JPR INFO
Journal of Pharma Research

Journal of Pharma Research
Available online through
www.jprinfo.com

Research Article
ISSN: 2319-5622

Histopathological and DNA Damage studies of Sting the Mice Liver treated with Paracetamol and Active Principle of Hybanthus Enneaspermus extract

Haseenabhanu. S*, Meenabai. G, Thyagaraju. K
Department of Biochemistry,Srivenkateswara university, Tirupati, Andhrapradesh, India.

Received on: 05-05-2014; Revised and Accepted on: 21-05-2014

ABSTRACT

In the present study mice were selected as models for testing the active principle whether it is having the regaining activity in tissues. The mice liver injured by giving crude paracetamol (500mg).Necrosis was observed. He Where as treated with active principles of Hybanthus enneaspermus no change was observed. when mice liver was damaged with paracetmol and treated with Hybanthus enneaspermus extract the regeneration of damaged tissue to normal was observed. Secondly, DNA damage was tested using commet assay.

Key words: *Mice liver, Histopathology, Cometassay, Paracetamol.*

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and is the third leading cause of cancer mortality [1]. Eighty percent of HCCs develop in the context of chronic liver diseases, as chronic liver injury generally induces liver fibrosis, followed by cirrhosis [2]. In an attempt to model this process paracetamol has been widely used to experimentally induce liver injury in rodents. A single dose of paracetamol leads to centrizonal necrosis and steatosis [3]. While prolonged administration leads to liver fibrosis, cirrhosis, and HCC [4]. Paracetamol impairs hepatocytes directly by altering the permeability of the plasma, lysosomal, and mitochondrial membranes. Highly reactive free radical metabolites are also formed by the mixed function oxidase system in hepatocytes, causing severe centrilobular necrosis [5, 6]. This model has been used extensively to examine the pathogenesis of cirrhosis.

Liver fibrosis is the pathologic result of ongoing chronic inflammatory liver diseases and is characterized by hepatic stellate cell (HSC) proliferation and differentiation to myofibroblast-like cells, which deposit extracellular matrix (ECM) and collagen. Quiescent HSCs are vitamin A storing cells in the space of Disse, and they account for about 15% of the total number of liver cells [7]. The activation of HSCs is mediated by reactive oxygen species and various cytokines, including transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, and platelet-derived growth factor (PDGF), as well as other factors which are released from the damaged hepatocytes and activated Kupffer cells [8]. The activated HSCs produce large amounts of ECM components, such as laminin and collagen type IV, in an accelerated fashion, resulting in fibrotic change of the liver. The number of HSCs was found to be increased in alcoholic liver disease and in other animal models of chronic liver disease [8]. HSCs are further characterized by their stellate-shaped morphology and expression of desmin and glial fibrillary acidic protein (GFAP). A novel subpopulation of HSCs in rats has been described to exhibit properties of progenitor cells and express CD133, originally thought to be a marker of endothelial progenitor cells (EPCs), hematopoietic stem cells, and other stem cells [9]. This finding gave rise to the hypothesis that CD133+ HSCs are up-regulated in chronically injured liver.

Epidermal growth factor (EGF), a polypeptide mitogen, and its tyrosine kinase receptor (EGFR) have been proposed to have vital roles in liver regeneration and transformation [10, 11]. EGF and EGFR are highly elevated in human cirrhotic livers [12]. However, to the best of our knowledge, the expression of EGF and EGFR in the injured liver in mouse models has not been fully investigated.

