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RESEARCH ARTICLE

Protective Effects of Ascorbic Acid Against Cadmium-Induced Toxicity in the Placenta and Fetus of Rats

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ABSTRACT

This study aimed to determine the protective role of L-ascorbic acid in a pregnant rat model of cadmium-induced toxicity. Cadmium is a toxic heavy metal that can seriously harm placenta and fetus tissue in pregnant women. Forty-two healthy female Wistar albino rats (250–300 g weight and 14–16 weeks) were randomly distributed into six equal groups ($n = 7$): control, cadmium 1 mg (CD1), cadmium 5 mg (CD5), ascorbic acid (AA), CD1+AA, CD5+AA. Cadmium was administered to pregnant rats by oral gavage every other day, and/or AA (200 mg) was administered every day. At the end of pregnancy (Day 21), blood, placenta, and fetuses were collected from rats. The results indicated that cadmium-induced oxidative stress by increasing the level of MDA and by decreasing the levels of GSH, SOD, and CAT activity in the serum of maternal. However, AA administration significantly decreased MDA levels and increased GSH levels, SOD, and CAT activity ($p < 0.05$). Cadmium (5 mg/kg) exposure significantly increased creatinine levels compared to AA and CD1+AA groups ($p < 0.05$). In addition, AA (200 mg/kg) significantly attenuated cadmium-induced histopathological alteration in the placental and fetal tissues. In conclusion, AA may prevent cadmium toxicity in maternal and fetal tissues, as it regulates oxidative imbalance in pregnant rat tissues and alleviates histopathological changes.

1 | Introduction

Cadmium (Cd) exposure is becoming an increasingly serious environmental hazard for both humans and animals as industrial development progresses. The population is exposed to Cd through contaminated drinking water and food, as well as cigarette smoke inhalation [1]. Pregnant women are more vulnerable to Cd toxicity due to increased Cd absorption as a result of increased iron absorption during pregnancy, as well as decreased Cd excretion [2].

Cadmium's ever-increasing presence is a threat to the general population, posing a greater risk not only to the mother, but also to the fetus and newborn during pregnancy and lactation [3]. Bush et al. reported that Cd accumulation due to smoking is associated with a decrease in the volume of fetal capillaries and an increase in the thickness of the villous membrane of the placenta [4]. The histopathological approach to Cd toxicity pathogenesis is thought to be an important tool for understanding the mechanism of teratogenicity and developmental toxicity, particularly embryonic lethality and delayed development and may benefit reproductive toxicity research [5].

The generation of reactive oxygen species (ROS) as a result of oxidative stress has been linked to a variety of pathological conditions such as apoptosis, cell overgrowth, and organ dysfunction [6]. Chater et al. reported that Cd increases membrane lipid peroxidation by increasing oxidative damage and causing

DNA damage in the liver [7] and kidney [8] tissues of pregnant rats. Ascorbic acid (AA) is an electron donor with a strong antioxidant effect in biological systems, which is important in preventing organ damage caused by oxidative stress [9]. In addition, AA appears to modulate systemic and leukocyte-derived cytokines, which can elicit pro- or anti-inflammatory responses [10]. Richter et al. reported that AA treatment may provide a useful intervention to improve placental function and protect fetal growth in pregnancy, which is complicated by fetal hypoxia [11].

In this study, we analyzed serum oxidative stress parameters and histopathological changes in the fetus and placental tissues of pregnant female rats exposed to cadmium toxicity and examined the ameliorative effects of AA.

2 | Materials and Methods

2.1 | Chemicals

L-ascorbic acid (99.99%) was purchased from Carlo Erba Reagent, France. Cadmium chloride ($CaCl_2$ 99.99%) was obtained from Merck, Germany.

2.2 | Animals

Forty-two Wistar-Albino female rats (250–300 mg and 14–16 weeks) were obtained from Pamukkale University Experimental Surgery Application and Research Center, housed under environmental conditions (21 ± 2°C), humidity ($45\% \pm 5\%$), with 12 h light-dark cycle and provided with standard pelleted rodent diet. All experimental procedures described below were approved by the Ethical Committee of Pamukkale University (approval number PAUHADYEK-2021/47). Animal care and all experimental procedures used were in accordance with those detailed in the Guide for Care and Use of Laboratory Animals.

2.3 | Experimental Design

Rats were divided into six experimental groups, each containing seven rats ($n = 7$); control, cadmium 1 mg (CD1), cadmium 5 mg (CD5), ascorbic acid (AA), CD1 + AA, and CD5 + AA. After a week of adaptation, female rats mated with male rats. The animals' vaginal smears were examined for the presence of sperm, and the vaginal smear that was found to be sperm positive was determined on the first day of pregnancy. Then, Cd (1 or 5 mg/kg) was administered to pregnant rats by oral gavage every other day, and/or AA (200 mg) was administered every day. The control group was administered saline by oral gavage. At the end of pregnancy (Day 21), blood, placenta, and fetuses were collected from rats under ketamine (87 mg/kg) and xylazine (13 mg/kg) anesthesia. Serum samples taken for biochemical analysis were stored in the freezer at −80°C, and tissue samples were fixed with 10% formalin for histopathological examination. Fetal placenta and other tissues of the fetus (vertebra, spinal cord, lung, heart, kidney) were taken for histopathological and immunohistochemical examinations.

