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Cadmium affects the development of somites in chick embryos (*Gallus gallus domesticus*) under in vitro conditions

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Abstract: Cadmium is one of the most toxic metals that has high environmental stability and can hardly be converted into substances of low toxicity. In the present study, the effects of cadmium on the development of somites of chick embryos under in vitro conditions were investigated. First, fertilized eggs were incubated until they reached the stage of 15–20 somites. The embryos were then separated from the yolk by using the window technique. The somites were separated by insulin needles under a stereomicroscope. The separated somites were then transferred to Dulbecco's modified Eagle's medium (DMEM) stock culture and treated with different concentrations of cadmium nitrate (0, 500, 1000, 2000, and 4000 ng/mL). After 72 h, the diameter of the nucleus, cell morphology, and extracellular matrix compounds were examined. Our results indicated that cadmium disrupted the developmental process of the somites. The microscopic studies of the somites, based on a histological technique, indicated that cadmium induced apoptosis in somite cells and also decreased the chondrogenesis and collagen synthesis. Therefore, somites are considered suitable temporary embryonic organs and interesting models for basic developmental study under in vitro conditions. Moreover, it might be possible to use somites in vitro for studying the effects of trace elements on living organisms.

Key words: Experimental model, chick embryo, somite, cadmium, toxicity

1. Introduction

The chick embryo is one of the most widely used animal models, especially in biomedical and embryological studies (1). It is readily available and can be manipulated in vitro by surgery. As a result of recent technical developments (2), researchers found similarity in the development patterns, accessibility for visualization, and experimental manipulation of chick embryos and mammalian embryos. Therefore, chick embryos have widely been used in different studies as a suitable alternative for mammalian embryos (2).

The somitic lineages are developed from the head mesoderm, the so-called paraxial mesoderm, or somitic mesoderm. Somites appear as paired epithelial blocks, which start anteriorly, immediately caudal to the otic vesicle, and run posteriorly on both sides of the neural tube and notochord to the caudal tip of the embryo (3). Tissues that surround the somites play the main role in the formation, proliferation, differentiation, and survival of somitic cells (2). Following a series of cell interactions with surrounding tissues, the somitic cells are altered from the epithelial form. Their abdominal-interior parts generate sclerotomal cells, which ultimately transform into cartilage and bone. The dorsal-lateral parts also produce dermomyotome, which forms muscle and skin (4).

In the chick embryo, the first pair of somites appear after 20 h of incubation, followed by the nerve plate formation. The rhythm of somite production is characteristic of the species. During somitogenesis, 52 pairs of somites were formed in the chick embryo from day 1 to day 5, with a pair of somites forming every 90 min at 37 °C (2).

Cadmium is a soft, silvery-white metal that is not usually present in nature. It is purified from zinc ore. Cadmium is one of the most toxic metals that has high environmental stability and can hardly be converted into substances of low toxicity. The process of removal of cadmium from the biosphere is very slow and it lasts for almost a decade or more (5). Cadmium is used as an anticorrosion agent, a stabilizer in PVC products, a neutron absorber in nuclear power machines, and also in the production of nickel-cadmium batteries. In addition, phosphate fertilizers also contain large amounts of cadmium, and it also accumulates in vertebrate organs like kidneys and liver. Marine invertebrates and algae build up high concentrations of cadmium and, as a result, it enters the food chain (6).

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In the majority of animal species, cadmium does not have a definite physiological function but exposure to this metal threatens biological systems in different ways. Kidneys are mainly affected by cadmium toxicity in humans. Skeletal injuries, bone mineralization, and carcinogenesis potential are examples of the destructive effects of cadmium (7). Other destructive effects such as mutagenic effects, fetal toxicity, apoptotic, and teratogenic potential have been proven in many species, including chicks (8). Somites, as masses of mesenchymal cells, are used in the present study to examine the effects of cadmium on their in vitro development.

