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Molecular mechanisms of Cisplatin- induced placental toxicity and teratogenicity in rats and the ameliorating role of N-acetyl-cysteine

Mohamed S. Hassan[a], Ashraf M. Morgan[a,*], Mohey M. Mekawy[a], M.A. Zeineb[b]

[a] Dept. of Toxicology & Forensic Med. Faculty of Vet. Med., Cairo Univ., Egypt
[b] Dept. of Zoology, Faculty of Science, Omar El-Moghtar Univ., Libya

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ABSTRACT

The aim of the present study is to investigate the molecular mechanisms of Cisplatin- induced placental toxicity and teratogenicity in rats and the ameliorating role of N-acetyl-cysteine (NAC). Cisplatin was administrated intraperitoneally at 5 mg/kg.b.wt as a single dose on the 12th day of gestation while NAC was administered orally throughout gestation either alone or in concomitant injection of Cisplatin at 200 mg/kg.b.wt. Cisplatin + NAC group showed reduction in the elevated morphological, visceral and skeletal abnormalities as well as the morphological and histopathological changes in placenta compared to Cisplatin - treated rats. Importantly, NAC attenuated Cisplatin-induced placental apoptosis through down-regulation of Fas and Caspase-3 genes expression. In conclusion, induction of placental apoptosis by overexpression of Fas and Caspase-3 genes gives a new insight into the mechanism of Cisplatin teratogenicity. The protective role of NAC, on the other hand, was characterized by attenuation of Fas and Caspase-3 genes- mediated apoptosis.

1[1] Introduction

Cisplain, the first generation anticancer platinum complex, is one of the most effective anticancer agents, displaying clinical activity against a wide variety of solid tumors (Siddik, 2003). It is currently available for the treatment of testicular, ovarian, and bladder carcinomas but its clinical usefulness has frequently been limited by undesirable side effects such as nephrotoxicity, gastrointestinal toxicity, ototoxicity, and neurotoxicity. In addition, low activity for certain kinds of cancers, such as breast and colon cancers, limits its clinical use (Cooley et al., 1994; Bruno et al., 2003).Therefore, extensive efforts have been made to develop new Cisplatin analogs with higher or equivalent antitumor activity and lower toxicity (WHO, 1992).

Van Cutsem et al. (2006) examined the phase III study of docetaxel and cisplatin plus fluorouracil (CF) compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer. They concluded that, adding docetaxel to CF significantly improved time-to-progression survival, and response rate in gastric cancer patients, but resulted in some increase in toxicity. Sequist et al. (2013) studied the phase III of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with epidermal growth factor receptor (EGFR) mutations. They concluded that afatinib is associated with prolongation of progression free survival when compared with standard doublet chemotherapy in patients with advanced lung adenocarcinoma and EGFR mutations.

Cisplatin plus gemcitabine was associated with a significant survival advantage without the addition of substantial toxicity. Cisplatin plus gemcitabine is an appropriate option for the treatment of patients with advanced biliary cancer, Valle et al. (2010). Wu et al. (2014) mentioned that afatinib versus cisplatin plus gemcitabine for first-line treatment of Asian patients with advanced non-small-cell lung cancer (NSCLC) harbouring EGFR mutations (LUX-Lung 6): an open-label, randomised phase 3 trial. Their result indicated that the first-line afatinib significantly improves progression-free survival with a tolerable and manageable safety profile in Asian patients with EGFR mutation-positive advanced lung NSCLC.

Cisplatin exposure has been linked to many adverse effects on female reproduction. This was recorded as ovarian toxicity (affects their morphology) (Nozaki et al., 2009); embryonal resorptions (Bajt and Aggarwal, 1985 and Muranaka et al., 1991); pre-implantation blastopathies and post-implantation embryotoxicity (Giavini et al., 1990); teratogenic and embryolethal effects (Muranaka et al., 1991 and Emanuela et al., 2006); extensive Cisplatin-DNA adduct formation in fetal brain and liver mitochondria (Giurgiovich et al., 1997) and renal malformations in treated chick embryos (Náprstková et al., 2003).

Cisplatin-procaine complex can cause embryotoxic effects if

* Corresponding author.
E-mail address: ashrafmorgan550@yahoo.com (A.M. Morgan).
[1] 1. Introduction

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administered during the period of organogenesis and early histogenesis (Ognio et al., 2003, 2006). Beside embryo-lethality, it induced growth retardation and malformations in surviving fetuses. A significant reduction of maternal weight gain was observed in pregnant mice after the administration of Cisplatin-procaine complex (Ognio et al., 2006).

The role of cysteine with and without other antioxidants (as vitamin E, Crocus sativus and Nigella sativa) in the prevention of Cisplatin toxicity was confirmed by various investigators as Değirmenci et al. (1996); El-Daly (1998), Lopez-Gonzalez et al. (2000), Feghali et al. (2001), Blakley et al. (2002), Schaaf et al. (2002), Previati et al. (2004) and Shalby et al. (2011).

Lopez-Gonzalez et al. (2000) mentioned that the mechanism of Cisplatin- induced ototoxicity is based on the generation of reactive oxygen species, which interferes with the antioxidant protection of the organ of Corti. Conversely, the protection of the cochlea with antioxidants (melatonin or an antioxidant mixture of alpha-tocopherol acid succinate, ascorbic acid, glutathione, and N-acetylcysteine) ameliorates the ototoxicity by Cisplatin.

Feghali et al. (2001) demonstrated *in vitro* studies that L-n-acetylcysteine (L-NAC) protected both auditory neurons and hair cells from the toxic effects of Cisplatin. Because it protects both of these inner ear structures, L-NAC may be potentially useful in protecting hearing, in general, from Cisplatin-induced damage. In addition, L-NAC has low systemic and mucosal toxicity. It also has a low molecular weight that may allow it to readily cross the round window membrane. All these characteristics make it potentially suitable for transtympanic application for the prevention of the ototoxicity of Cisplatin *in vivo*.

Previati et al. (2004) tested the effectiveness of butylatedhydroxytoluene (BHT), dithiothreitol (DTT) and N-acetylcysteine (N-Ac) as cyto-protectants, all of them reduced protein carbonylation to control levels and significantly protected OC-k3 from Cisplatin (CDDP) -induced cell death, with a higher protection when using the lipophylic antioxidant BHT.

It has been reported that treatment with taurine (TAU) or N-acetylcysteine (NAC) after Cisplatin (CP) administration significantly ameliorated CP-induced nephritic oxidation stress markers as compared with CP alone-treatment. On the other hand, treatment with TAU or NAC after CP administration significantly ameliorated CP-induced nephritic inflammation with possible attenuation of renal injury. The inhibition in oxidative stress and the elevation of the total antioxidant capacity as well as the inhibition of the inflammatory biomarkers by NAC or TAU after CP administration may play a central role in modulation of nephrotoxicity induced by CP (Shalby et al., 2011).

There are few trials investigating antioxidant supplementation in female reproduction. However, before clinicians recommend antioxidants, randomized controlled trials with sufficient power are necessary to prove the efficacy of antioxidant supplementation in disorders of female reproduction. Serial measurement of oxidative stress biomarkers in longitudinal studies may help delineate the etiology of some of the disorders in female reproduction such as preeclampsia (Agarwal et al., 2005).

