MEDICAL CHANNEL

Original Article

MORPHOLOGICAL CHANGES IN MITOCHONDRIA OF HEPATOCYTES BY DIFFERENT DOSES OF DICLOFENAC SODIUM IN RABBITS

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ABSTRACT

Objective: This study is therefore designed to observe quantitative electron microscopic changes in living liver after the therapeutic, double therapeutic and three times the therapeutic dose of Diclofenac sodium.
Study Design: It was an experimental study.
Introduction: Acute liver failure due to drug toxicity accounts 50% cases of hepatotoxicity. Most of these drug induced failures are either metabolic or idiosyncratic. As liver is the primary organ for drug metabolism the danger of toxicity is always present. Most of the NSAID cause liver injury to some extent which is usually dose dependant as in acetaminophen and usually reversible when the drug is withdrawn. Diclofenac sodium the commonly used NSAID sometimes causes severe liver injury and the injury is clinically when the patient has developed symptoms and signs. There are very few studies in which dose dependant alterations at sub-cellular level are studied
Methods: For this experimental study 88 male healthy rabbits were selected at IBMS of DUHS. The study period was from March to September 2009. The animals were divided into four groups each comprising of 22 animals. Group A served as control received normal saline 2 ml intraperitoneally, group B received diclofenac sodium 2mg/kg, group C received 4mg/kg and group D received 6mg/kg all intraperitoneally for two weeks. At the end of this experiment the animals were sacrificed, liver dissected out, cut into pieces fixed in 4% gluteraldehyde and then processed for electron microscopy.
Results: The changes in mitochondria were observed under E/M in different groups. It was found that the double and triple therapeutic doses produce significant changes in mitochondria in the form of swelling with damage to outer mitochondrial membrane, loss of cristae and appearance of densities. So it is suggested that this drug should be used with caution within therapeutic limits because it produces alterations in the sub-cellular structure even in non-toxic doses.
Conclusion: Diclofenac sodium damages the mitochondria of hepatocytes in therapeutic as well as slightly higher than therapeutic dose. So we should make the public aware of hazards of increasing use of this drug for minor musculoskeletal disorders.

Key words: Liver, Mitochondria, Hepatotoxicity, NSAID.

INTRODUCTION

Drug induced hepatotoxicity accounts for more than 50% cases of acute liver failure in the United States[1] and more than 75% cases of idiosyncratic reactions result in liver transplantation or death[2]. There are many drugs blamed, Diclofenac sodium is one of them. It is a strong analgesic and anti-inflammatory agent belonging to the group NSAIDs (Non-Steroidal Analgesic Anti-Inflammatory Drugs).

It is used in many painful conditions like osteo-arthritis, rheumatoid arthritis, its preoperative use in the form of patch or suppository effectively reduces the post-

operative pain[3, 4]. People are using it as self medicine and it's availability over the counter should also be for the controlled doses of drug within therapeutic limits. Therefore scientific evidence generated through the ultrastructural study would provide a very convincing evidence of general toxicity in the population.

Most drugs cause liver injury infrequently, but as the liver is the primary site of drug metabolism the likely hood of reactions are always present. Hepatic injury by NSAIDs is well known. Although diclofenac sodium among most widely used NSAIDs its use has been associated with some form of hepatotoxicity[5]. These reactions may be classified as idiosyncratic, hypersensitivity in some patients but in others there may be direct toxic effects of drug or its metabolites[6]. Roque But[7] in 1998 showed cytotoxicity of Diclofenac sodium when added to cultured human or rat hepatocytes.

As regards to sub-cellular structures, mitochondria are the first structure to be effected. Incubation of mitochondria with Diclofenac sodium causes mitochondrial swelling[8]. The endoplasmic reticulum is constantly altered during toxicity[9]. Rough endoplasmic reticulum (RER), major site of protein synthesis is also the primary site of damage by hepatotoxins.

MATERIAL AND METHODS

It was an experimental study for which 88 adult, healthy male rabbits were taken from the animal house of Institute of Basic Medical Sciences (IBMS), Dow University of Health Sciences (Karachi) after the proper approval of Ethical Committee. The period of study was from March to September 2009. The tissue processing was done in Dow Diagnostic & Research Laboratory (DDRL), while the slides were studied under Transmission electron microscope (Leica, Germany) in Sindh Institute of Urology & Transplantation (SIUT).