2.4 | Biochemical Assays

Blood samples were allowed to clot at room temperature and then centrifuged at 3000×g for 15 min. The clear serum was separated and used for biochemical assays. Renal Serum protein, urea, creatine, cholesterol, triglyceride (HUMAN, Wiesbaden, Germany), and glucose (BIOLABO, Maizy, France) levels were measured spectrophotometrically (Thermo Fisher Scientific Oy Ratastie 2, FI-01620 Vantaa, Finland).

Lipid peroxidation was assayed by measuring the level of MDA in serum. MDA levels were determined using the method described by Yagi [12]. The spectrophotometry analysis was performed at 532 nm for the evaluation of the product, and the results were recorded as nmol/L. The GSH content of serum was measured as nmol/L using the Ellman method [13]. The spectrophotometry analysis was performed at 412 nm for the evaluation. Superoxide dismutase (SOD) activity was measured as U/mL using the Sun et al. method [14]. The SOD activity was evaluated spectrophotometrically at 560 nm. Catalase (CAT) enzyme activity was performed according to the method of Aebi [15] and it was defined as U/mL. This method works by determining the rate constant (s/k) for the H_2O_2 decomposition rate at 240 nm.

2.5 | Histological and Microscopic Analyses

Samples of placenta and fetal structures were stained with hematoxylin and eosin. PAS staining was performed histochemical to show accumulations and basement membrane thickening. Moreover, 5-μm sections were taken from the selected paraffin blocks onto positively charged slides to study the S100-P antibody. The tissue samples taken were kept in an oven at 60°C overnight for deparaffinization and then were automatically stained with the Ventana Benchmark XT device using a routine procedure. The targeted proteins were made visible by using ready-made preparations (S100-P antibody [Polyclonal], Ventana, Roche, ready-to-use) on the automatically stained sections. Nuclear and cytoplasmic immunoexpression of S-100P in placental trophoblastic cells was considered positive [16].

2.6 | Statistical Analysis

Statistical analysis was performed using the SPSS 25.0 (IBM SPSS Statistics, IL, USA) statistical package. Data are shown as mean ± standard error mean (SEM), 95% confidence interval or percentage. GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) was used for graphical demonstration. One-way analysis of variance (ANOVA) was used to compare independent multiple groups, and the level of significance between groups was determined by the Bonferroni post hoc test. If the p value is less than 0.05, it was judged as

"significant," and if the p value is greater than 0.05, it was judged as "not significant."

3 | Results

3.1 | Biochemical Evaluation

As shown in Table 1, cadmium (5 mg/kg) exposure significantly increased creatinine levels compared to AA and CD1+AA groups in maternal rat serum ($p < 0.05$). AA administration decreased creatinine levels in CD1+AA and CD5+AA groups. There is no significant difference between all groups for glucose, cholesterol, protein, triglyceride, and urea levels.

As shown in Figure 1A, Cd (1 and 5 mg/kg) exposure significantly increased MDA level compared to the control group ($p < 0.05$). AA coadministration decreased MDA levels in the CD1+AA and CD5+AA groups. As shown in Figure 1B, Cd (1 and 5 mg/kg) exposure significantly decreased GSH level compared to the control group ($p < 0.05$). AA Coadministration increased GSH levels in CD1+AA and CD5+AA groups. As shown in Figure 1C, Cd (1 and 5 mg/kg) exposure significantly decreased SOD activity compared to the control group ($p < 0.05$). AA coadministration increased SOD levels in

TABLE 1 | The effect of ascorbic acid on glucose, cholesterol, protein, triglyceride, creatinine, and urea in CD-induced oxidative stress in rat serum ($n = 7$, mean ± SEM).

Groups	Glucose (mg/dL)	Cholesterol (mg/dL)	Protein (g/L)	Triglyceride (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)
Control	75.85 ± 12.85	66.19 ± 11.88	6.85 ± 0.82	86.66 ± 29.74	0.64 ± 0.21[ab]	42.33 ± 7.91
CD1	73.17 ± 7.51	69.88 ± 7.66	6.86 ± 1.05	76.12 ± 32.23	0.51 ± 0.22[ab]	54.29 ± 13.06
CD5	71.18 ± 8.06	68.06 ± 11.72	6.81 ± 1.76	64.70 ± 24.39	0.74 ± 0.97[a]	47.56 ± 20.13
AA	80.32 ± 12.62	67.76 ± 6.51	5.65 ± 0.85	86.96 ± 27.48	0.44 ± 0.29[b]	39.47 ± 19.83
CD1+AA	76.08 ± 13.26	75.93 ± 16.11	6.69 ± 1.18	101.96 ± 39.90	0.46 ± 0.27[b]	43.75 ± 13.67
CD5+AA	64.61 ± 7.49	72.06 ± 7.51	6.53 ± 0.35	81.51 ± 28.12	0.54 ± 0.19[ab]	51.24 ± 17.46

[a,b]In the same column, values with different letters show statistically significant differences in serum ($p < 0.05$).

