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Alterations in Pro-Oxidant And Antioxidant Status In Different Age Groups of Cadmium Exposed Male Common Indian Toads, *Bufo Melanostictus*

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ABSTRACT

The present study aims to investigate the prooxidant and antioxidant status in different age groups of Cadmium treated (Single dose of $CdCl_2$, 2.5 µg/g body weight, i. p.) male common Indian toads, Bufo melanostictus (presently Duttaphrynus melanostictus). In this study a significant increase in lipid peroxidation (LPO) Potential in the liver tissue of all the three age groups (young, middle aged and old) of Cadmium treated animal was found. The rate of elevation in LPO potential was found to increase with advancing age. A significant decrease in enzymatic antioxidant like superoxide dismutase (SOD) and Catalase (CAT) was found in liver of all the three age groups of toads exposed to Cadmium than their control counter parts. However the degree of inhibition of SOD and catalase was more pronounced in the old aged toads than that of the middle aged ones. Similarly cadmium treatment significantly declined the non-enzymatic antioxidant, Ascorbic acid (ASA) and Uric Acid (UA) status in liver tissue of all the three age groups of toads than that of their control untreated counterparts. The degree of depletion of ascorbic acid (ASA) and uric acid (UA) due to Cadmium treatment was minimum during maturation phase as compared to their younger and older counter parts. Therefore a more efficient antioxidant defense against the pro-oxidants due to cadmium treatment was found in the middle aged group of animals than that of their younger and older counter parts.

INTRODUCTION

Cadmium is a common environmental pollutant accumulating day by day as a result of industrial practices. Releasing from electroplating, galvanizing, fertilizer industries, zinc and lead mining, colour paints, plastics it accumulates in air, water, soil and when taken up by plants, it concentrates along the food chain. In addition to its extraordinary cumulative properties, Cd is also a highly toxic metal that can disrupt a number of biological systems usually at doses that are much lower than most toxic metals (Jarup et al., 1998; Nordberg et al., 2007). The molecular mechanism of Cd toxicity is incompletely understood. Studies showed that Cd may not generate reactive oxygen species (ROS) in tissues through the Haber-Weiss reaction (Yang et al., 1997) but can increase lipid peroxidation through generation of noxious radicals such as superoxide anion, hydroxyl radicals, nitric oxide and hydrogen Peroxide (Stohs et al., 2001). These radicals are highly reactive and thus stimulate lipid-peroxidation and deleterious modification of complex lipoprotein assemblies in bio-membranes and cellular dysfunction (Stohs et al., 2001). Waisberg et al., (2003) have demonstrated that Cd stimulates free radical production resulting in oxidative deterioration of lipid, proteins and DNA and initiating various pathological conditions in human and animals. Chronic exposure to Cd results in accumulation of the metal mainly in the liver and kidneys, as well as in other tissues and organs causing many metabolic and histological changes, membrane damage, altered gene expression and apoptosis (Shaikh et al., 1999; Casalino et al., 2002; Waisberg et al., 2003). Cadmium has an extremely long half-life period (of about 20 – 30 years in human body) and can affect various metabolic processes like energy metabolism, membrane transport and protein synthesis.