2. Materials and methods

2.1. Experimental design

Fertilized unincubated chick eggs were obtained from a local hatchery. The eggs were incubated at 38 °C with 70% humidity. This was done in order to bring them to the Hamburger–Hamilton 15–20 somite stage before commencing treatment. The culture medium, including 15% fetal calf serum (Biowest, USA), was prepared by adding 15 mL of fetal calf serum to 85 mL of Dulbecco's modified Eagle's medium (DMEM) stock culture (Biowest, USA) and 100 µL of antibiotics (penicillin and streptomycin) to prevent any risk of contamination.

To study the effects of cadmium nitrate (Merck) on somite development, different concentrations (0, 500, 1000, 2000, and 4000 ng/mL) were used (9). Chicken eggs were put at room temperature for 15 min to reduce yolk disintegration. Egg shells were then removed by the window technique and the embryos were separated from the yolk (1).

The embryos were examined by the manipulation technique (microscopic surgery) in order to isolate the somites (10). They were then separated from chick embryos under a stereomicroscope and the extra embryonic membranes were removed by insulin needles. The somite rows were transferred to a culture plate after multistage washing with physiological saline (11). Somites were then treated with different concentrations of cadmium nitrate for 72 h. The culture medium was changed every 24 or 48 h.

2.2. Tissue preparation

After 72 h of culture, the somites underwent histological examination. Bouin solution was used to stabilize the specimens. Samples were washed several times with the physiological serum after removal from the culture dish and left in a Bouin solution for 7 to 8 h. Ascending levels of ethanol (50%, 70%, 80%, and 96%) for 20 min and butanol (rinsing twice) for 40 min were used for tissue dehydration (12).

After dehydration, the specimens were left in melted paraffin in an incubator (58 °C) for a week. They were then put in metal molds containing liquid paraffin and encoded. The paraffin blocks were separated from the metal molds and kept in the freezer (−4 °C) until they were sectioned. Furthermore, the molded specimens were sectioned with 5 µm thickness.

2.3. Histological staining

Hematoxylin and eosin (13), picrofuchsin (14), picro-indigo carmine (15), toluidine blue (16), and alcian blue (17) stains were used to examine the histology of the somite sections. Hematoxylin and eosin staining was used to investigate the effects of the cadmium on somitic cell morphology (13), picrofuchsin and picro-indigo carmine staining were used to study the presence of collagen fibers (14,15), and toluidine blue and alcian blue staining were used to examine the proteoglycans and glycosaminoglycan of cartilage in the somite tissue (16,17).

2.4. Statistical analysis

The diameters of the nuclei were measured by ImageJ to find abnormality in the cells and also the lethal dose of cadmium nitrate for somitic cells (18). The results were presented as mean ± SD. Treatments were tested by one-way ANOVA followed by Tukey's test for pairwise comparisons of means. Significance was set at $P < 0.05$.

3. Results

3.1. Effects of cadmium on somitic cell morphology

Hematoxylin and eosin staining was used to study the effects of cadmium on somitic cell morphology. In the control group (Figure 1A) and at a concentration of 500 ng/mL the majority of cells had intact cell membranes and tense dispersal with spherical morphology. Somitic cells were located close to each other with an intact nucleus and low cytoplasm (Figure 1B). At a concentration of 1000 ng/mL, there was no change in cell density. At this concentration, spherical morphology of cells changed and some nuclear destructions were observed (Figure 1C). In 2000 ng/mL of cadmium nitrate, the changes in cell morphology increased and the nucleus had shrunk in somitic cells (Figure 1D). At a concentration of 4000 ng/mL, changes in cell morphology and nuclear destructions in the majority of the cell population were observed. Since nuclear destruction is a sign of apoptosis (18), our results indicated that the high concentration of cadmium nitrate induced apoptosis in somite cells (Figure 1E).

