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Research Article

GENDER DISPARITY OF N-NITROSODIETHYLAMINE INDUCED HEPATOCELLULAR CARCINOMA IN WISTAR ALBINO RATS

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Abstract

Cancer is particularly alarming in developing countries like India where life expectancy is increasing rapidly. Over the past 25 years the incidence and mortality rates of Hepatocellular carcinoma (HCC) are expected to double over the next 10–20 years.HCC is a transformation process of normal liver cell into a cancerous cell which involves initiation, promotion and progression stages.HCC was induced by administering 0.01% of N-Nitroso diethylamine (DEN) to animals through drinking water for about 15 weeks. DEN induces lethal cellular injuries & initiates carcinogenesis as a result of metabolic activation & transfer of an electrophilic species to DNA. With the help of available literature it can be hypothesized that gender difference is detectable in the occurrence of HCC and hence the present work is an attempt to prove the difference in the rate of cancer progression between the male and female rats when induced with DEN. The level of marker enzymes such as γ-Glutamyl transferase (γ-GT), 5'Nucleotidase was assayed to study the cancer progression while the level of lipid peroxidation(LPO) and protein carbonyl indicated the membrane damage in HCC bearing animals. The male animals showed profound biochemical alterations when compared to the female rats indicating its high HCC incidence rate.

Key words: Hepatocellular carcinoma, Diethylnitrosamine.

Cancer is a group of disease characterized by disregulated proliferation of abnormal cells that invade and disrupt surrounding tissues (1).It is a dynamic process that involves many complex factors which causes extensive morbidity and wide mortality in the human population. HCC is the fifth most-common malignancy in the world and is

the third most-common cause of cancer-related death worldwide (2, 3) .Hepatocarcinogenesis is a multistep process involving genetic and epigenetic alterations of various oncogenes, proto-oncogenes, and growth factors, as well as tumor suppressors. HCC arises due to chronic inflammation and subsequent liver fibrosis (4).

DEN is a widely occurring nitrosamine which is present in tobacco and various processed foods.It is one of the most important environmental carcinogens and primaly induces tumor of liver. It is normally used as hepatocarcinogen to induce liver cancer in animal models (5).In the present study DEN is used as a carcinogen to induce liver cancer since there was sufficient evidence of carcinogenic effect to classify DEN as possible human carcinogen (6). HCC was induced by administering 0.01% of DEN to animals through drinking water for about 14 weeks.

The primary event in DEN-induced carcinogenesis is DNA-damage leading to genetic mutations in healthy liver. Alkylation of DNA takes place at many sites (7) but attack at the O^6- position of guanine has received considerable attention and subsequent confirmation that the O^6-alkylguanine moiety is a miscoding lesion and may be responsible for the mutagenic and carcinogenic effects of dialkylnitrosoamines and related alkylating agents. (8,9)

HCC is considerably more common in men than women, with an incidence ratio of approximately 3:1 (10) Earlier studies have revealed that male rodents are more susceptible to chemically induced hepatocarcinogenesis (11). In order to understand the mechanisms underlying gender disparity in HCC, the researchers used DEN to induce cancer in mice. This resulted in HCC in 100 percent of male mice, but only in 10 to 20 percent of their females. They discovered that normal female mice given DEN produced far less Interleukin-6(IL-6) than the males and the incidence rate in males was found to be reduced by eliminating IL-6. Recently, increasing molecular mechanisms underlying the carcinogenic effect of both sex hormones were reported adding support to the gender disparity observed in hepatocellular carcinoma.

In DEN induced liver cancer free radical mediated oxidative stress is observed and hence LPO and Protein carbonyl was assayed in order to sort out the difference in the membrane damage caused by DEN in male and female rats. The level of marker enzymes such as LDH, 5' Nucleotidase, and **γ**-GT were assayed which serves as an indicator of cancer progression. The membrane permeability was found to be altered in DEN induced HCC and hence the

activity of ATPases was assessed in male and female rats. This comparative study of cancer induction was designed to place on record that males are more prone to liver cancer when compared to females.

