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Lectin histochemistry for detecting cadmium-induced changes in the glycosylation pattern of rat placenta

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Abstract

Cadmium (Cd) is an industrial and environmental pollutant that produces toxic effects on gametogenesis, pre- and post-implantation embryos, and the placenta. Because the effects of acute Cd intoxication on the placenta are not well understood, we investigated changes in its glycosylated components in Cd treated dams at days 4, 7, 10 and 15 of gestation using lectin histochemistry. $CdCl_2$ was administered to pregnant rats; control animals received sterile normal saline. Placentas were processed for DBA, Con A, SBA, PNA, UEA-I, RCA-I and WGA lectin histochemistry to evaluate changes in the carbohydrate pattern of the placenta that might modify cell interactions and contribute to embryonic alterations. Lectin binding was analyzed in the yolk sac; trophoblast giant cells; trophoblast I, II and III; spongiotrophoblast cells and endovascular trophoblast cells in the chorioallantoic placenta. Our lectin binding patterns showed that Cd caused alteration of SBA and DBA labeling of trophoblast-derived cells, which suggested increased expressions of α and β GalNAc. Cd also caused decreased UEA-1 binding affinity, which indicated fewer α-L-Fuc residues in placentas of Cd treated dams. The nonreactivity in trophoblast I of the control placentas incubated with Con-A contrasted with the labeling in placentas of experimental dams, which indicated increased expression of terminal α-D-Man, and α-D-Glc residues. We found that Cd altered the reactivity of placenta to several lectins, which indicated modification of the glycotype presented by the fetal component of the placenta. We report that Cd exerts a deleterious effect on the glycosylation pattern of the placenta.

Key words: cadmium, carbohydrate moieties, glycoproteins, lectin histochemistry, placenta, rat

Cadmium (Cd) is an industrial and environmental pollutant that produces toxic effects on male and female gametogenesis (Thompson and Bannigan 2008), placenta (Levin et al. 1987) and embryos during pre- and post-implantation stages (Thompson and Bannigan 2008). Likely underlying cellular mechanisms include altered cell adhesion, DNA damage, oxidative stress, apoptosis and estrogen mimicry (Thompson and Bannigan 2008, Samuel et al. 2011, Johnson et al. 2003).

Accumulation of Cd in human placenta and deleterious effects resulting from this are well documented (Bush et al. 2000, Jauniaux and Burton 2007, Kippler et al. 2010). Studies using laboratory rodents indicate that the placenta is particularly susceptible to toxic effects of Cd (Samariwickrama and Webb 1981, Lee et al. 2009, Turgut et al. 2007, Enli et al. 2010) including placental necrosis (Levin et al. 1987), decreased diameter and thickened basal lamina of the fetal blood vessels (Peereboom-Stegeman and Jongstra-Spaapen 1979), glycogen accumulation (Hazelhoff Roelfzema et al. 1987), clotting in capillaries, fibrin deposition and proliferation of giant cells in the labyrinthine zone (Padmanabhan 1986). Although the organization of the rodent maternal-fetal interface differs from human placenta, rats are the

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preferred model for investigating reproductive toxicology (Ain et al. 2006, Soares et al. 2012, De Rijk et al. 2002).

Expression of glycoproteins plays an important role in embryonic development and implantation, and in placenta development. Studies in rats treated with toxic compounds, such as 5-azacytidine, demonstrated altered placental glycoprotein patterns (Šerman et al. 2007). Lectins are carbohydrate-specific proteins that enable cellular recognition based on the molecular fit between pairs of complementary structures on the surfaces of interacting cells; one cell bears encoded biological information and the other can decipher the code (Lis and Sharon 1998). Lectins are structurally diverse and are of non-immune origin; they possess the unique property of recognizing and binding to carbohydrates specifically and reversibly (Gimeno and Barbeito 2004, Ghazarian et al. 2011).

Lectins are valuable tools for detecting and characterizing glycoconjugates and examining changes that occur on cell surfaces during physiological and pathological processes (Sharon and Lis 2004, Gabius and Kayser 2014). Bulmer and Peel (1996) reported that a wide variety of glycoconjugates were detected by lectins in rat trophoblast subpopulations on days 10, 12 and 15 of pregnancy. Because the effects of acute Cd exposure on the placenta are not well documented, we investigated changes in the glycosylated components of rat placenta in Cd treated dams at days 4, 7, 10 and 15 of gestation using lectin histochemistry.