3.2. Effects of cadmium on the density of collagen fibers

Picrofuchsin staining was used to evaluate the presence and density of collagen fibers after cadmium treatment. In the control group (Figure 2A) and at a concentration of 500 ng/mL (Figure 2B), the bright red color indicated the presence of collagen fibers within the samples. Moreover, the majority of the cells remained intact. At a concentration of 1000 ng/mL, formation of the collagen fiber decreased

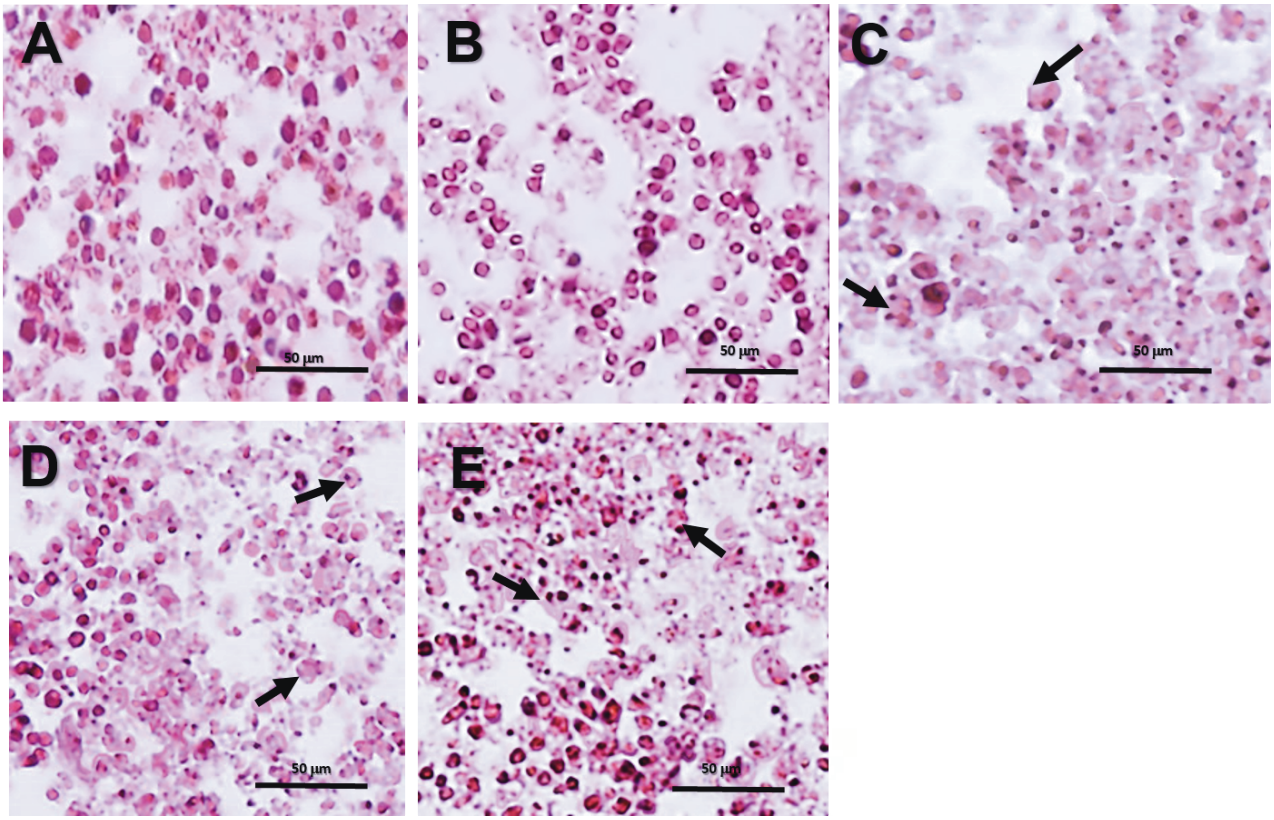

Figure 1. Somitic cells after 3 days of treatment with different concentrations of cadmium nitrate were stained with hematoxylin and eosin. Control (A), 500 ng/mL (B), 1000 ng/mL (C), 2000 ng/mL (D), and 4000 ng/mL (E). The arrows show apoptotic cells. All 1000× magnification.

(Figure 2C). At higher concentrations of cadmium nitrate, the formation of the collagen fiber was inhibited or delayed and most of the cell populations seemed to be apoptotic (Figures 2D and 2E).