Materials and Methods

Chemicals

Diethylnitrosamine (DEN) was purchased from sigma chemical company St. Louis, MO, USA. All other chemicals used were of analytical grade.

Animals

Male and Female Wistar strain rats weighing 160 ± 10 g were obtained by inhouse breeding from Dr.A.L.M PGIBMS, University of Madras, Taramani Campus. The rats were maintained under standard conditions of humidity, temperature (25 ± 2ºC) and light (12 ± 1 h light/dark). They were fed with standard rat pellet diet and had free access to water.The experimental designs were approved by the Institutional Animal Ethical Committee. (IAEC No.01/029/2010).

Experimental design

The animals were divided into four groups of six animals each.

Group I : Normal male control rats fed with standard diet and pure drinking water for 15 weeks.

Group II : Male rats induced with HCC by providing 0.01% N-Nitrosodiethylamine (DEN) through drinking water for 15weeks.

Group III : Normal female control rats fed with standard diet and pure drinking water for 15 weeks.

Group IV : Female rats induced with HCC by providing 0.01% N-Nitrosodiethylamine (DEN) through drinking water for 15weeks.

After the experimental period the animals were sacrificed by cervical decapitation and the liver tissues were immediately excised, weighed and processed for homogenization with motor driven Teflon coated homogenizer in ice-cold 0.1 M Tris-HCl buffer, pH 7.4 to get 10% homogenate. The liver tissue homogenate was used for the following biochemical estimations.

Biochemical Analysis

Total protein was estimated by the method of Lowry et al(12). The level of lipid peroxides was assayed by the method of Ohkawa *et al.*,1979. (13) The protein carbonyl content was quantified by the method of Levine *et al.* 2002(14). The activity of γ-glutamyl transpeptidase was estimated according to the method of Orlowski and Meister 1965(15). 5′-Nucleotidase was assayed by the method of Luly *et al.* 1972(16). Na^+, K^+-ATPase was estimated by the method of Bonting 1970(17). The activity of Ca^{2+}ATPase was assayed according to the method of Hjerten and Pan 1983(18), the activity of Mg^{2+} ATPase by the method of Ohinishi *et al*., 1982.(19).

Statistical Analysis

The values are expressed as mean ± standard deviation (SD). The results were computed statistically (SPSS software package, version 7.5) using one-way analysis of variance (ANOVA). Post hoc testing was performed for inter-group comparison using LSD comparison test.

Results

Figure-1 shows the changes in the body weight of the control and experimental groups. The female induced animals did not show a drastic decrease in the body weight indicating their ability to restore the body weight when compared to the male induced animals. A significant decrease ($p<0.001$) in body weight was observed in male induced animals when compared to the control animals.