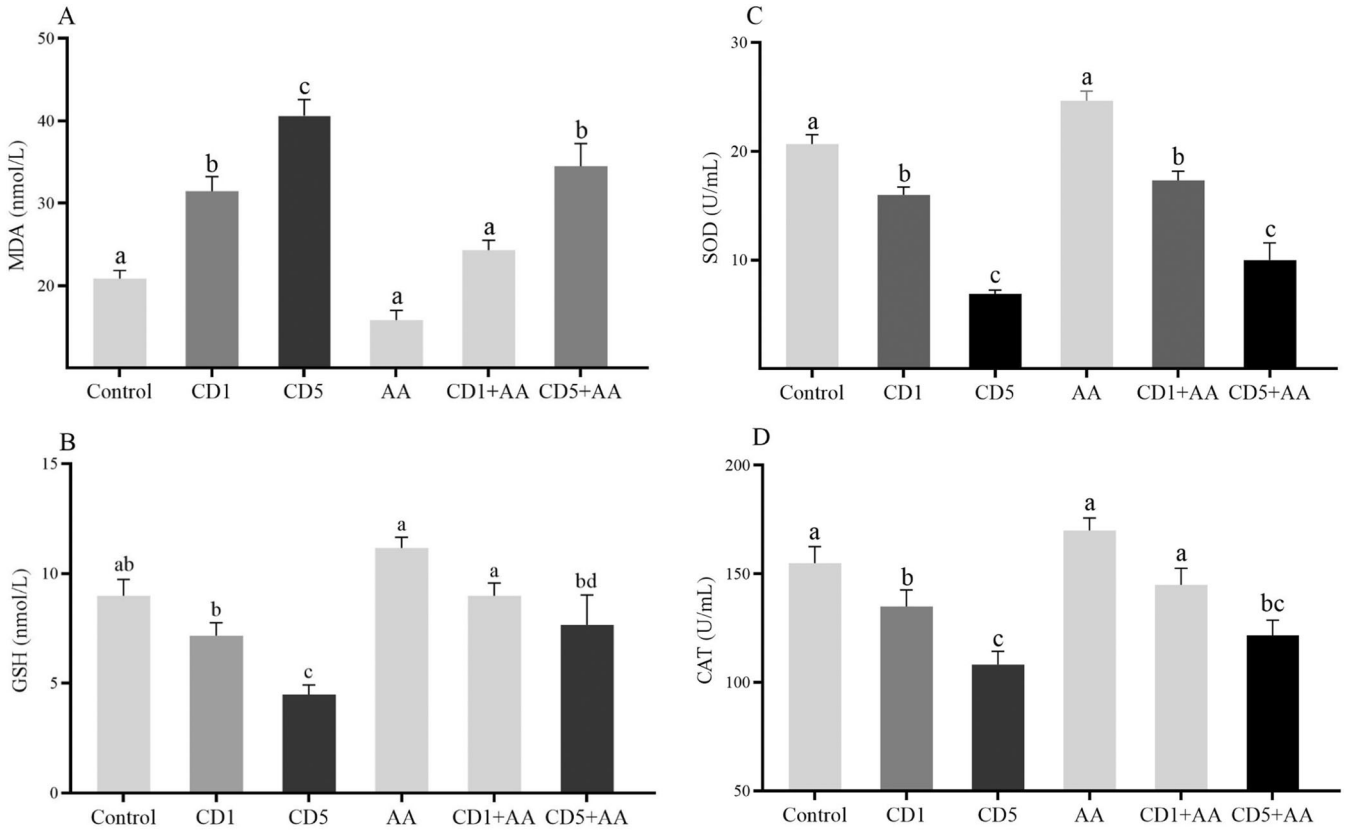

FIGURE 1 | Serum markers of oxidative stress ($n = 7$, for each group). (A) MDA levels in serum. (B) GSH levels in serum, (C) SOD activity in serum, (D) CAT activity in serum. Different letters indicate statistical differences between groups (a, b, c, d = $p < 0.05$). The values were expressed as means ± SEM.