Free radicals especially reactive oxygen species (ROS) like super-oxide radical ($-O^{\cdot}_2$). Hydrogen peroxide (H_2O_2) and hydroxyl radical (-OH) are generated in vivo as a consequence of oxidative metabolism in mitochondrial electron transport chain and a variety of cellular oxidases (Zhang et al., 2003). Since ROS are highly reactive they promote oxidation of biomolecules (lipid peroxidation, protein carbonylation, DNA modification) and are commonly referred to as pro-oxidants (Mates et al., 1999). Antioxidant enzymes like catalase (CAT), super oxide dismutase (SOD), glutathione peroxidase (GP) and non-enzymatic antioxidants like reduced glutathione, ascorbic acid, α- tocopherol, uric acid have been postulated to comprise the cellular defense system against biological oxidative damage (Halliwell and Gutteridge, 1989; Hermes-Lima and Story, 1993; Droge W., 2002). With increasing age increased generation of ROS have been found in most of the aerobic organisms (Sohal et al., 1990; 1994; 1995) and is considered as the primary factor responsible for age related accrual of molecular oxidative damage (Sohal, 2002). A situation called as oxidative stress can arise when the rate of ROS formation overwhelms the natural capacity of cellular antioxidant defenses so that net damage accumulates (Sies et al., 1983). Oxidative stress can occur under physiological situations such as ischemic / reperfusion and exhaustive exercise (Halliwell and Gutteridge, 1989; Ji, and Fu., 1992) as well as in response to extreme heat, cold or exposure to pollutants (Kapoor et al., 1990; Hausladen and Alscher, 1994). It is also generally agreed upon that oxidative stress plays important roles in acute Cd poisoning (Jie et al., 2009). Studies relating changes in oxidative stress parameters in cadmium exposed animals have been conducted mainly in adult endothermic animals i.e. mice or rat (Xu. et al., 2005; Acharya et al., 2007; Arash et al., 2013). However status of oxidative stress parameters in different age groups of Cd exposed ectothermic animals are rare.

Hence in the present study an attempt was made to assess the pro-oxidant and antioxidant status in liver tissue of Cadmium exposed common Indian toads, *Bufo melanostictus* (Schneider, 1799, presently known as *Duttaphrynus melanostictus)* (Frost et al., 2006) an ectothermic animal of different age groups to know their response towards cadmium toxicity.

MATERIALS AND METHODS

Animals and experimental conditions

In this study 36 male common Indian toads, *Bufo melanostictus* (presently *Duttaphrynus melanostictus*, Frost et al., 2006) of snout to vent length 2.5 cm to 9.9 cm and body weight of 2.5 g to 65 g collected from their natural habitat in and around Paralakhemundi (180 45' North latitude, 840 6' East longitude) localities were used. Age determination of these feral male common Indian toads were done by skeletochronology technique (Castanet, 1994; Smirina, 1994; Kumbar and Pancharatna, 2001) i.e. by counting the LAGs (Lines of arrested growth) in the matrix of their long bones and phalanges. Young toads of snout to vent (s-v) length 2.5 cm to 5.6 cm, body weight 2.8 g to 15 g were without any LAG (Lines of arrested growth) in their bone matrix of both long bones and phalanges and considered to be up to I year old as per skeletochronology technique (Castanet, 1994; Smirina, 1994; Kumbar and Pancharatna, 2001). Similarly sexually mature toads of s-v length 5.8 cm to 8.3 cm, body weight 18 g to 49 g were with 1 to 3 LAGs in the matrix of their long bones and considered to be 1+ to 4 years old and were grouped as middle aged toads. Matured toads of s-v length 8.5 cm to 9.9 cm, body weight 51 g to 65 g were with 4 to 5 LAGs in the matrix of their long bones and considered to be 4+ to 6 years old and were grouped as old toads. These docile toads become fully matured by 2 years and have a maximum life span of 6 years in male individuals in the wild (Nayak et al., 2007). Toads were maintained in a terrarium at room temperature (28± 20C) for one week (12 h light and 12 h dark/day) on a diet of living earthworm and water ad libitum.Toads from young, middle aged and old category were randomly divided into two groups separately of 6 toads each to form the control group and the experimental ($CdCl_2$ treated) group.Experimental animals from three different age (young, middle age and old) groups were injected single dose

of aqueous $CdCl_2$ solution intra-peritoneally at the rate of 2.5 µ g/g body weight, whereas control animals received equal amount of distilled water. On the 7th day after cadmium treatment animals were sacrificed for different biochemical estimations.

Tissue Preparation

Each specimen was killed by stunning its head and its snout-vent length and body weight were measured. Whole liver was dissected out, cleaned of adherent tissues in ice cold (40 ^{0}C) amphibian ringer's solution, weighed and processed immediately for different biochemical estimations.