Picro-indigo carmine staining was used to distinguish the nuclei from collagen fibers. In the control group (Figure 2F) and at a concentration of 500 ng/mL (Figure 2G), most of the nuclei were red and remained intact. Green color indicated the collagen fibers in the samples. At a concentration of 1000 ng/mL (Figure 2H) and higher of cadmium nitrate (Figures 2I and 2J) the collagen fibers greatly decreased.

3.3. Effects of cadmium on the cartilage glycosaminoglycans and proteoglycans

Alcian blue staining was used to identify the cartilage glycosaminoglycans in the somitic tissue. A blue complex indicated the presence of glycosaminoglycans in the control group (Figure 3A) and at a concentration of 500 ng/mL of cadmium nitrate (Figure 3B). At concentrations of 1000 ng/mL and higher of cadmium nitrate, glycosaminoglycan formation considerably decreased (Figures 3C–3E).

Toluidine blue staining was used to identify the cartilage proteoglycans in the somite tissue by a blue-purple complex. In the control group (Figure 3F) and at a concentration of 500 ng/mL of cadmium nitrate (Figure 3G) the proteoglycan can be observed. This colored complex gradually faded in 1000 ng/mL of cadmium nitrate, and at higher concentrations of cadmium nitrate no signs of proteoglycan formation were observed (Figures 3H–3J).

Higher magnification of images of somitic tissue with toluidine blue staining indicated that in the control group the cells were round with oval nucleus and in addition some cells were dividing (Figure 4A). When the samples were treated with a high concentration of cadmium nitrate (4000 ng/mL), as in Figure 1, signs of apoptosis such as changes in cell morphology and nuclear destruction (Figure 4B) and necrosis (Figure 4C) were observed.

3.4. Effects of cadmium on the cell nuclei

The nuclei of the somitic cells in the treated group at a concentration of 500 ng/mL of cadmium nitrate did not show any significant difference compared to those of the control group. At 1000 ng/mL concentration, the diameters of the nuclei were significantly smaller than those of the control group ($P < 0.001$). At a concentration of 2000 ng/mL, diameters of the nuclei were noticeably smaller than