CD1+AA and CD5+AA groups. As shown in Figure 1D, Cd (1 and 5 mg/kg) exposure significantly decreased CAT activity compared to the control group ($p < 0.05$). Coadministration with AA increased CAT levels at a statistically significant level in the CD1+AA group ($p < 0.05$), whereas it increased CAT levels in the CD5+AA groups.

3.2 | Histopathological Evaluation

As shown in Figure 2A–C, it has been demonstrated placental tissue and fetal lung, vertebra, spinal cord, and neural crest from the control group. Figure 2D,E shows the development of lung alveoli, intestinal system, kidney, and liver in the AA group.

Figure 3A shows PAS-positive vacuoles in fetal placental Hofbauer cells in the CD1 group. As shown in Figure 3B–D, due to cadmium toxicity, stromal edema, microvascular changes, congestion findings, vesiculation in cyto and syncytiotrophoblasts, vacuolar changes in Hofbauer cells, and necrosis findings in trophoblasts are observed in the placenta.

As shown in Figure 4A,B, histochemically performed PAS staining shows thickening of the vasculosyncytial membrane and thickened trophoblast and endothelial basement membranes. Figure 4C–E shows vacuolar changes, edema, necrosis, and vascular congestion in the placenta and the spinal cord and neural crest images in the CD5 group.

As shown in Figure 5A–C, AA coadministration reduced or eliminated Cd-induced pathological damage.

