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Age Related Protein Carbonylation In Different Organs Of Common Asian Toad, *Duttaphrynus Melanostictus*

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ABSTRACT

Protein carbonylation measured in terms of nano moles of 2, 4-DNPH incorporated /mg protein was found increasing significantly ($p < 0.001$) with advancing age in liver, kidney and brain of common Asian toad, *Duttaphrynus melanostictus*. There was significant positive correlation between carbonylated protein in different organs studied and increasing age of the animals. Liver showed highest, brain moderate and kidney with lower level of carbonylation in protein molecules. Since different organs were with different level of protein carbonylation, it appears some selected protein molecules are likely to be more carbonylated than others. Common Asian toads being ectothermic animals showed similar age related increasing trend of protein carbonylation like their endothermic mammalian counterpart indicating a common mechanism of accumulation of modified proteins during ageing.

INTRODUCTION

Ageing process causes accumulation of macromolecular damage in older individuals possibly due to low rate of repair or renewal of macromolecules. Accumulation of such damages lead to the derangement of cellular functions contributing to ageing and age associated diseases. Age associated accumulation of oxidative damage in mt-DNA correlates with the level of mt-DNA deletions observed in post mitotic tissues (Mecocci et al., 1993; Ames et al., 1995). Similarly oxidative damage to lipids (Lipid peroxidation) leads to loss of membrane properties and therefore, transport of metabolites. Oxidized proteins lead to aggregation, fragmentation,denaturation and cross linking by lipidperoxidation aldehydes like MDA (Malondialdehyde) and 4 HNE (4-hydroxy-non enal) (Bertrandfriguet and Luke Iszweda, 1997). Modifications introduced by oxidation of proteins make them more susceptible for proteolysis (Yu, 1994). Since there is an age related decline in protease activity there is a rise in level of damaged protein products with advancing age (Stadtman and Levine, 2000). The mechanism of oxidative modification of proteins involves mixed function of oxidation system (Stadtman, 1992) and oxygen free radical catalyzed reactions (Oliver et al., 1987). Many enzymes of different metabolic pathways are believed to be subjected to oxidative inactivation due to modification through oxidation of histidyl/ lysyl residues in catalytic sites. Some of the amino acid residues particularly proline, arginine and lysine are oxidized to carbonyl derivatives and estimation of carbonyl content of protein has been used as an estimate of protein oxidation during ageing.

Most of the age related studies on modifications of proteins are limited to some tissues in a few mammalian species. Sohal et al. (1993, 1994 and 1995) have reported increase in protein carbonyls with advancing age in hepatocytes of rat, brain and kidney of mice and also in brain tissues of gerbils. Serum protein carbonyl content was found to increase in an age related pattern in human beings (Kasapoglu and Ozben, 2001). Accumulation of oxidized and cross-linked proteins during aging of non-dividing human fibroblasts has been reported from Nicolle Sitte et al., 2000. The age related increase in oxidized protein is also associated with reduction in catalytic activity and heat stability of some enzymes in mammalian system (Stadtman and Levine, 2000; Sohal R.S., 2002). Bhattacharya et al., (2011) have also reported significant reduction in protein carbonylation in insoluble fraction of liver tissues of long lived mammalian species than their short lived counterparts. So from a comparative gerontological point of view it is necessary to know whether protein carbonylation is also a contributing factor in the ageing of organisms other than mammals' specifically ectothermic animals with different ageing patterns (Goss, 1974). Studies related to protein carbonylation with increasing age in ectothermic animals are very rare (Sohal et al., 1993; Jena et al., 1996) and almost nil with amphibians. So the present study aims to demonstrate whether protein carbonylation occurs in different organs like liver, brain and kidney of an ectothermic animal i.e. Common Asian toad, *Duttaphrynus melanostictus*.