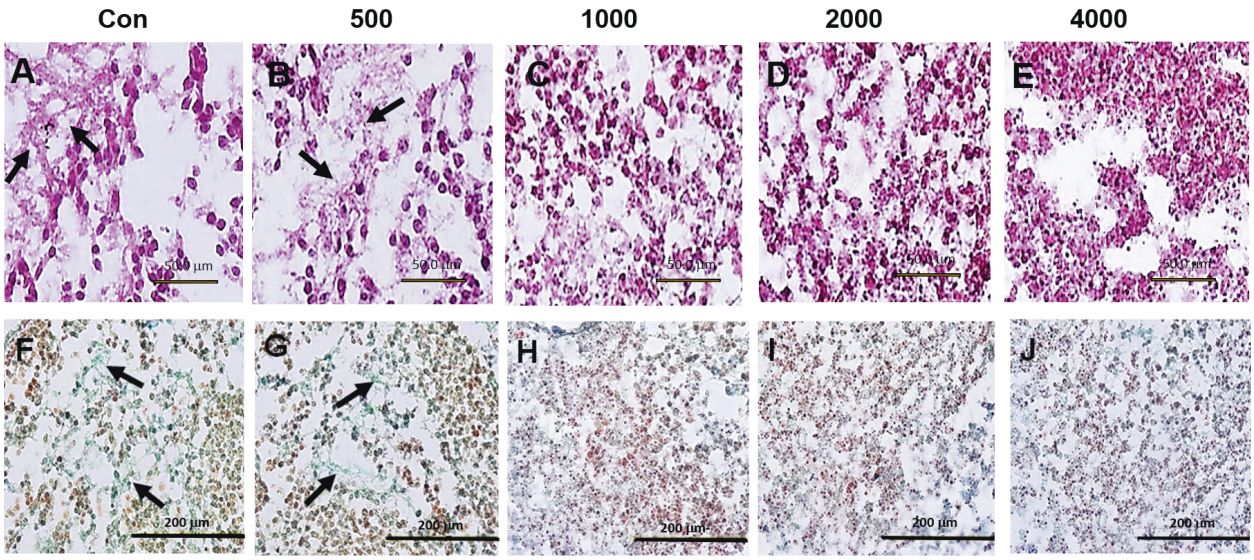

Figure 2. Cadmium nitrate inhibited collagen inductions in somitic tissues. Somitic tissues after 3 days of treatment with different concentrations of cadmium nitrate were stained with picrofuchsin and picro-indigo carmine. Control (A, F), concentration of 500 ng/mL (B, G), 1000 ng/mL (C, H), 2000 ng/mL (D, I), and 4000 ng/mL (E, J). The arrows show collagen fibers in the intercellular matrix. 1000× (A–E) and 400× (F–J) magnification.

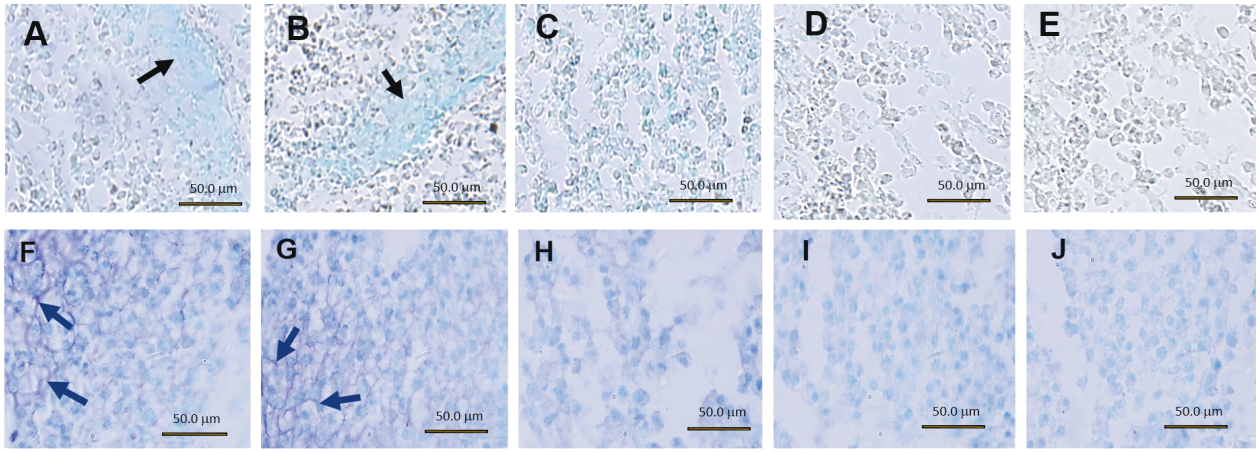

Figure 3. Cadmium nitrate inhibited cartilage glycosaminoglycans and proteoglycans. Somitic tissue after treatment with different concentrations of cadmium nitrate were stained with alcian blue for glycosaminoglycans (A–E) and with toluidine blue for proteoglycan (F–J) detection. Control (A, F), concentration of 500 ng/mL (B, G), 1000 ng/mL (C, H), 2000 ng/mL (D, I), and 4000 ng/mL (E, J). The arrows show the glycosaminoglycan and proteoglycans in the intercellular matrix. All 1000× magnification.

those of the control group ($P < 0.001$). The diameters of the nuclei at a concentration of 4000 ng/mL decreased significantly compared to those of the control group ($P < 0.001$). In the groups treated with 2000 ng/mL and 4000 ng/mL concentrations of cadmium nitrate, the size of nuclei was significantly smaller than that of the 1000 ng/mL concentration group ($P < 0.001$). Moreover, the diameters of the nuclei in the concentration of 4000 ng/mL of cadmium nitrate decreased remarkably compared with those of the group treated with a concentration of 2000 ng/mL ($P < 0.001$) (Figure 5).

