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GARCINIA HYDROXYLBIFLAVANONOL-1 (GB1) ISOLATED FROM SEEDS OF *GARCINIA KOLA* AMELIORATES THE TOXIC EFFECTS OF CADMIUM CHLORIDE ON THE OVARIES AND UTERUS OF ALBINO WISTAR RATS

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Abstract

In humans, cadmium toxicity in females especially during pregnancy can cause serious maternal and foetal morbidity and in extreme untreated cases, foetal mortality occurs. Studies aimed at the understanding the hormonal interplay following exposure to toxic compounds in females have been limited due to too few appropriate animal models. This study examined the possible protective effect of Garcinia hydroxylbiflavanonol (GB1) from seeds of *Garcinia kola* on cadmium chloride ($CdCl_2$) - induced reproductive toxicity in female Wistar rats. We observed that cadmium (Cd) accumulated in the uterus and ovaries of rats, significantly decreased ($p<0.05$) antioxidants [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH)], and significantly rose ($p<0.05$) the concentrations of malondialdehyde (MDA) in the uterus and ovaries of rats. Serum concentrations of estradiol, progesterone, follicle stimulating hormone and luteinizing hormone decreased significantly ($p<0.05$) following $CdCl_2$ administration. GB1 significantly ($p<0.05$) decreased Cd accumulation, MDA, H_2O_2 and significantly increased ($p<0.05$) SOD, CAT and GPx activities in the uterus and ovaries, and significantly increased ($p<0.05$) serum reproductive hormones except for LH. Taken together, these results suggest that GB1 exerts multiple mechanistic protective effects against cadmium toxicity probably attributable to its known antioxidant potentials.

Keywords: *Antioxidants, Cadmium chloride, Ovaries, Estradiol, FSH, LH, Progesterone, Garcinia bydroxylbiflavanolol-1, Uterus*

Introduction

Cadmium (Cd), a bio-accumulative non-essential element that is an environmental risk factor with various toxic effects in animals and humans enters the general environment from the natural weathering of materials, forest fires and volcanoes, but much larger amounts are released by human activities (Morrow, 2001). Cadmium chloride used in photography, photocopying, dyeing, calico printing, vacuum tube manufacture pigment production, galvanoplasty, lubricants, ice-nucleation agents and manufacture of special mirrors (Herron, 2003) may easily enter the environment. Long-term ingestion of large amounts of cadmium has been observed in Japan (Massanyi et al., 2005) and the exceptionally long half-life of cadmium in the human body of about 30 years (Kjellstrom, 1979), emphasizes the need for the effective monitoring and treatment of cadmium toxicity. Cadmium is also known to affect reproductive organs (Uwagie-Ero et al., 2018; Massanyi et al., 2005; Toman et al., 2002). In the blood and tissues, Cd stimulates the formation of metallothioneins and reactive oxygen species (ROS), thus causing oxidative damage in erythrocytes and in various tissues. This produces a loss of membrane stability and functions (Sarkar et al., 1998).

Oxidative stress is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen derived oxidants commonly known as reactive oxygen species (Zikic et al., 1998). The cellular damage in the gonads may be due to an improper balance between ROS generation and scavenging activities (Pajavic and Saicic, 2008). The scavenging potential in the gonad is normally maintained by adequate levels of antioxidant superoxide dismutase (SOD), catalase and glutathione (Shi et al., 1999). Long-term exposure to Cd increases lipid peroxidation and causes inhibition of SOD activity, indicating oxidative damage (Patra et al., 1999). The increase in lipid peroxidation (LPO) may be attributed to the alteration in the antioxidant defence system. This defence system includes the enzymes SOD, catalase, glutathione peroxidase (GPx), glutathione-s-transferase (GST) as well as glutathione, which normally protect against radical toxicity.

A number of plant compounds had been reported to exhibit a protective role against ROS and lipid peroxidation (Ahmed et al., 2000; Aqil et al., 2006). In southern India, several herbal products had been reported to fortify the reproductive systems of women and to mitigate oxidative stress due to ROS in the gonads after parturition (Karthikeyan and Rani, 2003).