METERIALS AND METHODS

Animals and age determination

In the present study male common Asian toads, (*Duttaphrynus melanostictus*) of snout-vent length 2.5 cm to 9.9 cm and bodyweight of 2.5 g to 65 g collected from Paralakhemundi localities (18° 45' North latitude, 84° 6' East longitude) were used after collection from their natural habitat.The toads were maintained in a terrarium at room temperature (28± 2° C) and 12 h light and 12 h dark/day. They were fed with live earthworms on alternate days and tap water was provided ad-libitum, before carrying out the experiment. These toads became fully mature by 2 years and have a maximum life span of 6 years in wild (Nayak et al. 2007; Sahoo D.D., 2012). The ages of individual toads were determined by counting lines of arrested growth (LAG) in their bone matrix of both long bones and phalanges following skeletochronology technique (Castanet, 1994; Smirina, 1994; Kumbar and Pancharatna, 2004). Male common Asian toads of snout to vent length up to 5.6cm and body weight less than 15g were without LAG in their long bones, sexually immature and as such they are said to be nearly one year old and grouped under young age group. Toads of snout-vent length ranging from 5.8 cm to 8.3 cm, body weight of 18 g to 49 g were sexually mature and with 1 to 3 LAGs in their long bones. They were considered to be 1+ year to 3+ or nearly 4 years old and were grouped as middle aged toad. Large sized toads of snout-vent length8.5 cm to 9.9 cm, and body weight 51 g to 65 g were sexually mature and with 4 to 5 LAGs in their long bones and phalanges. They were considered to be 4+ years to 5+ or nearly 6 years old and were grouped under old age group.

Preparation of tissue homogenates

The laboratory acclimated male common Asian toads (Duttaphrynus melanostictus) were killed by stunning on their head. The body weight and snout-vent length of individual toads were recorded. Then whole liver, kidney and brain were dissected out, cleaned of adherent tissues in ice cold (4°C) 0.25 M sucrose solution, weighed and processed immediately for different biochemical estimations.

A 3% tissue homogenate was prepared separately for each of the organs from individuals of different age groups in 0.25 M sucrose solution using a potter Elvehjem type of homogenizer with taflon pestle (Remi). The homogenates were transferred to pre-cooled centrifuge tubes and were centrifuged at 1000 x g for 10 minutes at 40C using a cold centrifuge (Remi). The supernatant of each sample was used for the estimation of protein carbonyl content and protein content.

Measurement of protein carbonyl content

Protein carbonyl content as an index of oxidized proteins in the sucrose soluble fraction of the homogenate of each sample was measured following the method of Uchida and Stadtman (1993). In this method the experimental tube contained and 0.8 ml of 0.1% (W/V) 2, 4-Dinitro phenyl hydrazine (2, 4-DNPH) in 2N HCl, whereas the control tube contained 0.8 ml of supernatant and 0.8 ml of 2N HCl only.

Both the control and experimental set of tubes were incubated at room temperature (28 ± 2^{0}C) for 1h. in a dark place with thorough shaking at every 15 minutes intervals. After the incubation period, 0.8 ml of ice cold 20% trichloroacetic acid (TCA) was added to all test tubes and the contents were centrifuged at 3000 rpm for 10 minutes. The supernatants were discarded and the residues were washed thrice with 1 ml of ethanol/ethyl acetate mixture (1:1 V/V) to remove free reagents. Then the residues were dissolved in 2 ml of 8 M guanidine hydrochloride prepared in 133 mM Tris buffer (pH 7.2) containing 13 mM EDTA.The extinction of the content present in the experimental tube was measured at 365 nm in a UV/Visible spectrophotometer (Systronics-119) against the content of the control tube. The protein carbonyl content was calculated from the molar extinction coefficient of 22 x 10^3x M-1 x cm-1 and the result was expressed as nano moles of 2, 4-DNPH incorporated /mg protein.

Estimation of protein content

Protein content of the tissue homogenates (Sucrose soluble fraction) of male common Asian toads of different age groups were estimated following the method of Lowry et al., (1951) using bovine serum albumin (Loba) as standard to express the protein carbonyl content in different tissues in terms of mg protein.