Measurement of Lipid Peroxidation

The tissue lipid peroxidation (LPO) level was measured as thiobarbituric acid reactive substances (TBARS) formed using tissue homogenate and thiobarbituric acid (TBA) following the method of Sestini et al., (1991) with minor modification as suggested by Jena et al., (1991).

0.5 ml of 2.5% ice cold aqueous tissue homogenate, 1.5ml of 1% orthophosphoric acid and 0.5 ml of 0.6% TBA were mixed in the experimental hard glass test tube with stopper and heated in a water bath for 45 min at 950C. A control tube was also run simultaneously with 0.5 ml distilled water instead of tissue homogenate. The mixtures were cooled to room temperature in running tap water. 3 ml of chloroform and 1 ml of glacial acetic acid was added to both control and experimental tubes and their contents were centrifuged at 1000 x g for 10 min. Extinction of upper phase of the supernatant from experimental tube containing TBARS was measured at 535 nm against the upper phase of the supernatant from the control. The amount of TBARS formed was calculated from the extinction co-efficient of 1.56 x 105 M-1.Cm-1 (Sinhuber et al., 1958) and was expressed as µ mol of TBARS formed /g tissue wet weight.

Assay of Antioxidant Enzyme Activities

Super-Oxide Dismutase (SOD)

A 2.5% (W/V) tissue homogenate was prepared in ice cold 50 mM phosphate buffer (pH-7.4) using a glass potter type homogenizer and centrifuged at 10,000xg for 10 minutes at 4^{0}C using a cold centrifuge (Remi). 1 ml of the supernatant was passed through a 5 ml column of sephadex-G-25 and the elute was used for assay of SOD (EC 1.15.1.1) activity according to the method of Das et al., (2000). In this method superoxide radicals are generated by photoreduction of riboflavin (Beyer and Fridovich, 1987) and its detection by nitrite formation from hydroxylamine hydrochloride as described by Elstner and Heupel (1976) and modified by Pattichis et al., (1994) using Greiss reagent. In brief superoxide radicals were allowed to react with hydroxylamine hydrochloride to produce nitrite which in turn acted upon sulphanilic acid to produce a diazonium compound that formed a red azo compound by reacting with napthylamine whose extinction was measured at 543 nm. SOD scavenges superoxide radicals produced by photo reduction of riboflavin. So nitrite formation in the reaction is inversely proportional to the amount of SOD present in the sample.

Catalase

To 2.5% (W/V) tissue homogenate prepared in ice cold 50mM phosphate buffer (pH-7.4), triton X-100 was added to a final concentration of 1% and centrifuged at 10,000 x g for 10 minutes at 4^{0}C using a cold centrifuge (Remi). The supernatant was used directly for assay of catalase (EC 1.11.1.6) activity by measuring the decrease in extinction of H_2O_2 at 240 nm (Aebi, 1974) using a molar extinction co-efficient of 43.6 m.-1cm.-1. Catalase activity was calculated and expressed as units /mg protein where one unit of catalase represents the amount of enzyme that consume 1 µ mole of H_2O_2 per minute. Protein content of the tissue homogenate (2.5%) prepared in distilled water was measured according to the method of Lowry et al., (1951) using Bovine serum albumin as standard.

Measurement of Non-enzymatic Antioxidant

Ascorbic Acid

Ascorbic acid was extracted after homogenizing and centrifuging the tissue with 6% cold trichloroacetic acid (TCA). The TCA supernatant was used to estimate ascorbic acid content following the method as described by Roe (1954) with slight modification as suggested by Tewary and Pandey (1964). Ascorbic acid in deproteinized tissue extract was oxidized to dehydroascorbic acid (DHAA) which irreversibly transformed into 2,3- diketogulonic acid(DKA) and coupled with 2, 4- dinitrophenylhydrazin to form a coloured product with H_2SO_4 .Its extinction was measured at 530 nm and the result was expressed as µ g/g tissue wet weight.