The animals were about three months of age and 900~1000 gm. in weight. They were divided into four groups A, B, C, and D each comprising of 22 animals. All the animals looked active and healthy. They were kept in separate cages and labelled by a coloured ribbon in the neck for groups, and numbered with marker. The food and water was ad libitum. The animals were weighed on day-1 and day-14th of the experimental period and their weight noted on a performa. Group A served as control, receiving normal saline 2 ml intraperitoneal / day, group B received Diclofenac sodium (injection Voren 75mg/3ml Yung Shin Pharma Ind. Co Ltd. Tachia Taiwan Roc) in the dose of 2 mg/kg body weight daily[10], group C received Diclofenac sodium in the dose of 4mg/kg body weight daily (double the therapeutic dose), and the group D received Diclofenac sodium 6 mg/kg/daily (three times the therapeutic dose), given intraperitoneally. On day 15 the animals were sacrificed by a blow on head.

The liver was then identified, and carefully examined in situ for any change in colour, size, contour and consistency. It was then cut into small pieces which were fixed in 4% gluteraldehyde and further processed for electron microscopy.

1. Electron Microscopy

All the tissue processing was done under a fume hood. Small pieces of liver tissues from each experimental rabbit of all four groups were transferred immediately to a pool of 4% gluteraldehyde fixative (cold at 4 ºC) for one hour then chopped to 1 mm^3 pieces.

Tissue pieces were again transferred to the same fixative for 24 hours. Tissues were then washed for two changes each of 10 minutes with Sorensen's phosphate buffer. They were post fixed in 1% osmium tetra oxide (4 ºC) for one hour and then washed in the same buffer. Tissues were then dehydrated in ascending grades of ethyl alcohol, 50%, 70%, 90% and then in absolute alcohol (twice) each for 10 minutes. They were then passed through two changes of acetone each of 10 minutes. They were then infiltrated gradually with half resin and half acetone at 37 ºC for one hour and in full resin again at 37 ºC for two hours in the incubator. Tissue sections were polymerized at 70 ºC overnight[11]. Semi-thin sections 300~400 nm (3-4ìm) were cut using glass knives by the aid of Leica ultramicrotome and stained with 1% toluidine blue, they were examined for tissue orientation. Ultra thin 100nm (1ìm) sections were cut with the glass knives on the same microtome (Leica Germany) and mounted on copper grids.

2. Staining

a) Grids were fixed on heat for 10 minutes then saturated with urannyl acetate (centrifuging rate was 15000 rev/sec) for 15 minutes. Grids were then passed through six washes each for one minute through 5 ml syringe attached with Millipore (-ve staining).
b) Grids were then stained with lead citrate. One drop of Lead citrate was put on the grid, centrifuged at 15000 revolutions per second, and then exposed to six washes with distilled water. Grids were dried in the oven at 37 ºC for 10 minutes.
c) Tissues were then examined under transmission electron microscope (TEM Leica, Germany).

Tissue changes observed were graded as 0 no change, +, ++, and +++ for mild moderate and severe change in mitochondrial membranes. At least five observations were taken for each animal and their mean calculated.

3. Statistical Analysis

SPSS version 17 (SPSs Inc. Chicago IL USA) was used for statistical analysis. A 'p' value less than 0.05 was accepted as statistically significant. Kruskal Wallis test was applied to see the difference between different groups[12].

OBSERVATIONS / RESULTS

There was no drop out of any animal. General observations in the animals were made about their physical activity, response to stimulus and food intake. The liver in all animals showed almost normal appearance on naked eye examination. Liver was reddish brown in colour with smooth surfaces, soft in consistency and with sharp margins.

Tissue sections on the grid were examined under electron microscope and following changes were noted.

Group A

The mitochondria were elongated and rod shaped, they were scattered all over the cell. Both the inner and outer mitochondrial membranes were distinctly visible with densities in matrix; while most of the cristae were arranged transversely (Fig 1). The mean value of changes observed in mitochondria in this group was 0.090+0.465 (Table-I).

Group B

Most of the mitochondria were elongated they were very well visualized scattered in the hepatocyte, some of the mitochondria were swollen and in these mitochondria the outer membrane was not properly visualized. The mean value of changes in this group was 0.318+0.862.