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. Linear regression analyses were also conducted to compare the changes of parameter with increasing age. All results were presented as mean ±SEM and differences were considered statistically significant when P < 0.05.

RESULTS AND DISCUSSION

Among the various types of macromolecular oxidative damage that arises during ageing, oxidative modifications of intracellular proteins have been suggested to play a key role in the causation of senescence associated losses in physiological functions. This is because oxidized protein often loses catalytic function and undergoes selective degradation (Stadtman and Levine, 2000). Several types of ROS induced protein modifications have been demonstrated (Shacter, 2000; Stadman and Levine, 2000) including loss of sulfhydryl (-SH) groups, formation of disulphide cross links, glyoxidation, lipid peroxidation adducts and protein carbonylations.

In the present study protein carbonyl content was found increasing significantly(P<0.001) (Table-1 and Fig.4) and showing positive correlation with advancing age in liver(Fig.1), kidney(Fig.2) and brain(Fig.3) of common Asian toads.The protein carbonyl content was found highest in liver, moderate in brain and lowest in kidney tissues in all the age groups(Table-1). It seems sensibilities of proteins for carbonlyation differ from organ to organ being influenced by their internal environment. Since protein carbonylation are generally thought to arise because of interaction with lipid peroxidation products (Uchida and Stadtman, 1993), the protein carbonylation and lipid peroxidation could be correlated in different organs. Reactive carbonyl compounds produced during lipid peroxidation like ALES (Advanced Lipid Peroxidation end Products) accumulate with ageing and age related diseases like neuro degenerative diseases, diabetes, atherosclerosis (Negre-Salvayre et al., 2008). Reactive carbonyl compounds (RCC) have been reported to form adducts, and cross linking on proteins, (Peterson and Doom, 2004). Lipid peroxidation potential of liver which has been reported to be higher than that in kidney tissue in our earlier report (Sahoo D.D. & Kara T.C, 2014) is in good agreement with our present findings. However higher lipid peroxidation potential in brain than that in liver as reported earlier (Sahoo D.D & Kara T.C, 2014) may be due to high PUFA (poly unsaturated fatty acid) content in the membranes of neurons and their high oxygen consumption.