4. Discussion

The chicken embryo is one of the widely used animal models, especially in the field of developmental biology and embryology (1). A large number of studies have been investigating the effects of pollutants on living organisms. This animal model was used broadly for investigating

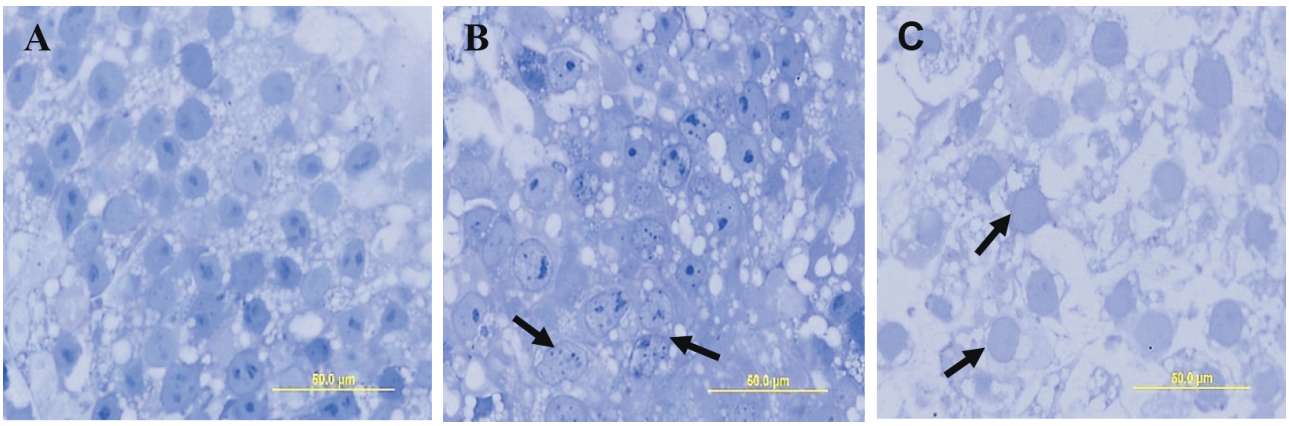

Figure 4. Effects of high concentration of cadmium on somitic cells. Somitic tissues were treated with 4000 ng/mL of cadmium nitrate, sectioned (1 µm), and stained with toluidine blue. Control (A) and sample (B, C). The arrows show the damaged cells. All 1000× magnification.

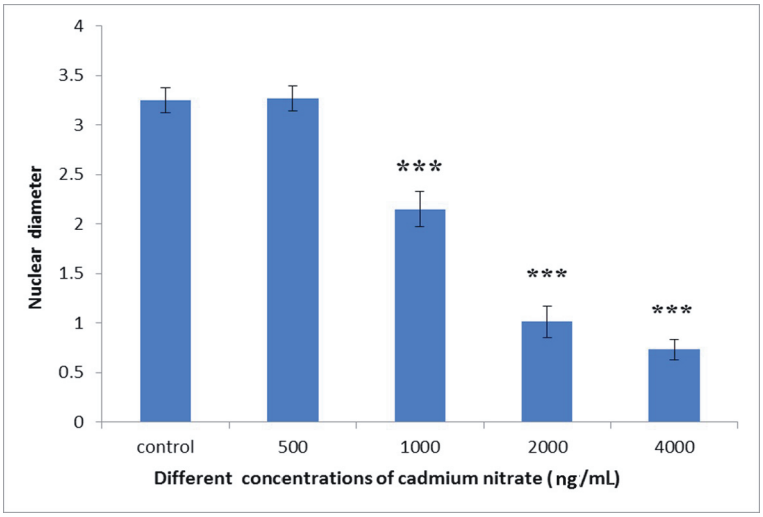

Figure 5. The diameter of nucleus in the somitic cell. Concentrations of 1000 ng/mL, 2000 ng/mL, and 4000 ng/mL were significantly different from the control group (***P < 0.001). There was no significant decrease in concentration of 500 ng/mL compared with the control group.

the effects of pollution on developmental biology. There are several reasons for choosing the chicken embryo. Along with being easily available, it has a vital system and warm-blooded amniotic developmental patterns. It can be manipulated through surgery, and the development of its organs is similar to that of mammals (2). Therefore, in the present study, we have used this animal model to investigate the effects of cadmium nitrate on chick embryonic somites in vitro.

