone density between cSLCS and intact control embryos, whereas the tibia length was significantly shorter in cSLCS embryos than in the intact control. These results suggest that calcium lactate supplementation in cSLCS supports bone formation in developing chicken embryos, but has adverse effects on the viability of embryos, particularly during the first week of embryo development.

Key words: calcium lactate, chick embryo, microcomputed tomography, shell-less culture, tibia

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Introduction

The development of a complete shell-less culture system (SLCS) of avian embryos, which enables the visualization of the phenotypic outcomes of the experimental manipulation of avian embryos, has long been a topic of interest. Traditionally, chicken embryos have been cultured using either egg windowing methods (Fisher and Schoenwolf, 1983; Andacht *et al*., 2004) or surrogate eggshell methods (Rowlett and Simkiss, 1987; Perry, 1988; Naito *et al*., 1990), in areas such as developmental biology (Matheus *et al*., 2019), embryo manipulation (Boulland *et al*., 2010), bioimaging (Funahashi and Nakamura, 2014), chorioallantoic membrane assays (Vu *et al*., 2018), and basic research on regenerative medicine (Chiba *et al*., 2010). In this respect, the hatching of Japanese quail chicks under a complete shell-less culture system (qSLCS) using polytetrafluoroethylene (PTFE) film supplemented with eggshell powder and calcium lactate was a significant achievement (Kamihira *et al*., 1998). However, because of the inability to directly observe developing embryos due to the opacity of the PTFE film, Tahara and Obara (2014) developed a modified culture method using transparent polymethylpentene (PMP) film, which enables the direct observation of developing chicken embryos under the SLCS (cSLCS).

In cSLCS, water-soluble calcium lactate was placed at the bottom of the culture vessel prior to the transfer of three-day-old embryos. However, it has been reported that the transfer and absorption of calcium and trace elements from the eggshell to the chicken embryo peaks around day 17 of the embryo development (Tuan, 1983; Torres and Korver, 2018). In addition, various developmental abnormalities (e.g., delayed embryo development, hypocalcemia, bone stunting,

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poor ossification, and dysmorphism) have been reported in avian embryos cultured *in vitro* without calcium supplementation (Tuan, 1980; Tuan and Ono, 1986; Jourdeuil *et al*., 2015). These experimental results indicate that the effects of calcium lactate on current cSLCSs need to be critically evaluated.

Therefore, the aim of the present study was to characterize the biochemical and morphological effects of calcium lactate supplementation up to day 17 of embryonic development in cSLCS.

Materials and Methods

Chicken Eggs

Fertilized White Leghorn (WL) eggs (MB line: National Livestock Breeding Center, Okazaki, Japan) maintained at the Tsukuba Plant Innovation Research Center (T-PIRC farm, University of Tsukuba, Japan) were used in the experiments.

Preparation of Culture Vessels

The cSLCS described by Tahara and Obara (2014) was used in the present study. A 250-mg portion of calcium DL-lactate pentahydrate (Cat #:031-00675, Fujifilm Wako Pure Chemical Co., Osaka, Japan) and 2.5 m*l* of sterilized distilled water (Otsuka Pharmaceutical Co., Tokyo, Japan) were added prior to the transfer of chicken embryos into the cSLCS.

Experimental Treatments

Eggs were preincubated in a forced-air incubator (P-008, Showa Furanki, Saitama, Japan), and maintained at 37.8℃ and 70% relative humidity for three days.

After incubation, embryos at stages 15−16 (Hamburger and Hamilton, 1951) were randomly assigned to one of the following four experimental treatment regimes:

Treatment-1 (Trmt-1): Three-day-old chicken embryos were placed into the cSLCS without supplementation with calcium lactate and distilled water.

Treatment-2 (Trmt-2): Sterilized distilled water (2.5 m*l*) without calcium lactate was placed on the PMP film in the cSLCS prior to the transfer of three-day-old chicken embryos.