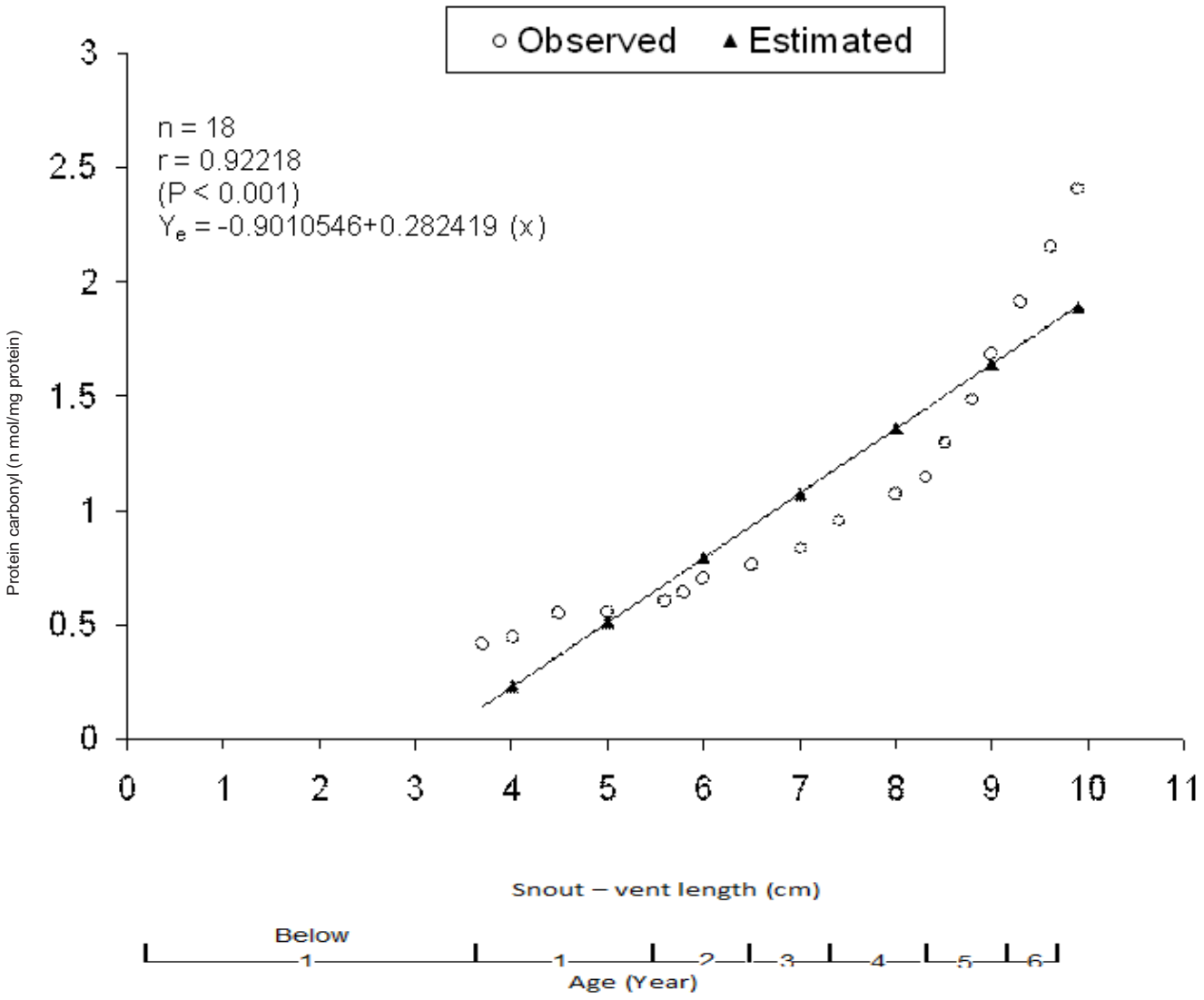

Fig. 1 : Linear regression analysis of age versus protein carbonyl content (nano moles of 2, 4-DNPH incorporated/mg protein) of liver of male common Asian toad, *Duttaphrynus melanostictus.*