1.1. Aim of Work

Cancer is a very dangerous disease which may be detected during pregnancy and Cisplatin, as an antineoplastic drug, could be urgently used in its treatment resulting in serious side effects on both dams and their embryos. In addition, the literature concerning the use of NAC as an antioxidant protector against Cisplatin- induced reproductive toxicity and teratogenicity are scanty. Therefore, our study aims at studying the molecular mechanisms of Cisplatin- induced placental toxicity and teratogenicity in albino rats and the ameliorating role of N-acetyl-cysteine in alleviating these toxic effects.

2. Materials and Methods

2.1. Materials

2.1.1. Animals

Eighty mature female albino rats, of body weight 180-200 g, were used as lab animals in this study. They were obtained from the National Institute of Ophthalmology, Giza, Egypt. Upon arrival, they were housed in metallic boxes with wood chip bedding and ad libitum access to clean tap water and balanced ration. Animals were maintained under hygienic conditions with natural air ventilation and a 12-hr light/dark cycle. All animals were kept under observation along two weeks before the start of the experiment for acclimatization.

2.1.2. Chemicals

All chemicals used in this study were analytically pure:

(1) The tested compound: Cisplatin (CP):
Trade Name: Cisplatin, Cisplatyl, Cytoplatino, Neoplatin, Placis, Platiblastin, Platinex, Platinol, Platistil, Platistin, Platosin.
(2) The tested protector: N-acetyl-l-cysteine (NAC):

2.2. Methods

2.2.1. Methods used for teratogenicity investigation

After quarantine period, the females were introduced into sexually mature and non-treated males and paired on a one-to-two basis usually in the early afternoon and left overnight. Pregnancy was confirmed in the following morning by the presence of sperms in the vaginal washing of each female and considered as the zero-day of pregnancy (Manson and Kang, 1994).

a) **Experimental design:**
The pregnant females were divided into four equal groups, 20 animals for each as following:
Group 1 (Control group): It was administered distilled water orally on days 0 - 20 of gestation and given physiological saline ip on day 12 of gestation.
Group 2 (Cisplatin group): It was given Cisplatin intraperitoneally at a dose of 5 mg/kg.b.wt as a single dose (Kopf-Maien and Erenswick 1985) on the 12th day of gestation.
Group 3 (NAC group): It was orally administered NAC in distilled water at a dose of 200 mg/kg daily on days 0-20 of gestation (Dickey et al., 2008).
Group 4 (Cisplatin + NAC group): It was administered NAC daily by oral intubation at a dose of 200 mg/kg.b.wt in distilled water on days 0-20 of gestation and injected with Cisplatin intraperitoneally at a dose of 5 mg/kg.b.wt as a single dose on the 12th day of gestation.
b) **Scheduled time and procedures of teratogenic examination:**
All control and treated female groups were killed under gaseous anesthetic chloroform just prior to the calculated date of delivery (at the 20th day of gestation). After that an incision was made in the abdominal wall to expose the abdominal viscera. The gravid uterus of each dam was exteriorized then the numbers of uterine implants, early and late resorptions, live and dead fetuses were counted and examined for morphological, visceral, and skeletal abnormalities according to the method described by Manson and Kang (1994).
The fetuses and placentas were weighed and examined for gross external abnormalities. Two placentas / litter were used for histopathological and genotoxicity investigations. The remained fetuses were divided into one third kept in Bouin's fixative for at least one week, after which, fetuses were sectioned using Wilson's free-hand razor blade sectioning technique as described by; searching for internal visceral malformations. The other two thirds were kept in ethanol for subsequent preparation for skeletal examination.

2.2.2. Methods used for the histopathological examination

The placentas intended for histopathological investigation were fixed in 10% neutral formalin and prepared for examination according to Bancroft and Stevens (1996).

2.2.3. Methods used for the genotoxicity investigation (Quantification of placental Fas and Caspase-3 genes mRNAs "apoptosis markers" levels of expressions using real-time RT- PCR

2.2.3.1. Total RNA extraction. RNA isolation was done by Total RNA Purification Kit (Jena Bioscience, Cat. No. PP-210S) according to the manufacturer instructions.Both the concentration and purity of RNA were determined by Nanodrop ND1000.

2.2.3.2. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). RT-PCR was done by using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Cat. No. #K1622).

2.2.3.3. Real-time PCR (qPCR). Real-time PCR (qPCR) was done by Luminaries Color HiGreen Low ROX qPCR Master kit (Thermo Scientific, Cat. No. # K0371).

Each assay included triplicate samples for each tested cDNAs and no-template negative control. Each reaction contained 30 pg/ml of each of the following primers: Caspase-3 5-CACGGTGATGAAGGAGTC-3; 5-GCAAGCCTGAATAATGAA-3, Fas 5-CAAACCAGCAACACCAAATGC-3; 5-CCGCAAGAGCACAAAGATTAGC-3, GAPDH- 5-TCACCATCTTCCAGG AGCGA-3; 5-CACAATGCCGAAGTGGTCGT-3.

Data were normalized by using GAPDH gene as a housekeeping gene (Morgan et al., 2017). Two-steps cycling protocol was adjusted as follows; UDG pretreatment at 50 °C for 2 minutes, Initial denaturation at 95 °C for 10 minutes then 45 cycles of denaturation at 95 °C for 20 seconds and annealing/extension at 60 °C for 45 seconds. Fluorescent data were acquired during each extension phase. The fold change compared to control samples was calculated using CT, ΔCT, ΔΔCT by Mxpro software Stratgene.

2.2.4. Methods used for statistical Analysis

Our data are recorded as percentages and means ± standard error (SE). Statistical significance of fetal and placental weights and gene expression were determined by one way ANOVA while Chi-square test was used for the comparison of the different morphological, visceral and skeletal anomalies between treated and control groups (SPSS; statistical package for social sciences 10.0 for windows) as adopted by Alan and Duncan (2001).

3. Results

The effects of Cisplatin exposure (ip at 5 mg/ kg on the 12th day of gestation) alone and with NAC (at a daily oral dose of 200 mg/kg on days 0-20 of gestation) on the morphology of the rat fetuses obtained from exposed dams are shown in Table 1 and presented in Figs. 1–6. Cisplatin produced significant elevation in the percentages of late resorption sites and dead fetuses compared with the control group.

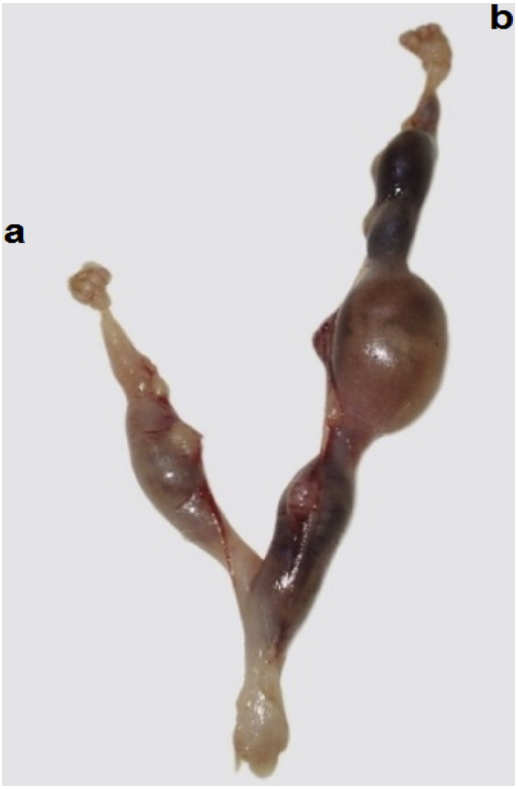

Fig. 1. Uterus of a pregnant female rat exposed to Cisplatin at 5 mg/ kg ip on 12th day of gestation showing resorption sites in the two horns (a) and a live fetus in the right horn (b).