Treatment-3 (Trmt-3): Sterilized distilled water (2.5 m*l*) with 250 mg of powdered calcium lactate was placed on the plastic film in the cSLCS prior to the transfer of three-day-old chicken embryos.

Intact control (IC): Fertilized shelled eggs were incubated in a forced-air incubator maintained at 37.8℃ and 70% relative humidity.

Care was taken to avoid physical damage to the embryos while transferring whole egg contents into the cSLCS (Fig. 1). The incubation conditions for fertilized embryos in a cSLCS have been described previously (Tahara and Obara, 2014). The viability of the embryos in the cSLCS was determined daily from day 3 (D3) to day 17 (D17) of incubation.

The duration of embryo incubation in the cSLCS was expressed in days, using the notation Di, where integer i=3, ... 17.

At D17, approximately 0.5 m*l* of blood was collected from a blood vessel located in the chorioallantoic membrane of the embryos. Plasma was separated from the blood by centrifugation, and the levels of lactic acid dehydrogenase (LDH), calcium (Ca), inorganic phosphorus (IP), glucose (GLU), aspartate aminotransferase (AST), total cholesterol (TCHO), and triglyceride (TG) were measured using an automated clinical chemical analyzer (DRI-CHEM V4000, Fujifilm Co., Tokyo, Japan). After blood collection, the embryos were fixed by being placed in a 10% formaldehyde neutral buffer solution (Cat #:062-01661, Fujifilm Wako Pure Chemical Co., Osaka, Japan).

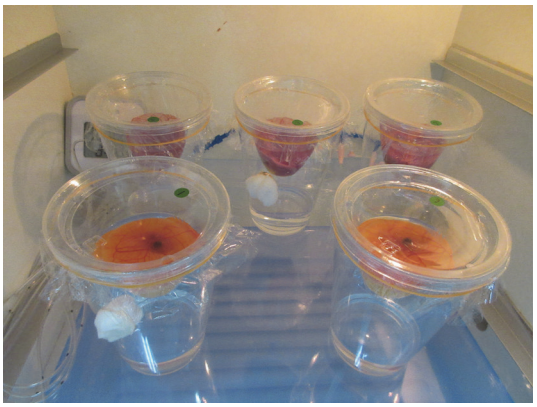

Fig. 1. **Outlook of chick shell-less culture system (cSLCS).**

Microcomputed Tomography (micro-CT) Analysis

After fixing, tibia samples were collected from D17 embryos and scanning images were acquired using a 3-D X-ray microscopic CT scanner (TDM-1000, Yamato Scientific Co., Tokyo, Japan) at 60 kV and 70 μA, using a tungsten target. After the segmentation threshold (109 mg/cm^3) was set to remove the soft tissue from the total tissue, the degree of ossification was quantified using the micro-CT analysis software TRI/3D-BON (Ratoc System Engineering Co., Tokyo, Japan).

Bone volume (BV, mm^3), total tissue volume (TV, mm^3), bone surface (BS, mm^2), bone volume per total tissue volume (BV/TV, %), bone surface per bone volume (BS/BV, 1/mm), and volumetric bone mineral density (vBMD, mg/cm^3) were determined by manual calculation.

Statistical Analysis

A split-plot design was used to analyze the viability of embryos using treatments as the main plot and incubation days as the sub-plot. Pairwise multiple comparisons between the treatments were conducted using Tukey's test.

Statistical analyses of blood plasma and a micro-CT analysis were performed using EZR Version 1.37 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R Version 3.4.1 (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander, which is designed to apply the statistical functions frequently used in biostatistics (Kanda, 2013). The statistical significance of the differences was determined using one-way analysis of variance (one-way ANOVA) for multiple comparisons, followed by Tukey's test.

Differences with $P<0.05$ were considered significant.

Institutional Approval

All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals of the University of Tsukuba, and the experiment was approved by the Animal Experiment Committee of the University of Tsukuba (Approval number 20-409).