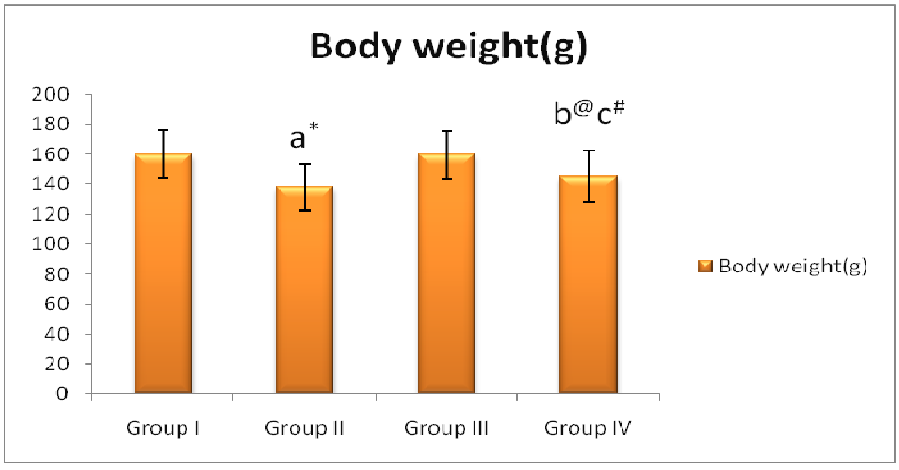

Figure 1: Changes in the body weight of the control and experimental groups

Each value is expressed as mean SD for six rats in each group

a:Group I compared with Group II

b:Group II compared with Group IV

c:Group III compared with Group IV

The symbol *represents statistical significance at p<0.001,#-p<0.01,@p<0.05

Figure 2 shows the level of LPO in the liver of control and experimental animals. It was found that the cancer bearing Group II animals showed a significant (p<0.001) increase in the LPO in the liver when compared with control animals (Group I). The female induced animals showed a lesser significance (p<0.05) in the levels of LPO when compared to the male induced animals.

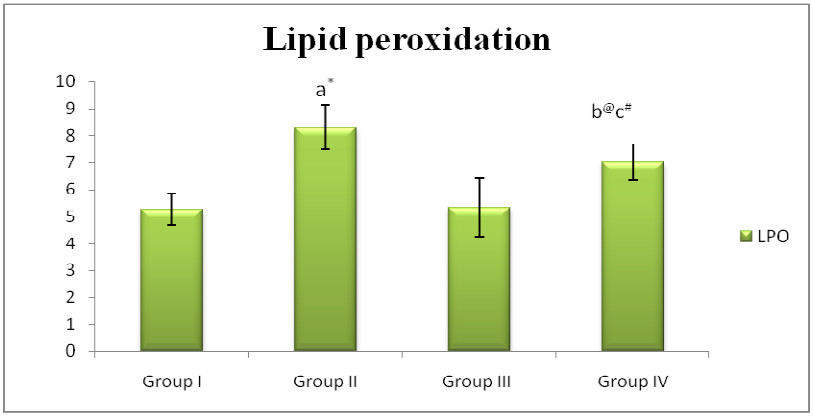

Figure 2: Level of lipid peroxides in control and experimental animals

Each value is expressed as mean SD for six rats in each group

LPO-n moles of MDH formed/mg protein.

a:Group I compared with Group II

b:Group II compared with Group IV

c:Group III compared with Group IV

The symbol *represents statistical significance at p<0.001,#-p<0.01,@p<0.05

Figure 3 gives an indication of the levels of protein carbonyl in the liver of control and induced animals. A significant increase (p<0.001) was observed in the cancer bearing male animals when compared to the normal animals.The significance observed while comparing the female HCC bearing animals with their controls was p<0.01.

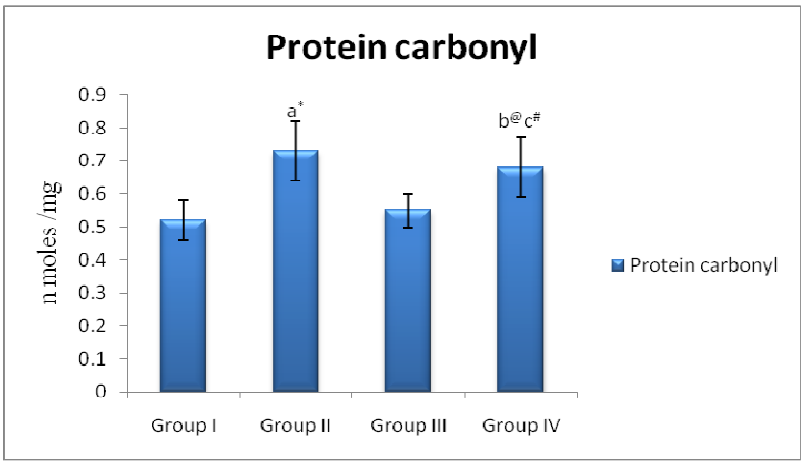

Figure 3: **Levels of protein carbonyl in the liver of control and induced animals**

