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Effect of Cadmium on Trophoblast Cell Proliferation and Apoptosis in Different Gestation Periods of Rat Placenta

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Abstract In this study, we aimed to show how cadmium (Cd) affects the trophoblast proliferation and differentiation in the placenta and the apoptotic activity in different gestational days and, hence, its effects of placental development with immunohistochemical and TUNEL techniques. Experimental model of our study consisted of placental development of control and Cd groups on 15, 17, 19, and 21th days of the gestation. Female rats in Cd groups were subcutaneously administered a single dose of 0.5 mg Cd/kg/day dissolved in sodium chloride as 2 mL/kg Cd chloride until the day they sacrificed. Embryo and placenta of female rats were separately removed on 15, 17, 19, and 21th days of the gestation in which the placental development takes place and placentas were processed for microscopic examinations. In the placentas of the control group, all layers were observed to be formed on the 15th gestational day and thereafter a continuous growth was monitored. In the Cd group also all layers existed from the 15th gestational day. However, they were smaller in size than control groups. Frequency of proliferating cell nuclear antigen (PCNA)-positive cells was decreased and the number of apoptotic cells was increased in all the gestational days related to Cd. In conclusion, Cd administered during the pregnancy was observed to cause abnormal placental development by disrupting the normal structure of the placenta, inhibiting the proliferation of trophoblast and increasing the number of apoptotic trophoblast cells.

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Introduction

Placenta plays a major role in the maintenance of fetal nutrition and pregnancy. Placenta is an important organ for risk evaluation in expectant mothers and the conceptus. Adequate placental growth and development are crucial to the development of the fetus, and dysfunctions of the placenta may be closely related to fetal developmental disabilities. Placental development disorders may cause results like miscarriage, preeclampsia, and fetal growth retardation in human being [1–3].

Placental development depends basically on co-occurrence of trophoblast proliferation, differentiation, and apoptosis. Order of tissue homeostasis, i.e., reconstruction and destruction depends on maintaining apoptosis/proliferation balance healthily [4]. Placenta may be used in human studies as a reliable, noninvasive indicator tissue for exposure to metals, particularly cadmium (Cd). Today, concentration of heavy metals like Cd and similar heavy metals in aquatic and air environments are widely present as a result of developments in various industrial fields, agricultural proliferation based on modern techniques. Especially toxic elements like Cd, potable waters, or foods contaminated by these heavy metals cause significant health problems in both humans and animals [5, 6].

Cadmium is a metal which is used widely in industry, has toxic effects on humans, and causes environmental pollution. Prevalence of usage areas and its high presence in cigarette make toxic effects of this metal a subject studied frequently. Important Cd sources affecting human life are cigarette smoke, refined nutrients, water pipes, coffee, tea, products released by burning coal, shellfishes, fertilizers used, dyes

and batteries containing Cd, and flue gases released in industrial production stages [7]. Occupational and environmental Cd exposure cause serious damages in the tissues like the placenta, kidney, liver, testis, heart, brain, and lungs [8–15]. Several investigations confirmed its transport across the placenta and accumulation in fetuses [16, 17].

The rat is still preferred as a model system in studies on embryology and reproduction toxicology. Both rat placenta and human placenta are hemochorial and share some unique features regarding uterine trophoblast invasion and spiral artery remodeling [18, 19].

We aimed to show how Cd affects apoptotic activity with trophoblast proliferation and differentiation in the placenta on different days of gestation and accordingly its effects on placental development with immunohistochemical and TUNEL techniques.

Materials and Methods

Animals and Experimental Groups

The animals were purchased from Trakya University Animal Care and Research Unit. The study was approved by the Institutional Animal Ethical Committee of Trakya University, Edirne, Turkey (Protocol Number: TUHDYEK-2012/21). Sixty-four female and sixteen male adult Wistar albino rats weighing 220–250 g were used in the study and none of them had mated previously. Two females and one male were kept overnight in one cage. A sperm-positive vaginal smear observed on the following morning was considered to indicate successful copulation. The sperm-positive day was designated as day 1 of pregnancy [20].