Results

As shown in Fig. 2, the viability of the embryos in Trmt-3 decreased significantly between D6 and D7 ($P<0.05$), whereas only a slight and non-significant decrease in embryo viability was observed in Trmt-2 during the same period. The embryo viabilities at D17 in IC, Trmt-1, Trmt-2, and Trmt-3 were 95.2%, 84.1%, 60.6%, and 26.0%, respectively.

As shown in Fig. 3-A, the average Ca concentrations in Trmt-1, Trmt-2, Trmt-3, and IC were 9.7, 9.5, 12.1, and 12.7 mg/d*l*, respectively. No significant differences in Ca concentration were observed between Trmt-3 and IC, whereas a significantly lower Ca concentration was observed in both Trmt-1 and Trmt-2 compared to Trmt-3 and IC ($P<0.05$). As shown in Fig. 3-B, the average IP concentrations in Trmt-1, Trmt-2, Trmt-3, and IC were 11.0, 9.1, 8.8, and 10.1 mg/d*l*, respectively. A significantly higher IP concentration was observed in Trmt-1 than in Trmt-2 or Trmt-3 ($P<0.05$), whereas no significant differences were observed between Trmt-2, Trmt-3, and IC ($P>0.05$). As shown in Fig. 3-C, the average LDH concentrations in Trmt-1, Trmt-2, Trmt-3, and IC were 521.5, 609.5, 830.2, and 685.3 U/*l*, respectively. A significantly higher LDH concentration was observed in Trmt-3 than in both Trmt-1 and Trmt-2 ($P<0.05$), whereas no significant differences were observed between Trmt-3 and IC ($P>0.05$).

As shown in Fig. 3-D, the average GLU concentrations in Trmt-1, Trmt-2, Trmt-3, and IC were 221.4, 233.8, 235.6, and 209.6 mg/d*l*, respectively. No significant differences were observed between the treatments ($P>0.05$).

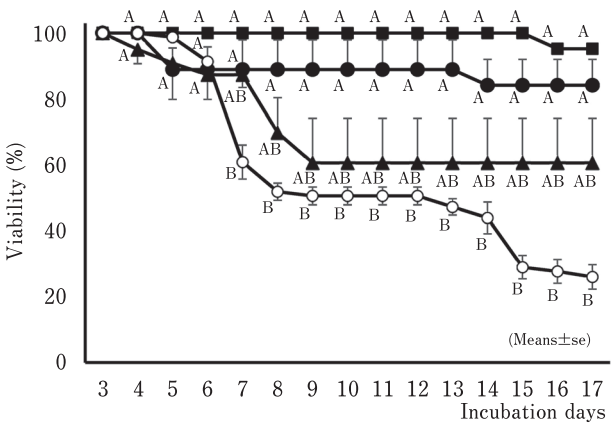

Fig. 2. **Viability of cultured embryos at D17 of incubation.** ■: Intact control ($n=21$ embryos), ●: Treatment-1 ($n=23$ embryos), ▲: Treatment-2 ($n=23$ embryos), ○: Treatment-3 ($n=47$ embryos).

As shown in Fig. 3-E, the average AST concentrations in Trmt-1, Trmt-2, Trmt-3, and IC were 32.5, 36.4, 40.0, and 39.0 U/*l*, respectively. A significantly lower AST concentration was observed in Trmt-1 ($P<0.05$), whereas no significant differences were observed among Trmt-2, Trmt-3, and IC ($P>0.05$).

As shown in Fig. 3-F, the average TG concentrations in Trmt-1, Trmt-2, Trmt-3, and IC were 252.3, 266.3, 322.8, and 360.4 mg/d*l*, respectively. No significant differences were observed between the treatments ($P>0.05$).

As shown in Fig. 3-G, the average TCHO concentrations in Trmt-1, Trmt-2, Trmt-3, and IC were 238.9, 249.1, 238.8, and 248.6 mg/d*l*, respectively. No significant differences were observed between the treatments ($P>0.05$).