The mean fetal and placental weights were significantly reduced in Cisplatin group compared with the control one. The percentages of the dwarf fetuses and fetuses that had s/c hemorrhage were significantly increased in Cisplatin group.

The effects of Cisplatin injection (i/p at 5 mg/ kg on the 12th day of gestation) alone and with NAC (at a daily oral dose of 200 mg/kg on days 0-20 of gestation) on the visceral organs of the obtained rat fetuses are shown in Table 2 and Figs. 7–10. They revealed that ip injection of pregnant dams with Cisplatin induced a significant elevation in the percentages of the offsprings that had visceral organs' anomalies compared to the control ones (Figs. 7–10).

Table 1
Morphological abnormalities of rat fetuses obtained from control and treated dams.

Group	Parameter												
	No. of pregnant dams	No. of uterine implants	Late resorption Sites		Dead fetuses		Live fetuses		Mean fetal weight (g)	Mean placental weight(g)	External morphological abnormalities		
											Dwarfism		S/c edema and Hemorrhage
			No.	%	No.	%	No.	%			No.	%	No. %
Control	20	200	0	0	1	0.5	199	99.50	4.09 ± 0.03	0.59 ± 0.003	0	0	0 0
Cisplatin	20	175	30	17.14[a]	45	25.71[a]	100	57.14[a]	3.09[a] ± 0.04	0.48[a] ± 0.003	25	14.28[a]	29 16.57[a]
NAC	20	194	0	0	2	1	192	98.96	3.98 ± 0.40	0. 58 ± 0.004	0	0	0 0
Cisplatin +NAC	20	183	29	15.85[a]	42	22.95[a]	112	61.20[a]	3.20[a] ± 0.109	0.50[a] ± 0.002	22	12.02[a]	25 13.66[a]

[a] Significant difference between control and treated groups at $p \leq 0.05$.

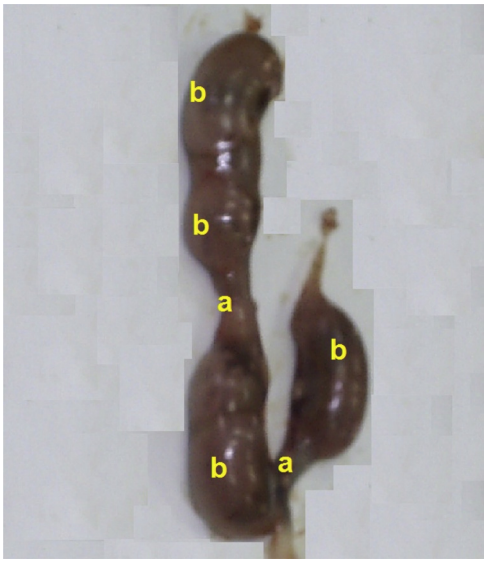

Fig. 2. Uterus of a pregnant female rat exposed to Cisplatin at 5 mg/kg ip on 12th day of gestation and NAC at 200 mg/kg on gestation days 0- 20 showing 2 resorption sites (a) and live fetuses (b) in the two horns.

Fig. 3. Two rat fetuses the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/kg ip on 12th day of gestation showing dwarfism and general morphological deformities (rounded head (a), micrognathia (b) and abnormal extremities (c).

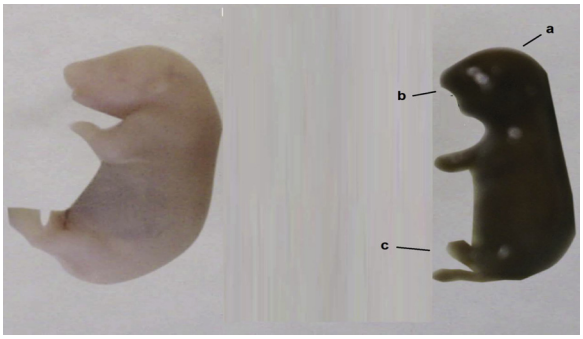

Fig. 4. Two rat fetuses the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/kg ip on 12th day of gestation showing general morphological deformities [rounded head(a), micrognathia (b) and abnormal extremities (c)] and S/C hemorrhage.

The effects of Cisplatin exposure (ip at 5 mg/ kg on 12th day of gestation) alone and with NAC (at a daily oral dose of 200 mg/kg on days 0-20 of gestation) on the skeleton of the rat fetuses obtained from exposed dams are shown in Table 3 and Figs. 11–15. Many skeletal abnormalities in the examined fetuses from Cisplatin- treated dam

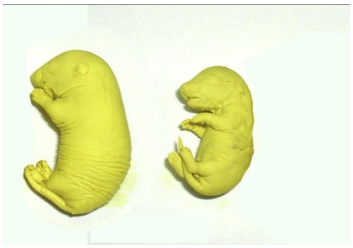

Fig. 5. Two rat fetuses the left one is control and the right one obtained from a treated pregnant dam with Cisplatin at 5 mg/kg ip on 12th day of gestation and NAC at 200 mg/kg on days 0-20 showing dwarfism.

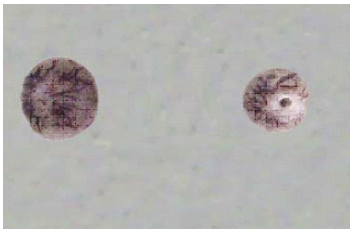

Fig. 6. Two placentas of a rat fetus; the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/ kg on 12th day of gestation showing reduction in size.

groups were observed in skull bones, sternum, phalanges, sacral and caudal vertebrae. They revealed that ip injection of pregnant dams with Cisplatin induced a significant elevation in the percentages of the offsprings that had skeletal anomalies compared to the control group.

Oral administration of NAC on days 0-20 of gestation in concomitant injection of Cisplatin (on the 12th day of gestation) showed reduction in the elevated percentages of offspring that had morphological, visceral and skeletal abnormalities compared with that recorded in Cisplatin - treated group but this effect was statistically insignificant.

NAC alone treated group did not show any significant variation in the percentages of rat fetuses that had morphological; visceral and skeletal abnormalities compared with those recorded in the control group.

The effects of Cisplatin treatment (ip at 5 mg/ Kg.) alone and with NAC (at a daily oral dose 200 mg/kg on gestation days 0- 20 of gestation) on the placental morphology and pathology are shown in Table 1, Figs. 6 and 16–21. They revealed that Cisplatin induced depression in the placental size (Fig. 6) with congestion and haemorrhages compared to the control. Also, it induced pathological alterations in the form of thickening and hyalinization in the giant cells; necrosis and apoptosis in the trophoblasts (Fig. 16) and labyrinth (Fig. 17); focal haemorrhage in labyrinth (Fig. 18) and edema surrounding the villi.