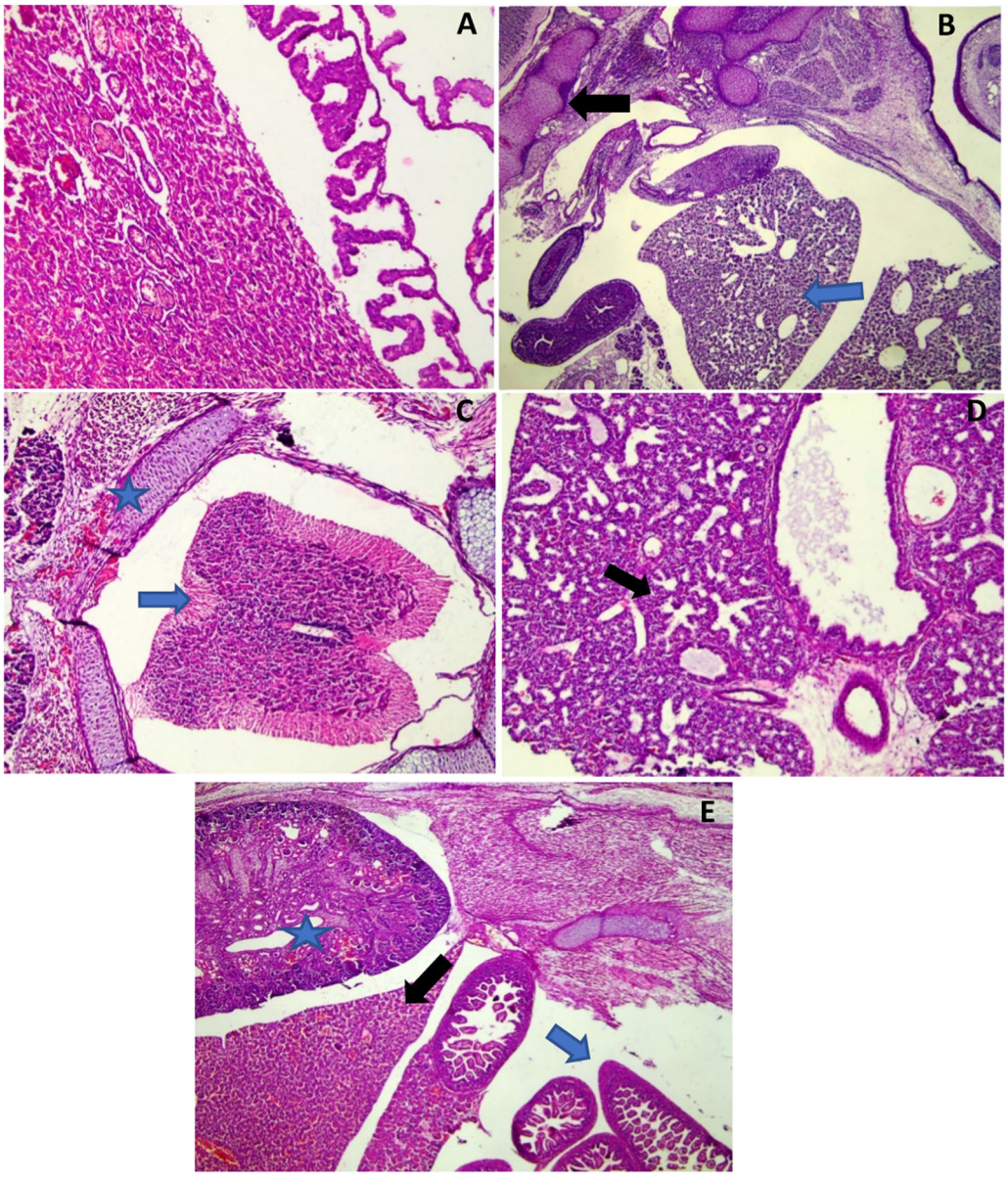

FIGURE 2 | (A) Placental tissue in the control group, H&E, ×100. (B) The fetal lung (blue arrow) and vertebra (black arrow) in the control group, H&E, ×40. (C) The fetal spinal cord (blue star) and neural crest (blue arrow) structure, in the control group, H&E, ×100. (D) It is seen that fetal lung alveolar development is more advanced in the AA group, H&E, ×400. (E) The fetal intestinal system (blue arrow), kidney (blue star), and liver (black arrow) development in the AA group, H&E, ×40.

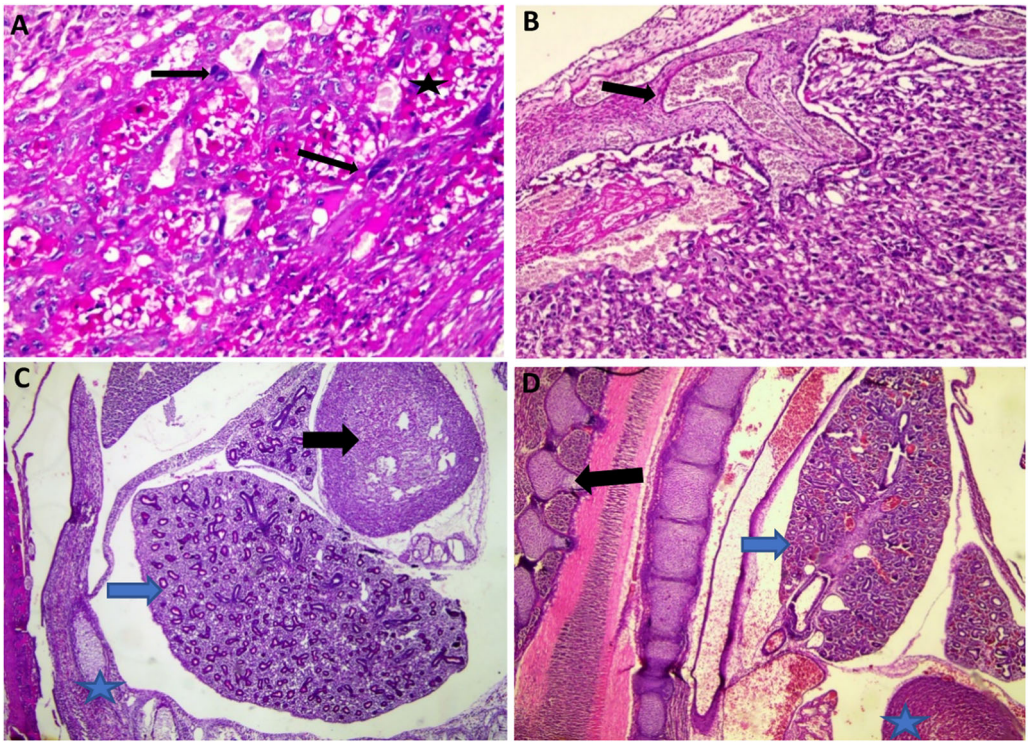

FIGURE 3 | (A) In the CD1 group, PAS-positive vacuoles in fetal placental Hofbauer cells, PAS, ×400. (B) In the CD1 group, vascular congestion and stromal edema in fetal placenta, H&E, ×40. (C) In the CD1+AA, the fetal lung (blue arrow), heart muscle (black arrow), and vertebrae (blue star), HE, ×40. (D) In the CD1, the fetal lung (blue arrow), heart (blue star), and vertebrae (black arrow), H&E, ×40.

S100 calcium-binding protein P (S100P) is produced in the placenta, substantially by trophoblast cells. It ensures cell proliferation. Cadmium reduces the level of S100P and inhibits development. Figure 6A shows S100P immunoexpression in trophoblastic cells in the normal placenta, whereas Figure 6B shows no S100P immunoexpression in the group exposed to cadmium toxicity. Figure 6C shows increased S100P immunoexpression in the CD5+AA group.

4 | Discussion

Cadmium is one of the very toxic industrial and environmental metals with a high volume of distribution and continues to pose a danger to human and animal health today [17]. Cadmium enters the redox cycle and depletes glutathione and protein-bound sulfhydryl groups, resulting in the production of reactive oxygen species such as superoxide ion, hydrogen peroxide, and hydroxyl radical. As a result, DNA damage and altered calcium and sulfhydryl homeostasis occur with increased lipid peroxidation [18]. It has been reported that the vitamin C supplement decreased the carcass cadmium burden and the cadmium content in the liver, kidneys, testicles, and muscles [19]. In our study, rats were given oral ascorbic acid and/or cadmium during pregnancy for 21 days and the maternal serum oxidative markers, biochemical parameters, and the histopathological changes in the fetus were examined.