As shown in Figs. 4-A and 4-B, no morphological abnormalities, such as bone bending deformities, were observed in the tibia of the chicks because of the treatments following a micro-CT analysis.

As shown in Figs. 4-C and 4-D, the high mineral deposition density area (red color) increased in size under Trmt-3. In addition, a three-dimensional (3-D) movie that was reconstructed using two-dimensional (2-D) mineral deposition density images also indicated an increase in the size of the high mineralization area under Trmt-3 (Fig. S1).

As shown in Fig. 5-A, the average tibia lengths in Trmt-1, Trmt-2, Trmt-3, and IC were 19.3±0.3, 18.6±0.5, 18.3±1.1, and 24.3±0.3 mm, respectively. Tibia length in the IC was significantly longer than in Trmt-1, Trmt-2, and Trmt-3 ($P<0.05$).

As shown in Fig. 5-B, the midpoint of the tibia was significantly thinner in Trmt-1 and Trmt-2 than in the IC ($P<0.05$), but no significant difference was observed between Trmt-3 and IC ($P>0.05$).

As shown in Fig. 5-C, the TV, BV, and BS values were significantly lower in Trmt-1, Trmt-2, and Trmt-3 than in the IC ($P<0.05$), but no significant differences were observed between the treatments ($P>0.05$).

For BS/BV, only Trmt-3 did not differ significantly from the IC ($P>0.05$; Fig. 5-C). The BV/TV was significantly reduced in Trmt-2 compared to that in the IC ($P>0.05$; Fig. 5-C).

For vBMD, only Trmt-3 did not differ significantly from the IC ($P>0.05$; Fig. 5-C). Supplementation of chicken embryos with calcium lactate resulted in a significant increase in the vBMD of the tibia of D17 embryos.

Discussion

It has been previously reported that the peak of calcium and trace element absorption from the eggshell to the chicken embryo is observed around D17 during the course of normal chicken embryo development (Tuan, 1983; Torres and Korver, 2018). In addition, the supply of pure oxygen into the cSLCS starting from D17 is mandatory for the embryos to hatch on D21 of incubation (Tahara and Obara, 2014), which is most likely due to the increase in internal respiration associated with the growth of embryos (Zhang and Burggren, 2012). Therefore, in the present study, the effects of calcium lactate