In Cisplatin + NAC treated group the placentas showed mild hyalinosis in the giant cells (Fig. 19) and ischaemia in the underlying labyrinth (Fig. 20). In addition, mild edema was observed in the surrounding villi (Fig. 21). The placentas of NAC group didn't show any pathological alterations.

Cisplatin exposure resulted in a significant increase in the level of placental Fas (10.50 folds) and Caspase-3 (22.20 folds) genes expression compared to control. However, NAC treatment induced an insignificant increase in the expression level of Fas (0.83 folds) gene expression and an insignificant decrease in the level of Caspase-3 (0.85 folds) gene expression compared to control. On the other hand, Cisplatin plus NAC exposure induced a significant decrease in the level of placental Fas (4.26 folds) and Caspase-3 (9.46 folds) genes expression compared to the Cisplatin alone- exposed rats (Table 4).

4. Discussion

Cis-diamminedichloroplatinum(II) or cisplatin is a DNA-damaging

Table 2
Visceral malformations of rat fetuses obtained from control and treated dams.

Group	Parameter														
	No. of examined fetuses	Malformations of													
		Head						Chest				Abdomen		Pelvis	
		Nares		Eyes		Brain		Heart		Lung		Liver		Kidneys	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Control	67	0	0	0	0	1	1.49	0	0	0	0	0	0	0	0
Cisplatin	48	19	39.58[a]	16	33.33[a]	15	31.25[a]	16	33.33[a]	14	29.16[a]	20	41.66[a]	18	37.5[a]
NAC	65	0	0	0	0	1	1.53	0	0	0	0	0	0	0	0
Cisplatin + NAC	51	17	33.33[a,b]	14	27.45 [a,b]	15	29.41[a]	13	25.50[a,b]	14	27.45[a]	18	35.29[a,b]	18	35.29[a]

[a] Significant difference between control and treated groups at $p \leq 0.05$.
[b] Significant difference between Cisplatin and NAC groups at $p \leq 0.05$.

Fig. 7. Transverse sections in the head of two rat fetuses, the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/kg ip on 12th day of gestation showing dilated nostrils.

Fig. 8. Transverse sections in the head of two rat fetuses, the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/kg ip on 12th day of gestation showing anophthalmia.

Fig. 9. Transverse sections in the chest of two rat fetuses, the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/kg ip on 12th day of gestation showing heart hypertrophy and intra-thoracic hemorrhage.

agent that is widely used in cancer chemotherapy. Its cytotoxic action is mediated by its interaction with DNA to form intra- and inter-strand crosslink adducts, which activate several signal transduction pathways, including those involving ATR, p53, p73, and mitogen-activated protein kinase (MAPK), and culminate in the activation of apoptosis (Jordan and Carmo-Fonseca, 2000; Siddik, 2003; Zhu et al., 2013). It has serious side effects as teratogenicity (Sorsa et al., 1985); nephrotoxicity (Horiguchi et al., 2006; Yao et al., 2007); neurotoxicity (Ta et al., 2006;

Fig. 10. Transverse sections in two rat fetuses, the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/kg ip on 12th day of gestation showing dilated renal pelvis.

Yao et al., 2007) and hepatotoxicity (Martins et al., 2008).

In the present study there was no evidence of maternal toxicity at the tested dosage level of Cisplatin during organogenesis. Cisplatin produced significant elevation in the percentages of morphological; visceral and skeletal anomalies compared to the control group.

These results are concomitant with those recorded by Köpf-Maier and Merker (1983) and Ognio et al. (2006) in mice; Keller and Aggarwal (1983) in mice and rats; Bajt and Aggarwal (1985) and Giavini et al. (1990) in rats. Also, our results are in agreement with those recorded by WHO (1991) which has been reported that platinum wire inserted into the uterus of rats was found to reduce the implantation of fertilized ova. An 83% reduction in the number of implantation sites in the affected uterine horn, compared to the unoperated horn, was found in rats unilaterally implanted on day 3.

It is argued that the apparent teratogenic inefficiency of cis-platinum on days 10 and 11 of murine pregnancy is caused by the inability of cis-platinum to pass the placental barrier at this stage of pregnancy (Köpf-Maier and Merker, 1983). This could explain the selection of the 12th day of gestation for Cisplatin ip injection in our study. Keller and Aggarwal (1983) reported that Cisplatin embryolethality in rats and mice was gestational stage-specific with the highest mortality corresponding to the period of rapid DNA replication in early organogenesis. It is proposed that the cause of Cisplatin-related embryonal resorption in rats may be due to decreases in serum concentrations of prolactin, luteinizing (LH), and progesterone hormones concentrations observed after drug treatment (Bajt and Aggarwal, 1985).

The recorded renal anomalies in our results are consistent with the results of Daugaard (1990); WHO (1992) and Horiguchi et al. (2006) who confirmed Cisplatin - induced nephrotoxicity. Anand and Bashey (1993) reported that Cisplatin is one of the most effective agents available for treating a variety of solid tumors but nephrotoxicity is the dose-limiting factor for the use of this drug. Moreover, the toxic effects of the drug in man and animals include ototoxicity, neurotoxicity and bone marrow suppression, but its chief dose-limiting side effect is nephrotoxicity (Arany and Safirstein, 2003; Boulikas and Vougiouka,

Table 3
Skeletal malformations of rat fetuses obtained from control and treated dams.

Group	Parameter											
	No. of Examined Fetuses	Skull Bones				Sternum		Phalanges		Sacral vertebrae		Caudal vertebrae	
		Wide open fontanel		Incomplete ossification of parietal and/or interparietal bones									
		No.	%	No.	%	No.	%	No.	%	No.	%	No	%
Control	133	1	0.75	0	0	0	0	0	0	0	0	0	0
Cisplatin	97	30	30.92[a]	22[a]	22.68[a]	28	28.86[a]	26	26.80[a]	20	20.62[a]	32	32.98[a]
NAC	129	2	0	0	0	0	0	0	0	0	0	0	0
Cisplatin +NAC	103	29	28.15[a]	20	19.41[a]	26	25.24[a]	24	23.30[a]	19	18.44[a]	30	29.12[a]

[a] Significant difference between control and treated groups at $p \leq 0.05$.

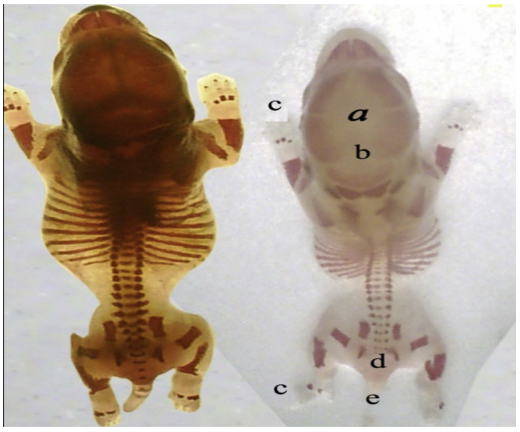

Fig. 11. Skeleton of two rat fetuses, the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/kg ip on 12th day of gestation showing wide open fontanel (a), incomplete ossification of parietal and interparietal bones (b), absence of phalanges (c), sacral (d), and caudal vertebrae (e).

