Sub-chronic diclofenac sodium induced alterations of alkaline phosphatase activity in serum and skeletal muscle of mice

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The present study has been carried on changes in activity of alkaline phosphatase in serum and gastrocnemius muscle of mice after sub-chronic use of diclofenac. Mice in experimental group received diclofenac (10 mg/kg body wt /day) for 30 days while control group received normal saline. Alkaline phosphatase was assayed in muscle and serum and its activity was localized histochemically in muscle. Results showed that diclofenac induced changes in specific activity of alkaline phosphatase at different periods of treatment variably compared to control group. Specific activity of alkaline phosphatase decreased significantly in gastrocnemius initially (48.74%), increased thereafter (132.96%) and slight decrease (13.97%) was noticed after 30 days. In serum, the specific activity of alkaline phosphatase decreased slightly after 10 days (18.78%), increased in the middle of the treatment period (132.04%) as well as showed increase (109.09%) compared to control group after 30 days stage of investigation. These findings were also confirmed by electrophoretic studies in muscle.

Keywords: Alkaline phosphatase, Diclofenac, Gastrocnemius, Serum, Sub-chronic

Diclofenac residues were found to be the cause for rapid population decline of Gyps vultures in southern Asian countries\(^1\). The toxic effects of diclofenac were known however the mechanism of action is still not fully understood. Nonsteroidal anti-inflammatory drugs (NSAIDs) continue to be prescribed as analgesics for patients with healing fractures even though these drugs diminish bone formation, healing, and remodeling\(^5\). Diclofenac is a widely used NSAID for treatment of a variety of inflammatory conditions such as rheumatoid arthritis, osteoarthritis and acute muscle aches. Diclofenac inhibits both cyclooxygenase (COX) isoenzymes, COX-1 and COX-2, by blocking arachidonate binding resulting in analgesic, antipyretic and anti-inflammatory pharmacologic effects\(^3\). The enzymes COX-1 and COX-2 catalyze the conversion of arachidonic acid to prostaglandin G2 (PGG2), the first step of the synthesis of prostaglandins and thromboxanes that are involved in rapid physiological responses. Delayed fracture healing and no body weight gain was reported in rats after long term treatment with diclofenac\(^4\). Diclofenac sodium causes a rare but potentially fatal hepatotoxicity that may be associated with the formation of reactive metabolites and subsequent adverse hepatitis effects may arise in certain individuals\(^5\). NS-398, a COX-2 specific inhibitor has been reported to interfere with the healing of injured skeletal muscle\(^6\). An inhibition of protein synthesis in rat skeletal muscle with three different cyclooxygenase inhibitors (aspirin, indomethacin and meclofenamate) was also reported\(^7\). The elevation in the serum level of alkaline phosphatase has been used as diagnostic index for skeletal muscle disorders\(^8\). Alkaline phosphatase showed an association with a number of muscle diseases including neuromuscular disorders, myopathies or other wasting conditions\(^9\). The objective of the study was to investigate diclofenac induced alterations of alkaline phosphatase activity in mice skeletal muscle.

Alkaline phosphatase (APase; E.C.3.1.3.1) represents a group of isozymes that are membrane-bound glycoproteins which catalyze the hydrolysis of inorganic and organic monophosphate esters at alkaline pH in vitro\(^10\). APase is ubiquitous in nature located in basal membrane of various tissues and also found in the serum\(^11\). Soluble forms of APase exist in the serum. Alkaline phosphatases are members of a rather diverse group of membrane proteins which are anchored to lipid bilayers in cell membranes by a phosphatidylinositol-glycan moiety attached to the

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carboxy terminus of the protein\textsuperscript{12}. Serum APase is a dimer, whereas the membrane-bound forms of APase are probably tetramers\textsuperscript{13}. Studies reported earlier have established that APase does form an important entity in the muscle cell\textsuperscript{14}. Patients suffering from hypophosphatasia, a genetic deficiency in APase activity, commonly suffer from severe rickets and osteomalacia\textsuperscript{15}.