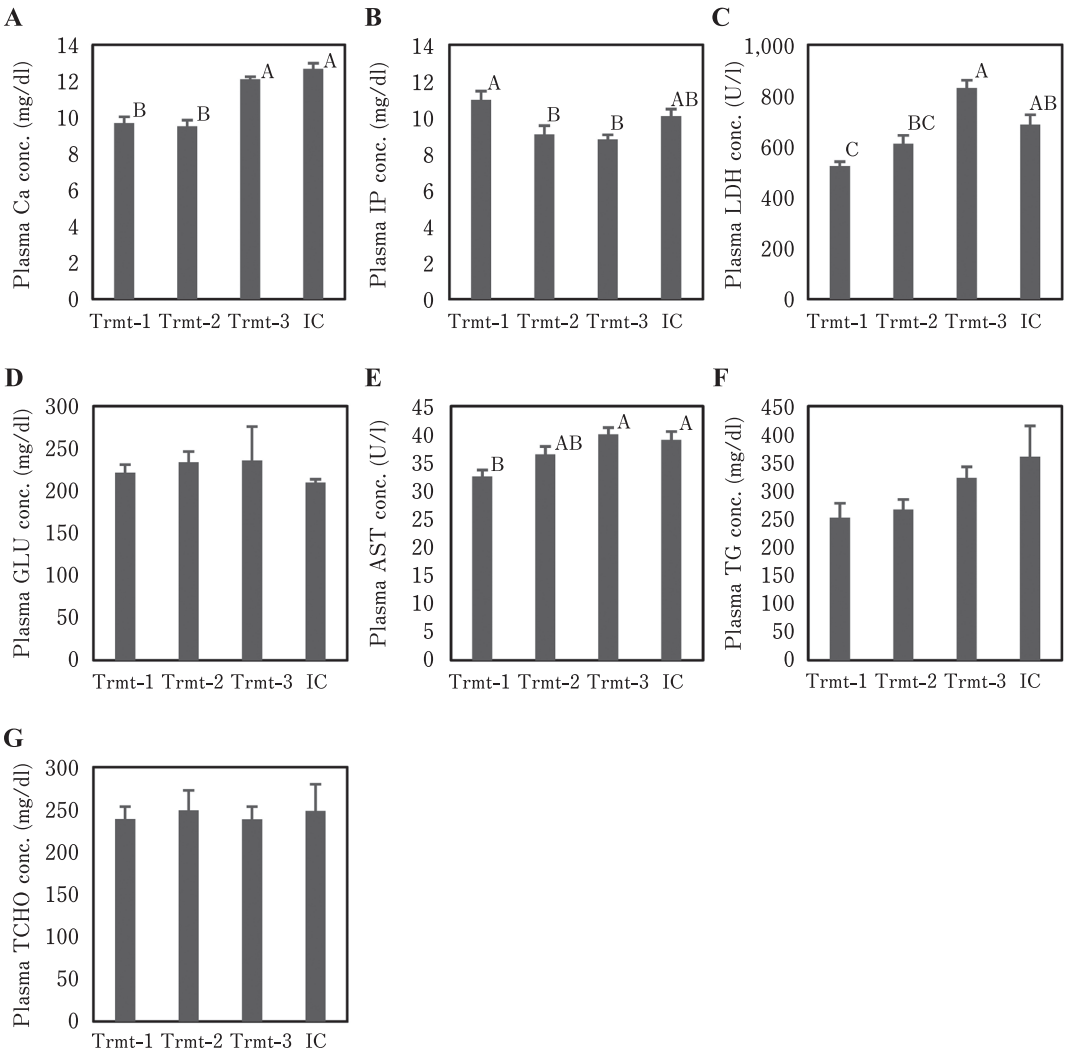

Fig. 3. **Concentrations of representative plasma components at D17 of incubation.** Abbreviations. Ca, calcium; IP, inorganic phosphate; LDH, lactic acid dehydrogenase; GLU, glucose; AST, aspartate aminotransferase; TG, triglyceride; TCHO, total cholesterol.

on the physiological and morphological parameters that influence the development of chicken embryos were examined up to D17 in the cSLCS, to avoid the confounding effects of calcium lactate and oxygen supplementation.

It has been reported that an external supply of both distilled water and calcium lactate is required for the development of normal chick embryos in cSLCSs (Tahara and Obara, 2014). In our preliminary experiment, the cessation of embryo development was observed within 24 h after supplementation with calcium lactate without distilled water in the cSLCS (data not shown). Therefore, it was concluded that the effects of calcium lactate and distilled water need to be evaluated independently.

The results showed a significant decrease in embryo viability between D3 and D9 of incubation in the cSLCS for both Trmt-2 and Trmt-3 ($P<0.05$), whereas no significant difference was observed for Trmt-1 during the same period. This result suggests that calcium lactate and distilled water are both toxic during the early stages of chick embryo development. However, this result is inconsistent with that of Tahara and Obara (2014), who reported a slight but insignificant decrease in embryo viability between D3 and D9 of incubation in the cSLCS. The reason for these inconsistent results is unclear, but it could be due to differences in the overall experimental conditions (e.g., chicken breed, egg size, hen's health, and manufacturer of the calcium lactate) between studies. Among these, the differences in the origin of fertilized eggs could be a major factor behind the discrepancy in embryo viability between studies using cSLCS; for example, commercially sourced fertilized eggs were used in both Tahara and Obara (2014) and Tahara *et al*. (2021) studies, whereas fertilized eggs produced by MB line, a parent stock introduced