Experimental model was carried on days 15, 17, 19, and 21 when placental development takes place in control and Cd-administrated groups (8 animals per group). 0.5 mg Cd/kg was dissolved in sodium chloride and then 2 mL/kg Cd chloride was administrated subcutaneously as a single dose per day to the rats in Cd group from the beginning of gestation until sacrificing them. After pregnant rats were sacrificed under intraperitoneally ketamine (90 mg/kg) and xylazine (10 mg/kg) anesthesia on gestation days 15, 17, 19, and 21, their embryos and placentas were taken separately. After this operation, embryo and placenta weights and embryo length were measured separately. Moreover, number of live litters is noted. The placenta tissues were individually immersed in Bouin's fixative, dehydrated in alcohol, and embedded in paraffin. Sections of 5 μm were obtained, deparaffinized, and stained with PCNA immunohistochemistry and TUNEL method.

Cadmium administration was performed by a single (per day) subcutaneous injection for it renders higher systemic concentrations compared to oral administration [21, 22]. Subcutaneous mode of administration was used in our present study as an intraperitoneal route of injections is not recommended during pregnancy [20, 23].

Immunohistochemical Evaluation

Immunocytochemical reactions were performed according to the avidin biotin complex technique described by Hsu et al. [24] The sections were incubated with specific mouse monoclonal anti-PCNA antibody (Cat. # MS-106-B, Thermo LabVision, USA).

The evaluation of the PCNA immunohistochemistry in all groups was performed using H-SCORE [25]. Briefly, sections were evaluated using a light microscope (Olympus CX-31-JAPAN) with a special ocular scale. Three randomly selected slides, each of five different fields at ×200 magnification, were evaluated for immunohistochemical labeling of PCNA. The labeling was scored in semiquantitative analyses that included the intensity of specific labeling in sections. The evaluations were recorded as percentages of labeled cells of all types in each of four intensity categories, denoted as 0 (no labeling), 1+ (weak labeling but detectable above control), 2+ (distinct labeling), and 3+ (intense labeling). For each tissue, an H-SCORE value was derived by summing the percentages of cells that were labeled at each intensity multiplied by the weighted intensity of the labeling: H-SCORE = $S\ Pi\ (i + 1)$, where i is the intensity score and Pi is the corresponding percentage of the cells. Two observers blinded to the experimental groups performed the H-SCORE evaluations, and the average score was used.

TUNEL Assay

Apoptosis were evaluated by the terminal dUTP nick end-labeling (TUNEL) assay. The TUNEL method, which detects fragmentation of DNA in the nucleus during apoptotic cell death in situ, was employed using an apoptosis detection kit (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, Cat. No. S7100, Millipore, USA).

Three randomly selected slides, each of five different fields at high-power lens visual fields (×40), and the apoptotic cells were identified by the intense dark nuclear staining. Data were expressed as the average of apoptotic cell numbers per sample.

Statistical Analysis

All statistical analyses were carried out using SPSS statistical software (S0064 Minitab Release 13, License Number: WCP1331.00197). All data were presented in mean ± SD. Differences in measured parameters among the groups were analyzed with a nonparametric test (Kruskal–Wallis). Dual comparisons between groups exhibiting significant values

were evaluated with a Mann–Whitney *U* test. These differences were considered significant when probability was less than 0.05.

Results

Morphometric Measures and Numbers of Live Litter

Weights of placenta and embryos for gestation days 15, 17, 19, and 21 were measured in both control and Cd groups. An increase was observed in weights of the placentas and embryos in the control group in parallel with gestation age. However, Cd was found to cause a significant weight loss on the all gestation days (Fig. 1).

Lengths of embryos were measured for gestation days 15, 17, 19, and 21 in both control and Cd groups. Increase in lengths was observed in the embryos of the control group in parallel with gestation age. However, Cd was found to decrease the lengths of embryo significantly in all gestation days (Fig. 1).

The number of live litters was noted for gestation days 15, 17, 19, and 21 in both control and Cd groups. Cd was found to decrease the number of live litters significantly on all gestation days (Fig. 1).

