



Bone mineral density is increased in the cadmium-induced omphalocele chick model by using three-dimensional micro-computed tomography

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Abstract

Purpose The cadmium (Cd) chick model has been described as a reliable model of omphalocele. Skeletal anomalies, including lumber lordosis, can be seen in the Cd chick model, as well as in the human omphalocele. Bone deformations, such as lordosis, are associated with high bone mineral density (BMD). Recently, three-dimensional microcomputed tomography (3DMCT) has been used to investigate skeletal development in small animal embryos. We used 3DMCT to test the hypothesis that the BMD is increased in the Cd-induced omphalocele chick model.

Methods After a 60-h incubation, chicks were exposed to either chick saline or Cd in ovo. Chick embryos were harvested at embryonic day 16.5 (E16.5) and were divided into control ($n=8$) and Cd ($n=9$). Chicks were then scanned by 3DMCT. The body volume, bone volume, bone/body volume ratio, bone mineral quantity and BMD were analysed statistically (significance was accepted at $p < 0.05$).

Results Bone mineral density (mg/cm^3) was significantly increased in the Cd group compared to control group (235.3 ± 11.7 vs 223.4 ± 4.6 , $p < 0.05$), whereas there was no significant difference in the bone/body volume ratio between the Cd group and the control group (0.7 ± 0.1 vs 0.6 ± 0.0). The body volume (cm^3) (0.3 ± 0.2 vs 0.3 ± 0.1), bone volume (cm^3) (0.2 ± 0.2 vs 0.2 ± 0.1), and bone mineral quantity (mg) (51.3 ± 41.6 vs 41.5 ± 16.5) were not significantly different between the two groups.

Conclusions Increased BMD may be associated with lordosis of the vertebral column in the Cd-induced omphalocele chick model, stimulating osteogenesis by activating the canonical Wnt signalling pathway.

Keywords Omphalocele · Cadmium · Chick embryo · Lordosis · Bone mineral density

Introduction

Omphalocele, one of the most common ventral body wall defects (VBWDs) in humans, is a midline defect in the anterior abdominal wall with herniation of the abdominal contents including liver and bowel, through the enlarged umbilical ring into the base of the umbilical cord and is confined

by an amniotic membrane. Omphalocele is accompanied by failure of return of the midgut hernia into the abdominal cavity, and it is, therefore, a primary failure of fusion of the anterior abdominal wall. This defect occurs in approximately 1 in 3000 live human births [1, 2].

Although experimental studies of VBWDs have relied on gene knockouts, surgical models and teratogen models, there are still few reliable animal models of omphalocele [3, 4]. Cadmium (Cd) has no nutritive function and is an established teratogen in a wide range of animal models, including the chick embryo [5, 6]. Previous work from our research team has established the Cd chick model, which is ideal for the investigation of omphalocele [7, 8]. These chick embryos were administered Cd at Hamburger–Hamilton (H–H) stages 16–17 [9] and developed an abnormal growth of the lateral plate mesoderm (LPM), resulting in the development of VBWD similar to the human omphalocele spectrum [6]. In this teratogen model, Cd appears to

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be a specific teratogen, with a predictable and reproducible rate of omphalocele phenotype occurring in approximately 40–50% of chicks [6, 10]. Interestingly, the Cd-induced omphalocele chick model has a lordosis of the vertebral column, a feature which is also seen in the human omphalocele [10, 11].

Lordosis of lumbar vertebrae plays a major role in changing the capacity of the abdominal cavity in the human omphalocele. The degree of lordosis in omphalocele patients is significantly greater than the degree of the controls. It has been described that the individual lordotic angle was significantly correlated with the size of omphalocele [11]. Although, the exact mechanisms of lordosis seen in the omphalocele still remains unknown, bone deformation, such as lordosis, has been reported to be associated with increased bone mineral density (BMD) [12].

Accurate visualization of three-dimensional structures requires comparative, functional and developmental studies of animal morphology. Nevertheless, few widely applicable methods exist for non-destructive whole-volume imaging of animal tissues [13]. Three-dimensional microcomputed tomography (3DMCT) has recently been innovated to investigate the morphogenesis in small animal embryos, including skeletal development [14, 15]. 3DMCT can produce high-resolution images of small embryonic organs and tissues, without destroying the specimens and with the ability to combine this technique with other techniques and imaging methods [13]. Although several studies using 3DMCT in chick embryos have been reported [16, 17], 3DMCT has never been used to characterize the phenotype of the Cd-induced omphalocele chick model.

The aim of our study was to visualize and analyse skeletal abnormalities in the Cd chick model by using 3DMCT and to investigate the hypothesis that the BMD is increased in the Cd-induced omphalocele chick model.