Each value is expressed as mean SD for six rats in each group
Protein carbonyl-n moles of DNPH incorporated/mg protein.
a:Group I compared with Group II
b:Group II compared with Group IV
c:Group III compared with Group IV
The symbol *represents statistical significance at p<0.001,#-p<0.01,@p<0.05

Figure-4: shows the activities of membrane bound ATPase in the liver tissues of control and experimental animals. Decrease in the activities of Na^+/K^+ATPase and Mg^{2+}- ATPase and increase in the Ca^{2+}ATPase was seen in cancer bearing Group II and Group IV animals. A lesser significance (p<0.05) was observed in HCC bearing female rats when compared to the male induced animals (p<0.001) but there was an alteration in the enzyme activities when compared to female control animals (p<0.01).

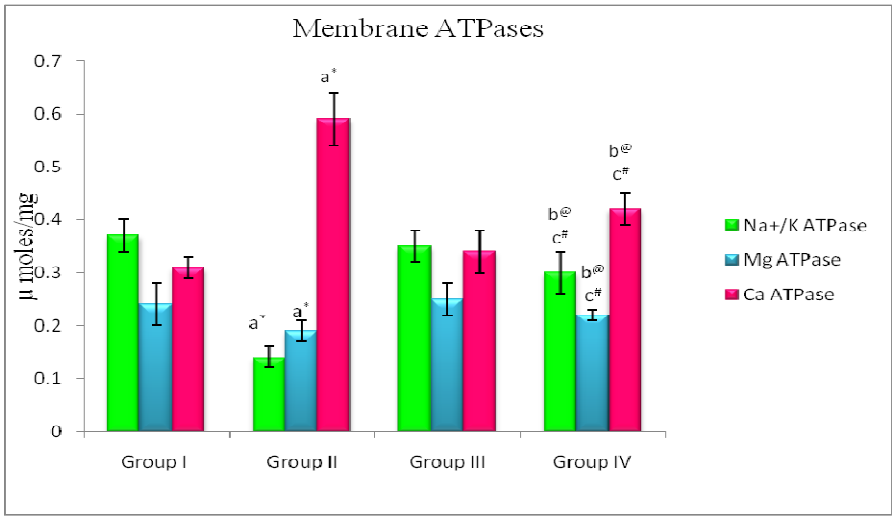

Figure 4: Activities of membrane bound ATPases in the liver tissues of control and experimental animals

Each value is expressed as mean SD for six rats in each group
ATPase-μ moles of phosphorus liberated/min/mg protein.
a:Group I compared with Group II
b:Group II compared with Group IV
c:Group III compared with Group IV
The symbol *represents statistical significance at p<0.001,#-p<0.01,@p<0.05

Figure-5: gives an indication of the level of the marker enzyme 5'Nucleotidase in the liver of control and induced animals. The change observed in female induced animals was much less when compared to male induced animals(p<0.05). A significant increase (p<0.001) was observed in the cancer bearing male rats when compared to the

normal.

