Histochemical Alterations in the Spleen of Rabbits Induced by Diclofenac Sodium (Voltaren)

[*]**Noory T. Taib and** [**]**Bashir M. Jarrar**
[*]*Department of Zoology, College of Science, King Saud University*
[**]*Department of Clinical Laboratory Sciences,*
College of Medical Applied Sciences,
Al-Jouf Branch, King Saud University

Abstract. A total of 30 male rabbits were exposed to intraperitoneal injection with a daily dose of diclofenac sodium (1.5 mg/kg body weight) for 70 days to investigate the resultant histological and histochemical alterations in the splenic tissues. This regime of chronic exposure to subtoxic doses of diclofenac sodium has produced no histological alterations in the spleen but caused histochemical alterations mainly in the form of haemosiderosis with elevation in the activity of alkaline phosphatase, 5-nucleotidase and lactate dehydrogenase. The activity of mitochondrial adenosine triphopsphatase was decreased while the activity of succinate dehydrogenase was not affected. The obtained results indicate that diclofenac sodium produces considerable histochemical alterations but little histological changes in the spleen.

Introduction

Diclofenac sodium is probably one of the most common non-steroidal compounds with analgesic, anti-inflammatory, antirheumatic and antipyretic properties [1]. This drug has an inhibitory effect on prostaglandin synthesis and used as an initial therapy for inflammatory and degenerative rheumatic diseases as well as for pain conditions such as musculoskeletal and post-operative pains and acute attacks of gout and ureteric colic [2-3]. Diclofenac is metabolized in the liver to 4-hydroxy diclofenac and other hydroxylated forms after glucoronidation and sulfation before being eliminated principally via urinary and biliary excretions [4-5]. Diclofenac (daily dose of 1.5 mg/kg body weight) is claimed to be faster acting than Ibuprofen, longer acting than Paracetamol and as safe as Ibuprofen [6].

There are many side effects observed with the use of this drug. It is not recommended for (paediatric uses) while its overdosing is potentially toxic. Diclofenac sodium causes a rare but potentially fetal hepatotoxicity that may be associated with the formation of reactive metabolites and subsequent adverse hepatitis effects may arise in certain individuals [5, 7, 8]. Diclofenac provokes proliferation of bile duct, hepatocellular degeneration, non-specific hepatitis with portal and lobular activity. It also elevates markedly transaminases levels, decreases the glycogen content of the hepatocytes and impaires ATP synthesis by the mitochondria with futile consumption of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) [9-11].

Some studies have shown that diclofenac sodium accounts for the thickening of the glomerular basement membranes together with mild focal tubular necrosis and intraluminal secretions in the proximal convoluted tubules [6, 12]. Other less frequent effects on the urinary tissues include haematuria, proteinuria, interstitial nephritis, lipid peroxidation and papillary necrosis [6, 13, 14]. Diclofenac has been also reported to damage moderately the seminiferous tubules and to impaire spermatogenesis [15].

There are limited studies on the histochemical alterations in the splenic tissues induced by chronic diclofenac sodium exposure. With this objective, a histological and histochemical study has been done on the spleen of adult rabbits killed at one week intervals up to 70 days of diclofenac sodium treatment.

Material and Methods

Experimental animals
A total of 30 adult male rabbits of three months age weighing 950-1000 gm obtained from Tahsin center, Riyadh, Saudi Arabia were used. Animals were kept in cages where each contained 2 animals.

Experimental design
Following a period of acclimatization (7 days), diclofenac sodium (Proanalysis-Merck, Germany) was administered intraperitonealy at the rate of 1.5 mg/kg body weight. The control group (n = 10) received sterile distilled water via the same route and all animals were maintained on standard laboratory animal diet pellets *ad libitum*. Animals body weight was checked regularly and dosages based on the body weight of each animal were calculated. Three animals (two exposed and one control) were killed by decapitation of the neck at weekly intervals during 70 days of treatment with diclofenac sodium.

Histological study
Fresh portions of the spleen from each rabbit were cut out rapidly, fixed in 10% neutral buffered formalin and Gender's fluid and then dehydrated with ascending grades of ethanol (70, 80, 90, 95 and 100%). Dehydration was then followed by clearing the tissue samples in 2 changes of chloroform before being impregnated with 2 changes of

melted paraffin wax, embedded and blocked out. Tissue sections (4-5 μm) were stained with haematoxylin-eosin, Mallory trichrome and prusian blue reaction according to the methods described by Pearse [16], Drury *et al.* [17], Bancroft and Stevens [18] and Kiernan [19] for the conventional histological procedure.

