Sub-Cellular Damage of Hepatocytes Caused by Different Doses of Diclofenac Sodium in Rabbit

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Abstract: Diclofenac sodium is a potent analgesic and anti-inflammatory drug that is extensively prescribed in treatment of rheumatoid arthritis, postoperative pain, and chronic pain associated with cancer. The present study was designed to elucidate the qualitative and quantitative changes in rough endoplasmic reticulum of hepatocytes after recommended single, double and triple therapeutic dose of Diclofenac sodium in rabbits. The aim of study is to minimize the indiscriminate use of this drug in community and among physicians. Experimental study was carried at Dow University of Health Sciences (DUHS) and Sindh Institute of Urology and Transplantation (SIUT) from March 2009 to June 2010. Eighty eight healthy animals of three months age and 900~1000 gm body weight were isolated from the animal house of DUHS. These animals were divided into four groups categorized into Group A, Group B, Group C, Group D, each containing twenty two animals. Diclofenac sodium were administered intraperitoneally with the daily doses of 2, 4, and 6 mg/kg body weight for 14 consecutive days in Groups B, C and D while the control group (Group A) received normal saline. Animals were sacrificed on day fifteen and livers were removed and fixed in 4% gluterldehyde. They were processed for electron microscopy and examined under transmission electron microscope. Data was collected and subjected for statistical analysis a 'P' value less than 0.05 was taken as significant. It was observed that Diclofenac sodium produces significant changes in hepatocytes. There was de-granulation and swelling of the cisternae of Rough Endoplasmic Reticulum (RER) when the dose is doubled and these changes were highly significant when the dose is increased to three times the therapeutic.

Keywords: Diclofenac sodium, Ultrastructure, Rough Endoplasmic Reticulum.

INTRODUCTION

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are well known for common musculoskeletal disorders [1] and these are the drugs of choice for osteoarthritis [2]. They are also used as an anti-carcinogen [3]. The widespread use of NSAIDs produced adverse effects including gastrointestinal (GI) toxicity which is common in both humans and animals, while hepatotoxicity associated with NSAIDs are quite uncommon [4-6].

Diclofenac Sodium, one of the members of NSAIDs is classified as a potent and widely used compound [7,8]. Although it is a best tolerated drug but acute hepatotoxicity has been reported even after the therapeutic use [9]. The risk of liver injury according to different investigators is 1~8 per 100000 patients per year and may cause diagnostic confusion [10]. This number is greatly enhanced by extremely widespread use of Diclofenac sodium in developing countries like Pakistan where the liver diseases are more prevalent among population.

The liver toxicity may range from mild alteration in liver functions to fulminant hepatitis or rarely death. The liver diseases are usually diagnosed on the basis of history, clinical examination, laboratory reports and finally the histopathological findings in biopsy specimen. Light microscopy is commonly used in the diagnosis of hepatotoxicity but injury at sub-cellular level can only be identified under electron microscope. [11]. Results can be useful in evaluating the risk / benefit ratio of hepatotoxic drug [12].

Hepatocytes are protected by different mechanisms; the structures which are most commonly involved in this mechanism are usually the target sites in sub-cellular injury. In hepatic toxicity both smooth and rough endoplasmic reticulum are usually damaged [13]. In Diclofenac sodium induced toxicity Rough Endoplasmic Reticulum (RER), the site of protein synthesis is primarily involved.

This study was thus undertaken to evaluate further the safety of Diclofenac sodium in relation to acute liver injury at sub-cellular level.

MATERIAL AND METHOD

This study was carried out in Institute of Basic Medical Sciences (IBMS), Dow University of Health Sciences (DUHS) during the period of March 2009 ~ June 2010. The study protocol was approved by the University Ethics Committee.

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Eighty eight (88) adult male rabbits of Switzerland species were selected for this experimental study. The animals were about three months of age and 900~1000 gm in weight, all looked active and healthy. They were kept in separate cages and labeled. Animals were fed and given water ad libitum. Experiments were performed in accordance with the animal health and welfare regulations of DUHS. Body weight was measured on first and last day of the experimental period. They were treated with the experimental drug according to following schedule:

Group A: (Control) 2 ml normal saline.

Group B: (Therapeutic Dose) Diclofenac sodium 2 mg / kg body weight [14].

Group C: (Double Therapeutic Dose) Diclofenac sodium 4 mg / kg body weight.