Garcinia hydroxylbiflavanonol-1 (GB1) is a natural flavonoid with reported antioxidant property, extracted from the seed of *Garcinia kola* (Guttiferae) common to West Africa. Previous phytochemical investigations of *G. kola* resulted in the isolation of cycloartenol, 24-methylene-cycloartenol and kolanone (Hussain et al., 1982) from the light petroleum extract and C-3/8"-linkedhydroxybiflavanonols from the ethylacetate extract of the seeds (Sonnenbichler et al., 1986). The bioflavonoid has been shown to improve the negative effects of oxidative stress in lipids, proteins and DNA Farombi et al. (2017). Its administration has been shown to be beneficial at the levels of metabolism, endocrine, testicular and ovarian activities (Uwagie-Ero et al., 2018, Farombi et al., 2017).

This study investigated the possible ameliorative effects of GB1 on cadmium chloride induced oxidative stress in the uteri and ovaries of female Wistar rats.

Methods

Plant studies

Source of plant material and identification

Mature seeds of *Garcinia kola* were purchased from a local Market in Calabar in October 2018. The seeds were identified by Michael Ekpo of the Department of Botany, University of Calabar, Calabar, Nigeria. A voucher specimen (UNICAL/BCM/017221) was deposited in the herbarium of the department of Biochemistry, University of Calabar, Calabar, Nigeria.

Extraction and fractionation of crude extract

The fresh seeds of *G. kola* were dried at room temperature and reduced to coarse powder by grinding. 1 kg of the pulverized plant material was defatted with 3 L of n-hexane in a Soxhlet apparatus (Büchi, Switzerland) for 24 h. The n-hexane was distilled off to afford 15.7 g of a yellowish-brown oily sample. The fat free plant material was then extracted with 80 % methanol for 72 h. This was

concentrated with a rotary evaporator to afford 140.2 g of methanol extract (brown sticky gum). 100 g of the methanol extract was suspended in distilled water and subjected to liquid-liquid partitioning with ethyl acetate (EA) to give 17 g of the EA fraction.

Extract fractionation and isolation of Garcinia hydroxybiflavanonol (GB1)

This was done as described by Nwaehujor et al (2015) and isolates were identified as previously described (Sonnebichler et al., 1986).

Animal experimental design

Female Wistar rats (170-210 g) were randomly assigned to 4 groups as follows; A= normal control, B =$CdCl_2$ only, C = $CdCl_2$+ GB1and D = GB1 only. Group A rats were orally administered 0.2 ml of 0.5% DMSO/day (vehicle), group B rats received $CdCl_2$ in drinking water (5 mg/kg b.w./day), group C rats received $CdCl_2$(5 mg/kgb.w.) in drinking water and 10 mg/kg b.w./day of GB1 administration orally, while group D rats were treated with GB1 (10 mg/kg b.w./day) only. GB1 and $CdCl_2$were dissolved in 0.5% DMSO and administered *per os* for 28 days. Experimental animals were kept in accordance with the guidelines for animal care as contained in the animal ethics handbook of the Faculty of Basic Medical Sciences, University of Calabar, Nigeria.

Assessment of serum testosterone (Test), luteinizing hormone (LH), follicle stimulating hormone (FSH), progesterone (Pro) and estradiol (E-ol) concentrations

Under mild anaesthesia, blood was collected from each female rat from the media cantus of the eye and allowed to stand for 30 min, thereafter, the rats were humanely euthanized. The blood was centrifuged for 10 min at 4,000 g and collected serum was assayed for testosterone (Test), luteinizing hormone (LH), follicle stimulating hormone (FSH), progesterone (Prog.) and estradiol (E-ol) concentrations using the enzyme linked immunoassay kits (ELISA) following the manufacturer's protocols (Immunometrics, London, UK).