In the present study, the effects of different concentrations of cadmium nitrate on somites' developmental process in vitro were investigated. Previously, it was shown that dietary exposure to cadmium (150 ng/kg of dietary cadmium chloride) caused histopathological changes in the testes, oxidative stress, endocrine disorders, and apoptosis in cocks (19). When pregnant mice were injected with 4 ng/kg cadmium chloride ($CdCl_2$) on days 7, 8, 9, and 10 of gestation, limb abnormalities and neural tube wall defects along with cell death in the neuroepithelium of the neural fold occurred in the offspring (20). Other studies showed that injection of $CdCl_2$ (50 M) in the egg air chamber of chick embryos caused morphological abnormalities, mainly in the cephalic region. This indicates the powerful teratogenic effects of cadmium on chick embryos (21). In addition, changes in the ventral body wall, neural tube, somites,

liver, and reproductive system development were reported (22). Somites are temporary organs that contribute to the development of the skeletal muscles, cartilage, tendons, endothelial cells, and dermis of the embryo (23). In the current study, the effects of a higher dosage of cadmium nitrate on somitic cell abnormality were observed in vitro, which could cause the further malformations in organs that were observed in previous studies. Therefore, in the present study, investigating the effects of cadmium on the somitic cells provides a new perspective on how cadmium affects the early stages of development.

We measured cell nucleus diameters to determine cadmium nitrate's effect on cell morphology. The relationship of the mean of the nuclear area and the ratios of nuclei to cytoplasm correlated with the severity of cancer in human colon tissues (24). Moreover, it has been demonstrated that mean nuclear size and cell size may be helpful for the assessment of tumor malignancy, tracking intracellular changes in tissues, and potentially monitoring tumor response to treatment in vivo (25). In the current study, in the somites treated with cadmium nitrate at a concentration of 1000 ng/mL or higher, a significant decrease in the diameter of the cell nucleus was observed. These results indicated the potential of cadmium to cause cancerous cell development.

In the present study, we showed that collagen fibers synthesis was stopped or delayed by increasing the cadmium nitrate concentrations. Previous studies indicated the possible effects of cadmium on collagenases. It has been shown that the collagen metabolism and collagen content was reduced by inhibitory effects of cadmium (26). Cadmium reduces the hydroxyproline content in the chick embryo. The results indicated that the inhibitory effect of cadmium on collagen synthesis was largely due to the inhibition of collagenous peptide synthesis (without inhibition of its hydroxylation) (27). These effects were also reported in rat osteosarcoma cells (ROS 17/2.8). It was also concluded that cadmium causes a dose-dependent decrease in collagen synthesis (28). Our results confirmed the inhibitory effect of cadmium on collagen synthesis. The density and spatial arrangement of the three-dimensional collagen structure define mechanical tissue properties and can act as a guide for cell migration (29). In the present study, the reduction of collagen in somites may play a role in disturbing cell migrations during development and also cause abnormality in somatic-derived tissues and organs.

The reduction of cartilage glycosaminoglycan and proteoglycans by the effect of cadmium nitrate has been shown here. It was reported that cadmium reduced the cartilage-forming potential of the tissue in the developing limbs of mice (30). In another study, it was demonstrated that exposure to cadmium and lead reduced the densitometric parameters and total thickness of articular cartilage in rats (31). These observations are consistent with our findings suggesting that cadmium may inhibit glycosaminoglycan and proteoglycans of cartilage. Increasing the concentration of cadmium nitrate will remove or delay these factors. These results may explain why the in vivo effects of cadmium on cartilage are widely emphasized.

In conclusion, we investigated the effects of different concentrations of cadmium nitrate on the development of somites in the chick embryo. After separating and culturing somites, the effects of different concentrations of cadmium nitrate were examined on day 3 of somitic culture. The results indicated that cadmium nitrate causes failure in the synthesis of collagen and chondrogenesis. Moreover, it seems to increase the risk of cell death. Our study revealed that somites, suitable in vitro models, can be used for determining the effects of heavy metal cytotoxicity on cell morphology, analyzing toxic metal elements, and evaluating the effects of toxicity on the environment. This can be due to the high sensitivity of embryonic tissues to teratogenic factors.

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