Uric Acid

Uric acid content in the tissue was estimated following the method of Buchanan et al. (1965), modified from Beale (1954). 0.5 ml of 5% tissue homogenate prepared in ice cold 50 mM phosphate buffer (pH-7.0) was deproteinized by adding 4 ml of N/23 H_2SO_4 and 0.5 ml of 5.6% sodium tungstate to get protein free supernatant following centrifugation at 1000 x g for 10 minutes. Uric acid content in the 3 ml of deproteinized supernatant was estimated from the extinction measured at 720 nm of the product formed after adding 0.2 ml of phosphotungstic acid reagent (PTR) and 1 ml of 0.6N NaOH to it, using the standard curve prepared with known conc. of uric acid. The values were expressed as µ g/g tissue wet weight.

Statistical Analysis

Multiple group comparisons were assessed using one way analysis of variance (ANOVA). Comparisons between two groups were performed by student's't'-test. All results were presented as mean ± S.E.M and differences were considered statically significant when $p < 0.05$.

RESULTS AND DISCUSSION

A number of evidence indicate that transition metals act as catalysts in the oxidative deterioration of biological macromolecules and therefore the toxicities associated with these metals may be due to oxidative tissue damage. Metals such as iron, copper, cadmium, chromium, lead, mercury nickel and vanadium exhibit the ability to produce reactive oxygen species, resulting in lipid peroxidation, DNA damage, depletion of sulfhydryl (-SH) groups of proteins and altered calcium homeostasis (Stohs and Bagchi, 1995). Metal ions perturb the cellular prooxidant – antioxidant balance leading to disruption of normal cellular functions (Dalton et al., 1996). Among various transition metals, cadmium is an abundant nonessential element that is generating concern due to its accumulation in environment and it can manifest cytotoxic and genotoxic effect on different organs in animals (Hartwig, 1998; Jagetia and Adiga, 1994). Cadmium has an extremely long half-life period (20 – 30 years in human body) and is highly cumulative especially in organs like liver, kidney, lungs, bones (Nordberg and Nordberg, 1988; Manca et al., 1991). Prooxidants like reactive oxygen species (ROS) are often implicated in Cd toxicity and carcinogenesis (Jie et al., 2009). Jie et al., (2009) have also reported Cd induced generation of ROS, accompanied by activation of redox sensitive transcription factors (NF-KB, AP-1 and Nrf 2) and alteration of ROS – related gene expression. However Cd induced oxidative damage by disturbing the prooxidant – antioxidant balance in different tissues in relation to aging processes has been rarely reported.

The present study was conducted to assess the Cadmium generated prooxidant and antioxidant status in different age groups (young, middle aged and old) of common Indian toad, Bufo melanostictus; an ectothermic animal model.

In this study a significant ($p < 0.001$) increase in lipid peroxidation (LPO) potential in liver tissue of all the three age groups (young, middle aged and old) of toads was found (Table-1, Fig.-1) following a single dose of cadmium treatment (2.5µ g/g body weight, i. p.). The rate of elevation in LPO potential was found increasing with advancing age of the animal studied (Table-1). Since cadmium exposure causes lipid peroxidation in many tissues as reported earlier (Giralut et al., 1998; Sarkar et al., 1998; Ognjanovic et al., 2003; El-Demerdash et al., 2004; Acharya et al., 2007; Arash et al., 2013) it has been suggested that Cd may induce oxidative stress by producing ROS like -OH, $-O^-_2$, H_2O_2 (Waisberg et al., 2003; Jie et al., 2009). The present finding is in good agreement with the reports made earlier in endothermic animals and accepts the common view that the cadmium induces formation of ROS which enhances the prooxidant status of different organs.