Material and methods

Animals

Our use of animals was in accordance with the guidelines for care and use of laboratory animals established by the Bioethics Committee of the Veterinary Sciences School (Universidad Nacional del Centro de la Provincia de Buenos Aires). Twenty-four four-month-old 242.8 ± 22.3 g virgin female Wistar rats were obtained from the Veterinary Sciences School. Rats were housed at 22 ± 2° C, 50–60% humidity and with a 12:12 h light:dark cycle. Animals were supplied pelleted food and water *ad libitum*. Maternal weight, water and food consumption were monitored throughout gestation. No animals exhibited signs of stress or toxicity during mating, gestation or during the experiment. Females were mated with 6-month-old males (2:1). The presence of sperm in the vaginal orifice was considered day 0 of gestation (Ain et al. 2006).

Chemicals and treatments

$CdCl_2$ anhydrous powder was purchased from Sigma Chemical Co. (St. Louis, MO). Solutions were prepared freshly in sterile distilled water; all doses were 10 mg Cd^{+2}/kg body weight. The dose was selected based on earlier reports (Levin et al. 1987, Baranski 1985, Padmanabhan 1986, Padmanabhan and Hameed 1990, Zhao et al. 1997, Salvatori et al. 2004). Cd was administered by a single subcutaneous injection, which produces higher systemic concentrations than oral administration (IARC 1993, Zalups and Ahmad 2003). Intraperitoneal injection of Cd is not recommended during pregnancy in laboratory animals because it involves a risk of inadvertent administration into fat or muscle or into internal organs (Gaines Das and North 2007, Nampoothiri and Gupta 2008).

Dams were divided randomly into four groups of six animals: group 4/20 (inoculation day/sacrifice day, both in relation to gestation day) was injected with $CdCl_2$ on day 4 of gestation, group 7/20 was injected with $CdCl_2$ on day 7 of gestation, group 10/20 was injected with $CdCl_2$ on day 10 of gestation and group 15/20 was injected on day 15 of gestation. Doses were 10 µg Cd^{+2}/g body weight diluted with distilled water to 3 µg Cd^{+2}/µl. Control animals were injected with a corresponding volume of sterile normal saline (0.30 ml/100 g body weight) on days 4, 7, 10 **or** 15 of pregnancy.

Uterus and placenta collection

On day 20, dams were anesthetized with 1.4% isoflurane in 100% oxygen and euthanized by decapitation according to the protocol approved by the Bioethics Committee of the Veterinary Sciences School. Uteruses and placentas were removed and fixed in 10% buffered formalin and processed for routine histological paraffin embedding. Briefly, samples of uteruses and placentas were dehydrated through a series of alcohol (70, 85, 95 and 100%), cleared in xylene, infiltrated and embedded with paraffin, then sectioned at 5 µm. A section of every sample was stained with hematoxylin and eosin (Bancroft and Gamble 2008).

Lectin histochemistry

The procedure for lectin histochemistry has been described earlier (Fernández et al. 2000, 2014).

Briefly, sections of uterus were deparaffinized, rehydrated through a descending series of ethanols, incubated in 0.3% H_2O_2 in methanol (30 min) at room temperature, rinsed several times in 0.01 M pH 7.2 phosphate-buffered saline (PBS), and immersed in PBS containing 0.1% bovine serum albumin (BSA) for 15 min. Sections then were incubated with biotinylated lectins (Vector Laboratories Inc., Burlingame, CA) for 1 h followed by incubation with avidin-biotin-peroxidase complex (ABC) (Vector Laboratories Inc.) for 45 min. The lectin concentration was 30 µg/ml in PBS for all lectins, except for PNA, which was 10 µg/ml. The lectins were diluted with a 0.1 % BSA. The seven biotinylated lectins, their abbreviations and carbohydrate specificities are given in Table 1.

Using the standard procedure in our laboratory, horseradish peroxidase streptavidin SA-5704 (Vector Laboratories, Inc., Burlingame, CA), used as a detection system, was incubated for 30 min at room temperature. Slides were rinsed three times in PBS for 5 min each time. Liquid 3,3′ diaminobenzidine tetrahydrochloride (DAB) was used as chromogen (DakoCytomation, Carpinteria, CA). Specimens were rinsed in distilled water, dehydrated through graded ethanols, cleared in xylene and mounted in Permount (Fisher Scientific, Hampton, NH). All sections were counterstained with Mayer's hematoxylin. In addition, slides from control placentas were stained with eosin. Negative controls included blocking the lectins by incubating them with their blocking sugars (0.1 −0.2 M in PBS) for 1 h at room temperature before application to the sections and by omitting lectins from the incubation and using only their diluent (0.1% BSA)

The lectin binding intensity was scored using a semiquantitative scale as follows: (0) label absent, (1) weakly positive, (2) moderately positive and (3) strongly positive (Munson et al. 1989, Jones et al. 2007, Fernández et al. 2014). The structures examined at the maternal-fetal interface included the yolk sac in the inverted yolk sac placenta; trophoblast giant cells; trophoblast I, II and III; spongiotrophoblast cells and endovascular trophoblast cells in the chorioallantoic placenta.