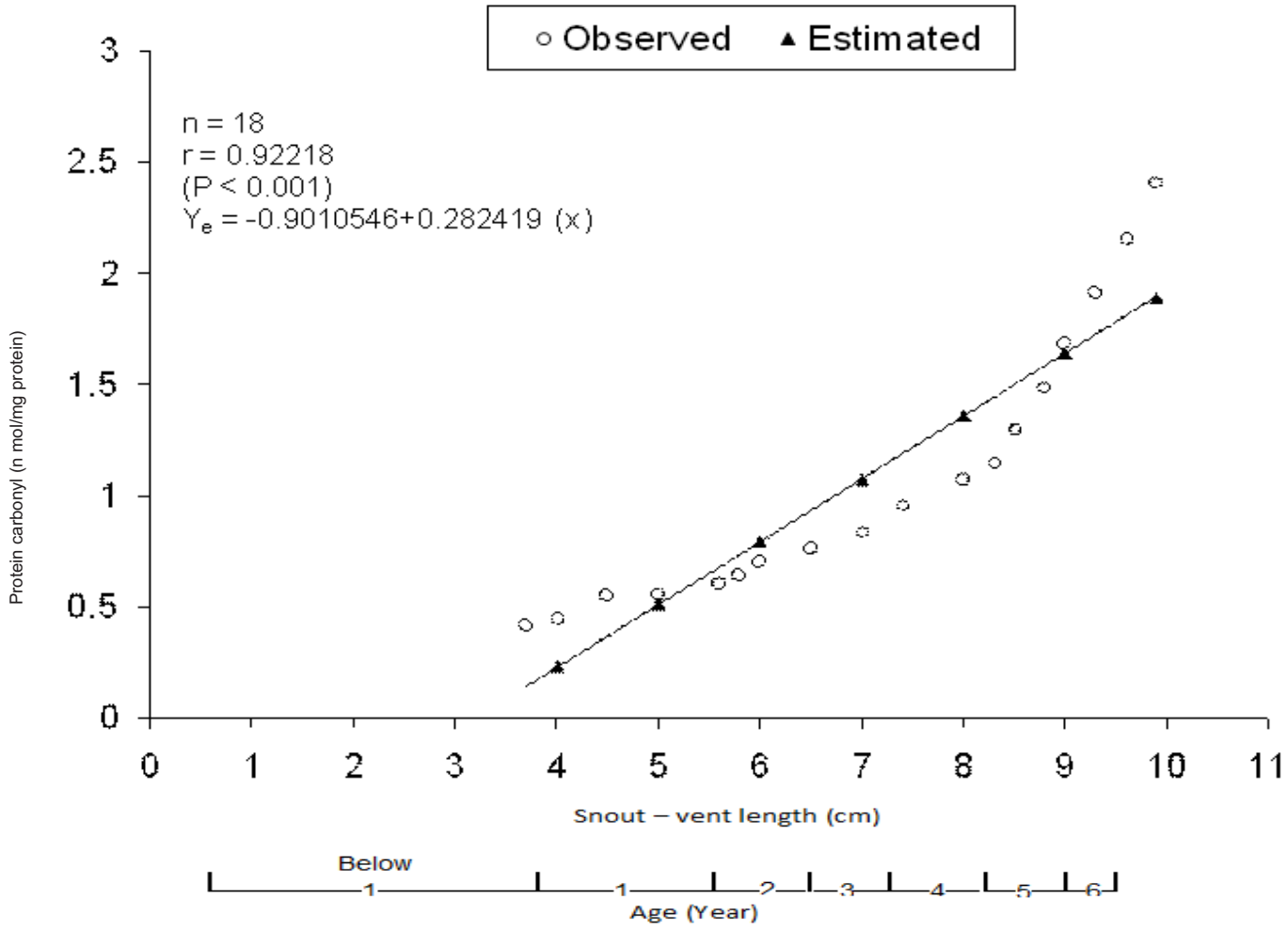

Fig.2 : Linear regression analysis of age versus protein carbonyl content (nano moles of 2, 4-DNPH incorporated/mg protein) of kidney of male common Asian toad, *Duttaphrynus melanostictus.*