Histochemical study

Portions of fresh spleen from each rabbit were cut out rapidly, frozen in liquid nitrogen and stored in air tight tubes at -80°C until use. Cryostat sections (8-10 μm in thickness) were cut at -25°C. Unfixed fresh frozen cryostat sections were used for the histochemical characterization of the investigated phosphatases according to the following methods:

Alkaline Phosphatase (ALPase): The demonstration of ALPase was based on the modified medium described by Mayahara *et al.* [20]. The medium consisted of 10 mM β-glycerophosphate dissolved in 80 mM Tris-HCl buffer (pH 9.2) to which was added 3.9 mM magnesium chloride and 2.0 mM lead acetate. Sections were developed in yellow ammonium sulfide and mounted in glycerine jelly.

5-nucleotidase (AMPase): The reaction detecting AMPase was based on the metal salt method modified by Dawson *et al.* [21]. The medium consisted of 2 mM lead nitrate, 200 μM 5-adenosine monophosphate (5-AMP), 1 mM levamisole (alkaline phosphatase inhibitor), 1 mM manganase chloride , 0.1% gelatin, and 280 mM sucrose in tris maleate buffer (pH 7.4). The resultant lead phosphate was converted to lead sulfide by the addition of ammonium sulfide and mounted in glycerine jelly.

Mitochondrial ATPase (Mg^{2+}-ATPase): The activity of this enzyme was demonstrated by the method of Wachstein *et al.* [22]. The medium consisted of 0.83 mM adenosine triphosphate (disodium salt) dissolved in 0.2 M Tris-maleate buffer (pH 7.4), and 1 mM magnesium sulfate. Sections were post-treated in 2 mM lead nitrate, developed in yellow ammonium sulfide and mounted in glycerine jelly. The reaction of Mg^{2+}-ATPase was controlled by incubation set of sections in the above medium without magnesium sulfate.

Dehydrogenases: Succinate and lactate dehydrogenases were histochemicaly demonstrated in the present study according to tetrazolium method [23, 24]. The final incubation medium for each enzyme consisted of the nitro blue tetrazolium (NBT) stock solution, the substrate solution and the employing co-enzyme. The NBT stock solution consisted of 2.5 ml NBT solution (4 mg/ml), 2.5 ml of 0.2 M Tris buffer (pH 7.4), 1 ml of 0.05 M magnesium chloride and 3 ml of distilled water. Potassium cyanide (100 mM) or sodium azide (100 mM), respiratory chain inhibitors of cytochrome systems, was added to the final incubating medium of the bound dehydrogenases. Phenazine methosulphate, an intermediate electron acceptor, was added to the incubating medium at the rate of 1 mg/ml of the medium.

Results

No mortality or clinical signs due to diclofenac sodium intoxication were observed for any of the treated rabbits over the entire experimentation period. In addition, no

increase in the ratio of spleen: body weight, in comparison to the control animals, or splenomegaly was recorded.

Compared with the respective control rabbits, diclofenac sodium produced no distinct histological splenic changes. No alterations were seen in the histological components of the red pulp, periarteriolar lymphoid sheath (white pulp), trabeculae and capsule of the spleen of treated rabbits in comparison with that of the control ones. A considerable increase in the number of resident macrophages in splenic cords of the treated rabbits was noticed (Fig. 1). More Prussian blue positive granules were observed in the cytoplasm of the macrophages of rabbits exposed to diclofenac sodium for 6 weeks compared with the untreated rabbits (Fig. 2). Haemosiderosis was not recognized in the periarteriolar lymphoid sheeth components.
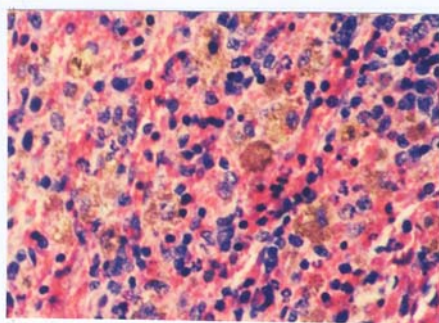

Fig. 1. Light micrograph of spleen showing an increase in the resident macrophag. Diclofenac treated rabbit. Eight weeks post-treatment. H&E. x270.
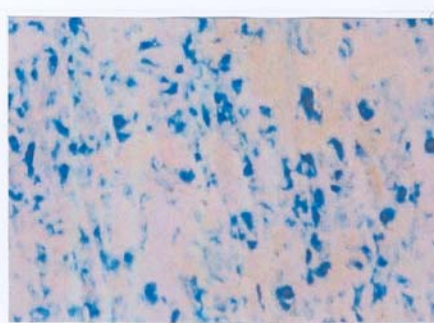
Fig. 2. Light micrograph of spleen showing extensive haemosiderosis. Diclofenac treated rabbit. Six weeks post-treatment. Prusian blue reaction. x450.