Oxidative stress is associated with various pathological conditions that cause apoptosis, cell overgrowth, and organ dysfunction, and AA has ameliorating effects on this damage [20]. Exposure to cadmium induces lipid peroxidation products such as increased MDA and decreased antioxidant defense systems such as GSH, SOD, and CAT causing tissue and organ damage [21]. Enli et al. suggest that Cu supplementation may protect against Cd toxicity in liver, kidney, and placental tissues of pregnant rats and fetuses [22]. Similarly, our findings indicated that cadmium increased serum MDA levels while decreasing GSH, SOD, and CAT levels, a dose-dependent manner. However, AA administration had an ameliorative effect against oxidative damage. It was shown that a significant increase in blood urea and serum creatinine was found in pregnant rats administered cadmium [23]. In the present study, creatinine levels increased at a Cd dose of 5 mg/kg. AA administration decreased maternal serum creatinine levels. The growing embryo needs glucose to meet its energy needs. Yi et al. demonstrated that mice exposed to environmental Cd exposure during pregnancy developed hyperglycemia in adolescence and impaired glucose tolerance in adulthood following maternal Cd exposure during pregnancy [24]. However, cadmium did not affect glucose levels of rats in our study. These differences could be related to animal species, cadmium administration methods, and application doses.

Díaz et al. showed that cadmium administration to pregnant rats induced maternal toxicity and embryotoxicity including teratogenic effects revealed by maternal lesions and also embryonic and fetal death [25]. It has also been reported that rats exposed to Cd caused weight loss and histological changes in the maternal placenta, ovaries, and liver [26]. Similarly, our findings revealed that cadmium exposure caused histopathological damage to the fetal