In the current study, we exposed mice to paracetamol to create fibrosis, cirrhosis, and HCC and assessed histopathology, EGF expression, and HSC populations. We observed that CD133+ HSCs are recruited during chronic liver injury in paracetamol -treated mice. Multiple HCCs were found in the livers of all mice after one week of paracetamol treatment; however, the pathological findings and EGF expression patterns in the injured liver were different from those previously reported in humans, suggesting that species-specific differences exist with respect to the histopathological and molecular pathogenesis of chronic liver injury. In mice the transformation was induced by treating with paracetamol, pathological changes were studied in liver treated with paracetamol, plant extract alone, plant extract and paracetamol and control mice. Comet assay did to see the percentage of DNA damage. The alkaline version of the single cell gel electrophoresis assay (comet assay) is widely used to evaluate the genotoxic potential of chemicals and environmental contaminants that can induce oxidative stress leading to cellular injury. The comet assay was carried out to determine the protective role of the *Hybanthus enneaspermus* against paracetamol induced DNA damage in whole blood of animals. The percentage of damaged cells and the average tail lengths of comets in experimental groups were measured.

MATERIALS & METHODS

A total of 42(6months old) male albino mice weighing 25±50g obtained from Bangalore. The treatments were provided for seven days as follows.

Table No. 1: Groups for the Treatment of liver

GROUPS	TREATMENTS
GROUPI	Control
GROUPII	Paracetamol(300mg/kgbw in saline, ip)
GROUPIII	Ethanolic HE extract(100mg/ kgbw in CMC, orally)
GROUPIV	Active fraction (50mg) +Paracetamol(300mg)/kgbw

Histopathology:

Fixation and staining control and treated samples. The tissues were isolated from control and treated mice very gently rinsed with physiological saline to remove blood and debris if any adhering to them. They were fixed in bovins solution until processing (in case of liver 5% formalin). The tissues were washed running tap water, to remove bovin's solution. After dehydrating

***Corresponding author:**
Haseenabhanu. S
Department of Biochemistry,
Srivenkateswara university, Tirupati, Andhrapradesh, India.
**E-Mail: haseenabhanu.ph.d@gmail.com*

through a graded series of alcohols, the tissues were cleaned in methyl benzoate and embedded in paraffin wax. Sections were cut at 6µ thickness and stained with haematoxylin [13] and counter stained with eosin dissolved in 95% alcohol. After dehydration and cleaning, sections were mounted in Canada balsam. Histological examinations of these tissues were observed under the light microscope (10x X 40x).

Detection of DNA damage by Alkaline single cell gel electrophoresis (Comet Assay):

Possible DNA damage induced by over dosage of paracetamol at different doses was detected using the alkaline single cell gel electrophoresis (Comet Assay) following a simplified protocol with slight modifications [14, 15] (Endoh et al., 2002; Singh et al., 1988).

***Reagents*:**

1. 1% Normal melting agarose.
2. 1% Low melting agarose.
3. 0.5% Low melting agarose.
4. Lysing solution: 36ml of lysing buffer (73.5g of NaCl, 18.612g of EDTA, 5g of Sodium lauryl sarcosinate, 0.6g of Tris dissolved in 500ml of distilled water and adjust the pH to 10.0), 4ml DMSO and 400µl TritonX-100.
5. Electrophoresis buffer: 30ml of 10N NaOH and 5ml of 0.5M EDTA made up to 1000ml with distilled water and adjust the pH to 10.0
6. Neutralizing buffer: 24.228g of Tris dissolved in 500ml of distilled water and pH was adjusted to 7.5.