Immunohistochemical Findings

Positivity was observed in giant cells, spongiotrophoblast, and labyrinth trophoblast cells in PCNA immunostaining in the control group on gestation days 15 and 17 (Fig. 2(1a, b and 2a, b). Positivity in giant cells, spongiotrophoblast, and labyrinth trophoblast cells was observed to decrease in PCNA immunostaining in the Cd group on gestation days 15 and 17 as compared with the control group on the same gestation day (Fig. 2(1c, d and 2c, d)).

PCNA immunostaining in the control group on gestation days 19 and 21 was observed in giant cells, spongiotrophoblast, labyrinth trophoblast cells, and glycogen cells. (Fig. 3(1a, b and 2a, b)). As a result of Cd administration on gestation days 19 and 21, PCNA positivity in giant cells, spongiotrophoblast, labyrinth trophoblast cells, and glycogen cells was observed to decrease as compared with the control group on the same gestation day (Fig. 3(1c, d and 2c, d).

When PCNA immunostaining-positive cell frequency was assessed in accordance with H-SCORE analysis, it was observed to show a decrease in the control group placentas in parallel with gestation age and Cd administration was observed to decrease the number of PCNA-positive cells (Fig. 4).

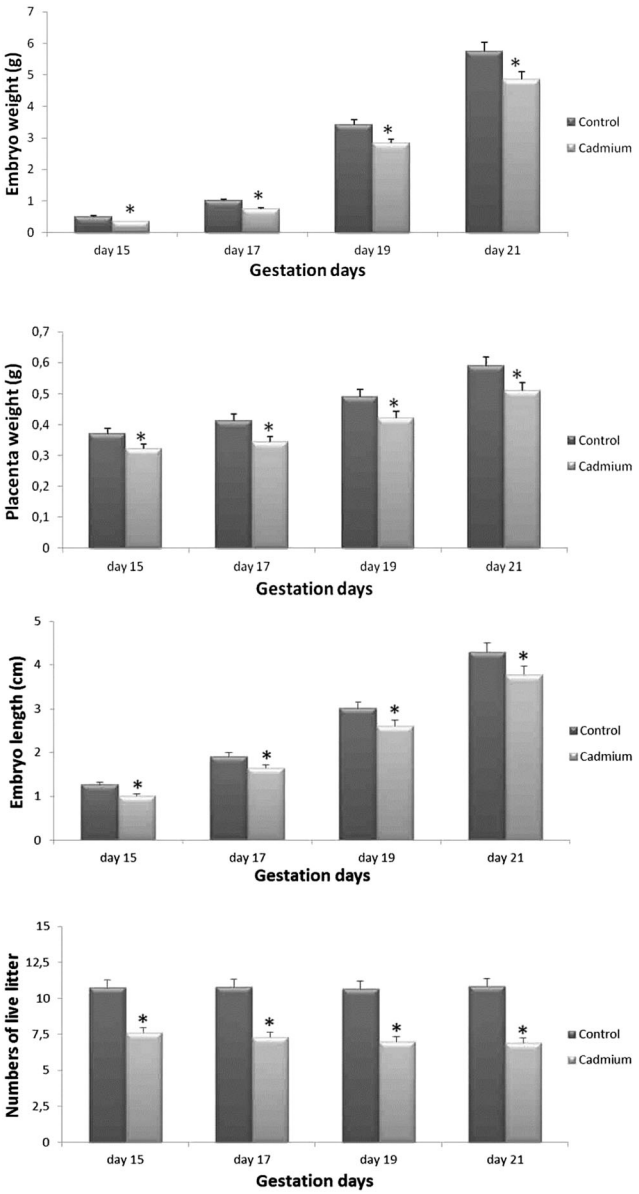

Fig. 1 Effects of subcutaneous injection of 0.5 mg/kg Cd body weight on different days of gestation in the rat. Weights of embryo and placenta, embryo size, and numbers of live litter were significantly different Cd groups compared with control groups. *$p < 0.05$

TUNEL Findings

TUNEL-positive cell was observed rarely in the placentas of rats in the control group on gestation days 15, 17, 19, and 21. As a result of Cd administration on different gestation days (15, 17, 19, and 21), the number of TUNEL-positive cells in rat placenta was increased compared with the control group on the same gestation day (Fig. 5).