Cadmium exposure has been reported to decrease the enzymatic antioxidant (SOD and catalase) activities in various tissues of rats (Nagraj et al., 2000; Casalino et al., 2002; Yalin et al., 2005; Acharya et al., 2007; Ognjanovic et al., 2008; Arash et al., 2013). In the present study significant decrease ($p < 0.001$) in SOD (Table-1, Fig.-2) and catalase (Table-1, Fig.-3) activities in liver tissue of Cd treated toads of different age groups is in accordance with the earlier report. The decline in SOD activity is presumably due to strong interaction of Cd with the metal binding moieties (Cu, Zn and Mn) of SOD (Casalino et al., 2002). Alternatively, Cd may alter the protein conformation by interacting with the enzyme (Nagraj et al., 2000).Cadmium also has been reported to inhibit the activity of many enzymes by binding to their sulfhydril groups or by inhibiting the protein synthesis (Shaikh et al., 1999; Waisberg et al., 2003). Reduced activities of catalase after cadmium treatment may be due to increased level of tissue prooxidants (ROS) or secondary to the effect on SOD (Kono and Fridovic, 1982). The present study also showed more reduced activities of SOD and catalase in old toads than their young and middle aged counter parts (Table-1) after Cd treatment. This may be due to increased accumulation of Cd in old toads causing gradual time related zinc deficiency and so decreased SOD activity (Bin and Garfinkel, 1994). Decreased activities of antioxidant enzymes during old age (Kashiwagi et al., 2005; Kellogg and Fridovich, 1976) might have contributed to the above process.

Cadmium treatment has been reported to deplete ascorbic acid (Hijova and Nistiar, 2005; Acharya et al., 2007; Ognjanovic et al., 2008) and uric acid (Hijova and Nistiar, 2005) in liver of rats. In the present study a significant decrease ($p < 0.001$) in ascorbic acid (Table-1, Fig.-4) and uric acid (Table-1, Fig.-5) content was found in different age groups of cadmium treated toads. The rate of decrease in these antioxidant small molecules after Cd treatment was found more in senile toads than their younger counter parts (Table-1). It is known that increased accumulation of Cd in the liver induces lipid peroxidation

Table 1 Effect of $CdCl_2$ (2.5 µ g/g body weight i. p.) on lipid peroxidation (LPO) level, SOD, catalase, ascorbic acid (ASA) and uric acid (UA) content of liver tissue of male common Indian toads of three different age groups.

Animals of different age groups	Experimental Conditions	LPO (µg moles of TBRAS/g tissue	SOD (units/mg protein)	Catalase (units/mg protein)	ASA (µ g/g tissue)	UA (µ g/g tissue)
Young (Up to 1 yr.)	Control	0.137± 0.016	4.12±0.32	5.86±0.13	230.02±13.16	374.01±7.95
	Experimental	0.250± 0.02**	2.05±0.16***	2.31±0.10***	120.16±13.15***	153.00±7.27***
	% of change	+ 82%	-50%	-60%	-47%	-59%
Middle Aged(1+ to 4 yrs.)	Control	0.208±0.007	5.30±0.42	6.50±0.12	186.01±10.54	429.98±9.06
	Experimental	0.403±0.02***	2.75±0.306***	3.08±0.14***	125.50±10.70**	240.00±14.00***
	% of change	+94%	-48%	-52%	-33%	-44%
Old (4+ to 6 yrs)	Control	0.400±0.022	2.02±0.24	4.71 ± 0.33	119.88±7.31	340.95±16.31
	Experimental	0.796±0.05***	0.85±0.117**	1.7± 0.19***	63.83±6.65***	128.00±14.11***
	% of change	+99%	-58%	-64%	-47%	-62%

Data are expressed as mean ±S.E.M., (n=6). The significant differences calculated from those in control group are designated as ** (P<0.01), *** (p<0.001).

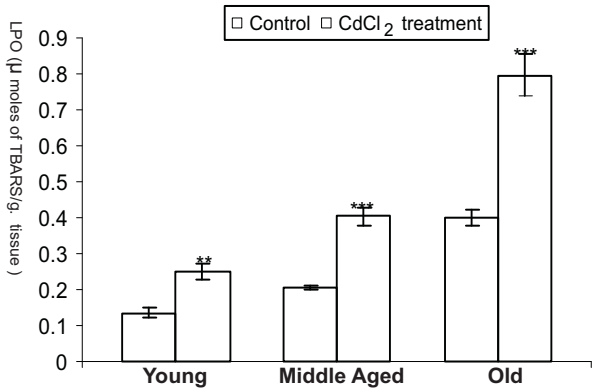

Fig.1: Effect of $CdCl_2$ (2.5 µ g/g body weight i. p.) on LPO (lipid peroxidation) of liver of male common Indian toad, of three different age groups. Data are expressed as mean S.E.M., (n=6). ** (p<0.01); *** (p<0.001) different