***Procedure*:**

At the end of the treatment with paracetamol, blood was collected freshly from the retro-orbital plexus and used for the assay. Half frosted microscope slides were coated with 1% normal melting agarose in physiological buffer saline (PBS).The slides were then allowed to dry at room temperature protected from dust and other particles. An aliquot of 10µl of fresh blood was mixed with 140 µl of 1% low melting point agarose in Milli-Q water. This mixture was then layered on the top of the pre-coated slide and covered with a 24 X 50mm cover slip and kept on ice to allow the agarose to solidify. After the agarose had solidified on ice for at least 10-15min, the cover slip was gently removed and a third layer of 0.5% low melting agarose was layered on the top of the second layer and covered with a cover slip and kept on ice for 5-10 min. After the agarose had solidified, the cover slip was gently removed and the slides were carefully immersed in a freshly prepared ice-cold lysing solution. After lysis overnight at 4°C the slides were placed in an electrophoresis unit and the buffer reservoirs were gently filled with fresh electrophoresis buffer to level of 0.25 cm above the microscope slides, and incubated for 20min at 4°C to allow the unwinding of DNA. Keeping the same temperature, the slides were, subjected to electrophoresis, (25 V, 400 mA) for another 25min. After electrophoresis, the slides were placed on a tray to remove alkali and detergents and neutralized with neutralizing buffer for 10min. Excess liquid was carefully removed from each slide using a paper towel. The microscope slides were carefully dried at room temperature avoiding dust and other particles and then stored in a sealed container until the day of image analysis. The dried microscope slides were stained with ethidium bromide in water (20µg Ml^{-1}; 50 µl/ slide). The slides with a cover slip were examined at 400X magnification under a fluorescence microscope and the photomicrographs of cells were taken. 150-200 randomly selected cells (5-7 zones/slide) in each slide were counted (4 slides/ animals in each group) to determine the number of damaged cells and then the percentage of damaged cells were calculated using the formula: % damage= (Number of damaged cells/ Total number of cells counted) X 100.

The length of the comet tail was determined by using an occulometer affixed in the eye piece of the microscope. The comet tail length was measured between the edge of the comet head and the end of the comet tail, calculated in micrometers [16] (Sebastien et al., 2003). The results were expressed as:

1. percentage of cells with tail(tailed cells) in each group was scored and
2. Average tail length due to DNA migration in each group.

RESULTS & DISCUSSION

The control mice liver showed normal architecture, normal hepatic central vein and hepatocytes with centrally placed nuclei (**Fig. 1**). Histological analysis of liver tissues of mice treated with paracetamol alone showed severe condition of central vein, hyperplastic nuclei, more granularity of the cytoplasm, cloudy swelling changes (**Fig. 2**). Liver tissues of mice treated with *Hybanthus enneaspermus* alone showed hepatocytes with centrally placed prominent nucleus, sinusoids and with central vein (**Fig. 3**). Liver tissues of mice treated with *Hybanthus enneaspermus* extract and paracetamol mixture showed infiltration, recovered sinusoids space and increased size of hepatocytes (**Fig. 4**).