Ovarian and Uterine Antioxidant analysis

To prepare the tissues, one gram (1 g) of rat ovary/uterus was homogenized in 3 mL of 20 mM posphate buffer (pH 7.4) by using Heidolph homogenizers with a Teflon pestle. 10 µL 0.5M BHT in acetonitrile was added to 1 mL of tissue homogenate to prevent sample oxidation. The precipitate was removed by centrifugation (2000rpm). An aliquot of the sample was removed and the sample was frozen immediately at -20°C prior to testing. 0.2 mL of the homogenate was used for assay. Tissue lipid peroxidation was measured by the method of Devasagayam and Tarachand (1987). The malondialdehyde content of the samples was expressed as nmoles of MDA formed/mg protein. Superoxide dismutase (SOD) enzyme was assayed according to the method of Marklund and Marklund (1974). The activity of catalase was assayed by the method of Sinha (1972). The activity of catalase was expressed in Units/mg protein (one unit is the amount of enzyme that utilizes 1 µmole of hydrogen peroxide/min) while glutathione peroxidase (GPx) activity was determined by the method of Rotruck *et al*. (1973). The amount of GSH in the total homogenate was measured according to the method of Sedlak and Lindsay with some modifications. 1500 μL of measurement buffer (200mM Tris-HCl buffer containing0.2mM EDTA at pH 7.5), 500μL of supernatant, 100μL of 5, 5-dithiobis (2-nitrobenzoic acid) (10 mM), and 7900 μL of methanol were added to a tube and vortexed and incubated for 30min in 37°C. The absorbance was measured at 412nmusing a spectrophotometer. The standard curve was obtained by using reduced glutathione.

Assessment of Cadmium concentration in the ovaries and uteri

All the samples were analysed for cadmium content with the technique of Atomic Absorption Spectrometry (AAS). The spectrometer used was Type Varian, Spetr-200 with limit of detection for cadmium 0.006 ppm (ug/l)

Data and Statistical analysis

Data (n = 5) obtained were presented as mean±SEM and analysed using one-way analysis of variance (ANOVA) and posthoc comparisons were carried out using either Dunnett's t-test or Tukey's test (where appropriate) on GraphPad Prism version 5.01. Values of $P < 0.05$ were considered significant in the study.

Results and Discussion

Exposure to non-essential metals is known to negatively affect some systems of the body by prompting oxidative stress. In this study, we examined the short term direct (ovarian and uterine oxidative stress) and indirect (pituitary secretion of reproductive hormones) effects of $CdCl_2$ exposure on the female reproductive system and possible amelioration with GB1 (Figure 1A – E).Studies have shown that cadmium (Cd) accumulates in tissues such as the liver (Nwokocha et al., 2011, 2012a, b), resulting in significant oxidative stress in these tissues (Farombi et al., 2012). Some of these tissues include the kidneys (Klaassen *et al.*, 2009), vascular endothelium (Prozialeck *et al.*, 2006), mammalian and poultry liver (Klaassen *et al.*, 2009; Arroyo *et al.*, 2012; Li *et al.*, 2013), hepato-pancreas of shrimp (Wu and Chen, 2005) as well as subcellular compartments of oysters (Sokolova *et al.*, 2005), among others. Since Cd absorption and excretion are reported to be slow, it can accumulate over time and cause toxic effects (McLellan et al., 1978). The ovary is one of the target tissues affected by Cd bioaccumulation (Samuel et al., 2011).

Being vital in terms of female reproduction, it was worthwhile to assess the likelihood of Cd accumulation in the uteri and ovaries after short-term exposure (Figure 2F and 3F). Uterine and ovarian concentrations of Cd increased significantly in our study, showing direct and indirect effects of $CdCl_2$ (Figure 2F and 3F). It is direct since Cd replaces Ca^{2+} and Zn^{2+} by mimicking their physiological processes in the cells (Valko et al., 2005). It is indirect since Cd in non-reproductive glands (e.g. the hypothalamus and pituitary) negatively affects reproductive function through suppressed release of FSH and LH (Hoyer, 2005). Our result is consistent with reports of Höfer et al. (2009) who observed high Cd accumulation in the uteri after 3 days of oral gavage, and 4 weeks of *ad libitum* access in drinking water, respectively. Clinical (Varga et al., 1993) and experimental (Piasek and Laskey, 1994; Jancóvá et al., 2006; Massanyi et al., 2007) data have reported that Cd accumulates in the ovaries following oral exposure. In our study, post-treatment with GB1 offered significant protection (Figure 2F and 3F), and it is likely that the mechanisms involved in GB1's suppression of Cd accumulation in the ovaries and uteri are (i) alteration of $CdCl_2$ absorption in the gut and/or (ii) enhancement of its excretion through the kidneys.