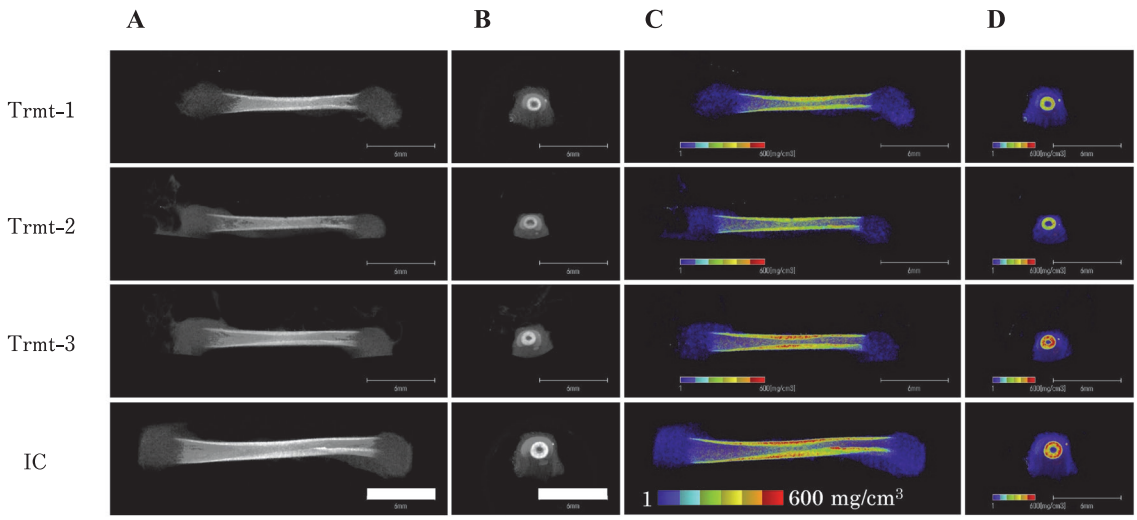

Fig. 4. **Micro-CT images of embryonic tibia at D17.**
A and B: Two-dimensional micro-CT images (scale bar＝6 mm).
C and D: Two-dimensional mineral density images.
A and C: Side sections of a whole tibia.
B and D: Cross sections at the midpoint of the tibia.
Gradient color scales in Fig. C and D indicate bone mineral density, where blue represents the lowest mineral density (1 mg/cm^3), followed by light blue, green, yellow, and orange representing mineral density values in ascending order, and red representing highest mineral density (600 mg/cm^3).

from the National Livestock Breeding Center, Okazaki, Japan, were used in the present study.

The viability of embryos in Trmt-3 started to decrease from D13, but no significant difference was observed between Trmt-2 and Trmt-3 ($P>0.05$). Although the reason for the decrease in viability was also unclear, this result indicates that the conditions in the cSLCS used in this study were not ideal for this critical stage of embryonic development. Nevertheless, it has been reported that supplementation of calcium lactate in cSLCS is essential for the hatching of chicks (Kamihira *et al*., 1998; Tahara and Obara, 2014).

To examine the physiological effects of calcium lactate supplementation on the development of chicken embryos, the major blood plasma components were analyzed at D17. No significant difference in Ca concentrations was observed between Trmt-3 and IC ($P>0.05$), whereas the values were significantly lower in both Trmt-1 and Trmt-2 than in IC ($P<0.05$). This result indicates that calcium lactate was absorbed by the embryos in the cSLCS, and is consistent with the results of previous studies (Tuan, 1980; Tuan and Ono, 1986). Although the blood IP concentration was reported to be higher in Japanese quail in a qSLCS than in intact embryos (Tuan and Ono, 1986), no significant difference was observed in the blood IP concentration among IC, Trmt-2, and Trmt-3 ($P>0.05$) in the present study. Because Ca and IP are both essential minerals for the normal growth of an embryo, further research is necessary to determine the interaction between these two minerals.