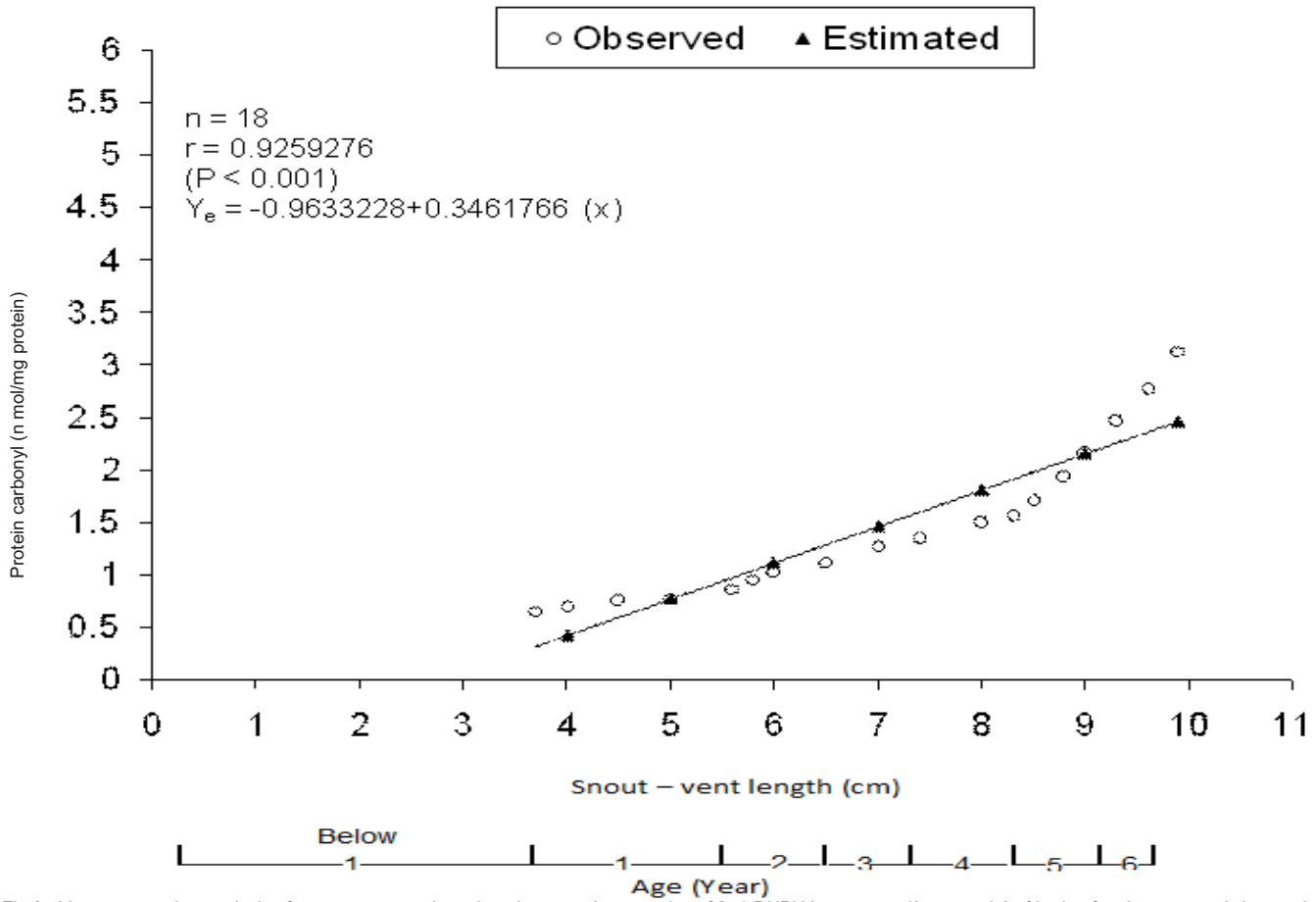

Fig.3 : Linear regression analysis of age versus protein carbonyl content (nano moles of 2, 4-DNPH incorporated/mg protein) of brain of male common Asian toad, *Duttaphrynus melanostictus*