Group C

In this group many mitochondria were found to be swollen, few had ruptured outer membrane with increased densities in the

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TABLE-I

Animal No.	Group A (Control)	Group B Diclo 2mg/kg)	Group C (Diclo 4mg/kg)	Group D (Diclo 6mg/kg)
1	0	0	2	2.00
2	0	1	1	1.00
3	0	0	0	1.00
4	1	0	0	0.00
5	0	1	0	2.00
6	0	0	3	1.00
7	0	0	0	2.00
8	0	0	1	3.00
9	0	0	0	1.00
10	0	1	0	0.00
11	1	0	1	2.00
12	0	0	2	3.00
13	0	0	0	1.00
14	0	0	1	2.00
15	0	1	2	0.00
16	0	0	0	1.00
17	0	1	1	2.00
18	0	0	1	3.00
19	0	0	0	0.00
20	0	0	0	1.00
21	0	1	1.	1.00
22	0	1	0	1.00
Mean	0.090	0.318	0.727	1.363
Std Dev	0.28748	0.46577	0.86244	0.93154

TABLE-II
KRUSKAL-WALLIS TEST
RANKS

	Groups	N	Mean Rank
Observations	A	22	28.91
	B	22	37.43
	C	22	47.64
	D	22	64.02
	Total	88	

TEST STATISTICS[A,B]

	Observations
Chi-Square	29.090
df	3
Asymp. Sig.	.000

matrix. The mean value of changes observed here was 0.727+0.931.

Group D

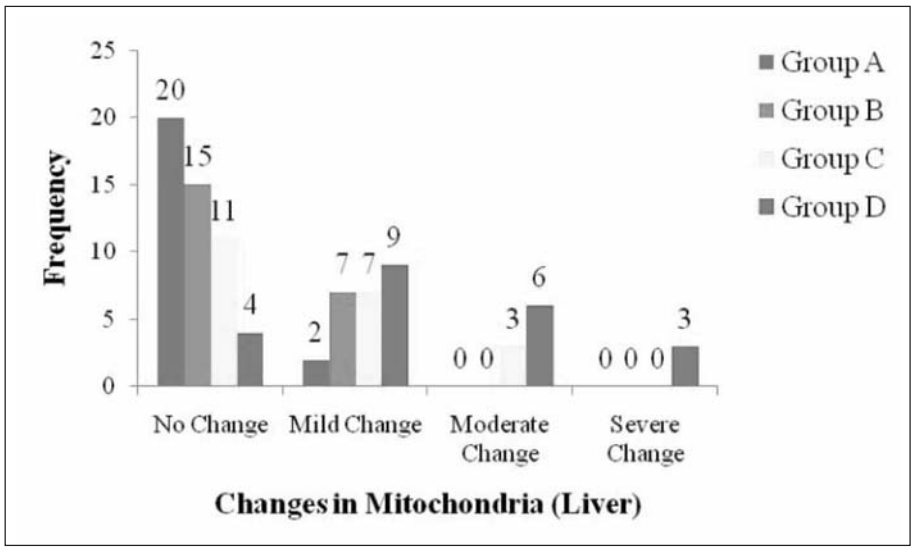
In this group ultrastructural abnormalities were evident in the hepatocytes. There was marked swelling of the mitochondria in many of the cells. In some of the mitochondria the outer membrane was ruptured (Fig 2), there was loss of the cristae in inner membrane while increased densities inside the matrix were noted; the mean value of changes in mitochondria in this group was 1.363+0.931.

STATISTICAL ANALYSIS

Kruskal Wallis test was applied to see the significance of results and it was found that p-value was highly significant (Table-II).

DISCUSSION

In the present study we investigated the possible linkage between the dose of Diclofenac sodium and injury to cell organelles, to understand the mechanism of hepatotoxicity at ultrastructural level. There are definite mitochondrial changes which highlight the



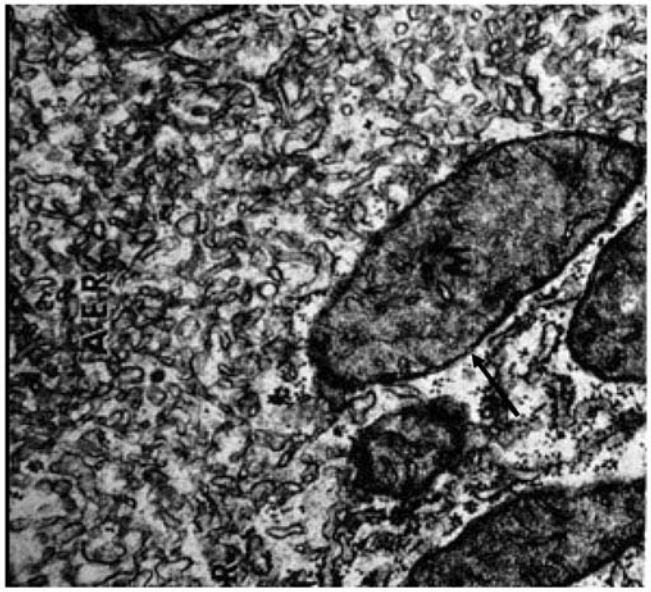


Figure 1: Electron micrograph of the rabbit liver (group A) treated with normal saline showing normal mitochondria of hepatocytes with intact outer and inner membranes (arrow)