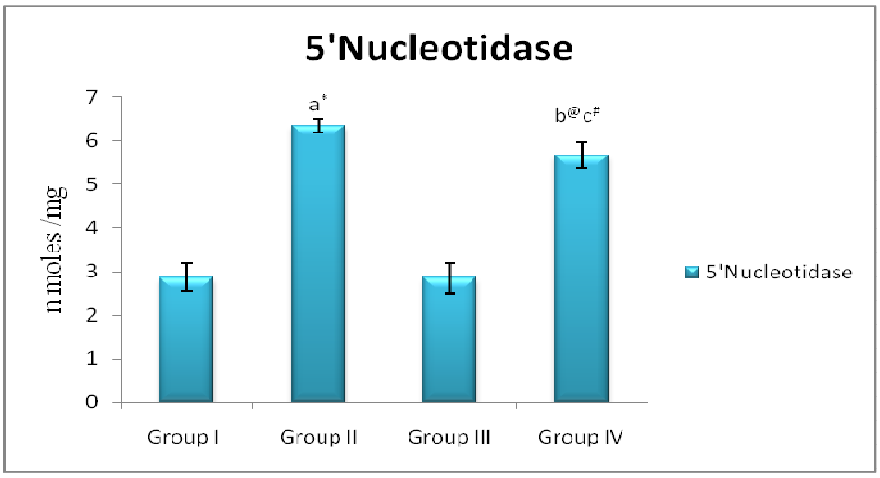

Figure 5: Level of 5'Nucleotidase in control and experimental animals.

Each value is expressed as mean SD for six rats in each group
5-N-n moles of p-inorganic phosphorus formed/min/mg protein.
a:Group I compared with Group II
b:Group II compared with Group IV
c:Group III compared with Group IV
The symbol *represents statistical significance at p<0.001,#-p<0.01,@p<0.05

Figure 6 shows the increase in γ-Glutamyl transferase activity in the cancer bearing Group II & Group IV animals when compared with the normal control animals. The enzyme activity was differentially altered in the female induced animals with a significance of p<0.01 when compared to normal female controls and this increase was lower than that observed in male induced animals.(p<0.001) .The female HCC bearing animals showed less significance when compared to male induced animals.(p<0.05).

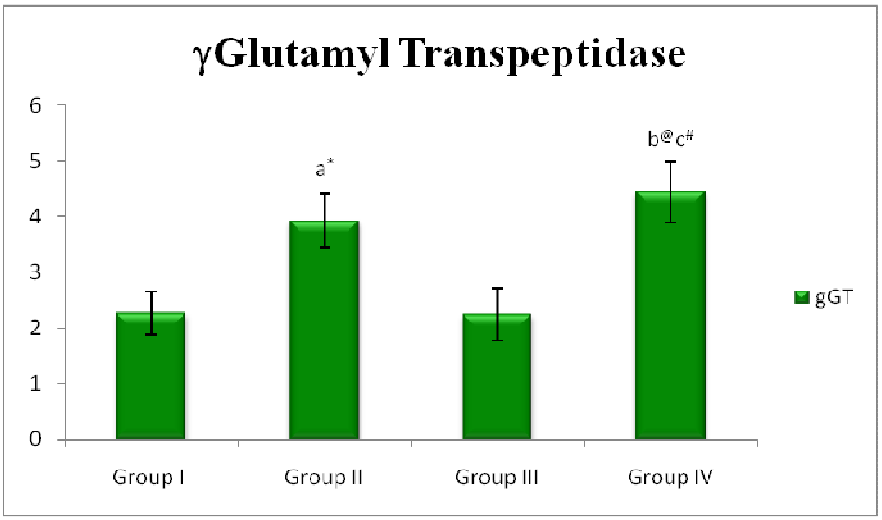

Figure-6: Level of γ- Glutamyl transpeptidase activity in the control and experimental animals.

Each value is expressed as mean SD for six rats in each group
GT-n moles of p-nitroaniline formed/min/mg protein.
a:Group I compared with Group II
b:Group II compared with Group IV
c:Group III compared with Group IV
The symbol *represents statistical significance at $p<0.001$,#-$p<0.01$,@$p<0.05$