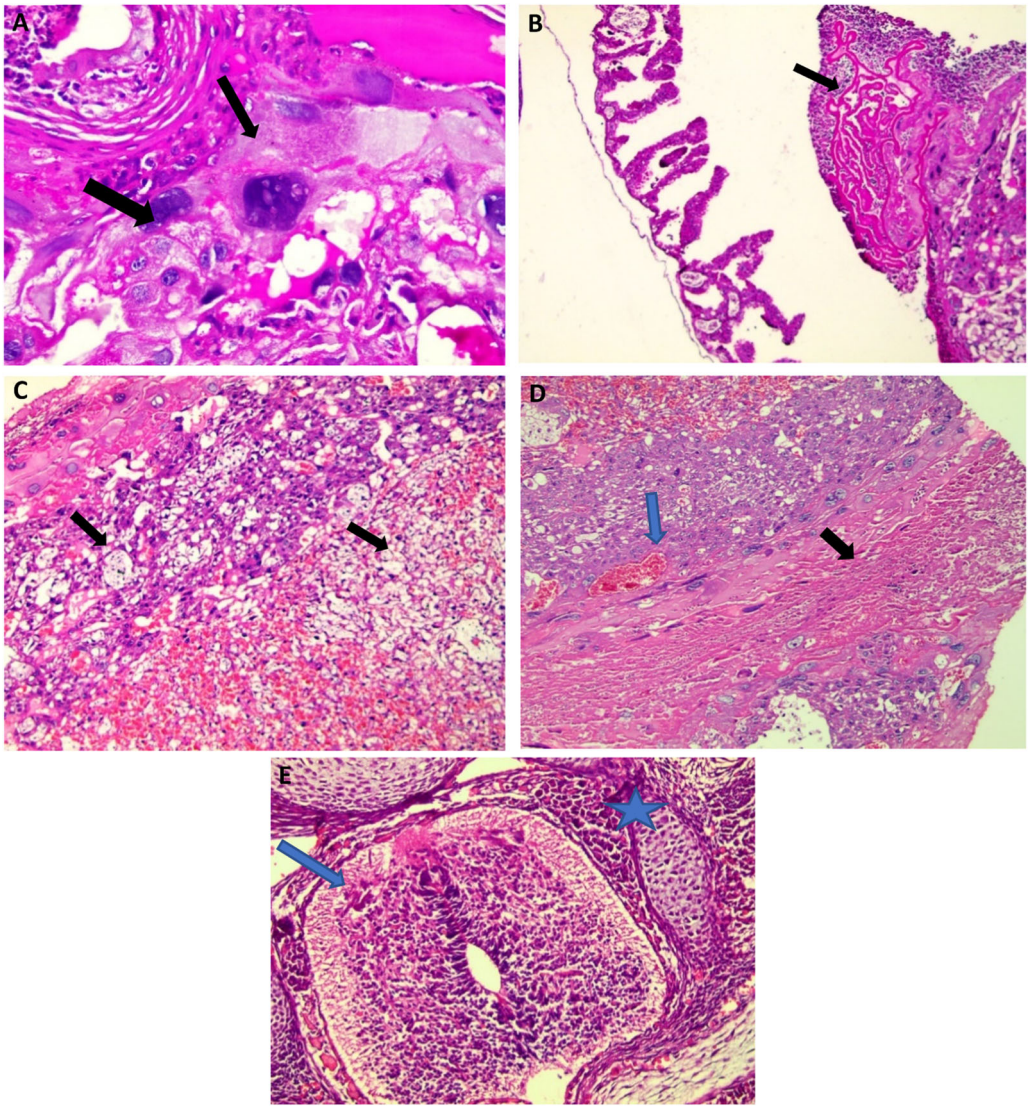

FIGURE 4 | (A) In the CD5 group, vesiculation in cyto and syncytiotrophoblasts in fetal placenta in cadmium toxicity, PAS, ×400. (B) In the CD5 group, vasculocysitial membrane thickening in fetal placenta in cadmium toxicity, PAS, ×40. (C) In the CD5 group, vacuolar changes and edema in the placenta in cadmium toxicity, H&E, ×100. (D) In the CD5 group, placental necrosis (black narrow) and vascular congestion (blue arrow) in cadmium toxicity, H&E, ×100. (E) In the CD5 group, the fetal spinal cord (blue star), and neural crest (blue arrow) in cadmium toxicity, H&E, ×200.

placenta and organs, a dose-dependent manner. Wang et al. reported that Cd treatment caused placental thickening in the media of vessel walls and degeneration and excessive perivillous fibrin deposition in the placental labyrinth in rats [27]. It has been demonstrated that although Cd deposition was observed in these trophoblasts, the cells with highest affinity to Cd were cytotrophoblasts, and the most severely damaged cells were spongiotrophoblasts [28]. Our findings, such as thickening of the vasculosyncytial membrane and thickening of the trophoblast and endothelial basement membrane, were detected in the histochemically applied PAS staining. AA treatment reduced or eliminated these pathological findings in rats exposed to cadmium toxicity. S100P, a protein that is encoded by the S100P gene, is an interesting and promising marker that can enable the detection of many aggressive cancers in the early stages [29]. Zhou et al. demonstrate that cadmium inhibits S100P expression and cell proliferation in the placenta, meanwhile, inhibiting placental trophoblast cell proliferation through targeting S100P [30]. We showed that S100P immunoexpression is seen in trophoblastic cells in the normal placenta. The S100P immunoexpression was not observed in the group exposed to cadmium. However, AA administration increased S100P immunoexpression in cadmium toxicity.

5 | Conclusions

This study found that exposure to cadmium caused oxidative imbalance in pregnant rats, resulting in oxidative stress and pathological damage to the placenta and fetus. We revealed that AA oral use during pregnancy in cadmium exposure reduces oxidative stress in rats and ameliorates fetal and placental tissue damage. Our findings support that AA supplementation may have a protective effect against Cd-induced oxidative stress and tissue damage in the pregnant rat's placentas and fetuses.