Results

We observed no significant clinical symptoms in the groups exposed to Cd during the treatment period. We observed varied intensities and patterns of lectin binding in the placentas of both control and Cd exposed dams. The control placentas exhibited no staining after pre-incubation with the carbohydrate specific for each lectin to block binding; this inhibition confirmed the specificity of lectin binding. The lectin binding patterns are summarized in Table 2 and illustrated in Figs. 1–7.

Affinity for SBA lectin in control placentas (Fig. 1A) was observed only in the yolk sac and trophoblast endovascular tissue. Although no labeling was observed in most of the areas examined In the Cd exposed placentas, when lectin binding was observed, its intensity varied in trophoblast I, II and III (Fig. 1B) and in spongiotrophoblast cells. Finally, the endovascular trophoblast cells of all groups exposed to Cd exhibited strong affinity for SBA lectin.

DBA labeling was absent in control placentas (Fig. 2A). The labeling pattern in all Cd treated dams was similar to SBA in placentas exposed to Cd; however, the intensity of staining was greater in the spongiotrophoblast cells (Fig. 2B), less in trophoblast I, and absent in trophoblast II and III of placentas compared to controls.

PNA labeling was absent in virtually all areas of control and Cd exposed placentas (Fig. 3A, B), except for moderately positive labeling in tropho-

Table 1. Lectins and their specificities

Source of lectin	Abbreviation	Sugar specificity
Glycine maximus	SBA	α- and ß- GalNAc
Dolichos biflorus	DBA	α-D-GalNAc
Arachis hypogaea	PNA	ß-D-Gal (ß1-3)> D-Gal NAc
Canavalia ensiformis	Con-A	α-D-Man and α-D-Glc
Ulex europaeus-I	UEA-I	α1,2-linked fucosyl residues
Triticum vulgaris	WGA	ß-D-GlcNAc and NeuNAc
Ricinus communis-I	RCA-I	ß-Gal

Man, mannose; Glc, glucose; GlcNAc, N-acetyl glucosamine; Gal, galactose; GalNAc, N-acetylgalactosamine

Table 2. Intensity of lectin binding in placentas of control and Cd treated rats

	Yolk sac	Giant cells	Trophoblast I	Trophoblast II and III	Spongiotrophoblast	Trophoblast endovascular
SBA						
control	1	0	0	0	0	2
group 4/20	0	0	2	1	0	3
group 7/20	0	0	1	1	0–1	3
group 10/20	0	2	2–3	1	0–1	3
group 15/20	0	0	3	2	1	3
DBA						
control	0	0	0	0	0	0
group 4/20	0	0	0	0	2	2–3
group 7/20	0	0	0	0	2–3	2
group 10/20	0	0	1	0	2–3	2–3
group 15/20	1	0	1–2	0	2	2
PNA						
control	0	0	2	0	0	0
group 4/20	0	0	0	0	0	0
group 7/20	0	0	0	0	0	0
group 10/20	1	0	0	0	1	0
group 15/20	0	0	0	0	0	0
Con-A						
control	3	2	0	2	2	2
group 4/20	2	1	2	1	1	2
group 7/20	2	0–1	2–3	1–2	1	1–2
group 10/20	2	0–1	3	1	1–2	2
group 15/20	2	1–2	3	1	1–2	2
UEA-1						
control	0	2	2	1	2	0
group 4/20	1	1	0	0	0–1	0
group 7/20	1	0	0	0	0	0
group 10/20	0–1	0	0	0	0	0
group 15/20	0–1	0	0	0	0–1	0
WGA						
control	2	2	2	1	2	2
group 4/20	1	1	1–2	2	1	2
group 7/20	2–3	2	2–3	2	2	2
group 10/20	2	1–2	3	2	2	2
group 15/20	2	1– 2	3	1	1	2
RCA-1						
control	1	3	3	1	2	1
group 4/20	2–3	3	2	1–2	2	0
group 7/20	2–3	2	2	1	2	0
group 10/20	2–3	2	1 - 2	1–2	1	0
group 15/20	2	2–3	2	1–2	2	0

blast I of control dams and weak labeling in the spongiotrophoblast of group 10/20.