Furthermore, the number of TUNEL-positive cells in the placental tissues in both control and Cd groups were determined in Fig. 6. The number of apoptotic cells in Cd groups

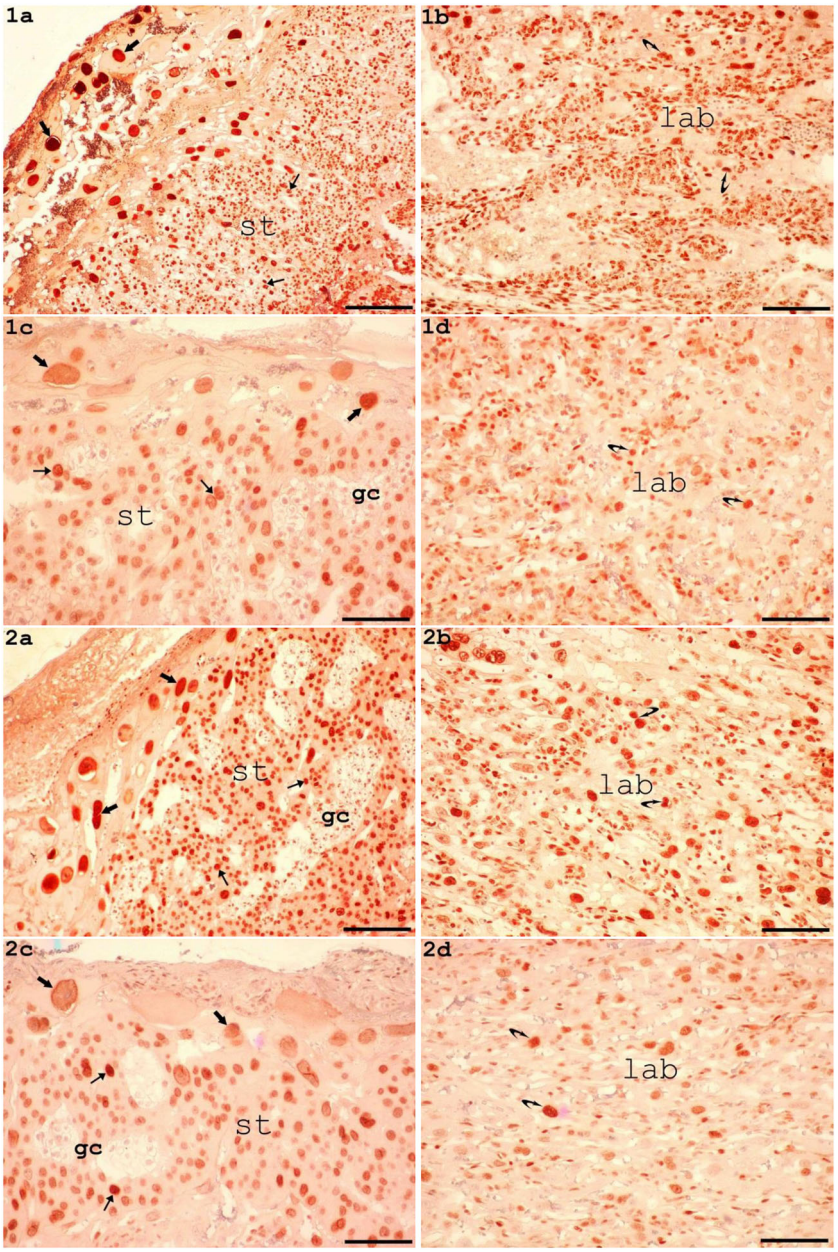

Fig. 2 PCNA immunolabeling on days 15 and 17 of pregnancy. (*1a*, *b*) Very strong positive PCNA immunostaining intensity was observed in spongiotrophoblast cells (*thin arrow*), trophoblast giant cells (*thick arrow*), and labyrinth trophoblast cells (*thin arrow*) in PCNA in the control group on gestation day 15. (*1c*, *d*) PCNA immunostaining intensity was decreased in all zones of the Cd group compared with the control group on the same day. (*2a*, *b*) PCNA immunostaining intensity in the control group on day 17 of pregnancy. (*2c*, *d*) PCNA immunostaining intensity was decreased in the Cd group compared to the control group on the same day. *Lab* labyrinth layer, *st* spongiotrophoblast layer, *gc* glycogen cells. Immunoperoxidase, hematoxylin counterstain, *1a*, *1c*, *2a*, *2c*: *scale bar* 200 μm; *1b*, *1d*, *2b*, *2d*: *scale bar* 100 μm

was significantly higher as compared with that in control groups (Fig. 6).

Discussion

Placenta functions between mother and fetus during gestation. Thus, any pathological case affecting mother or fetus affects regular functions of the placenta and causes morphological changes. Some lesions developing in the placenta may cause fetal life negatively. Various factors affecting mother and fetus may leave traces in the placenta. Sometimes the placenta's own structural changes may be a factor in the deterioration of fetal status [26].

In the rat, the placenta is chorioallantoic placenta and consists of two fetal compartments: labyrinth zone and junctional zone [27]. The junctional zone is positioned at the maternal interface, whereas the labyrinth zone is at the fetal interface. The junctional zone is a cellular compartment consisting of at least two trophoblast subtypes: spongiotrophoblast and glycogen cells. Control of junctional zone growth, and of the overlying giant cell layer, may be partly regulated by Nodal, as the spongiotrophoblasts and giant cell layer expand when Nodal is disrupted [28]. These two zones differ in function, where the junctional zone is involved in endocrine and invasive functions, the labyrinth zone forms the main transport barrier and controls maternal to fetal transfer of nutrients. Both the labyrinth and junctional zones are comprised of trophoblast cells.