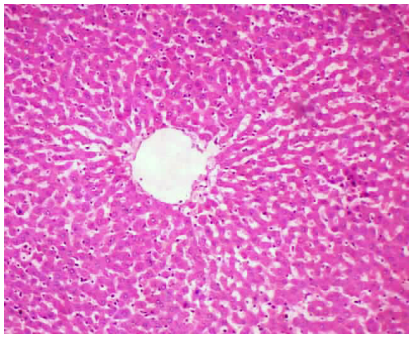

Fig. 1: Control Liver

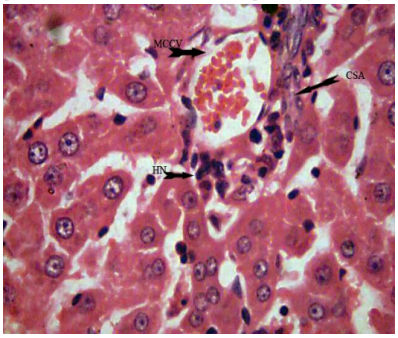

Fig. 2: Paracetamal Treated Liver

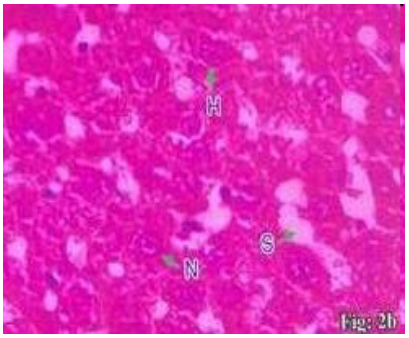

Fig. 3: EEHE Alone Treated Liver

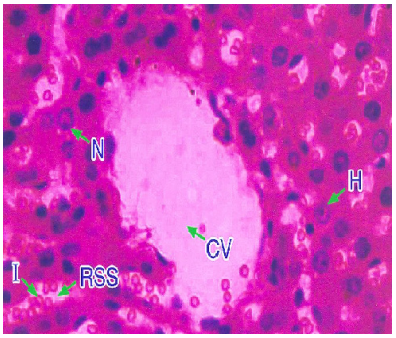

Fig. 4: Active Fraction + Paracetamol Treated Liver

Fig. 1-4: The changes in Normal and Treated Mice Liver

Comet assay:

Blood drawn from the control mice (Group I) during acute treatment showed very few (4.32%) damaged cells. Paracetamol induced (Group II) showed 59.12% cells, having a distinct comet tail which came down significantly to 34.61% in *Hybanthus enneaspermus* (200mg/ kgbw) and paracetamol (Group VI) treated and 14.32% in *active fraction* (50mg/ kgbw) and paracetamol (Group VII) treated animals.

The tail length was also measured to assess the damage to erythrocyte. In the control group (Group I) the magnitude of the comet tail was very low and increased after paracetamol treatment. It might cause DNA damage of the cells that involved in greater elongation and diffused comet tail formation, resulting in an increase of tail momentum in single-gel-electrophoresis.

The microscopical image resulting from paracetamol (300mg/ kgbw) induced damaged blood cells (Group II) are comets with small or non-existent head and large, diffused tails in acute phase treatment. The average tail length was increased about 89.23 in (Group II) animals compared with (Group I) animals. Whereas the tail lengths decreased by 41.6% in EEHE (100mg/ kgbw) and Paracetamol (Group III) treatments compared with (Group II) animals. The average tail length was decreased about 47.6% in (Group II) animals compared with (Group I) animals. Whereas the tail lengths decreased by 66.3% in EEHE (100mg/ kgbw) and Paracetamol (Group III) treatments compared with (Group II) animals.