We observed Con-A labeling in all components of the placentas of the control groups that ranged from moderately to strongly positive, except for the trophoblast **I** (Fig. 4A). The lectin binding intensity was less in the Cd treated groups (Fig. 4B) than in controls except for the trophoblast endovascular tissue, where the intensity was similar. Trophoblast I of group 7/20 exhibited moderate to intense labeling.

UEA-1 exhibited moderate binding affinity in the placentas of control dams (Fig. 5A), except for absence of labeling in the yolk sac and the endovascular tissue of the trophoblast. Labeling was slight in placentas of Cd treated dams (Fig. 5B). The yolk sac

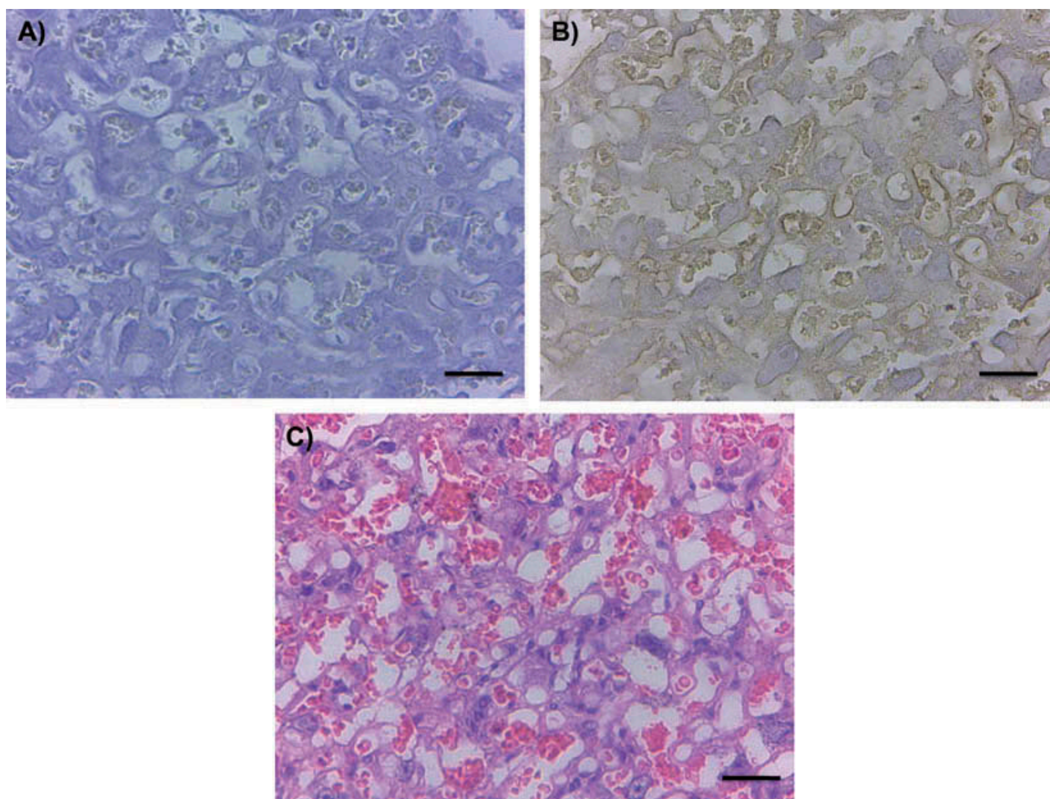

Fig. 1. SBA labeling of labyrinth zone of placentas of control (A) and Cd treated dams of group 10/20 (B) showed no labeling and moderate labeling, respectively. For clarity, an H & E stained section of the labyrinth zone of placenta of a control dam (C) is shown. The labyrinth zone shows maternal blood spaces containing non-nucleated red blood cells (asterisk) and embryo-fetal blood vessels (arrowhead) containing nucleated red blood cells. Interstitial trophoblast cells are located among the vasculature. Scale bars = 10 μm.

exhibited no labeling in control dams and weak or no labeling in Cd exposed dams.

WGA binding intensity was variable among the structures of the control and experimental placentas evaluated (Fig. 6A, B). In control placentas, all components showed weakly positive labeling except for trophoblast II and III. Placentas of treated dams showed strong labeling in trophoblast I in older placentas.

RCA-1 lectin labeled variably all of the areas evaluated in placentas of control and Cd-treated groups (Fig. 7A, B). A remarkable exception was the lack of labeling in trophoblast endovascular tissue of the experimental groups.