No significant differences were observed in the blood LDH concentrations of Trmt-3 and IC, which indicates that calcium lactate supplementation had an influence on LDH activity in embryos. In contrast, it has been reported that embryonic LDH activity tends to be higher in SLCSs without the addition of calcium, than in IC (Tuan and Ono, 1986). The reasons for this controversial result need to be determined in the future. LDH is an escape enzyme that converts lactate to pyruvate for gluconeogenesis, or to acetyl-CoA for metabolism, and is found in almost every cell of the body. It can be hypothesized that calcium lactate supplementation could have influenced the enzymatic cascade to stimulate LDH activity in cSLCS.

The plasma AST concentration was significantly lower in the Trmt-1 group than in the IC group ($P<0.05$). In general, AST levels are increased in the presence of liver damage, and are primarily used as an indicator of liver function in humans. Although a significant difference was observed in the AST concentration between IC and Trmt-1, it was considered to have no clinical implications because the AST values were within the normal range. No statistically significant differences were observed in the GLU, TCHO, and TG concentrations between the treatments ($P>0.05$).

To evaluate the effects of calcium lactate supplementation on bone formation in cSLCS, morphometry of the tibia at D17 was performed using micro-CT analysis.

Qualitatively, a morphologically normal tibia was observed at D17 in Trmt-3. Furthermore, the micro-CT analysis indicated no significant difference in vBMD between Trmt-3 and IC ($P>0.05$), whereas the values were significantly lower in both Trmt-1 and Trmt-2 than in IC ($P<0.05$). Bone miner-