Estrogen and progesterone are very important female reproductive hormones. Studies have demonstrated the role of steroid hormones such as estradiol and progesterone in the development and differentiation of reproductive tissues, and fertility preservation (da Silva Faria et al., 2010). The gonadotropins (FSH and LH) secreted by the anterior pituitary gland are required for the synthesis of estradiol and progesterone, since the former has a stimulatory effect on the ovaries to secrete estradiol and progesterone (Priya et al., 2004). Estradiol is known to enhance granulosa cells' sensitivity to circulating FSH and LH, by raising steroid hormone synthesis in the granulosa cells (analogous to the FSH, LH - Leydig cell - Testosterone co-operation). Consistent with previous reports (Zhang et al., 2008; Mansour and Ramadan, 2010; Samuel et al., 2011), this study showed that estradiol and progesterone were significantly decreased in the $CdCl_2$only group, compared to the control and GB1 treated groups (Figure 1A and 1B)

The observed decrease in estradiol and progesterone concentrations in the $CdCl_2$ group (Figure 1A and 1B) may be partly attributable to the decreased stimulatory effects of FSH and LH on the gonads. An earlier study using male post-pubertal rats showed that Cd accumulated in the hypothalamus and the anterior pituitary (Lafuente et al., 2000), causing oxidative stress-induced apoptosis of gonadotropins of the anterior pituitary (Wei et al., 2007). This may be partly responsible for the decreased serum FSH and LH concentrations in this study (Figure 1C and 1D), since the number of viable gonadotrophs may have a direct negative effect on the concentrations of FSH and LH. The observed decrease in serum FSH and LH concentrations following $CdCl_2$ exposure in our study is consistent with previous studies (Pillai et al., 2003; Priya et al., 2004; Al-Gnami and AL-Lebawi, 2014).

These results showed that GB1 significantly improved circulating levels of estradiol, progesterone, FSH and LH in $CdCl_2$ exposed rats significantly. The mechanism underlying this significant improvement in serum concentrations of

estradiol, progesterone, FSH and LH in the GB1 treated groups may not be far from reducing oxidative stress. GB1's ability to reduce Cd accumulation and its attendant increase in antioxidants in the ovaries may be responsible for the significant improvement in hormonal profile.

The role of antioxidants involves neutralizing of excess free radicals to protect cells from toxic effects and contribution to disease prevention (Douglas et al., 2000). In this study, a significant decrease in SOD activity was observed in the ovaries and uteri ofCd only challenged group animals (Figure2A and 3A). However, treatment with GB1 was observed as being significantly beneficial (P <0.001). SOD has proven a useful probe for studying the free radicals in reactions involving oxygen, since it acts as a defence against oxidative tissue damage by dismutation of superoxide radicals (Ognijanovic et al., 2003). SOD also plays an important role in the regulation of the luteal function during pregnancy (Pajavic and Saicic, 2008). Long-term exposure to Cd increases lipid peroxidation and causes inhibition of SOD activity, resulting in oxidative damage to liver, kidney and testes (Patra et al., 1999). Normally, GPx is known to be present in high concentrations in ovarian tissues (Mattison et al., 1983). Significant (P<0.05) reduction in GPx activity was observed in the ovaries and uteri of the negative control group compared to normal control, but treatment with GB1 presents some amelioration (Figure 2B and 3B). The activity of another antioxidant enzyme, catalase (CAT), was also significantly (P<0.05) lowered, but GB1 attempts to restore CAT activities (Figure 2C and 3C). This is in accordance with the findings of previous studies that Cd inhibits the activities of the majority of enzymes involved in antioxidant systems (Casalino et al., 2002; Jamall and Sprowls, 1987), inducing an increased production of free radicals, lipid peroxidation and destruction of cell membranes (Casalino et al., 1997; Kostic et al., 1993). A similar trend to GPx was also noticed in GSH activity in the ovaries and uteri of the negative control group compared to normal control (Figure 2D and 3D). Glutathione (GSH) is the cofactor for lipid hydrogen-peroxide detoxifying enzyme.