Group D: (Three times Therapeutic Dose) Diclofenac sodium 6 mg / kg body weight.

Drugs were administered intraperitoneally. At the end of study period all animals were dissected, the liver was removed, diced into small pieces of 1 mm^3 sizes and was fixed in 4% gluteraldehyde. Sorensen's phosphate buffer was used to wash the tissue two times each 10 minutes. Dehydration process was completed by passing the tissue through alcohol solutions in ascending concentrations i.e. 50%, 70%, 90% and then absolute alcohol 10 minutes each. Two changes of 10 minutes with acetone were applied, then the tissue pieces were infiltrated in half resin and half acetone at 37 ºC for one hour and then in full resin for two hours. Polymerization was allowed for overnight [15].

For tissue orientation semi thin sections (3~4 µm) were cut by glass knives with the aid of Leica ultra microtome they were stained with toludine blue and examined under light microscope. Ultra-thin 100 nm (1 µm) sections were cut by the same microtome, stained with uranyl acetate and lead citrate and examined under transmission electron microscope (TEM Leica, Germany).

This method is same as carried out in the experiments previously published [16]. The changes were graded as 0 = No change, 1 = Mild change, 2 = Moderate change, and 3 = Severe change.

Statistical Analysis

SPSS (version 17) was used to analyze the collected data for statistical analysis. Post hoc tests were applied to see the differences. A 'p' value less than 0.05 was taken as statistically significant.

RESULTS

All animals remained alive, but those of groups C and D had loss of appetite, their response to stimulus was also slow.

Qualitative Changes

Group A

The acinus arrangement was quite normal. RER was scattered among the other organelles. Granules of polyribosomes were attached to the outer surface of cisternal membrane (Figure **1**).

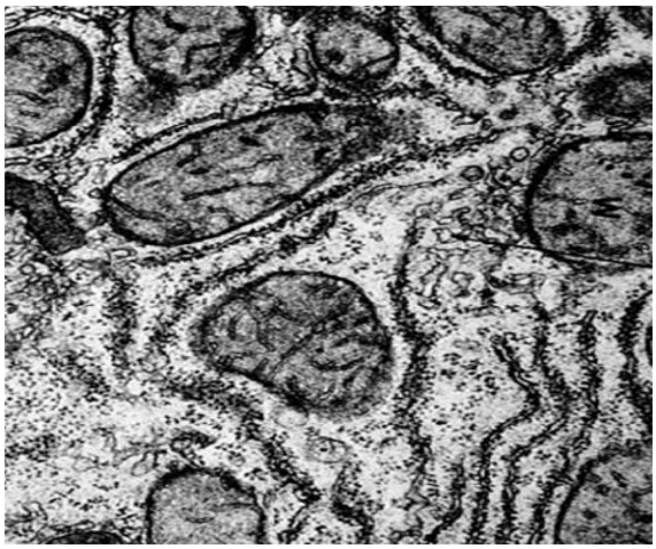

Figure 1: Changes in the Rough Endoplasmic Reticulum (RER) of Hepatocytes.

Group B

At places degranulation of Rough Endoplasmic Reticulum was evident which persisted in some lobules.

Group C

There was widespread swelling of Rough Endoplasmic Reticulum in Group C animals. There was detachment of ribosomes in some of the cisternae.

Group D

In Group D animals, dilatation and vesiculation of cisternae of Rough Endoplasmic Reticulum was most prominent. Partial loss of ribosomes was also observed. Some of the cisternae were transformed into vesicles by fragmentation (Figure **2**).

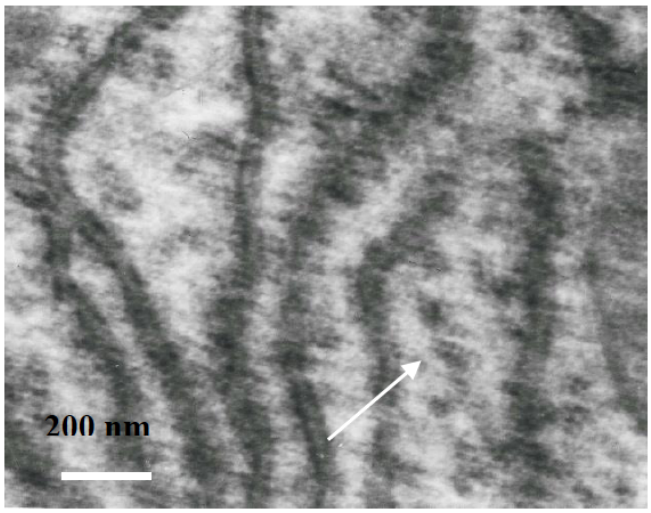

Figure 2: Electron micrograph of the rabbit liver treated with Diclofenac sodium 6mg/kg (group D) showing degranulation of RER (arrow) in hepatocytes.