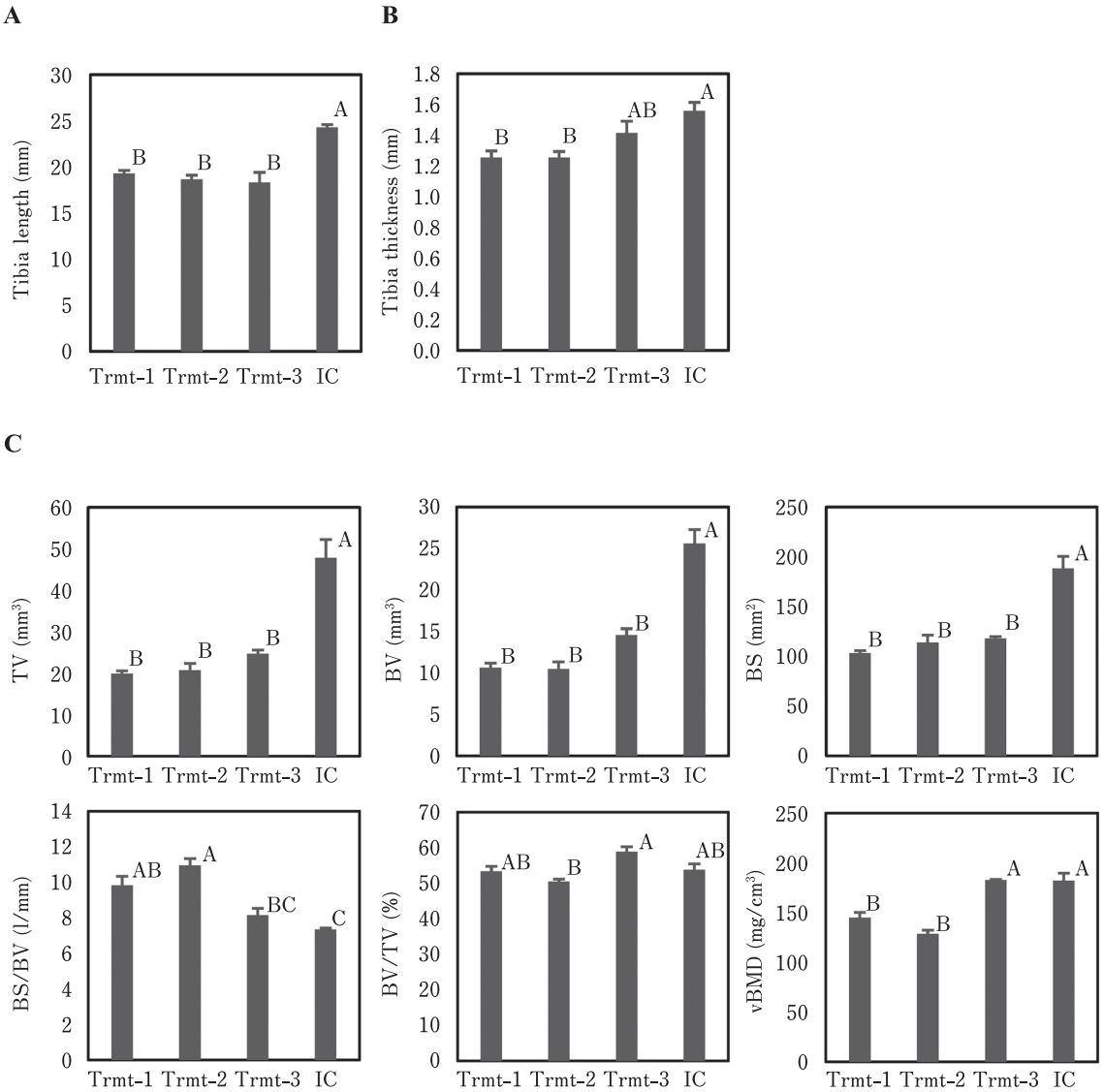

Fig. 5. **Quantification of physical properties of tibia at D17 using micro-CT analysis.**
A, Tibia length; B, Tibia thickness at the midpoint of long axis.
Abbreviations. TV, tissue volume; BV, bone volume; BS, bone surface area; BS/BV, bone surface area per bone volume; BV/TV, bone volume per tissue volume; vBMD, volumetric bone mineral density.

alization of chick embryos has been reported to be promoted considerably between D14 and D17 (Yair *et al*., 2012). In the absence of an external calcium supply in SLCS, on the other hand, skeletal abnormalities of embryo associated with a lower blood calcium concentration have been reported (Jourdeuil *et al*., 2015). Therefore, it was hypothesized that calcium lactate supplementation to SLCS in the present study contributed to the development of a morphologically normal tibia at D17.

On the other hand, quantitative parameters such as tibia length, TV, BV, and BS (Fig. S2) were all significantly reduced in chick embryos in cSLCS, compared with IC ($P<0.05$). A smaller embryo size has also been reported in quail embryos in qSLCS (Kamihira *et al*., 1998). A delay in embryo development is considered to be one of the reasons for the smaller embryo size in SLCS (Ono and Tuan, 1986). Experimental treatment with phenobarbital, an antiepileptic drug, has been reported to shorten the fetal long bones by exerting inhibitory effects on chondrocyte proliferation and bone mineralization (Yan *et al*., 2016). Therefore, the development of a tibia with normal morphology and density associated with subnormal quantitative parameters observed in the present study, could be attributed to the effects of unidentified inhibitory factor(s) on chondrocyte proliferation in cSLCS.

Recently, normal chicks were hatched by supplementing calcium carbonate as a calcium source in cSLCS (Tahara *et*

al., 2021). Since the circulatory system is the universal means of transporting minerals in vertebrates, the effects of two different sources of calcium on the blood calcium concentration, and influences on mineral metabolism and bone formation in a cSLCS, need to be critically evaluated in future studies.

In summary, it was shown that calcium lactate supplementation in cSLCS supports bone formation in developing chicken embryos, but has adverse effects on the viability of embryos, particularly during the first week of embryonic development.

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Conflicts of Interest

The authors declare no conflict of interest.

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