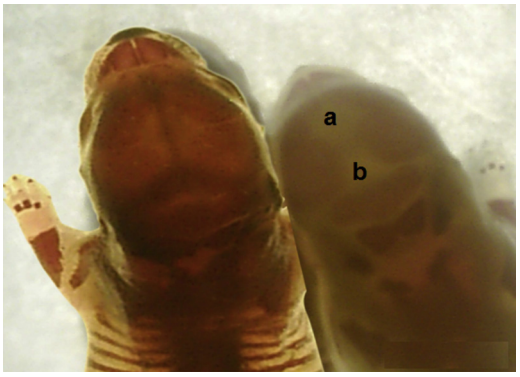

Fig. 12. Skeleton of two rat fetuses, the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/ Kg on 12th day of gestation and NAC at 200 mg/ kg on gestation days 0- 20 showing wide open fontanel(a) and incomplete ossification of parietal and interparietal bones(b).

2003 and Sastry and Kellie, 2005).

The kidney selectively accumulates Cisplatin and its analogues to a higher degree than other organs, probably through mediated transport (Arany and Safirstein, 2003; Kawai et al., 2005). Morphologically, the nephrotoxicity induces necrosis of the terminal portion of the proximal tubule and apoptosis, predominantly in cells in the distal nephron. Cisplatin treatment also induces extensive death of cells in the proximal and distal tubules and loop of Henle (Arany et al., 2004 and Taguchi et al., 2005).

Cisplatin nephrotoxicity has been demonstrated to be mediated by DNAase1 (Basnakian et al., 2005). This enzyme is a highly active renal endonuclease, and its silencing by antisense is cytoprotective against the in vitro hypoxia injury of the kidney tubular epithelial cells (Basnakian et al., 2005). Also, Cisplatin nephrotoxicity is due to a complex metabolic pathway that activates the drug to a potent kidney toxin. Among the earliest reactions of the kidney to Cisplatin is the activation of the mitogen-activated protein kinase (MAPK) cascade (which is involved in the action of most non-nuclear oncogenes, and is responsible for cell response to growth factors), and molecular responses typical of the stress response (Arany and Safirstein, 2003).

It has been proposed that Cisplatin is metabolised to a reactive toxic thiol through a biotransformation pathway that requires gamma glutamyltranspeptidase (γ-GT), aminopeptidase N (AP-N), renal dipeptidase (RDP) and C-S lyase (Hanigan et al., 1994; Townsend and Hanigan, 2002; Townsend et al., 2003b; Zhang and Hanigan, 2003). The biotransformation pathway is suggested to involve the following steps: Cisplatin–GSH conjugates are secreted into the proximal tubule lumen by MRP2 (Kamazawa et al., 2000) and then cleaved by γ-GT, creating a cysteine–glycine conjugate that is cleaved to a cysteine conjugate by the cell surface aminopeptidases, AP-N or RDP. Cisplatin–cysteine conjugates are suggested to be reabsorbed via renal cysteine heterodimeric amino acid transporters such as system-L, system-b0,+ or system-xc− (Okuno et al., 2003) and then metabolised by C-S lyase to toxic reactive thiols resulting in nephrotoxicity (Townsend et al., 2003b).

It is likely that Cisplatin is conjugated with reduced glutathione (GSH) in a non-enzymatically mediated reaction in the liver and is presented to the kidney as a Cisplatin–GSH conjugate. Cisplatin can form monoplatinum and diplatinum conjugates with GSH, cysteinyl–glycine and N-acetyl-l-cysteine (NAC) in cell-free solutions (Bernareggi et al., 1995; Townsend et al., 2003a). These Cisplatin conjugates have yet to be detected in rat or human plasma following Cisplatin administration, but it is proposed that they result in nephrotoxicity via one of two routes, either via loss of free GSH (Lau, 1999; Satoh et al., 2000), or through cellular biotransformation resulting in toxic metabolite formation (Townsend et al., 2003b; Zhang and Hanigan, 2003). All these mechanisms could explain the recorded kidney anomalies (dilated renal pelvis) in our study.

The recorded hepatomegally in the Cisplatin - treated fetuses in our results confirms the observed Cisplatin hepatotoxicity by Martins et al. (2008). They attributed this effect to the membrane rigidification; decreased GSH/GSSG ratio, ATP, GSH and NADPH levels; lipid peroxidation; oxidative damage of cardiolipin and protein sulfhydryl groups. Moreover, cell death by apoptosis was also demonstrated. The findings strongly suggest the participation of the mitochondrial signalling pathway in this process.

In coincidence with the observed brain anomalies in the exposed offspring in our results, Ta et al. (2006) found that neurotoxicity

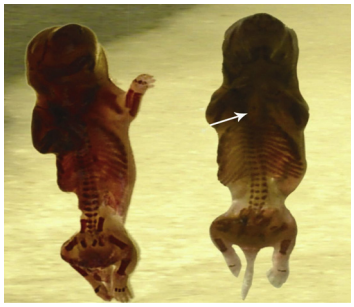

Fig. 13. Skeleton of two rat fetuses, the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/kg ip on 12th day of gestation showing reduced number of sternbrae.

Fig. 14. Skeleton of two rat fetuses, the left one is control and the right one obtained from a pregnant dam treated with Cisplatin at 5 mg/ Kg on 12th day of gestation showing absence of hind limb phalanges.

Fig. 15. Skeleton of two rat fetuses, the left one is control and the right one obtained from a pregnant dam with treated Cisplatin at 5 mg/ Kg on 12th day of gestation showing absence of sacral (a) and caudal (b) vertebrae.

occurred in up to 30% of Cisplatin treated patients and was dose-limiting for this drug. They also observed that Cisplatin treatment was associated with high levels of Pt-DNA binding and apoptosis of dorsal root ganglion (DRG) neurons in vitro.

The placenta plays a critical role in regulating the exchange of various substances as nutrients, hormones, and other molecules essential for the maintenance of pregnancy and normal fetal development between the maternal and fetal circulation throughout gestation. It also protects the developing fetus from potentially detrimental environmental xenobiotics. However, this barrier is incomplete as drugs and toxins can diffuse across the placenta (Syme et al., 2004).

Our results revealed that ip treatment of pregnant dams with Cisplatin induced significant decrease in placental weight, degenerative micromorphology which is indicative for necrosis and apoptosis in

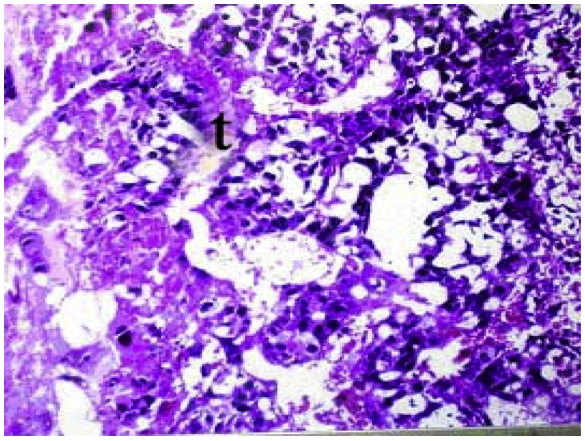

Fig. 16. Placenta of a rat fetus obtained from a pregnant dam treated with Cisplatin at 5 mg/ Kg on 12th day of gestation showing necrosis in the trophoblasts (t) (H & E, X 40).