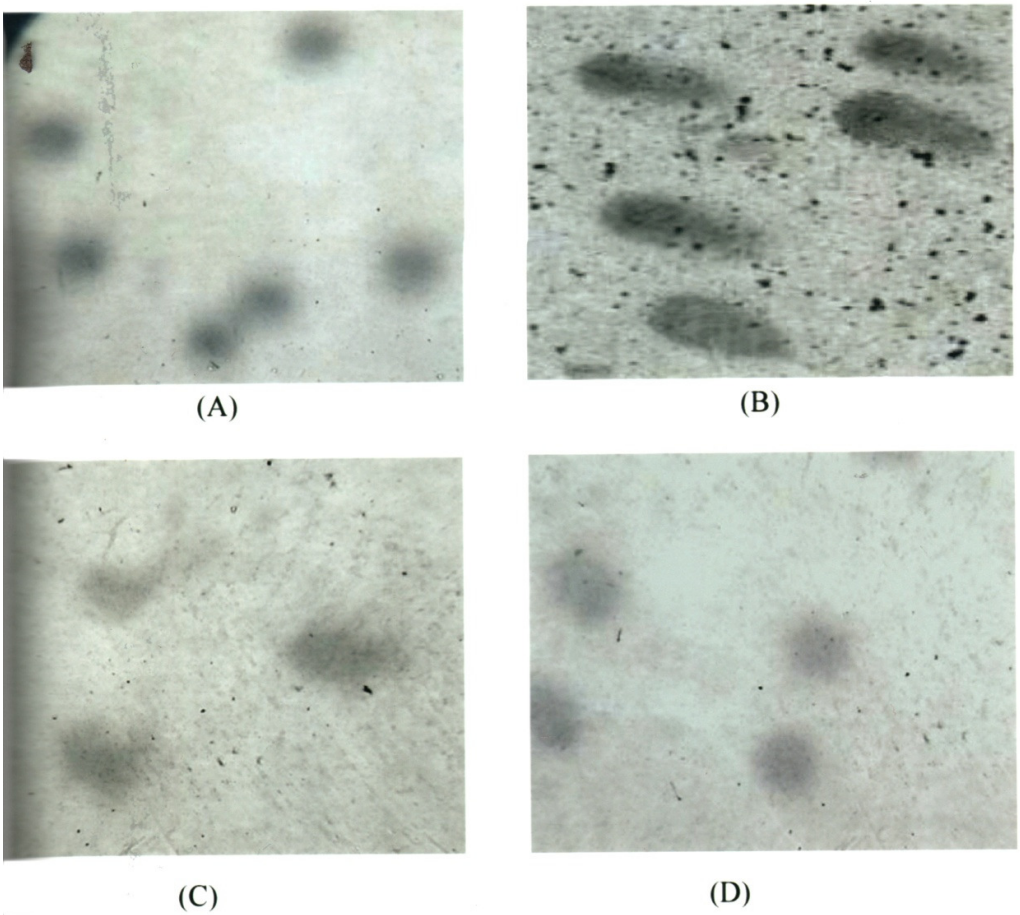

Fig. 5: A-D: Effect of EEHE on DNA damage in Blood Lymphocytes of control and Treated Mice during Acute Treatment.
A: normal mice**; B:** Paracetamol treated; **C:** HE alone **D:** B & C combination

A. Microphotograph of lymphocytes of control (Group I) in animals showed no significant DNA migration (damage), X 400.
B. Microphotograph of DNA damage in the animals treated with paracetamol (Group II) at a dose of 300mg/kg bw. Showing DNA migration with distinct comet tail formation, X400.
C. Microphotograph of DNA damage in the animals treated with EEHE (100mg/ kg bw) + paracetamol (300mg/kg bw) (Group III) showing less migration of DNA and tail formation with shorter tail length, X400.
D. Microphotograph of DNA damage in the animals treated with *active fraction* (50mg/ kg bw) + paracetamol (300mg/kg bw) (Group III) showing no or less migration of DNA and tail length is very short compared to control group, X400.

Table: 2 Assessment of the protective activity of EEHE against paracetamol induced damage in blood lymphocytes of mice

Group	Damaged cells showing comet (%)	Average tail length (µM)
I	4.32 ± 1.02	6.91± 0.23
II	59.13 ± 1.62	64.15± 4.01
II	34.61± 1.21	37.5 ± 2.04
IV	**14.32± 0.89**	**21.6 ± 3.08**

Values are expressed as Mean±SE by Duncan's multiple range test (DMRT). P<0.01.

CONCLUSION

In the present investigation, the observations suggest that there was a high statistical difference in the level of DNA damage between oxidative stress bearing mice and active fraction treated mice. Histopathological studies showed the regeneration nature of the medicinal plant *Hybanthus enneaspermus* .i.e the liver injured by crude paracetamol showed regeneration of tissue and it is normal.It was found that treatment with active fraction reduced the extent of paracetamol DNA damage (gentoxic stress) of blood cells in comparison with paracetamol treated groups in a duration and dose dependent manner. These results show that active fraction

provides a beneficial effect against paracetamol induced oxidative damage. The protective effect may be due to the active compounds 1,3 butadienes and unsaturated carboxylic compounds.

ACKNOWLEDGEMENT

This work was supported by a research grant received From UGC, Government of India.

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How to cite this article:

Haseenabhanu. S, Meenabai. G, Thyagaraju. K: Histopathological and DNA Damage studies of Sting the Mice Liver treated with Paracetamol and Active Principle of Hybanthus Enneaspermus extract. J. Pharm. Res., 2014; 3(5): 88-91.

Conflict of interest: The authors have declared that no conflict of interest exists.

Source of support: Nil