Discussion

LPO is regarded as one of the basic mechanism of cellular damage caused by free radicals. It is a factile process arising from the reaction of free radicals with lipids leading to cellular injury and deterioration of cellular constituents including lipid, proteins and nucleic acids..In the present study, induction with DEN causes a increase in activated oxygen species which in turn promotes lipid peroxidation (20). DEN is the most important environmental carcinogen among nitrosamine in interacting with membrane lipids and consequently inducing free radical formation (21) Byproducts of lipid peroxidation have been shown to cause profound alterations in the structural organization and functions of cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane bound enzymes and loss of essential fatty acids. Free radicals react with lipids causing peroxidation resulting in the releases of products such as malondialdehyde, hydrogen peroxide and hydroxyl radicals. An increase in lipid peroxides indicates serious damage to cell membranes, inhibition of several important enzymes, reduced cellular function and cell death . The products of lipid peroxidation include Malonialdehyde, hydroperoxides, hydroxyl alkenes and hydroxyl radicals. MDA has known to cause mutagenesis in various tissues by forming DNA adducts. Plasma MDA level has been regarded to be an indicator of lipid peroxidation (22).

In the present study, a profound increase of MDA was observed in the male induced animals whereas in female induced animals there was a slight increase in the plasma MDA levels due to the protective mechanism in females by the presence of estrogen. This shows that the progression of liver cancer in females is delayed when compared to males.

Proteins are also attacked by ROS directly or indirectly through LPO. Protein radicals can lead to modification in enzyme activities. (23) .Damage caused to the membrane transport proteins may produce cellular ionic

homeostatis and lead to alterations in intercellular calcium potassium that will trigger a series of change in cells (24).The alteration of protein may allow target protein to be attacked by proteinases .The carbonyl group is used widely as a marker for ROS mediated protein oxidation. Reactive oxygen species (ROS) are known to convert amino groups of protein to carbonyl moieties.(25) .Oxidative modification of protein leads to increased recognition and degradation by proteases and loss of enzymatic activity.Using PCC as a marker, it could be demonstrated that oxidative damage to proteins correlates well with aging and the severity of some diseases (26).

In the present study, the induction with DEN may lead to protein/oxidative damage which in turn alters enzyme activity, structure and signaling pathways in the male rats.

G-Glutamyl transferase is an ectoenzyme which play an important role in the degradation of glutathione. It catalyses the transfer of glutamyl group from a larger variety of peptide donors to a wide range of aminoacids. (27).It is involved in the uptake of glutathione by breaking down the extra glutathione and its aminoacid content available to the cell.G-GT serves as a specific marker for the prognosis of carcinogenesis events. GGT plays a key role in the gamma-glutamyl cycle, a pathway for the synthesis and degradation of glutathione and drug and xenobiotic detoxification

In the present study, the induction with DEN causes an increase in the □-GT values in the male induced animals indicating the cancer progression whereas a moderate increase was observed in the female induced animals.

5'Nucleotidase is present at the bile canalicular and sinusoidal surface of plasma membrane of hepatocytes. It will hydrolyze nucleotides with a phosphate group of carbon atom 5 of ribose. Damage of hepatic cells may cause leakage of 5'Nucleotidase into extracellular fluid and then into circulation.In the present study the increase in 5'nucleotidase activity might be due to shedding of plasma membrane vesicles associated with malignant liver cells.

Membrane bound enzymes such as Na^+/K^+-ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase are responsible for the transport of sodium/potassium, Calcium and Magnesium ions across the cell membranes at the expense of ATP by hydrolysis.They maintain the ionic gradient between aqueous intra and extracellular phases. The activities of Na+/K+ ATPase, Mg ATPase has found to be inhibited in various cancer bearing animals. In the present study a significant decrease ($p<0.01$) in the activities of ATPases in the liver tissues was observed in the cancer bearing Group II and

Group IV when compared to their respective controls.It may be due to the increased lipid peroxidation .Peroxidation of membrane lipids initiates the loss of membrane integrating and membrane bound enzyme activities which leads to disruption in cellular homeostatis.(28).Inhibition of cellular homeostatis may cause impaired signal transduction, altered cellular metabolism and increase in membrane fluidity. The decrease in the activities of Na^+/K^+-ATPase and Mg^{2+}ATPase in liver cancer bearing animals is due to increased production of free radicals leading to cell injury.(29).