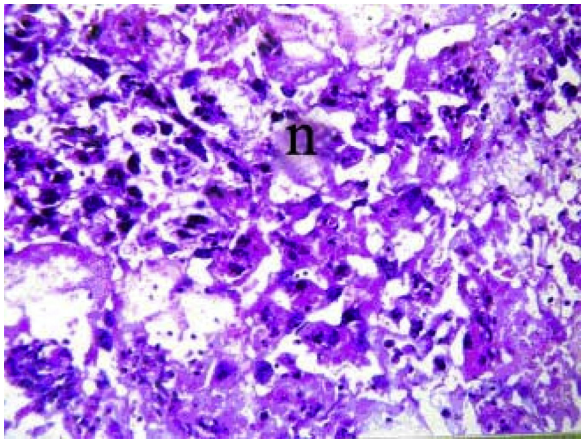

Fig. 17. Placenta of a rat fetus obtained from a pregnant dam treated with Cisplatin at 5 mg/ Kg on 12th day of gestation showing necrosis in labyrinth (n) (H & E, X 40).

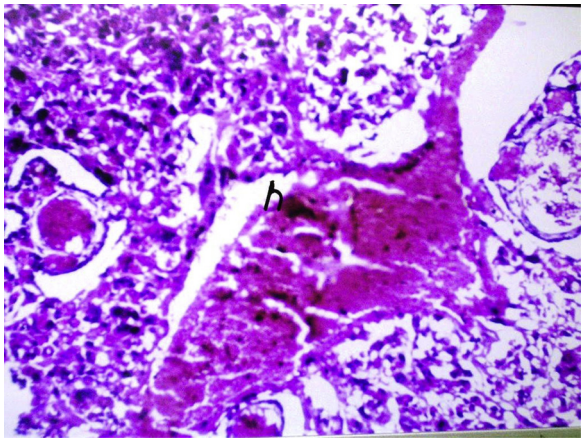

Fig. 18. Placenta of a rat fetus obtained from a pregnant dam treated with Cisplatin at 5 mg/ kg on 12th day of gestation showing focal haemorrhage in labyrinth (n) (H & E, X 40).

placental cells. We found that the placentae of Cisplatin - exposed rats showed severe pathological alterations in the form of thickening and hyalinization in the giant cells associated with necrosis in the trophoblasts, and labyrinth. There was focal haemorrhage in the labyrinth and oedema arround the villi compared to the control group. Similarly,

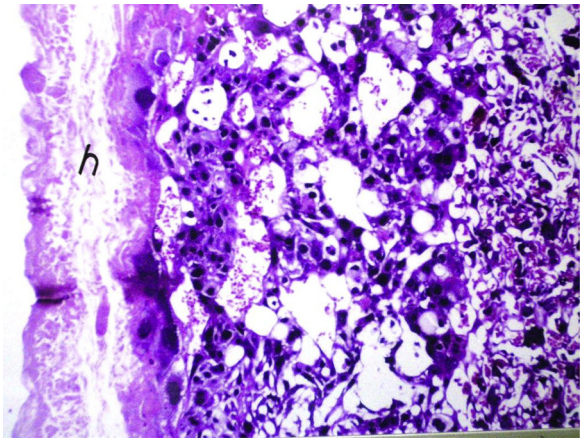

Fig. 19. Placenta of a rat fetus obtained from a pregnant dam treated with Cisplatin at 5 mg/ Kg on 12th day of gestation and NAC at 200 mg/kg on gestation days 0- 20 showing mild hyalinosis in giant cell layer (h) (H & E, X 40).

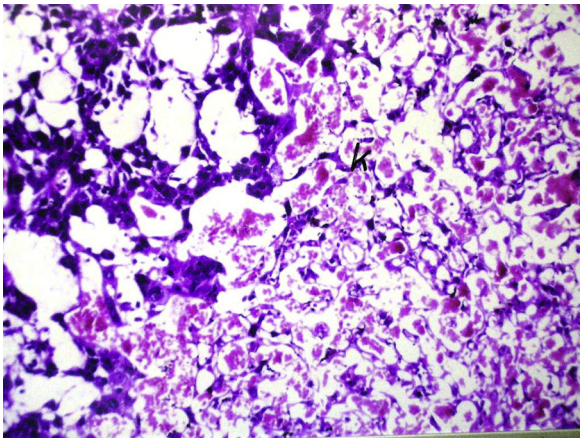

Fig. 20. Placenta of a rat fetus obtained from a pregnant dam treated with Cisplatin at 5 mg/ Kg on 12th day of gestation and NAC at 200 mg/kg on gestation days 0- 20 showing ischemia in labyrinth (k) (H & E, X 40).

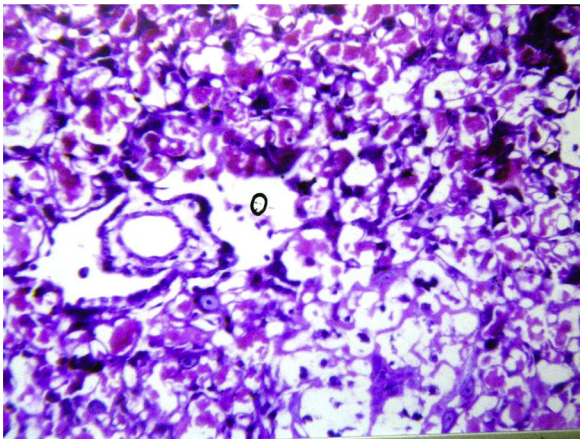

Fig. 21. Placenta of a rat fetus obtained from a pregnant dam treated with Cisplatin at 5 mg/ kg on 12th day of gestation and NAC at 200 mg/kg on gestation days 0- 20 showing mild oedema (O) surrounding the villi (H & E, X 40).

Furukawa et al. (2013) examined the sequential histopathological changes in the placenta from rats exposed to cisplatin. Cisplatin was intraperitoneally administered at 2 mg/kg/day during GDs 11-12 (GD11,12- treated group), or GDs 13-14 (GD13,14-treated group), and the placentas were sampled on GDs 13, 15, 17 and 21. Fetal mortality rates were increased up to approximately 65% from GD 17 onward, and fetal weights were decreased on GD 21 in the GD11, 12-treated group. A reduction in placental weights was detected from GD 15 onward, and the placentas on GD 21 were macroscopically small and thin in both treated groups. Histopathologically, in the GD13,14-treated group, an increase in apoptotic cells was detected on GDs 15 and 17 in the labyrinth zone, and on GD 21 in the basal zone, resulting in labyrinth zone hypoplasia. By contrast, in the GD11, 12-treated group, an increase in apoptotic cells was detected on GDs 13, 15 and 17 in the labyrinth zone, and during the experimental period in the basal zone. A decrease in Phospho-Histone H3 positive cells was detected on GD 13 in the labyrinth zone and basal zone, resulting in hypoplasia of the labyrinth zone and basal zone. In addition, a marked decrease in glycogen cell-islands in the basal zone was also detected on GDs 15 and 17. There was a reduction in interstitial invasion of glycogen cell-like trophoblasts into the metrial gland on GD 15, resulting in metrial gland hypoplasia. Therefore, they considered that cisplatin administration in pregnant rats induces growth arrest of the labyrinth zone and basal zone, leading to small placenta. It is assumed that metrial gland hypoplasia is secondarily induced by the failure of glycogen cell island development associated with basal zone hypoplasia.