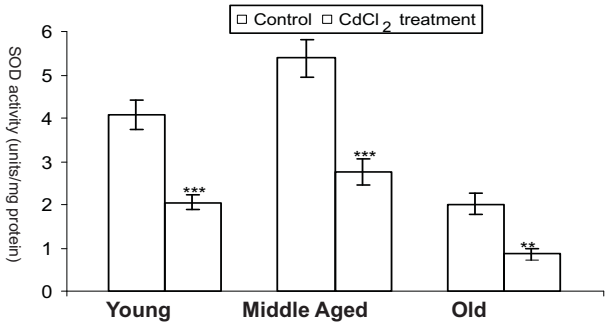

Fig.2: Effect of $CdCl_2$ (2.5 µ g/g body weight, i. p.) treatment on SOD activity of liver of male common Indian toad, of three different age groups. Data are expressed as mean±S.E.M. (n=6). One unit of SOD represents the amount of enzyme capable of inhibiting 50% of nitrite formation under assay condition. **(P<0.01),***(P < 0.001) different

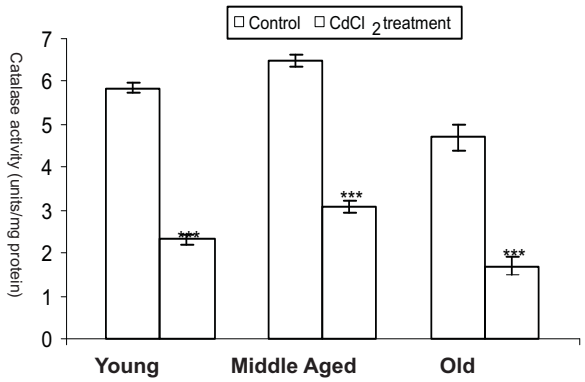

Fig.3: Effect of $CdCl_2$ (2.5 µg/g body weight, i. p.) treatment on catalase activity of liver of male common Indian toad of three different age groups. Data are expressed as mean ±S.E.M. (n=6). One unit of catalase represents the amount of enzyme that consumes 1 mole of H2O2 per minute. ***(P < 0.001) different from control.

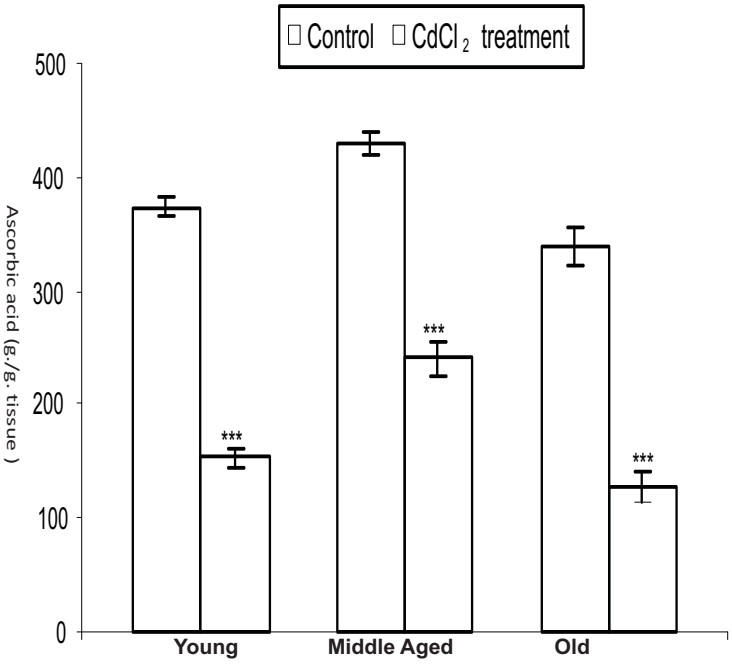

Fig.4: Effect of $CdCl_2$ (2.5 µg/g body weight, i. p.) treatment on ascorbic acid content of liver of male common Indian toad of three different age groups. Data are expressed as mean ± S.E.M. (n=6). **(P < 0.01), **(P < 0.001) different from control.