ROS usually initiates the attack on cell membranes, thus, causing a significant peroxidation of the polyunsaturated fatty acids (PUFAs) of lipid membranes. The increase in H_2O_2 generation may have initiated the peroxidation of the lipid membranes of the uteri and ovaries, leading to the formation of large concentrations of MDA (Figure 1D). There are numerous reports in animal models showing that Cd intoxication significantly increases the malondialdehyde (MDA) levels. Our findings corroborated that $CdCl_2$ caused significant elevation of MDA values (P<0.05), which treatment with GB1 crashed significantly in the uterus (P<0.05) (Figure 2E and 3E).

Taken together, our study provides some beneficial properties of the naturally occurring antioxidant, GB1 on female reproductive activities in rats. Our results are consistent with the previous findings that *N. sativa* oil increases glutathione (GSH) and SOD (El-Abhar et al., 2003). *C. longa* protects against ischemia-induced changes by increasing the antioxidant defence mechanisms (Dikshit et al., 1995). *B. juncea* administered to animal groups increased activity of SOD and catalase. It also sharply increased the activity of glutathione reductase (GSH), GPx and GST in the experimental group compared to the controls (Khan et al., 1996).

CONCLUSION

$CdCl_2$, a rich source of the Cd, and representative of non-essential bio-accumulating metals/metalloproteins, which reports show rapidly accumulates following acute oral administration in pregnant rats producing deleterious changes, also showed its adverse effects in serum hormones as well as oxidative stress factors in the uteri and ovaries of non-pregnant mice used in this study. Efforts at finding natural compounds that have abilities of averting these debilities have not yielded many benefits. But, for the first time, we report that garcinia hydroxybiflavanonol-1 (GB1), a natural antioxidant obtained from methanol seed extracts of *G. kola* possesses an ability to ameliorate $CdCl_2$-induced oxidative stress in test female Wistar rats, especially the changes manifesting in the uteri and ovaries. The mechanism by which it does so, and signalling pathways involved is still elusive, but it is the pivot of our future research endeavour.