Table 4
The levels of placental Fas and Caspase-3 genes expression of rat fetuses obtained from control and treated dams.

Gene	Groups	Relative Quantitation (RQ)
Fas	Cisplatin	10.50 ± 1.3[a]
	NAC	0.83 ± 0.08
	Cisplatin plus NAC	4.26 ± 0.51 [a]
Caspase-3	Cisplatin	22.20 ± 2.25 [a]
	NAC	0.85 ± 0.09
	Cisplatin plus NAC	9.46 ± 1.10 [a]

All values are presented mean ± SE. (n = 3 rats/ group).
[a] Significantly different from control group at $P < 0.05$.

Köhler et al. (2015) applied platinum-based neoadjuvant chemotherapy to 21 consecutive patients with cervical cancer diagnosed in their second trimester. At the time of delivery by cesarean delivery, platinum concentrations in umbilical cord blood and amniotic fluid were 23-65% and 11-42% of the maternal blood, respectively, indicating its transplacental passage to the fetus.

In accordance with the observed placental histopathological changes, the recorded teratogenic effects following Cisplatin exposure in our results could be attributed to transplacental transfer of Cisplatin from mother to the fetus after 12 days of gestation where its MWt is as small as 300.05 Dalton (Köpf-Maier and Merker, 1983).

Many anticancer agents have been shown to be mutagenic, teratogenic and carcinogenic in experimental systems. Two recent case-referent studies among hospital personnel have pointed to slightly increased risks of disorders in pregnancy outcome; one of the studies has shown an excess of spontaneous abortions and other malformations in children of females with a history of work with anticancer agents (Sorsa et al., 1985).

Cisplatin is known to alter DNA in various ways, including DNA adduct formation (Giurgiovich et al., 1997). Also, the genotoxic effect of Cisplatin (chromosome aberrations and sister chromatid exchanges) has been observed in testicular tissues of human patients (Gundy et al., 1989) and spermatozoa of rats (Hooser et al., 2000) resulting in embryotoxicity and developmental defects in their offspring. High levels of Cisplatin-DNA adducts have previously been observed at term in mitochondrial DNA (mtDNA) from organs of pregnant rats, and from their offspring, after administration of a single injection of 15 mg Cisplatin /kg.b.wt to the pregnant rat on day 18 of gestation. Cisplatin-induced mitochondrial toxicity in maternal rat kidney is severe, while damage to mitochondria in fetal kidney and liver, occurring as a result of the transplacental drug exposure, appeared to be mild (Gerschenson et al.,

2001). Giurgiovich et al. (1997) demonstrated extensive Cisplatin-DNA adduct formation in brain and liver mitochondria of fetal rats exposed transplacentally and suggested that mitochondrial DNA in some organs may be a particular target for Cisplatin genotoxicity.

Sergi et al. (2004) and Siddiqui et al. (2008) suggested that oxidative stress caused by Cisplatin may, in part, be contributing to Cisplatin -induced genotoxic damage (Coughlin and Richmond, 1989). Therefore, Cisplatin - induced teratological changes could be attributed to the alteration in genes regulating cell metabolism and oxidative stress.

The recorded teratogenic (specially brain) and placental histopathological lesions of Cisplatin-treated groups in our study could be related to the direct cytotoxicity of this metal and/or indirectly via free radicals production. Sullivan and Krieger (1992) and Sergi et al. (2004) suggested that Cisplatin metabolism may generate reactive oxygen species (ROS), which in turn can lead to enhanced brain lipid peroxidation. The developing brain is highly rich in unsaturated fatty acids (Clandinin, 1999), free redox active iron (Connor, 1994), whereas it contains low antioxidant defence (Tuppo and Forman, 2001) and thus more prone to oxidative insults.

Coughlin and Richmond (1989) mentioned that Cisplatin and most other platinum compounds induce damage to tumors via induction of apoptosis; this is by free radical-mediated activation of signal transduction leading to the death receptor mechanisms as well as mitochondrial pathways (Apoptosis is responsible for the characteristic nephrotoxicity, ototoxicity and most other toxicities of the drugs (Boulikas and Vougiouka, 2003). Cisplatin can react in a nonenzymatic manner with water in vivo to form monoaquo and diaquo species following dissociation of the chloride groups. These metabolites extensively bind to protein (> 90%) and thus have minimal cytotoxicites but the non-protein bound, ultrafilterable reactive species are cytotoxic (Sullivan and Krieger, 1992).

Oral administration of N-acetyl-cysteine (NAC) in concomitant injection of Cisplatin showed reduction in the elevated morphological abnormalities compared to Cisplatin - treated rats. This reduction was statistically insignificant. Also, administration of the tested protector (NAC) showed insignificant reduction in the elevated percentages of offsprings that had visceral and skeletal anomalies in Cisplatin + NAC group compared to Cisplatin one. NAC administration with Cisplatin ameliorated most of the observed placental pathological alterations. The placentas showed mild hyalinosis in the giant cells, ischaemia in the underlying labyrinth and mild edema in the surrounding villi.

Cisplatin has been reported to induce cellular damage at several structural levels (Fuertesa et al., 2003). Besides its primary target DNA, cisplatin has been shown to interact with some transport proteins on the cellular membrane and in the cytoplasm (Spierings et al., 2003). For example, cisplatin has been shown to cluster and activate FAS in a FAS ligand-(FASL) independent manner (Micheau et al., 1999), sequentially activating caspase-8, -3 and -6 (Seki et al., 2000). The caspase-dependent intrinsic pathway is one of the main death pathways activated by specific cellular damage. In this pathway, FAS plays a predominant role by forming death inducing signaling complex (DISC) composed of FAS, FAS associated death domain (FADD) and caspase-8 (Siegel et al., 2000; Chan et al., 2000). Oral treatment with Cisplatin resulted in over expressions of placental Fas and Caspase-3 genes mRNAs (apoptosis markers). The up-regulation of Fas and Caspase-3 genes in these cells suggested activation of the Fas-mediated pathway.

Henkels and Turchi (2009) supported a model in which Cisplatin-induced programmed cell death in Cisplatin- sensitive and - resistant Human Ovarian Cancer cells was proceeded via caspase-3-independent and-dependent pathways, respectively. It was the result of dissimilar upstream signaling events. The execution of apoptotic events that precede target protein proteolysis and subsequent chromosomal DNA degradation were assessed where proteolytic degradation of procaspase-3 was observed in both types of cells following Cisplatin treatment,

Mese et al. (2000) reported that caspase-3 activation mediates apoptosis induced by Cisplatin, and its induction could represent a novel approach to the effective treatment of malignant tumors. Wei et al. (2010) concluded that Fas over-expression reverses resistance of small cell lung cancer cells, possibly due to the increased cell sensitivity to apoptosis and the decreased expressions of glutathione S-transferase π (GST-π) and excision repair crosscomplementing-1 (ERCC1).