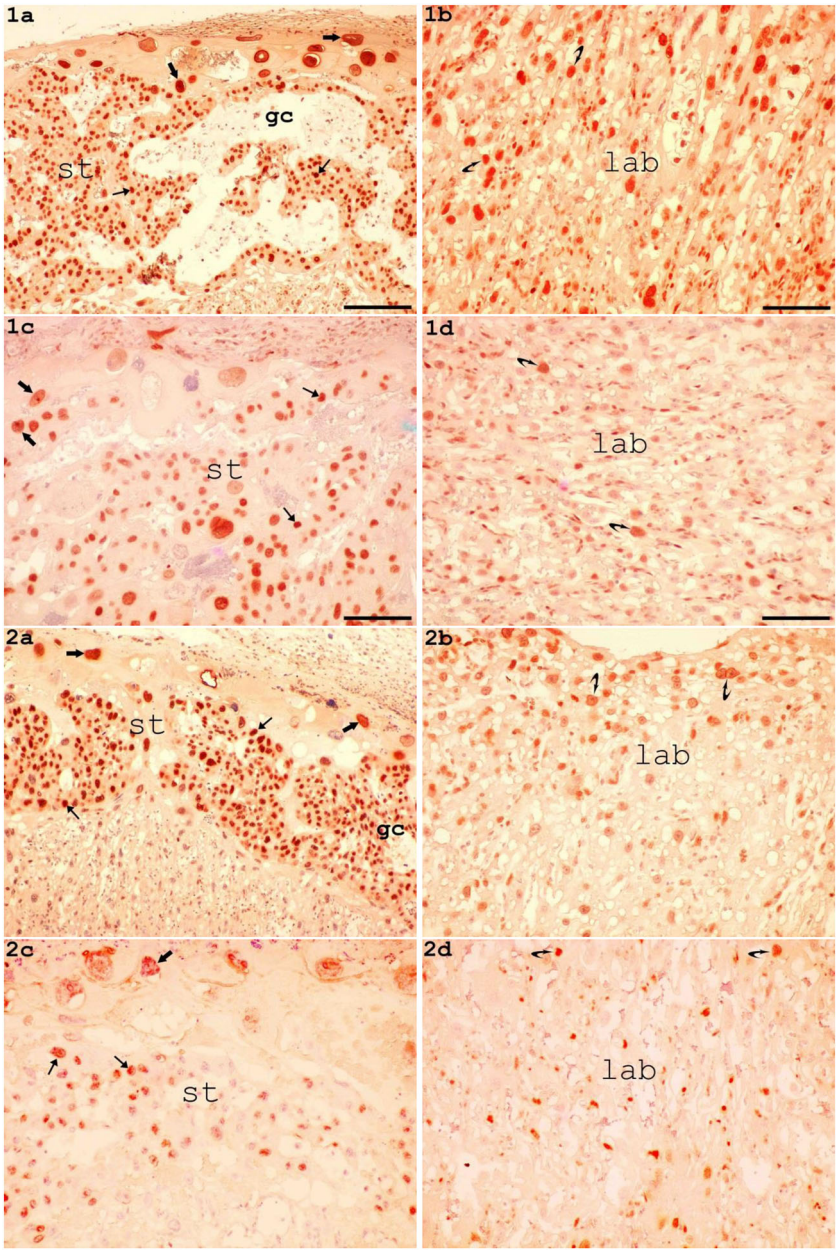

Fig. 3 PCNA immunostaining on days 19 and 21 of pregnancy. (*1a*, *b*) Strong positive PCNA immunostaining intensity was observed in spongiotrophoblast cells (*thin arrow*), trophoblast giant cells (*thick arrow*), and labyrinth trophoblast cells (*thin arrow*) in PCNA in the control group on gestation day 19. (*1c*, *d*) PCNA immunostaining intensity was decreased in all zones of the Cd group compared with the control group on the same day. (*2a*, *b*) PCNA immunostaining intensity in the control group on day 21 of pregnancy. (*2c*, *d*) PCNA immunostaining intensity was decreased in the Cd group compared to the control group on the same day. *Lab* labyrinth layer, *st* spongiotrophoblast layer, *gc* glycogen cells. Immunoperoxidase, hematoxylin counterstain, *1a*, *1c*, *2a*, *2c*: *scale bar* 200 μm; *1b*, *1d*, *2b*, *2d*: *scale bar* 100 μm