Discussion

We used lectin histochemistry to determine the effects of Cd on the carbohydrate composition of the maternal-fetal interface in rats. Our findings for control placentas were consistent with an earlier report (Bulmer and Peel 1996) These investigators identified a glycosylation pattern for trophoblast-derived cell subpopulations, i.e., absence of reactivity to DBA in all structures and absence of reactivity to PNA except trophoblast I. We used lectin histochemistry to determine the effects of Cd on the carbohydrate composition of the maternal-fetal interface in rats. Our results for control placentas were consistent overall with an earlier report (Bulmer and Peel 1996) that demonstrated differential reactivity of 17 lectins between specific regions of the rat uterus during pregnancy. Our finding of no reactivity to DBA in any of the cell populations and tissues that we studied are consistent with the earlier report (Bulmer and Peel 1996). Our findings also are consistent with the earlier report regarding the non-reactivity to PNA (Bulmer and Peel 1996), exception for trophoblast I in our study.

The lectin binding patterns that we observed showed that Cd caused alteration of the SBA and DBA labeling of trophoblast-derived cells, which suggested increased expressions of α- and β-GalNAc. Cd also caused decreased UEA-1 binding affinity, which indicated fewer α-L-Fuc residues in

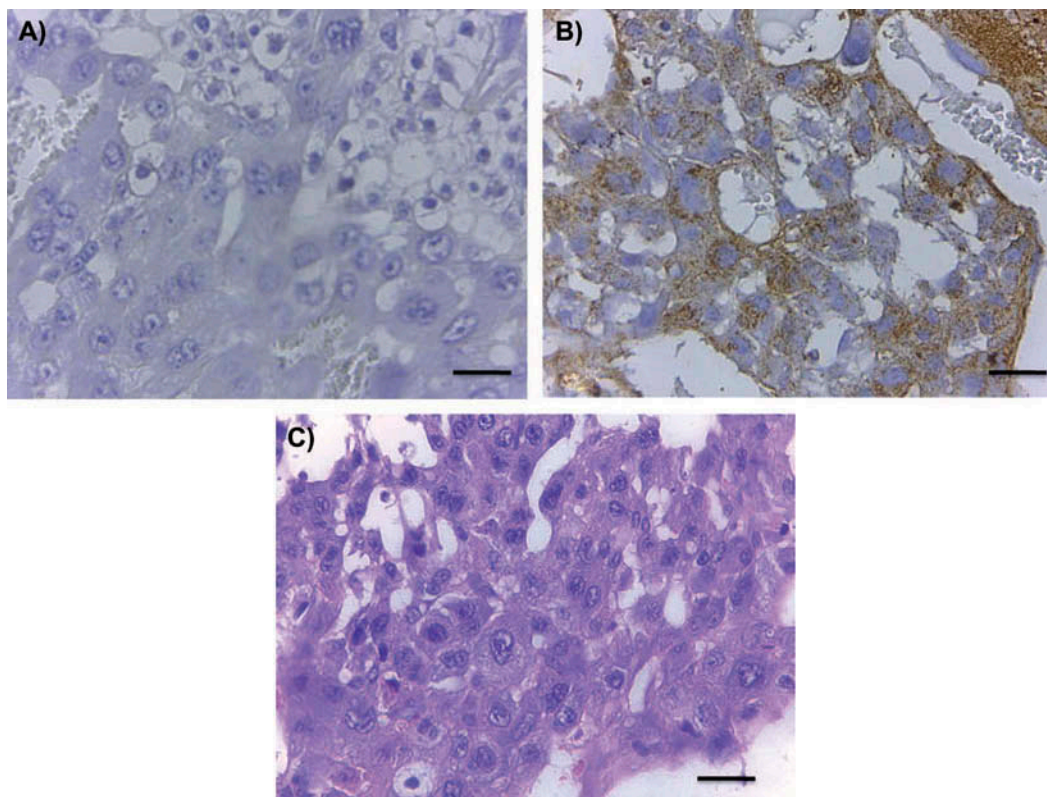

Fig. 2. DBA labeling of spongiotrophoblasts of placentas of control (A) and Cd treated dams of group 10/20 (B). The control placentas were negative and Cd treated placentas showed strong labeling. An H & E stained section of the spongiotrophoblast of placenta of a control dam is shown for reference (C). Spongiotrophoblast cells are the main constituents of the trophospongiosum or junctional zone; fetal capillaries do not penetrate it. Channels depicted are maternal blood vessels. Scale bars = 10 μm (A), 5 μm (B and C).