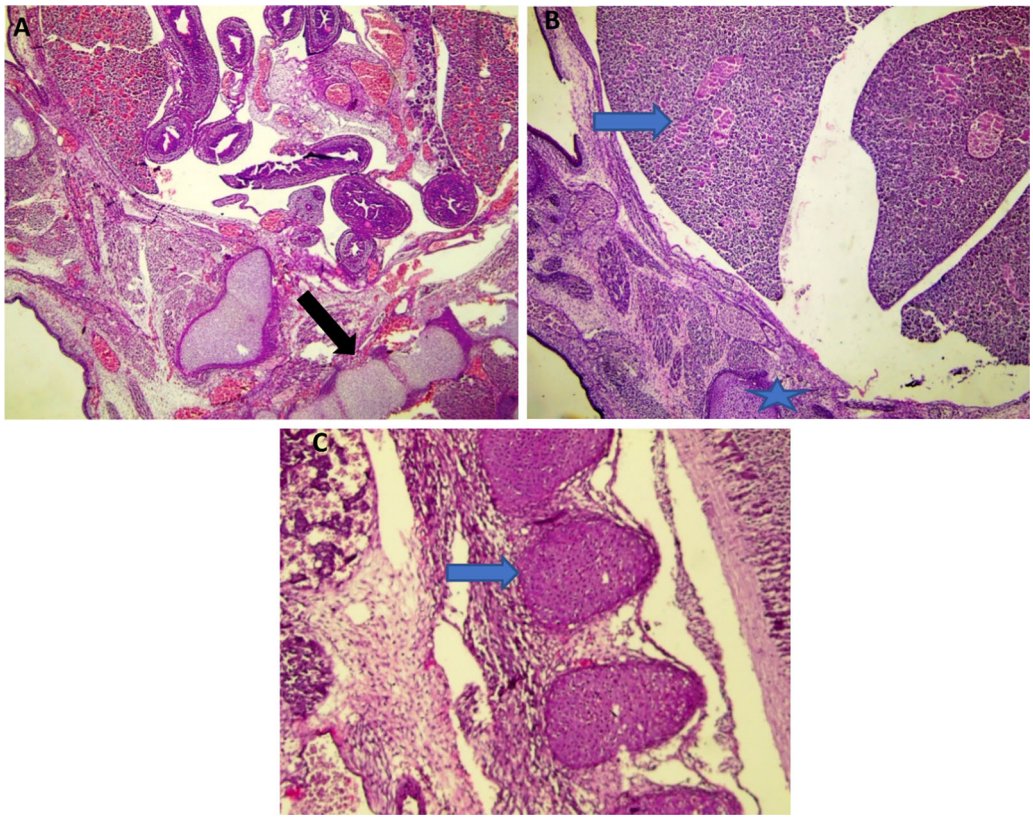

FIGURE 5 | (A) In the CD5+AA group, vertebra (black arrow), fetal structures, PAS, ×40. (B) In the CD5+AA group, liver (blue arrow), spinal cord (blue star) H&E, ×40. (C) In the CD5+AA group, vertebra (blue arrow), H&E, ×100.

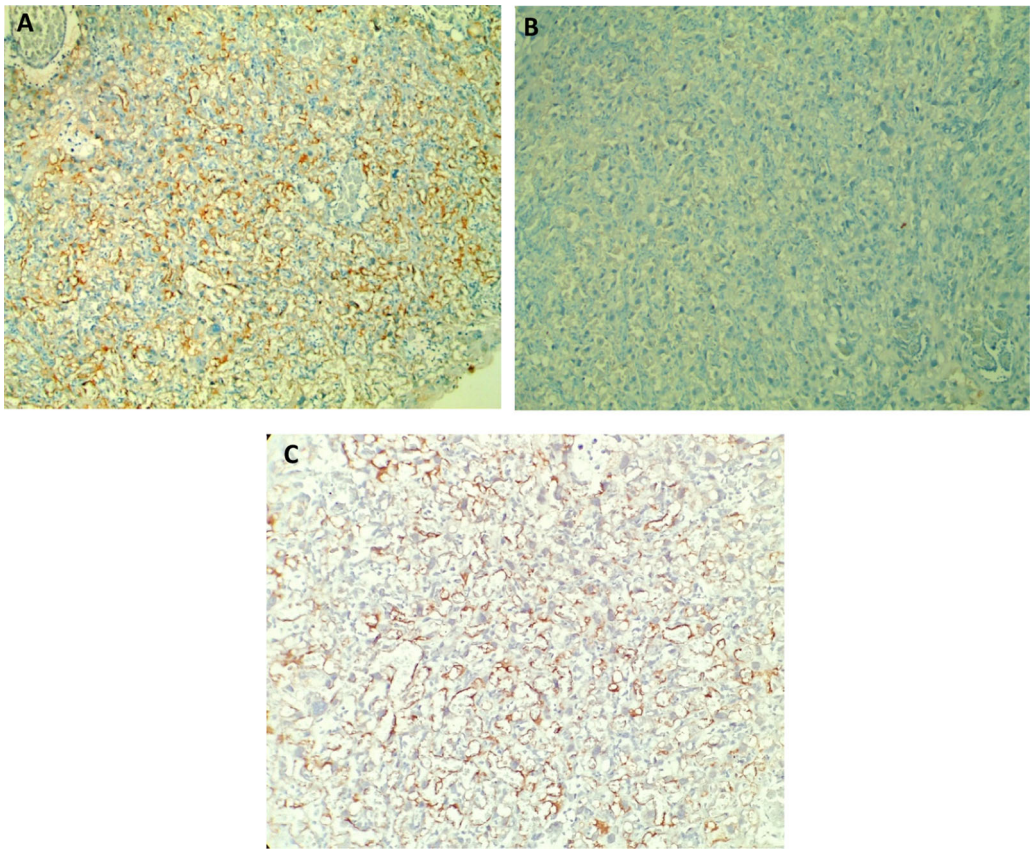

FIGURE 6 | (A) Increased S100P immunoexpression in the control group, placenta, IHC, ×100. (B) Decreased S100P immunoexpression in CD5 group, placenta, IHC, ×100. (C) Increased S100P immunoexpression in CD5+AA group, placenta, IHC, ×100.

Author Contributions

This work was carried out in collaboration with all authors. M.B., M.F.D., S.G. designed the study and performed the animal experiments. M.F.D. and M.B. analyzed the data and wrote the manuscript. Y.A.K. performed the pathological examination and analysis. F.K. performed biochemical analyses.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data generated or analyzed during this study are included in the manuscript.

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