Materials and Methods

Animals—Healthy male mice of Balb-c strain (36) weighing about 24-27 g were procured from Central Research Institute (CRI), Kasauli, (Himachal Pradesh), India. Animals were maintained in hygienic conditions in a well ventilated room of animal house of Department of Bio-Sciences of H P University, Shimla with D:L cycle of (12:12) h and a temperature of 25 ± 2°C. Animals were provided with commercial feed (Hindustan Lever Ltd., New Delhi, India) and water ad libitum. All procedures, including the maintenance of the animals had the approval of Institutional Animals Ethics Committee of the University (IAEC approval no: IAEC/Bio/2009/6-HPU).

Drug administration—Diclofenac sodium was purchased from Sigma Aldrich Co., USA. Stock solution was made in distilled water. Further dilutions were done according to the body weight records of the animals. All the chemicals used were of analytical grade. To investigate the changes in the specific activity of APase in control and experimental group, mice (18) received diclofenac intramuscularly at the dose rate of 10 mg/kg body wt. /day for 10, 20 and 30 days while 18 of the control group received normal saline. The drug dose chosen was higher than the recommended therapeutic dose (8 mg/kg) for mice. Mice were sacrificed by cervical dislocation, the next day at the end of the experiment. Gastrocnemius muscle was excised and serum was collected at each stage of the investigation.

Histological study—Bouin fixed gastrocnemii were embedded in paraffin wax after dehydrating in ascending grades of alcohol and clearing in xylene. Further, 7-8 µm thin sections were processed for Haematoxylin-Eosin staining. Lastly, after clearing in xylene, sections were mounted in DPX and photographed.

Histochemical localization—APase was localized histochemically in cryostat cut sections of the gastrocnemius muscle by Gomori’s calcium cobalt sulphide method\textsuperscript{16-18} with slight modifications. Briefly, tissues were fixed overnight in neutral calcium formol and next day, processed for histochemical staining. The cryostat cut sections were preincubated in 100 mM Tris-maleate buffer containing 1% MgCl\textsubscript{2} overnight at room temperature. Thereafter, the preincubated sections were incubated in the substrate solution (pH 8.9) containing sodium β-glycerophosphate for half an h at 37°C and preceded according to Gomori’s method\textsuperscript{16}. These were quickly dehydrated in ascending grades of alcohol and mounted in Canada balsam and photographed.

Biochemical assay—The muscle homogenate was prepared in ice cold distilled water which was centrifuged at 1664 x g for 20 minutes at 4°C. The supernatant was used for the enzyme assay. Serum was collected and centrifuged at 2599 x g for 15 min at 4°C. Muscle and serum APase was assayed by the method of Weil and Russel\textsuperscript{19}. Briefly, sodium β-glycerophosphate was used as substrate. The homogenates were incubated with the substrate solution (pH 8.9) for one h at 37°C. The reaction was stopped by 10% TCA. Finally, the solution was centrifuged at 936 x g at 4°C and the supernatant was collected for determination of enzyme activity. The color intensity was read at 650 nm in a Hitachi 150-ophrey Double Beam Spectrophotometer. The amount of phosphate released was plotted against known concentrations of KH\textsubscript{2}PO\textsubscript{4}. Protein content was estimated according to method of Lowry et al.\textsuperscript{20}. The specific activity of alkaline phosphatase was calculated.

Homogenization of the tissue—Muscle homogenate was prepared according to the method previously described\textsuperscript{21-22} with slight modifications. Tissue was homogenized in 5 volumes of Tris buffer, 10 mM, pH 7.4 containing 1% triton X-100, 2 mM MgCl\textsubscript{2} and 0.025 mM ZnCl\textsubscript{2}. Homogenate was centrifuged at 15,000 x g for 20 min. The pellet was rehomogenized, centrifuged at 15,000 x g for another 20 min. Supernatants were combined and again centrifuged at 20,000 x g for 30 min. All the steps in this procedure were carried out at 4°C.

Electrophoresis—It was conducted according to the method of Epstein et al.\textsuperscript{23} with some modifications. Supernatants were used for electrophoretic studies. Proteins were estimated according to Lowry et al.\textsuperscript{20}. The enzyme was resolved on 7% separating and 5% stacking gel. Equal amount of protein was loaded in each lane. The enzyme was localized using activity staining technique earlier described by some workers\textsuperscript{24} with certain modifications. Gels were analyzed densitometrically.
Statistical analyses—Values are represented as mean ± SEM. Results were analysed using Student’s ‘t’ test.