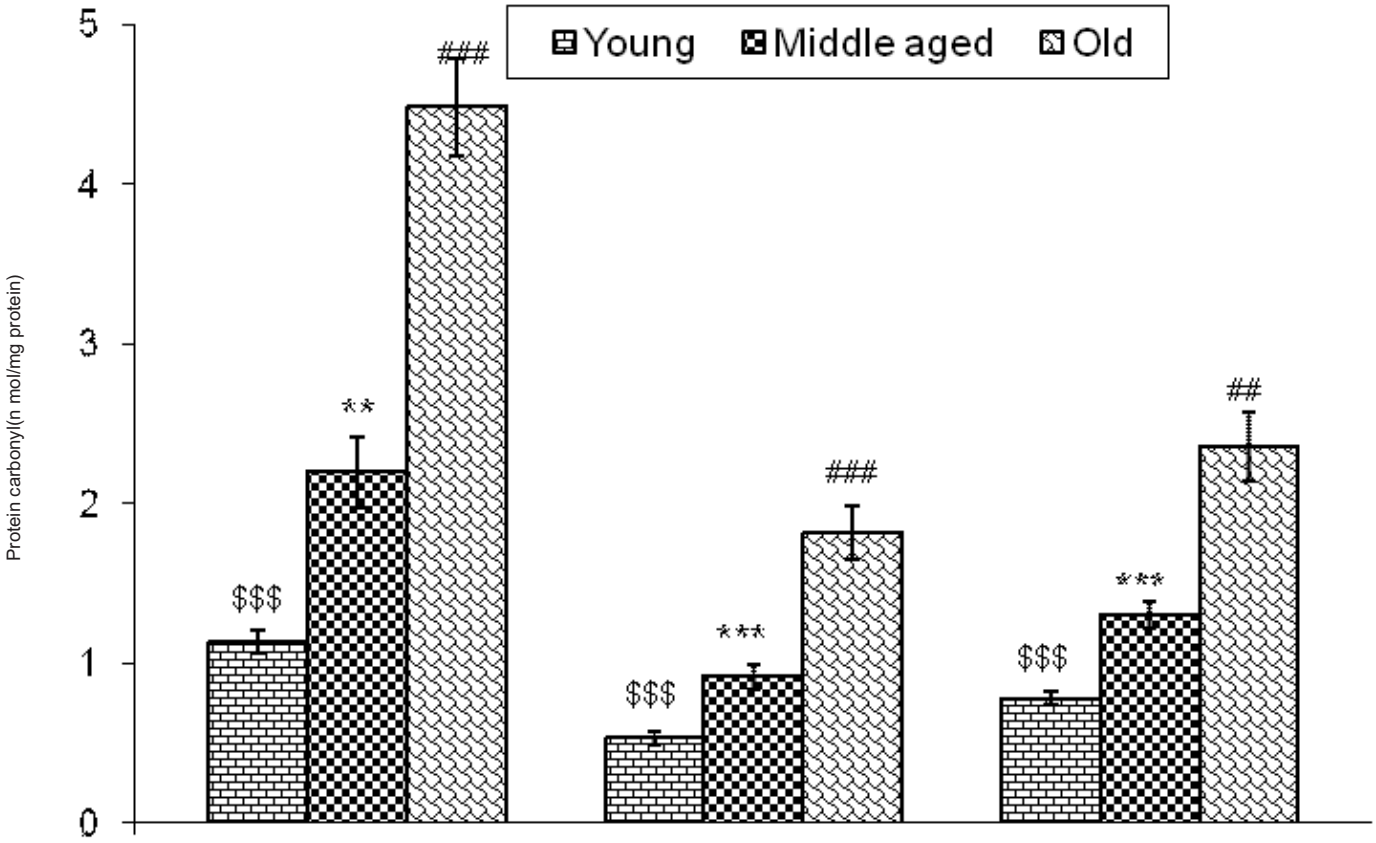

Fig.4 :Effect of age on protein carbonyl content (nano moles of 2, 4-DNPH incorporated/mg protein) of liver, kidney and brain of male common Asian toad, *Duttaphrynus melanostictus*.Data are expressed as mean S.E.M. (n=6).

**Significant difference between Young and Middle aged (P<0.01)
***Significant difference between Young and Middle aged (P<0.001)
##Significant difference between Middle aged and old (P<0.01)
###Significant difference between Middle aged and old (P<0.001)
$$$Significant difference between old and young (P<0.001).

Table:1 Age related changes in protein carbonyl content of liver, kidney and brain of male common Asian toad, *Duttaphrynus melanostictus.*

Age group (years)	Liver	Kidney	Brain
	Protein Carbonyl content (n mole of 2, 4-DNPH incorporated/mg Protein)	Protein Carbonyl content (n mole of 2, 4-DNPH incorporated/mg Protein)	Protein Carbonyl content (n mole of 2, 4-DNPH incorporated/mg Protein)
Young (Up to 1 year)	1.13 ± 0.073 $$$ (6)	0.53 ± 0.037 $$$ (6)	0.78 ± 0.045 $$$ (6)
Middle aged (1^+ to 4 years)	2.20 ± 0.222 ** (6)	0.913 ± 0.072 *** (6)	1.30 ± 0.083 *** (6)
Old (4^+ to 6 years)	4.49 ± 0.306 ### (6)	1.82 ± 0.169 ### (6)	2.36 ± 0.218 ## (6)