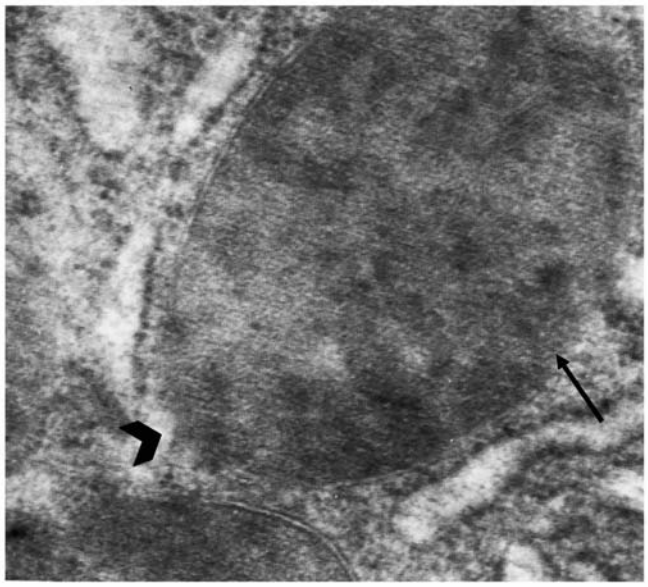


Figure 2: Electron micrograph of the rabbit liver treated with Diclofenac sodium 6 mg/kg (group D) showing mitochondria of hepatocytes with damaged outer membrane (arrowhead) and indistinct inner membrane (arrow)

damage which may not manifest overtly in all cases however. The pathogenesis of low incidence but high severity of Diclofenac hepatotoxicity is unknown. One reason may be the lack of animal model studies. Individual patient with specific susceptibility factor will eventually determine whether a patient will tolerate the drug, or will develop toxic response rarely.

In the hepatocyte there is biotransformation of drug, which yields water soluble products that are excreted in urine or bile[13]. This hepatic biotransformation involves oxidative pathways primarily by way of cytochrome P-450 enzyme system[14]. The drug binds or otherwise disables respiratory chain enzymes or mitochondrial DNA resulting in oxidative stress, with ensuing anaerobic metabolism, lactic acidosis and triglyceride accumulation. A decrease in ATP concentration and damage to mitochondrial membranes lead us to consider a mitochondrial bioenergetics dysfunction to be one of the possible events causing the Diclofenac toxicity[7].

On the other hand Dunk and Danan favour a metabolic mechanism[15]. In this proposed mechanism, liver cell injury at the molecular level has been studied. Diclofenac sodium is a lipophilic and weakly acidic compound. Biochemically it is phenylacetic acid derivative with carboxylic acid moiety, and has a diphenylamine backbone with secondary amine[5].

This secondary amine also contributes to its uncoupling activity in mitochondria and is involved in the production of oxidoreductive stress. In this regard our results are in conformity with Breen who reports five patients of acute hepatitis with proven Diclofenac

toxicity[16].

Disappearance of matrix granules in group D animals shows that mitochondria are extremely sensitive to the drug, both at normal dosage and especially in overdose. These results are in accordance with Desmet and Vos[17] who have described disappearance of matrix granules as early and reversible phenomena.

As far as the severity of lesion in group D animals is concerned results are comparable with Purcell et al[18] who found correlation (by biochemical methods) between the dose of drug and severity of liver damage.

CONCLUSION

Hepatotoxicity related to Diclofenac although not very common but when it occurs it is life threatening. So it is important for the physicians to be vigilant to the hepatotoxic potential of diclofenac sodium. An increased awareness, surveillance and reporting of events will lead to better understanding of risk factors.

The toxicity is either idiosyncratic or metabolic in vast majority of cases, so further work is needed to identify the patients at greater risk and we should also make the public aware of hazards of increasing use of this drug for minor musculoskeletal disorders. In this regard pharmaceuticals have also notified healthcare professionals to measure the liver enzymes periodically in patients taking diclofenac sodium for long term[19].

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