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Figures

Serum hormones levels following treatments

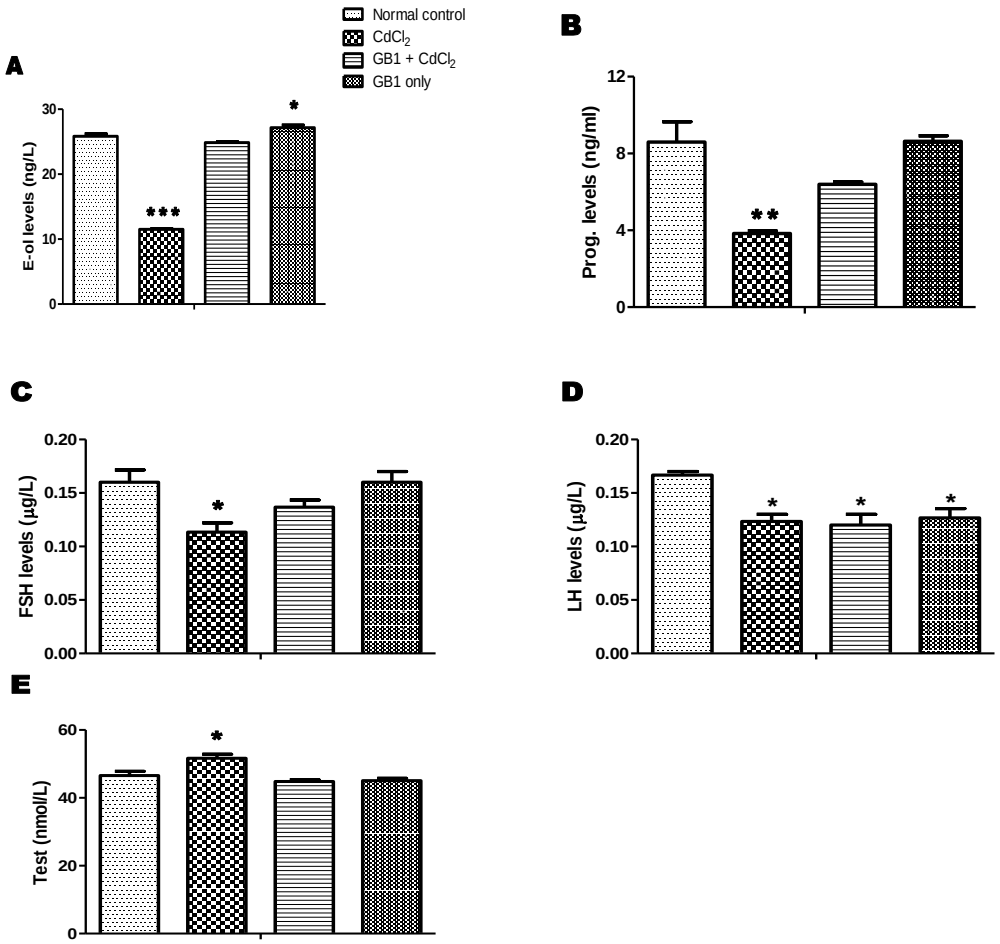

Figure 1 (A-E): Serum concentrations of hormones Estradiol (E-ol, A); Progesterone (Prog., B); Follicle Stimulating Hormone (FSH, C); Luteinizing Hormone (LH, D); Testosterone (Test, E).

Measurement of oxidative stress factors of the ovaries

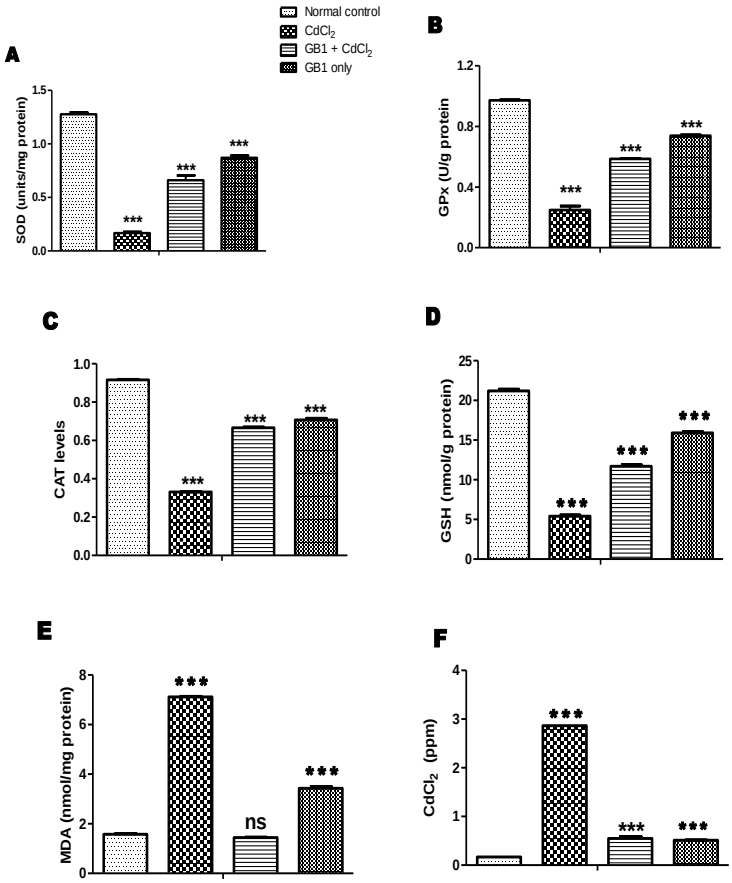

Figure 2 (A-F): Assay of Oxidative stress factors (ROS) and $CdCl_2$ concentrations in ovaries: (Superoxide dismutase (SOD, A); glutathione peroxidase (GPx, B); Catalase (CAT, C); glutathione (GSH, D); Malondialdehyde (MDA, E); Cadmium chloride ($CdCl_2$, F)