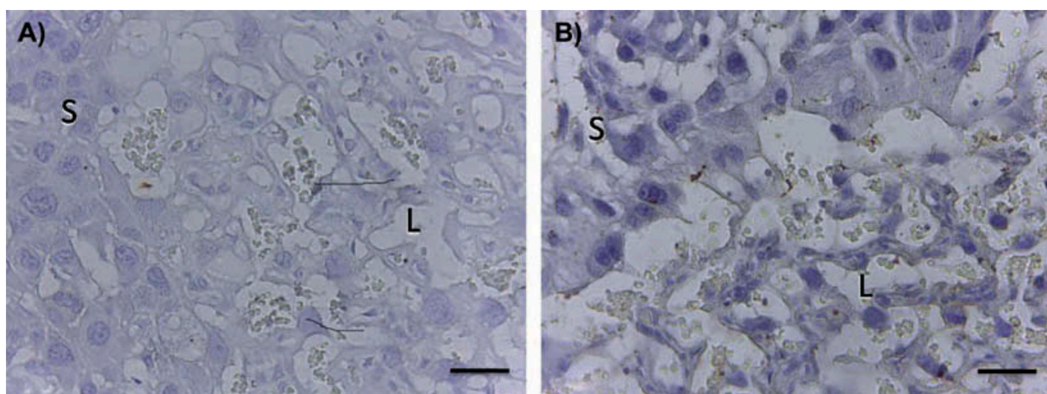

Fig. 3. Spongiotrophoblast (S) and labyrinth zone (L) of control (A) and Cd treated dams of group 7/20 (B) showed no PNA labeling. Scale bars = 10 μm.

placentas of Cd treated dams. The nonreactivity in trophoblast I of the control placentas incubated with Con-A differed from the positive labeling in placentas of experimental dams, which indicated increased expression of terminal α-D-Man, and α-D-Glc residues.

Although transient, the initial interaction between trophoblast and uterine epithelium in species with hemochorial placentation is important for successful implantation (Aplin and Jones 2012). Molecular recognition between the trophoblast and the endometrium involves direct intercellular

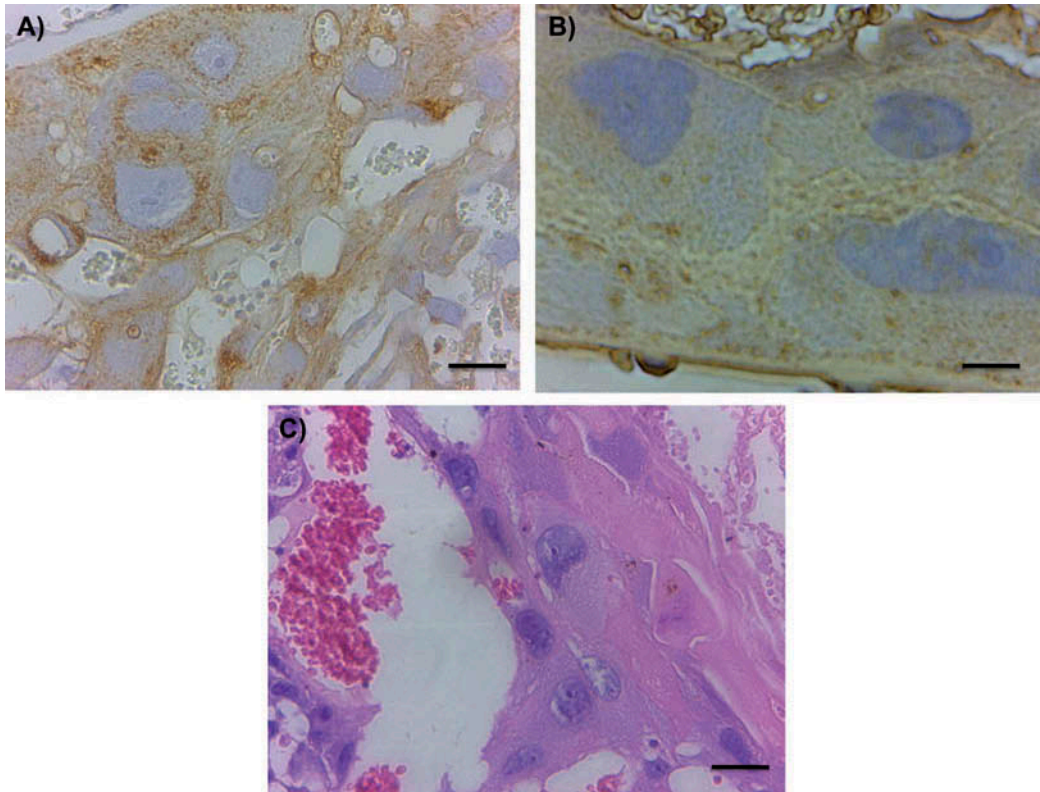

Fig. 4. Giant cells of control (A) and Cd treated dams of group 7/20 (B) labeled with Con-A showed moderate and weak labeling, respectively. H & E stained giant cells of placenta of a control dam are shown for reference. Giant cells are mononucleated and their cytoplasm is vacuolated and slightly basophilic. Scale bars = 50 μm (A and C) and 100 μm (B).