Ca^{2+}ATPase maintain the concentration gradient of Ca^{2+} between cytosol and extracellular fluid. Many ATPases, including Ca^{2+} ATPase contain essential sulfhydryl groups. Impairment of this enzyme may be due to peroxidative stress which may act on sulfhydryl groups present in active site of Ca^{2+} ATPase (30).Thiol modification (loss of protein sulfhydryl groups) has been recognized as critical event in cytotoxicity (31). Damage to thiol moieties may result in inhibition of Ca ATP function and increase in calcium concentration may result. (32).

In the present study a profound increase ($p<0.001$) in the Ca^+ ATPase was observed in cancer bearing Group II and Group IV animals when compared with the control animals. The increased activity may be due to the cytotoxic effect of DEN ,but still because of the protective effect existing in female rats the alteration in enzyme activity observed was quite less($p<0.05$) when compared with the male rats.

Conclusion

The present work was designed in order to assess the gender disparity of liver cancer in wistar albino rats.From our present study it was found that male and female rats showed significant biochemical alterations when treated with DEN.A significant increase was observed in the levels of LPO and protein carbonyl indicating the membrane damage. Decrease in the levels of Na^+/K^+ ATPase ,Mg^{2+} ATPase and increase in Ca^{2+}ATPase was observed which was due to the changes in the membrane permeability. But when compared to the male induced animals the female induced animals showed only slight biochemical alterations indicating the protective mechanism existing in female due to the presence of the hormone estrogen and increased expression of anti-inflammatory agent IL-6.

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References

1. Chatterjee.Resistance or sensitivity to anticancer drugs.An emerging arena involving stress proteins GPR78.Recent Research developments in cancer.Transworld research network,2002,Vol.4,pp161-176.
2. Blair A, Kazerouni N.Reactive chemicals and cancer, Cancer Causes Control.1997, Vol.8, pp473-90.
3. Bosch FX, Ribes J, Cleries R, Diaz M.Epidemiology of hepatocellular carcinoma. Clin Liver Dis, 2005, Vol.9, pp191.
4. Cabibbo G, Craxi A.Epidemiology, risk factors and surveillance of hepatocellular carcinoma. Eur Rev Med Pharmacol Sci, 2010,Vol.14, pp352-355.
5. Bhosale P, Motiwale L, Ingle AD, Grade RV and Roa KVK.Protective effect of rhodotorula glutinis NC7M 3353 on the development of hepatic preneoplastic lesions. Curr.Science, 2002,Vol.83,pp303-308.
6. Verna L, Whysner J and Williams GM.Nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA adduct formation, mutagenicity, and tumor initiation.Pharmacol Therapy,Vol.71,pp57-81.
7. Pegg AE.Formation and metabolism of alkylated nucleosides: Possible role of carcinogenesis by nitroso compounds and alkylating agents.Adv. Cancer Res., 1977, Vol.25,pp195.
8. Loveless A.Possible relevance of 0-6- alkylation of deoxyguanosine to the mutagenecity and carcinogenicity of nitrosamines and nitrosamides. Nature, 1969, pp 223-206.
9. Gercham LL & Ludlum DB.The properties of O^6-methylguanine in templates for RNA polymerase. Biochim. Biophys. Acta, 1973, pp 308-310.
10. Kew MC. Epidemiology of hepatocellular carcinoma. Toxicology, 2005, Vol 35, pp181–182.
11. Firminger HI, Reuber MD.Influence of adrenocortical, androgenic, and anabolic hormones on the development of carcinoma and cirrhosis of the liver in AXC rats fed N-2-fluorenyldiacetamide. J. Natl. Cancer Inst, 1961, Vol. 27, pp559- 595.
12. Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ. Protein measurement with the Folin's phenol reagent. J. Biol.

Chem.,1951, Vol 193,pp265-276.