Quantitative Changes

The mean of changes in RER was calculated (Table **1**), they were non-significant in Groups A, B and C while highly significant changes were observed in hepatocytes of Group D animals (Table **2** & Figure **3**), 'p' value was highly significant <0.005.

Table 1: Quantitative Changes in all Groups

	Group A	Group B	Group C	Group D
Mean	0.14	0.27	0.55	1.36
Std Dev	0.34	0.54	0.66	0.98

Table 2: Post Hoc Tests – Changes in RER of Hepatocytes – Multiple Comparisons (Dunnett T3)

	Group B	Group C	Group D
Group A	0.940	0.093	0.000
Group B		0.533	0.000
Group C			0.024

The mean difference is significant at the 0.05 level.

DISCUSSION

Hepatotoxicity is a potential complication of most prescribed drugs, possibly due to the central role of the liver in drug metabolism. The present study indicated that Diclofenac sodium induced marked histopathlogical alterations at cellular as well as sub-cellular level in the liver tissues of rabbits. At the sub-cellular level there was swelling and proliferation of

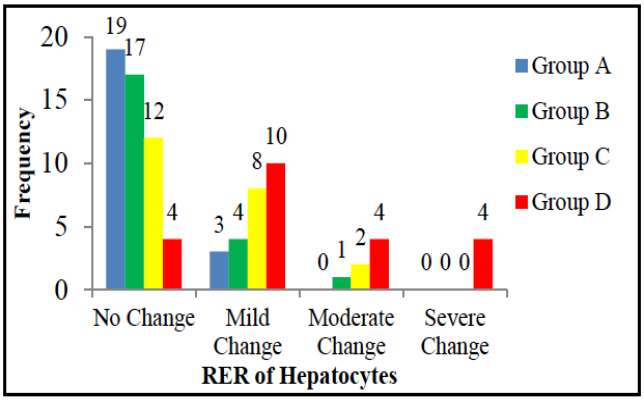

Figure 3: Frequency of Changes in the Rough Endoplasmic Reticulum (RER) of Hepatocytes.

Smooth Endoplasmic Reticulum (SER) and detachment of ribosomes from Rough Endoplasmic Reticulum and finally apoptosis / necrosis of hepatocytes.

There are only few *in vitro* experimental studies to document ultrastructural changes in hepatocytes after exposure to Diclofenac sodium. Long term exposure of cultured hepatocytes to Diclofenac sodium or repeated doses of the same have been proved to influence the metabolism of primary hepatocytes [17]. Diclofenac sodium administered to rabbit in therapeutic, double and triple dose for 14 days by intraperitoneal routes produced ultrastructural changes in the liver cells. They were non-significant in Groups B and C while highly significant changes were observed in Group D animals. Dilatation of Rough Endoplasmic Reticulum and degranulation of ribosomes were the prominent features.

The damage to hepatocytes is noticed in many hepatic lobules. This can be explained as the metabolites of Diclofenac N,5-$(OH)_2$ and 5-OH diclo have the tendency to accumulate in these cells, this damages the microsomes which is the main event in the toxicity of hepatocytes induced by Diclofenac sodium [18]. Manov *et al.* 2006 also implicates the highest apoptotic effect with the formation of 5-OH Diclofenac [11].

This study also supports the study of Yapar K, where Diclofenac sodium was injected to albino mice in different doses for 5 days and toxic effects were identified in liver by biochemical methods [19]. Our study is in conformity with Gulsein *et al.* who have observed dose dependent hepatocyte damage in rats under light microscope [20].

CONCLUSION

In conclusion, Diclofenac sodium damages the Rough Endoplasmic Reticulum of hepatocytes. Its prolong use may cause permanent damage to these cells of liver leading to fibrosis and in extreme cases cirrhosis of liver. We should make the public aware and discourage the use of this drug for minor musculoskeletal problems. Other tissues of the body can be studied for light microscopic as well as ultrastructural changes induced by the use of this drug.

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