Labyrinth trophoblast cells of the labyrinth zone are the main type of trophoblast cell that regulates the transport of nutrients and wastes across the placenta [27]. One of the main functions of the junctional zone is to act as an endocrine compartment. To maintain progesterone secretion from the corpus luteum, the spongiotrophoblasts of the junctional zone and secondary giant cells produce prolactin-like hormones (PLP-M and PLP-N), lactogens, and cytokines during pregnancy [29–31]. Another suggested function of the junctional zone is to limit the growth of maternal endothelium into the fetal placenta [32].

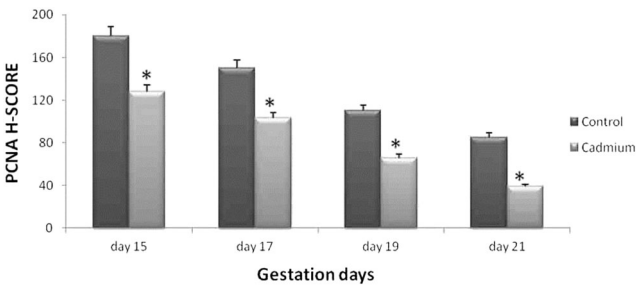

Fig. 4 PCNA H-SCORE for gestation days 15, 17, 19, and 21 were measured in both control and Cd groups. The differences were statistically significant. *$p < 0.001$

The placenta serves as the point of contact between maternal and fetal circulation. It functions as the means by which all necessary nutrients are delivered to the fetus, as well as a barrier to prevent passage of toxic substances, including metals. If the latter is accomplished by binding of the metals