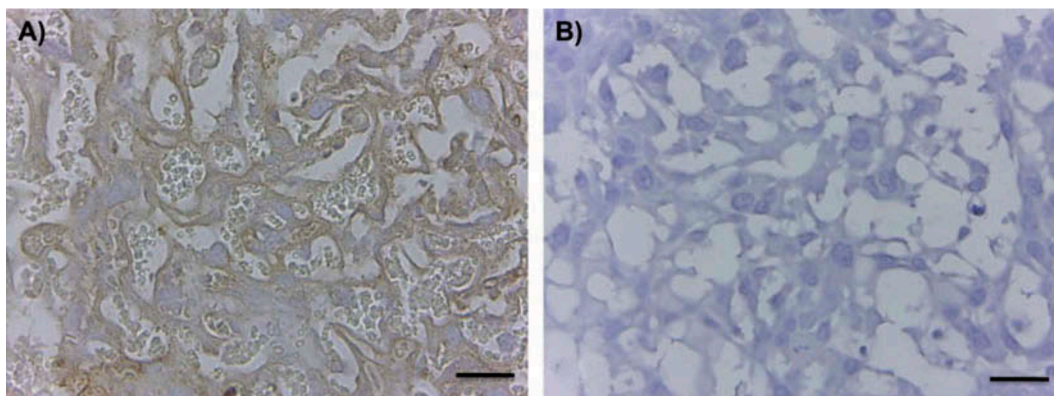

Fig. 5. UEA-1 labeling of labyrinth zone of placentas of control (A) and Cd treated dams of group 15/20 (B) show moderate labeling (A) and no labeling (B), respectively. Scale bars = 10 μm.

contact that is caused by calcium-activated adhesion proteins such as tenascin and E-cadherin (Slater et al. 2002). Jones and Aplin (2009) introduced the concept of a fetomaternal glycocode: fetal and maternal surfaces each have a specific pattern of glycosylation and the proper glycotype must be recognized by a complementary apposing surface for successful implantation. We found that Cd modified the affinity for several lectins, which so produced a glycotype that was altered from that presented by the fetal component of the control placentas. Cd also caused changes in the lectin binding of the uterine epithelium during early pregnancy (gestation days 4 and 7, data not shown), which suggests that Cd may affect both fetal and maternal glycotypes.

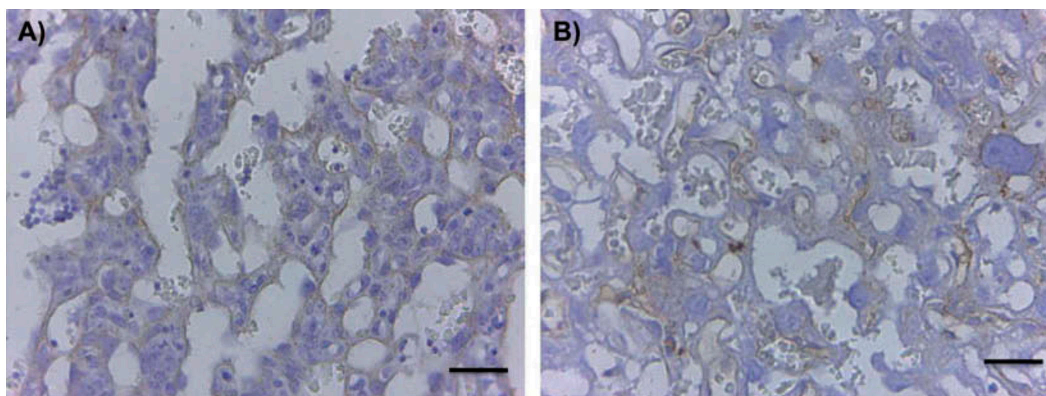

Fig 6. WGA labeling of labyrinth zone of placentas of control (A) and Cd treated dams of group 10/20 (B). Both zones showed moderate labeling. Scale bars = 10 µm.