The histochemical characterization, distribution patterns and sites of activities of the investigated enzymes in the control and treated rabbits were as follows:

Alkaline phosphatase: In the control spleen, a strong activity of ALPase was seen in the foamy cells and macrophages of the red pulp while no activity for this enzyme was detected in the periarteriolar lymphoid sheaths. In the treated spleens, a significant increase in the activity of ALPase was observed in the foamy cells and marophages as well (Fig. 3).

5-nucleotidase (AMPase): In the control spleens, the activity of AMPase was confined mainly to some components of the splenic cords. A considerable increase in the activity of AMPase was noticed in the splenic cords of the treated rabbits (Fig. 4).

Mitochondrial adenosine triphosphatase (Mg^{2+}-ATPase): A moderate cytoplasmic activity of Mg-ATPase was recognized in the dendritic macrophages of the red pulp of the control spleens. The reaction of Mg^{2+}-ATPase in the spleens of diclofenac treated rabbits showed significant reduction in comparison with the respective control ones. The reduction in the activity was clear after five weeks of treatment (Fig. 5).
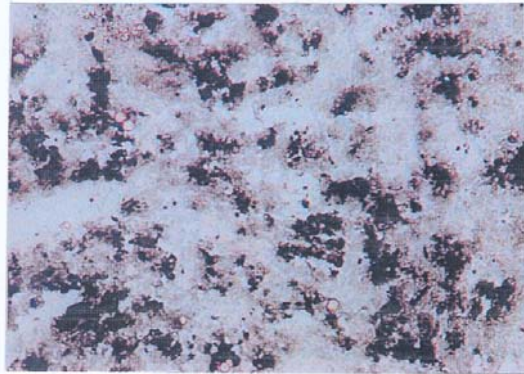
Fig. 3. Light micrograph of spleen showing significant activity of ALPase in the macrophages. Diclofenac treated rabbit. Ten weeks post-treatment. x450.
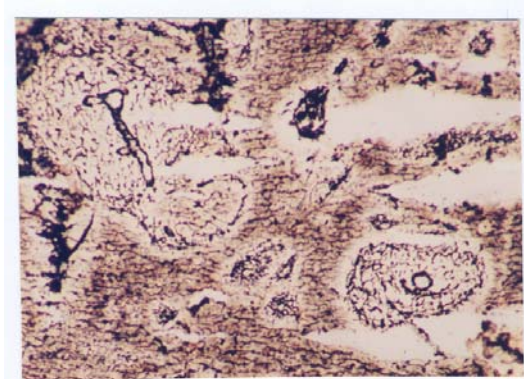

Fig. 4. Light micrograph of spleen showing considerable activity of 5-nucleotidase. Diclofenac treated rabbit. Metal salt method. Eight weeks post-treatment. x450.
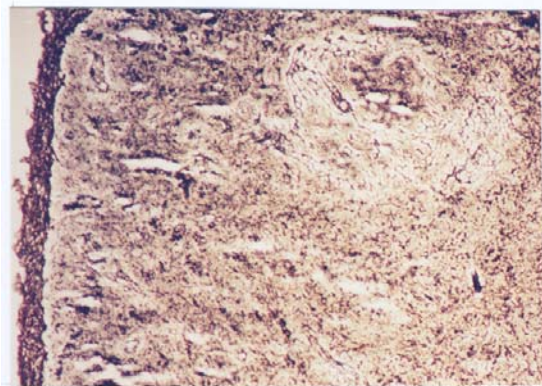

Fig. 5. Light micrograph of spleen showing the activity of the mitochondrial ATPase. Diclofenac treated rabbit. Wachstein method. Five weeks post-treatment. x270.

Succinate dehydrogenase (SDH): In the control spleens, a considrable activity of SDH was demonstrated in the trabeculae, follicular arterioles of the white pulp, the penicillar arteries and to less extent in the splenic capsule. No significant change in the activity of SD in the spleen of diclofenac exposed rabbits was observed.

Lactate dehydrogenase (LDH): The spleens of the control rabbits showed a strong reaction for LDH in the trabeculae, splenic capsule and splenic arteries. A significant increase in the activity of LDH was detected in the spleen of rabbits exposed to diclofenac sodium in comparison to the untreated control animals (Fig. 6).
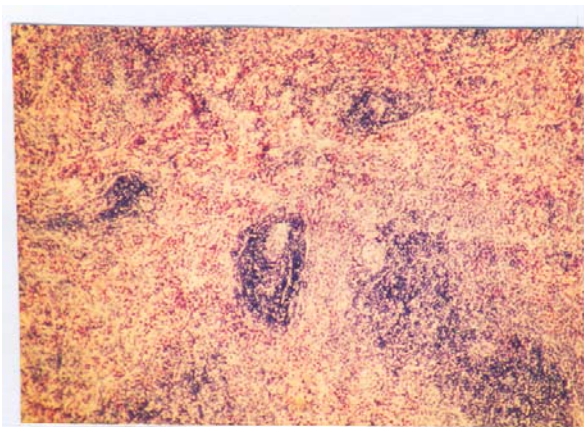

Fig. 6. Light micrograph of spleen showing the activity of the lactate dehydrogenase activity. Diclofenac treated rabbit. Tetrazolium method. Ten weeks post-treatment. x270.