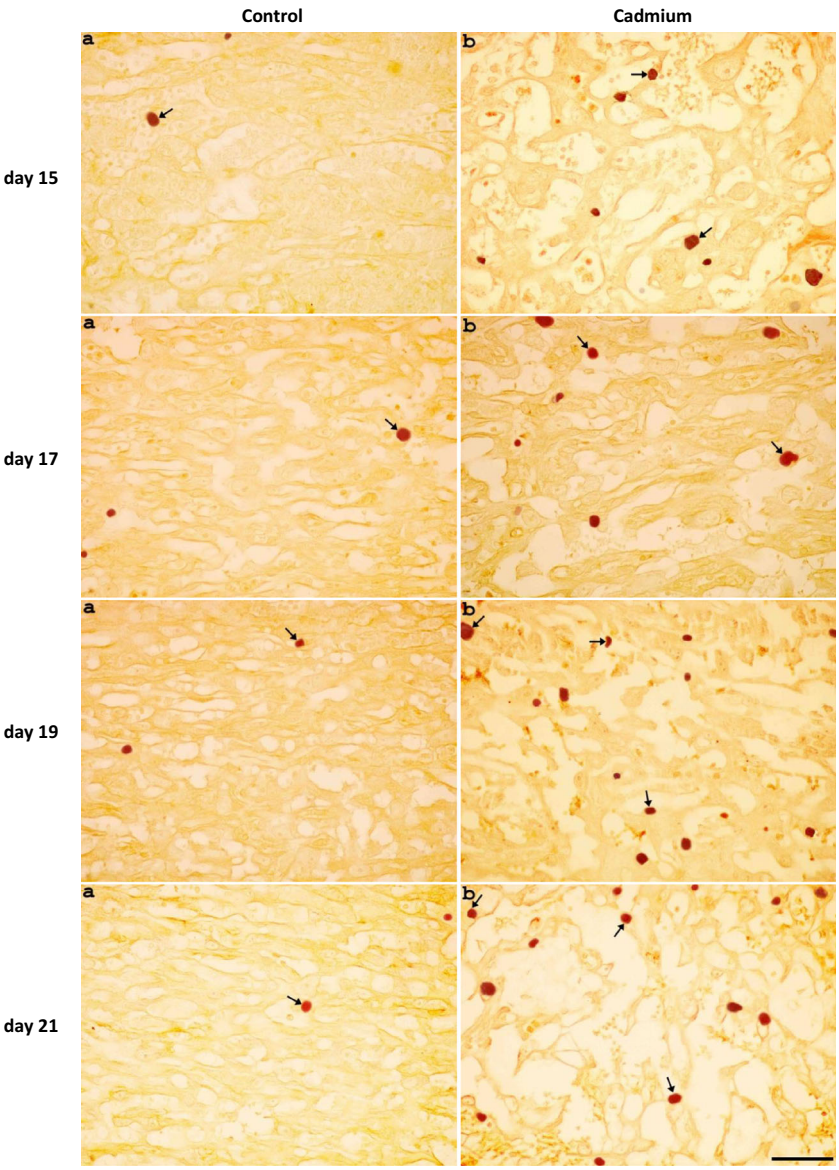

Fig. 5 Light microscopy of placenta tissues on days 15, 17, 19, and 21 of pregnancy for TUNEL. TUNEL-positive cell was observed rarely in the placenta of rats in the control group on gestation days 15, 17, 19, and 21. As a result of Cd administration on gestation day 15, 17, 19, and 21, the number of TUNEL-positive cells in rat placenta was to significantly increase as compared with the control group on the same gestation days. *Arrow*: TUNEL-positive cells (TUNEL staining, *scale bar* 50 μm)

to the placenta, it may interfere with placental function, in particular, the transport of essential trace elements required for fetal growth and development [33, 34].

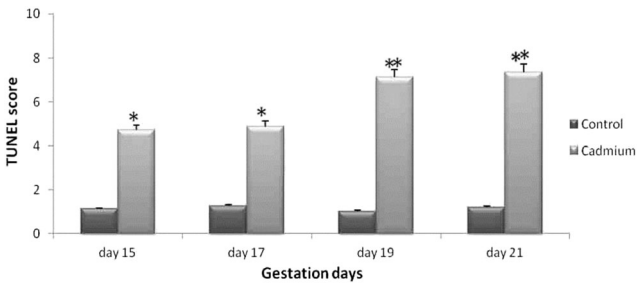

Fig. 6 TUNEL score for gestation days 15, 17, 19, and 21 were measured in both control and cadmium groups. The differences were statistically significant. *$p < 0.01$, **$p < 0.001$

Primary exposure sources of Cd are industrial pollution, nutrients, and smoking [35]. Considering the Cd level per cigarette from 120 ng to 2 μg [36], a smoker may be exposed easily to 1.5–60 fg Cd every day. Accordingly, placental accumulation of this metal causes both structural and functional changes in the placenta. Cd may harm the fetus by affecting metabolisms of elements like zinc, copper, iron, and selenium [6].