13. Ohkhawa H, Ohishi N, Yogi K. Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. Analyt . biochem, 1979, Vol.95, pp351.
14. Levine RL Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radic Biol Med, 2002, Vol**.**32,pp790–796
15. Orlowski K and Meister A, Isolation of γ-glutamyl transpeptidase from dog kidney. J. Biol. Chem, 1965, Vol 240, pp338-347.
16. Luly, P., O. Barnabei and E. Tria, Hormonal control *in vitro* of plasma membrane bound Na^+/K^+ ATPase of rat liver, 1992, Biochem. Biophys. Acta.,Vol. 282,pp447-452.
17. Bonting SL .Sodium-potassium activated adenosine triphosphatase and cation transport, In: Bittar EE, editor. Membranes and ion transport, 1970. Vol. 1, pp257–363.
18. Hjerten S, Pan H. Purification and characterization of two forms of a low-affinity Ca2+-ATPase from erythrocyte membranes. Biochim Biophys Acta, 1983, Vol. 728, pp281–288.
19. Ohnishi T, Suzuki T, Suzuki Y, Ozawa K. A comparative study of plasma membrane Mg2+-ATPase activities in normal, regenerating and malignant cells. Biochim Biophys Acta, 1962, Vol 684, pp67–74.
20. Thirunavukarasu C and Sakthisekaran D.Effect of selenium on N-nitrosodiethylamine induced multistage carcinogenesis with reference to lipid peroxidation and enzymic antioxidants.Cell Biochem Funct., 2001,Vol.19, pp27-35.
21. Nakae D, Kobayashi Y, Akai H and Nabuaki. A Involvement of hydroxy guanine formation in the inhibition of rat liver carcinogenesis by low dose levels of N-Nitrosodiethylamine, Cancer Res., 1997, Vol .57, pp1281-1287.
22. Pompella A, Romani A and Benditti M Comporti loss of membrane protein thiols and lipid peroxidation of allyl alcohol hepatotoxicity, Biochem, Pharmacol., 1991.Vol. 41, pp1225-1259.
23. Kerr JFR,Wyllie AH and Currie AR.A basic biological phenomenon with wide ranging.Brit.J.Cancer, 1972,Vol.26,pp 239-257.

24. Davies K. J., Goldberg A. L. Proteins damaged in extracts of red blood cells. J. Biol. Chem, 1987, Vol 262, pp 8220-8226.
25. Chavko M., Harabin A. L.Regional lipid peroxidation and protein oxidation in rat brain after hyperberic oxygen exposure. Free Radical Biol. Med, 1996, Vol.20, pp 973-978.
26. Chevion M, Berenshtein E, Stadtman ER. Human studies related to protein oxidation: protein carbonyl content as a marker of damage. Molecular Nutrition Research Group, Nestle Research Center, Vers chez les Blanc, Lausanne, Switzerland.
27. Siest G, Courtay C, Oster T, Michelet F, Visvikis A, Diederich M, Wellman M. "Gamma-glutamyltransferase: nucleotide sequence of the human pancreatic cDNA. Evidence for a ubiquitous gamma-glutamyltransferase polypeptide in human tissues". Biochem. Pharmacol. 1992, Vol. 43, pp 2527–2533. doi:10.1016/0006-2952(92)90140-E. PMID 1378736.
28. Skekhoren MA and Bonting SL. Transport ATPases; properties and functions. Physiol. Rev., 1981, Vol 61, pp 1-76.
29. Jain K S and Sohet S B .Calcium potentates the peroxidation of erythrocyte membrane. Lipids, 1981, Vol.642, pp 46-54.
30. Pascoe S A, Olafsdottir K and Reed DJ .Vitamin E protection against chemical induced injury.Maintenance of cellular protein thiols as a cytoprotective mechanism, Arch. Biochem. Biophys, 1987, Vol.256, pp150-158.

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