Materials and methods

Embryo growth and treatment

White-leghorn chicks were hatched from fertilized eggs obtained from a local hatchery (Shiraishi Farm, Saitama, Japan) and incubated at 38 °C in a forced air incubator at 70% relative humidity for 60 h. After 60 h of incubation, chick embryos that had reached Hamburger–Hamilton (H–H) stages 16–17 [9] of embryological development were treated in ovo with either 50 µL of 5 µM Cd or 50 µL of chick saline using a method described by Thompson et al. [10]. On embryonic day 16.5 (E16.5), chicks were harvested and divided into the Cd group ($n=9$) confirming the omphalocele phenotype and the control group ($n=8$).

3DMCT imaging and skeletal analysis

Chick embryos at E16.5 were placed in 4% paraformaldehyde for several days. After this preparation, we scanned the embryos using a 3DMCT system (Hitachi Healthcare, Tokyo, Japan). CT images were acquired using the following parameters: 50 kVp tube voltage, 500 µA tube current, 48 mm axial field of view and 96 µm isotropic voxel size. The image files were converted from a proprietary format into DICOM format using LaTheta software (version 3.22). Volume-rendered 3-dimensional CT images were reconstructed using VGStudio MAX (Volume Graphics, Aichi, Japan). We assessed the skeletal morphology of chick embryos in various sections and reconstructed in 3D image. Finally, we statistically analysed the body volume, bone volume, bone mineral quantity and bone density, respectively. The overall BMD was considered as the bone mineral content per cubic volume (mg/cm^3) based on the following equation. The cubic volume was calculated as the integrated value of “the planar dimensions of the area of the cross-sectional tomographic image” and “the inter-slice intervals between the cross-sectional tomographic images”. All numerical data in the results of the skeletal analysis are presented as the mean \pm standard deviation. Differences between the two groups were tested by using Mann–Whitney’s U test. Statistical significance was accepted at p value < 0.05 .

Results

In the surface mode in 3DMCT, abdominal contents including the liver and bowel were seen in the herniation through the VBWD in the Cd chick model (Fig. 1b), whereas no herniation was observed in control chicks (Fig. 1a). In the skeletal mode of 3DMCT, lordosis of the vertebral column and malformed lower limbs were observed in the Cd chick model (Fig. 2b), whereas a c-shaped lumbar curvature and a normal position of the lower limbs were seen in control chicks (Fig. 2a). BMD (mg/cm^3) was significantly increased in the Cd group compared to the control group (235.3 ± 11.7 vs 223.4 ± 4.6 , $p < 0.05$). However, there was no significant difference in the bone/body volume ratio between the Cd group and controls (0.7 ± 0.1 vs 0.6 ± 0.0). The body volume (cm^3) (0.3 ± 0.2 vs 0.3 ± 0.1), bone volume (cm^3) (0.2 ± 0.2 vs 0.2 ± 0.1), and bone mineral quantity (mg) (51.3 ± 41.6 vs 41.5 ± 16.5) were not significantly different between the two groups, respectively (Table 1).

Discussion

Understanding the morphological development of embryos in a 3D frame work is fundamental to developing new surgical strategies for congenital malformations. 3DMCT has

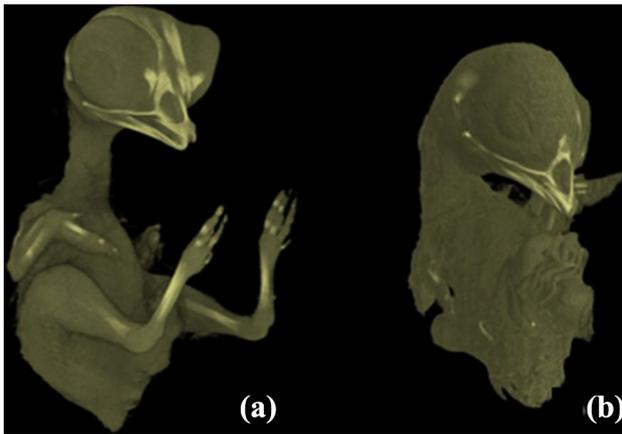


Fig. 1 **a** In controls, the surface mode in 3DMCT showed no herniation of the abdominal viscera or smooth surface of the ventral body wall. **b** In the surface mode 3DMCT of the Cd chick model, the abdominal contents, including the liver and bowel, was evident in the herniation of the defect