Data are expressed as Mean S.E.M., Number in parentheses indicates animals used.
Significant difference between young and middle aged - **(P < 0.01), *** (P < 0.001).
Significant difference between middle aged and old - ## (P < 0.01), ### (P < 0.001)
Significant difference between old and young - $$$ (P < 0.001)

Susceptibility of brain for high lipid peroxidation has been reported in endothermic animals like rat, because of its high PUFA content which peroxidizes easily (Cotman and Peterson 1989; Sahoo and Chainy, 1997). Consequently protein carbonyl content in brain, expected to be higher than that in liver tissues is actually with lower value than that in liver (Table-1). This may be due to high antioxidant protection in brain (Jena et al., 1996; Guilherme et al., 2006) and proteasome activity. Ascorbic acid which is a potent antioxidant (Ames et al., 1993; Chatterjee et al., 1995) and acts as a free radical trap (Sandnes, 1991) has been reported to be in higher concentration in brain than in liver tissue of common Asian toads (SahooD.D., 2012). Jena et al., (1996) have reported about the better protective action of ascorbic acid in brain than in liver tissue of male garden lizard, *Calotes versicolour*. They have also speculated more antioxidant protection in brain and kidney than in liver tissue against oxidative modification of proteins.

It is suggested that oxidative modification to proteins plays an essential role in ageing. Oxidative damage of specific proteins links oxidative stress and age related losses in physiological functions (Sohal, 2002). An increase in the rate of post translational modification of proteins involving oxidation, deamination, glycation and a decrease in the rate of degradation of modified proteins by proteasome result in the accumulation of abnormal proteins during ageing (Stadtman et al., 1993). A series of studies by Stadtman and his associates have demonstrated that amounts of protein carbonyl contents increases with age in several different mammalian tissues (Stadtman, 1995; Stadtman and Barlett, 1997). In the present study significant increase in protein carbonyl content and its positive correlation with advancing age in liver, kidney and brain tissues of common Asian toads are in conformity with the observations reported earlier in endothermic animals (Stadtman, 1992; Stadtman et al., 1993). Chin-Yuan-Hsu et al., (2008) have also reported concomitant elevated protein oxidation and decrease in antioxidant defense with increasing age in annual fish, Nothobranchius rachovii. In our ealier report (Sahoo, D.D., 2012) lipid peroxidation was found to increase significantly with age in common Asian toads. The results of present study are in conformity with our earlier report as lipid peroxidation products cause extensive modification of cellular protein (Grune and Davies, 2003). Ageing process which is associated with increase in oxidative stress, gradual decrease in antioxidant defense and decreased proteasome activity (Gracy et al., 1991; Friguet, 2006) might have caused age related increases in protein carobonylation in different organs observed in this study. Moreover, decreased degradation of modified proteins and consequently their accumulation could result direct inhibition of proteasomes (Grune and Davies, 2003). Breusing et al., (2009) have also reported about the inverse correlation of protein carbonylation and proteasome activity in rat liver, lungs and kidney tissues. So, the results in this study are in conformity with the previous findings and there may be a common mechanism responsible for age related increase in protein carbonylation in both ectothermic and endothermic animals.

CONCLUSIONS

In conclusion there was significant increase in the accumulation of protein carbonyl contents in liver, kidney and brain tissues with advancing age in male common Asian toad (*Duttaphrynus melanostictus*). Among all the organs investigated liver was with highest, brain moderate and kidney was with lowest level of protein carbonyl content. In vivo oxidative modifications of cellular proteins appears to be the most common mechanism leading to accumulation of carbonylated proteins during ageing of both ectothermic and endothermic animals. However degree of protein carbonylation was found different from one organ to other. So it appears only a selected number of proteins are prone to carbonylation. How far protein carbonylation promotes senescence in different species of animals needs an assessment and further extension of studies.

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