Discussion

The results of the present investigation showed that the subtoxic chronic diclofenac sodium exposure has no apparent effects on the splenic tissue. This might suggest that no deleterious action is exerted by diclofenac on the cellular components of the spleen. However, the drug under investigation increased the preciptation of haemosiderin in the red pulp of the treated rabbits. This might indicate an effect of diclofenac sodium on the haematopoiesis, probably in terms of increased destruction rate of erythrocytes. However, this effect is still to be elucidated.

The current results showed that diclofenac has no effect on the activity of succinate dehydrogenase but enhanced the activity of lactate dehydrogenase. Succinate dehydrogenase participates in the aerobic oxidation of carbohydrates in the citric acid cycle and is bounded to the inner mitochondrial membrane [25]. The stability of succinate dehydrogenase activity after diclofenac exposure possibly indicates that this drug does not impaire mitochondrial functions and carbohydrates oxidation in the aerobic metabolic processes especially the electron transport. This finding may also indicate that there is no effect on the oxidative phosphorylation of the cellular

components of the splenic tissues. The increase in the activity of lactate dehydrogenase which plays an important role in the intermediary metabolism as a link between amino acid metabolism and the citric acid cycle where it converts lactate into pyruvate [25] might indicate a role for diclofenac in increasing this process.

The results of the present work showed that diclofenac sodium enhanced the activitiy of alkaline phosphatase and 5-nucleotidase but decreased the activity of mitochonderial adenosine triphosphatase. This finding suggests increased transmembrane transport of diclofenac where alkaline phosphatase is involved in the absorption and transportation across these membranes [26]. On the other hand, alkaline phosphatase is a meta-enzyme and contains zinc as an integral part [27]. The non-specific increase in the activity of alkaline phosphatase by diclofenac exposure may be a result of the incorporation of the drug in the place of zinc atoms leading to an increase in the activity of this enzyme. 5-nucleotidase is a dephosphorylating enzyme related to cyclic AMP and involved in the catabolism of nuclear material in disintegrating cells and specifically dephosphorylates 5-monucleotides [28]. Hence, the present results might suggest a possible role of this enzyme in the catabolism of nucleic acids in the cells disintegrated by diclofenac effect. Mitochondrial adenosine triphosphatase is inhibited by any substance that blocks sulfohydryl (SH) group. The decrease in the activity of mitochondrial adenosine triphosphatase due to diclofenac exposure may be resulted from its interaction with the sulfohydryl groups and the oxidative phosphorylation of the cells.

Alterations in the activity of some splenic phosphatases and dehydrogenases due to diclofenac exposure were demonstrated in the present study. This might lead us to conclude that the changes in the activity of these enzymes might represent adaptive responses to the changes in the catabolism of the splenic cells or indicate partial impairments of their functions induced by diclofenac.

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'قسم علم الحيوان، كلية العلوم بالرياض، جامعة الملك سعود

'قسم علوم المختبرات الطبية، كلية العلوم الطبية التطبيقية بالجوف، جامعة الملك سعود

ملخص البحث. استعمل في هذا البحث ٣٠ أرنباً بالغاً من أجل تحري التغيرات الكيميانسيجية التي يحدثها عقار ديكلوفناك الصوديوم المعروف بالفولتارين. تم إعطاء الجرعات عن طريق الحقن بتجويف البطن لمدة ٧٠ يوماً متتالياً بمعدل ١.٥ ملجم/كجم من وزن الجسم.

أظهرت نتائج هذا البحث أن التعرض لجرعات مزمنة تحت عتبة السمية من عقار ديكلوفناك الصوديوم لا تتسبب بإحداث تغيرات نسيجية بالطحال لكنها تحدث به تغيرات كيميانسيجية تتمثل بالأساس لصبغيات الهيموسـدرن وزيـادة في وتـيرة البلعمـة الخلويـة، إضـافة إلى ارتفـاع في نـشاط كـل مـن الفوسـفاتاز القاعـدي ونيكلوتيداز– ٥ ونازع هيدروجين اللاكتات بينمـا تتـسبب بالمقابـل في خفض نـشاط أدونـسين ثلاثي الفوسـفاتاز ولم يحدث أي تغير على الإطلاق في نشاط نازع هيدروجين السكسنات. يمكن الاستنتاج من نتائج هذا البحـث أن عقار ديكلوفناك الصوديوم يحدث تغيرات كميانسيجية بالطحال بينما التغيرات النسيجية التي يحدثها جد محدودة.