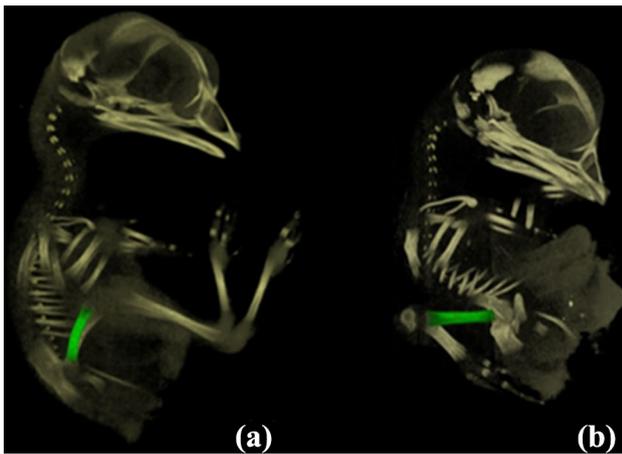


Fig. 2 **a** In the skeletal mode 3DMCT in controls, a c-shaped lumbar curvature and a normal position of the lower limbs (green) were observed. **b** In the Cd chick model, the skeletal mode of 3DMCT showed lordosis of the vertebral column and malformed lower limbs (green)

the potential to produce quantitative 3D images of small animals, and it is ideal for mineralized tissue, including bones. With the 3DMCT imaging system, we were able to

analyse high-resolution images of small organs and tissues in embryos, producing sections from any orientation that we required such as sagittal, coronal and transverse sections. Although most of the studies using the Cd chick model have been focusing on molecular mechanisms [7, 8, 18], Thompson et al. [10] have previously demonstrated skeletal morphogenesis by using skeletal alizarin staining in the Cd affected chick model. In this study, we clearly visualized for the first time the skeletal anomaly in the Cd chick model by using 3DMCT. The 3DMCT imaging technique is cost-effective in comparison to serial histological analysis, with little sample preparation and with relatively simple data post-processing [14].

Although the exact molecular mechanisms of lordosis of lumbar vertebrae seen in the omphalocele have not been thoroughly elucidated to date, the canonical Wnt signaling pathway is known to play a key role in the osteoblast response and increased osteogenic activity, arising from the upregulation of Wnt signalling, leads to increased BMD [12]. It has been previously reported that one of the canonical Wnts, Wnt6, is upregulated in the Cd-induced omphalocele chick model [19]. In this study, BMD was significantly increased in the Cd chick model compared to controls. We, therefore, speculate that increased BMD is associated with lordosis of the vertebral column in the Cd chick model, stimulating osteogenesis by activating the canonical Wnt signalling pathway. Conversely, reduced BMD has been known to be associated with osteopenia [12]. Randall et al. have previously reported that among 58 children with gastroschisis, there were 4 with osteopenia, whereas none of 8 children with omphalocele had osteopenia [20]. In this study, we observed significantly increased BMD in the Cd-induced omphalocele chick model. Therefore, our results in this study also support the concept that osteopenia is not characteristic in the omphalocele patients with increased BMD, different from gastroschisis patients.

Dorsal malpositioning of hind limbs seen in this 3DMCT study has known to be caused by the lateral plate mesoderm grown dorsally [6]. In the Cd chick model, it has been reported that abnormal cell death in the lateral plate mesoderm with cellular changes and reduction in proliferation in the periderm occur in association with failure of the lateral plate mesoderm to fold ventrally, causing omphalocele phenotype [21]. Abnormalities in cell

Table 1 Numerical data of the skeletal analysis by 3DMCT

	Body volume (cm ³)	Bone volume (cm ³)	Bone/body volume ratio	Bone mineral quantity (mg)	Bone mineral density (mg/cm ³)
Control	0.3 ± 0.1	0.2 ± 0.1	0.6 ± 0.0	41.5 ± 16.5	223.4 ± 4.6
Cd	0.3 ± 0.2	0.2 ± 0.2	0.7 ± 0.1	51.3 ± 41.6	235.3 ± 11.7*

**p* < 0.05 vs control

adhesion have been observed concurrent with this process, with initial disassociation of peridermal cells and subsequent inappropriate adhesion of periectodermal tissue that comes into apposition. Because cell adhesion molecule function is intimately related to signalling [22], abnormal signalling mechanisms could come into play at this stage, causing lordosis of the vertebral column. Thus, we believe that the effects of cadmium on the chick model, causing omphalocele, lordosis and hindlimb malpositioning, are specific and all act via a single mechanism.

Taken together, the results of this study indicate that 3DMCT has the potential to replace serial histology as the reference standard in many in vitro studies, and it provides a practical approach to obtain quantitative information during some longitudinal investigations in vivo. Therefore, we believe that 3DMCT will become a standard tool in many laboratories in the future, owing to the clear 3D images and the quantitative data that it provides.