In animal experiments, Cd was observed to have teratogenic and developmental effects [37]. In these experiments, low birth weight [38, 39] was shown for the babies born as a result of Cd exposure in pre-gestational period and during gestation. Huel et al. [40] reported that low birth weight was seen in the babies of mothers who were exposed to Cd in 1984. In other studies performed, an increase was shown in the concentration of placental Cd in the women smoking during their gestation

period. This increase was found to be associated with low birth weight [41, 42]. In our study, we found that placenta and embryo weights decrease significantly as compared with the control group as a result of Cd administration on the gestation days covered by our experiment. Our findings are compatible with the findings of previous studies [43, 44].

As in other organs of human body, cell development, and function in placenta depends on the balance between cell proliferation and cell death. PCNA was stated to be expressed most in early gestation, less in mid-gestation, and least at term [45]. Furthermore PCNA expression in the placentas of rats was found also to decrease in the forthcoming days of gestation. Intensive PCNA positivity in placenta on gestation days 11 and 13 was reported to decrease on gestation days 17 and 21 [25]. Trophoblast proliferation being necessary for normal placental development shows a decrease as a result of some pathological cases. Unek [46] stated that PCNA positivity decreased significantly as compared with the control group in the placentas of rats where IUGR was formed. In the study performed by Acar [25] PCNA immunopositive cell frequency was reported to decrease in the control group and diabetic rat placentas in parallel with gestation age but this positivity was reported to increase more as a result of diabetes. In our study number of PCNA immunopositive cells in placenta was seen to decrease in parallel with gestation age. Our findings supported the information that as long as placenta approaches term, it loses its proliferative characteristic [45]. After Cd administration PCNA positivity was seen to decrease significantly as compared with the control group.

Apoptosis is a programmed death of the cells which are not needed as required by intercellular relations in developed organisms and of which functions are deteriorated without harming environment. Apoptotic mechanism and programmed cell death are present during whole life from embryo period. In the placenta samples taken from first and last trimester normal gestation in humans apoptosis was shown in all cell types and large portion of apoptotic cells (>50 %) was ascertained to be trophoblasts. There was a general view that as long as gestation progresses, increased apoptosis is observed as a natural result of aging [47, 48].

In animal experiments, placental apoptosis is induced by such things as glucocorticoid (3), lipopolysaccharide [49], T-2 toxin [50], busulfan [51], and some anticancer drugs [52, 53]. In a previous study [46], TUNEL-positive cells found in fetal and maternal part of the placenta were counted on gestation day 20 and the number of apoptotic cells in the maternal part was reported to be higher than the ones in the fetal part. In another study [54], apoptotic cells in rat placenta on gestation day 18 were reported to rarely exist. Apoptosis which is necessary for normal development of the placenta was reported to increase extremely as a result of applications performed externally or the cases affecting the healthy placenta and embryo [53, 55, 56]. In another study [56], rat placenta of ethylnitrosourea was shown to cause apoptosis in the labyrinth layer. In our study, we found a rare amount of apoptotic cells in the control group in accordance with literature. As a result of Cd administration, the number of apoptotic TUNEL-positive cells in the labyrinth layer was found to increase significantly and this increase was found to be much more in gestation days 17 and 19 as compared with gestation days 15 and 17.

Consequently, our study shows that Cd administrated during gestation causes abnormal placental development by disordering the apoptosis/proliferation balance of trophoblast cells. We think that to bring the data obtained from our study in literature will make a contribution to studies that will follow.

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