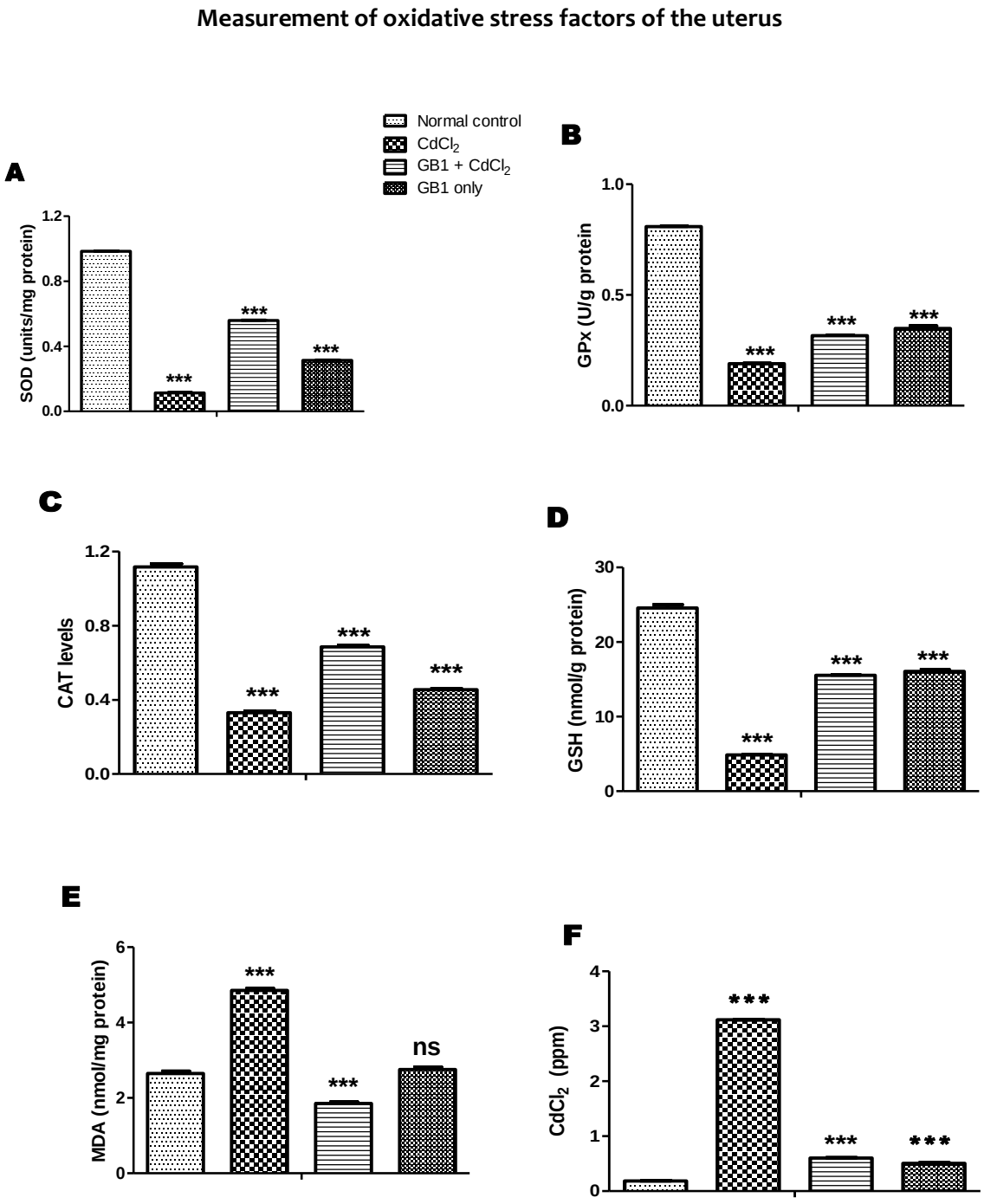

Figure 3 (A-F): Assessment of Oxidative stress factors (ROS) and $CdCl_2$ concentrations in the uteri of test animals – SOD (A); GPx (B); CAT (C); GSH (D); MDA (E); $CdCl_2$ (F).