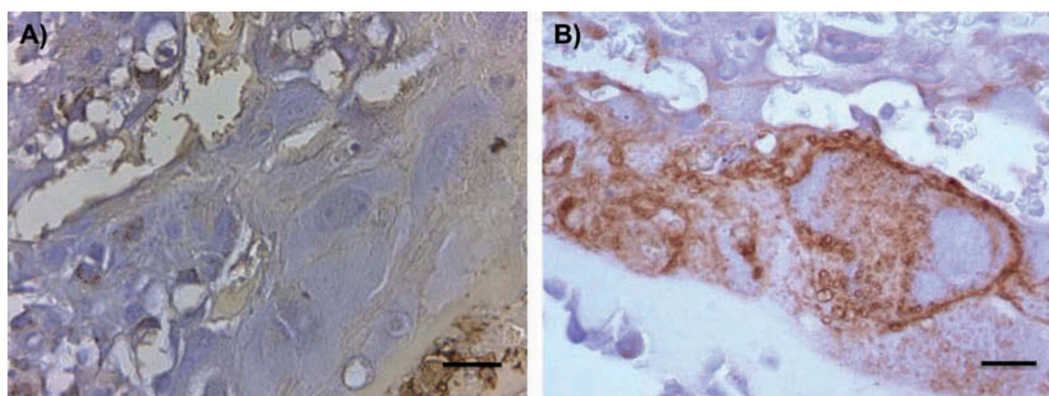

Fig. 7. Giant cells of placentas of control (A) and Cd treated dams of group 4/20 (B) labeled with RCA-1. Cells of control placenta showed moderate labeling, whereas cells of Cd exposed placentas showed strong labeling. Scale bars = 200 µm.

Cd induced changes in the fetal glycotype may have other consequences as well. Surface glycoproteins have been associated with many physiologic and pathologic processes (Gabius and Kayser 2014) including cell death (Lichtenstein and Rabinovich 2013, Templeton and Liu 2010). Murine trophoblast cells are susceptible to the deleterious effects of Cd (Thompson and Bannigan 2008). Cd induced cell death of rat placental trophoblast cells has been reported (Lee et al. 2009, Pillet et al. 2006), which leads to placental toxicity. In addition, Cd affects steroid synthesis and possesses estrogen- and androgen-like activities (Johnson et al. 2003, Darbre 2006, Byrne et al. 2009, Kortenkamp 2011). Lee et al (2009) reported that Cd affected development adversely by causing decreased placental and fetal weights and the number of live fetuses, and increased numbers of resorptions, dead fetuses and post-implantation losses. Using the same experimental design, we found earlier that Cd increased the number of embryonic resorptions in Cd treated dams (Díaz et al. 2014). The local remodeling of the glycocalyx of both fetal and maternal components of the placenta caused by Cd may have contributed to embryonic death.

Aplin and Jones (2007) reported that among hemochorial placentas of the lesser hedgehog tenrec *(Echinops telfairi)*, spotted hyena *(Crocuta crocuta)*, nine-banded armadillo *(Dasypus novemcinctus)*, human *(Homo sapiens)* and guinea pig *(Cavia porcellus)*, DBA binding was restricted to the cytotrophoblast of the lesser hedgehog tenrec. Stewart et al. (2000) reported no DBA affinity in mouse placenta. We found that many placental components exhibited no binding for DBA in either control or experimental placentas; however, some structures of the treated groups, such as the endovascular tissue of the trophoblast and spongiotrophoblast, were stained.

Implantation of the embryo into the uterine wall is a requirement for development. The role of glycoproteins in the trophoblast-uterine

interaction during implantation has been studied (Aplin and Kimber 2004). The glycan portions of glycoproteins are synthesized in a non-template process.

Both genetic and environmental factors participate in shaping the final structure of the glycoproteins (Lauc et al. 2014). Genetic factors comprise the "glyco-genes," i.e., the genes that codify glycosyltransferases and glycosidases as well as other genes that are involved in the complex biosynthetic pathways of glycans (Lauc et al. 2014).

Many glyco-genes that play roles in normal development of the placenta are regulated by epigenetics (Nelissen et al. 2011, Rugg-Gunn 2012, Lauc et al.2014). It has been demonstrated that Cd can induce various epigenetic changes in mammalian cells both in vitro and in vivo (Wang et al. 2012). The possibility that Cd exerts an epigenetic effect on the glycosylation pattern of the rat placenta cannot be ruled out.

We used lectin histochemistry to both define and monitor the damaging effects of Cd and to map characteristics of the carbohydrate moieties in fetal placental components. Our findings demonstrated that Cd administration at different times during pregnancy caused changes in the glycosylation pattern of the fetal component of the rat placenta.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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