In future research, by using several simple contrast stains, it is hoped that 3DMCT will provide versatile, high-contrast, quantitative 3D images of tissues and the vasculature of live small animals. Especially in small embryos, the methods can be used on a wide variety of animal species fixed and preserved by the most common methods. Stained tissues can be scanned in liquid, and further analysis can be performed on the same samples. Versatile X-ray contrast staining represents an additional option for volumetric imaging of non-mineralized tissues, and it is hoped that this method will prove useful in comparative, developmental, and evolutionary research in addition to skeletal abnormalities, as investigated in this study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal procedures were reviewed and approved by the Juntendo University School of Medicine Animal Care and Use Committee (Institutional Review Board no. 1295).

References

- Axt R, Quijano F, Boos R et al (1999) Omphalocele and gastroschisis; prenatal diagnosis and perinatal management. A case analysis of the years 1989–2007 at the Department of Obstetrics and Gynecology, University of Homburg/Saar. *Eur J Obstet Gynecol Reprod Biol* 87:47–54
- Barisic I, Clementi M, Hausler R et al (2001) Evaluation of prenatal ultrasound diagnosis of fetal abdominal wall defects by 19 European registries. *Ultrasound Obstet Gynecol* 18:309–316
- Van Dorp DR, Malleis JM, Sullivan BP, Klein MD (2010) Teratogens inducing congenital abdominal wall defects in animal models. *Pediatr Surg Int* 26(2):127–139
- Sitarek K (2001) Embryolethal and teratogenic effects of carbendazim in rats. *Teratog Carcinog Mutagen* 26:673–680
- Menoud P, Schowing J (1987) A preliminary study of the mechanisms of cadmium teratogenicity in chick embryo after direct action. *J Toxicol Clin Exp* 7:77–84
- Thompson J, Bannigan J (2001) Effects of cadmium on formation of the ventral body wall in chick embryos and their prevention by zinc pretreatment. *Teratology* 64(2):87–97
- Fujiwara N, Duess J, Puri P, Thompson J (2013) Disturbance of SHH signalling pathway during early embryogenesis in the cadmium-induced omphalocele chick model. *Pediatr Surg Int* 29(2):165–170
- Doi T, Puri P, Bannigan J, Thompson J (2008) Downregulation of ROCK-I and ROCK-II gene expression in the cadmium-induced ventral body wall defect chick model. *Pediatr Surg Int* 24(12):1297–1301
- Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. *J Morphol* 88(19):49–92
- Thompson J, Bannigan J (2007) Omphalocele induction in the chick embryo by administration of cadmium. *J Pediatr Surg* 42(10):1703–1709
- Nagaya M, Kato J, Niimi N, Tanaka S (2000) Lordosis of lumbar vertebrae in omphalocele: an important factor in regulating abdominal cavity capacity. *J Pediatr Surg* 35(12):1782–1785
- Hardcastle SA, Dieppe P, Gregson CL, Arden NK (2014) Osteophytes, enthesophytes, and high bone mass: a bone-forming triad with potential relevance in osteoarthritis. *Arthritis Rheumatol* 66(9):2429–2439
- Metscher BD (2009) MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC Physiol* 9:11
- Holdsworth DW, Thornton MM (2002) Micro-CT in small animal and specimen imaging. *Trends Biotechnol* 20(8):S34–S39
- Kanczler JM, Janos M, Smith EL, Roberts CA et al (2012) A novel approach for studying the temporal modulation of embryonic skeletal development using organotypic bone cultures and microcomputed tomography. *Tissue Eng Part C Methods* 18(10):747–760
- Kim JS, Min J, Recknagel AK, Riccio M et al (2011) Quantitative three-dimensional analysis of embryonic chick morphogenesis via microcomputed tomography. *Anat Rec (Hoboken)* 294(1):1–10
- Heidrich A, Schmidt J, Zimmermann J, Saluz HP (2013) Automated segmentation and object classification of CT images: application to in vivo molecular imaging of avian embryos. *Int J Biomed Imaging* 2013:508474
- Doi T, Puri P, Bannigan J, Thompson J (2010) Disruption of non-canonical Wnt/CA²⁺ pathway in the cadmium-induced omphalocele in the chick model. *J Pediatr Surg Int* 45(8):1645–1649
- Doi T, Puri P, Bannigan J, Thompson J (2009) Alteration of canonical Wnt pathway in the cadmium-induced omphalocele in the chick model. *Reprod Toxicol* 28(2009):121–124
- Randall TL, Jean PG, Ann A (1993) Musculoskeletal involvement in children with gastroschisis and omphalocele. *J Pediatr Surg* 28(4):584–590
- Thompson J, Hipwell E, Loo HV, Bannigan J (2005) Effects of cadmium on cell death and cell proliferation in chick embryos. *Reprod Toxicol* 20(4):539–548
- Gumbiner BM (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84(3):345–357

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