However, NAC co-treatment with Cisplatin resulted in down-regulation of Fas and caspase-3 genes. This indicated that NAC provided an effective protection for the placental cells against apoptosis by an extrinsic pathway which initiated by activation of Fas/FasL pathway. In coincidence with these results, Blakley et al. (2002) and Schaaf et al. (2002) confirmed the protective role of NAC against Cisplatin- induced toxicities through attenuation of oxidative stress mitigated inflammation as well as enhancement of the antioxidant defenses and prevention of apoptosis.

Several apoptotic pathways have been defined, including the extrinsic (death receptor) pathway, the intrinsic (mitochondrial) pathway, and the perforin/ granzyme pathway. The extrinsic pathway mainly induces apoptosis via Fas/FasL-mediated caspase-8 activation (Elmore, 2007). The level of Fas expression was increased by Cisplatin treatment, indicating that Cisplatin may mediate the extrinsic pathway. These findings indicated that Cisplatin may induce apoptosis-related changes. Also, the Cisplatin elicited teratogenic effects could be related to its transplacental transfer and induction oxidative stress causing apoptosis in placental cells (Köpf-Maier and Merker, 1983; Furukawa et al., 2013; Köhler et al., 2015).

The role of some chemicals and drugs in the prevention of toxicity due to Cisplatin was explored by various authors as Anderson et al. (1990) who studied the role of cellular glutathione in the prevention of toxicity due to Cisplatin in mice treated with buthioninesulfoximine (BSO), a selective inhibitor of gamma-glutamylcysteinesynthetase (and therefore of glutathione synthesis) and with glutathione and glutathione monoisopropyl ester. Pretreatment of mice with BSO enhanced the lethal toxicity of Cisplatin by about twofold. Administration of glutathione ester (dose, 2.5-7.5 mmol/kg) protected against lethal Cisplatin toxicity; glutathione was also effective, but much less so. Glutathione ester, in contrast to glutathione, is effectively transported into cells and split to glutathione intracellularly.

Our results confirmed the role of cysteine (a sulphydryl compound and glutathione precursor) in amelioration of toxicity due to Cisplatin treatment as reported by Feghali et al. (2001), Dickey et al. (2008) and Shalby et al. (2011). Feghali et al. (2001) *in vitro* studies demonstrated that L-n-acetyl-cysteine (L-NAC) protected both auditory neurons and hair cells from the toxic effects of Cisplatin. Because it protects both of these inner ear structures, L-NAC may be potentially useful in protecting hearing, in general, from Cisplatin-induced damage. In addition, L-NAC has low systemic and mucosal toxicity. It also has a low molecular weight that may allow it to readily cross the round window membrane. All these characteristics make it potentially suitable for transtympanic application for the prevention of the ototoxicity of Cisplatin in vivo.

Dosing and frequency of administration of NAC for protection against Cisplatin (CDDP) nephrotoxicity was investigated in rats. Two models of toxicity were tested: a single high dose of CDDP (10 mg/kg) ip, and multiple low dose treatments (1 mg/kg ip twice a day for 4 days, 10 days rest, then repeated). Both models of CDDP administration produced renal toxicity. The protective properties of NAC were affected by the dose and frequency of administration (Dickey et al., 2008).

Shalby et al. (2011) found that NAC had antioxidant and anti-inflammatory effects against Cisplatin -induced nephrotoxicity. They concluded that inhibition in oxidative stress and the elevation of the total antioxidant capacity as well as the inhibition of the inflammatory biomarkers by NAC after Cisplatin administration may play a central role in modulation of nephrotoxicity induced by Cisplatin. In agreement with our aim, the role of cysteine, together with other chemicals, in the prevention of toxicity due to Cisplatin was studied by various authors as

El-Daly (1998), Lopez-Gonzalez et al. (2000), Blakley et al. (2002), Schaaf et al. (2002) and Previati et al. (2004).

Concurrent administration of cysteine together with vitamin E, Crocus sativus and Nigella sativa reduced the toxicity of Cisplatin in rats. When administered i.p. for 5 alternate days with 3 mg/kg Cisplatin, cysteine (20 mg/kg) together with vitamin E (2 mg/rat) an extract of Crocus sativus stigmas (50 mg/kg) and Nigella sativa seed (50 mg/kg) significantly reduced blood urea nitrogen (BUN) and serum creatinine levels as well as Cisplatin-induced serum total lipids increases. The serum activities of alkaline phosphatase, lactate dehydrogenase, malate dehydrogenase, aspartate aminotransferase and alanine aminotransferase of Cisplatin-treated rats were significantly decreased, whereas the activities of glutathione reductase and isocitrate dehydrogenase were significantly increased (El-Daly, 1998).

Blakley et al. (2002) and Schaaf et al. (2002) confirmed the effectiveness of the antioxidants, alpha-tocopherol (TOCO) and NAC in reducing the elevation of ROS levels and consequently ameliorating the development of Cisplatin -induced cytotoxicity.

Previati et al. (2004) tested the effectiveness of butylatedhydroxytoluene (BHT), dithiothreitol (DTT) and NAC as cyto-protectants, all of them reduced protein carbonylation to control levels and significantly protected OC-k3 from Cisplatin-induced cell death, with a higher protection when using the lipophylic antioxidant BHT. This result confirmed that treatment with NAC may be less efficient than other protectors against Cisplatin - induced teratogenicity.

5. Conclusion

It can be concluded that Cisplatin is teratogenic on exposure during the organogenesis period especially on the 12th day of gestation in rats. It induced several morphological, visceral and skeletal anomalies in the obtained fetuses with severe pathological alterations in the placentas. The recorded Cisplatin- induced teratogenocity in rats may be related to its free radical production and induction of oxidative stress and consequently its genotoxic activity where it enhanced placental Fas and Caspase-3 genes expression levels. This is considered a fundamental part of the outward pathway of cell apoptosis, prompting apoptosis in the placental cells. Thus, the upregulation of these genes has an essential role in the extrinsic pathway of cell apoptosis and depletion that was reflected as structural anomalies in the fetus. On the other hand, treatment with NAC throughout the whole length of gestation induced a protective effect against Cisplatin - induced teratogenicity and placental pathology. It elicited anti-apoptotic activity by downregulating these two genes, thereby protected the placenta and fetus from Cisplatin -induced apoptosis and teratogenesis, respectively.

Ethics approval and consent to participate

The Local Committee for Faculty of Veterinary Medicine, Cairo University approved the design of the experiments and the protocol.

Availability of data and material

There are no restrictions to the availability of any materials and data upon request.

Declaration of Competing Interest

The authors declare that there are no competing interests associated with the manuscript.

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Author contribution

Dr. Mohamed S. Hassan conceived the study and designed the experiment. Dr. Ashraf M. Morgan reviewed all the results, carried out data analysis, and drafted the manuscript. Dr. Mohey M. Mekawy reviewed the manuscript. Zeineb, M.A performed the experiments and carried out the biochemical and immune analysis. All authors read, revised, and approved the final manuscript.

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