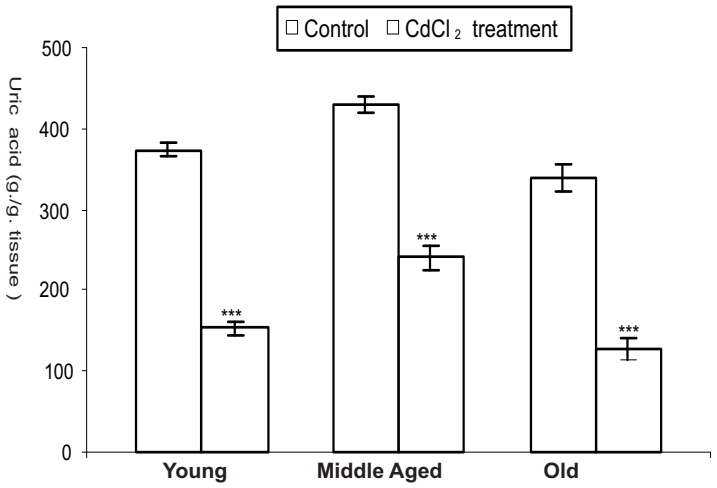

Fig.5: Effect of $CdCl_2$ (2.5 µg/g body weight, i. p.) treatment on uric acid content of liver of male common Indian toad, *Bufo melanostictus* of three different age groups. Data are expressed as mean ±S.E.M. (n=6). ***(P < 0.001) different from control.

Dialdehyde (MDA) and 4-Hydroxy Non-Enal (4-HNE)) which consequently inhibits the enzyme L-gulonolactone oxidase (Chatterjee et al., 1973; Hudecova and Ginter, 1992) that is necessary for synthesis of ascorbic acid which is a potent scavenger of ROS. So in senile toads decreased ascorbic acid content in their liver may be due to increased accumulation of cadmium and / or due to aging process (Ruth et al., 1984; Rikans et al., 1996; Patnaik and Kanungo, 1966). The antioxidant action of ascorbic acid is more complex. It may complex cadmium or redox metal ions displaced by cadmium to prevent lipid peroxidation. It may also involve regeneration and / or reduction of other antioxidants like uric acid, vitamin E. In this study significant decrease ($p < 0.001$) in uric acid content of liver of Cd-treated toads of all the age groups (Table-1, Fig.-5) is in accordance with the earlier report (Hijova & Nistiar, 2005). The high rate of decrease in uric acid content in the liver of old toads (Table-1) may be due to low ascorbic acid content in that tissue and/or due to aging (Lee et al., 1995; King et al., 1997).

CONCLUSION

In conclusion cadmium treatment was found to cause increased generation of prooxidants (ROS) causing oxidative stress in liver of different age groups of common Indian toads. In addition to oxidative assault of biomolecules, antioxidant enzymes and non-enzymatic antioxidants were found depleting. This may be due to interaction of Cd with the metal-binding moieties of enzymes and inhibition of synthesis of non-enzymatic antioxidants by increased accumulation of lipid peroxidation products (MDA, 4-HNE). However the degree of depletion of antioxidant enzymes and non-enzymatic antioxidants was minimum in middle aged toads than their young and old counter parts. Increased cadmium accumulation in old toads and age related decrease in antioxidants might have forced the old toads into more oxidative stress. The response shown here in this study by an ectothermic animal model is very much similar to that in endothermic animals as reported earlier. This may be due to the terrestrial mode of life and comparatively better management of body temperature in toads.

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