Results
Toxic effects of diclofenac treatment were observed in mice gastrocnemius in terms of altered morphological and biochemical levels. The histopathological changes pointed towards massive tissue damage. Diclofenac treatment caused numerous pathological changes during different stages of investigation as compared to the control group. Lightmicrographs of control mice gastrocnemius depicted normal fascicles surrounded by perimysium encasing group of myofibers having relatively uniform pattern. Individual muscle fibers showed endomysium around them, each one having normal peripheral arrangement of the nuclei (Fig. 1A and 1B). Diclofenac treatment for 10 days on the other hand resulted in structural disorganization in terms of degenerating fibers, atrophied fibers and inflammatory cell infiltration (Fig. 1C). After 20 days of drug treatment, merging of fibers was noticed which had irregular limiting membranes, many fibrolysed as well as abnormally hypertrophied fibers were also observed. Previous peripheral arrangement of the nuclei delineated which occupied central position now: (Fig. 1D). More disorganized structure of the muscle was observed after 30 days of diclofenac treatment. Chains of darkly stained nuclei were noticed in interfibrillar regions. Muscle fibers lost proper shape and uniformity due to loss of perimysial and endomysial boundaries, many atrophied fibers were noticed to be without nuclei, degeneration continued as well as merged fibers were also noticed (Fig. 1E and 1F).

With regard to histochemical studies, the micrographs of gastrocnemius muscle exhibited darker staining with intense APase activity around the sarcolemmal regions in the control group. Further, section from control group showed normal fibers with intact boundaries having a uniform pattern (Fig. 2A); whereas the intensity of enzyme positive areas was found to be lighter in the section from treated group after 10 days of treatment. Degenerating fibers and merging sarcolemma were also noticed. The enzyme activity was found to be more pronounced around the smaller fibers as compared to the hypertrophied ones where enzyme activity seemed to be less (Fig. 2B). After 20 days of treatment with diclofenac, the increased number of hypertrophied as well as degenerating fibers with degeneration foci were observed (Fig. 2C). Also lesser enzyme activity around the merging sarcolemma was seen as compared to control. After 30 days, weaker staining for APase was observed; many fibers were found without sarcolemma being merged with each other along with somewhat larger and intact fibers showing more enzyme activity in comparison to other degenerating fibers. However, some atrophic fibers also had darker staining for APase. Fibrolysis was also noticed along with lesser enzyme positive areas (Fig. 2D).

These histochemical findings were also confirmed from biochemical assay where APase activity in gastrocnemius decreased significantly as compared to control after first stage of investigation. Activity of APase in gastrocnemius showed a decrease from 0.0143 ± 0.0014 µM Pi/µg of protein/h to 0.0073 ± 0.0003 µM Pi/µg of protein/h after 10 days of treatment. Significant increase was observed in alkaline phosphatase activity after 20 days of treatment from 0.00364 ± 0.00016 µM Pi/µg of protein/h to 0.00484 ± 0.00024 µM Pi/µg of protein/h. After 30 days of treatment, slight decrease in APase activity [0.0022 ± 0.0001 µM Pi/µg of protein/h to 0.0019 ± 0.00014 µM Pi/µg of protein/h; was found (Fig. 3)].

The biochemical findings were further supported by electrophoretic study (Fig. 4). An image analysis of gels showed that in treated group, alkaline phosphatase activity decreased significantly as compared to biochemical assay where APase activity in gastrocnemius showed a decrease from 0.0143 ± 0.0014 µM Pi/µg of protein/h to 0.0073 ± 0.0003 µM Pi/µg of protein/h after 10 days of treatment. Significant increase was observed in alkaline phosphatase activity after 20 days of treatment from 0.00364 ± 0.00016 µM Pi/µg of protein/h to 0.00484 ± 0.00024 µM Pi/µg of protein/h. After 30 days of treatment, slight decrease in APase activity [0.0022 ± 0.0001 µM Pi/µg of protein/h to 0.0019 ± 0.00014 µM Pi/µg of protein/h; was found (Fig. 3)].
noticeable increase in the enzyme activity (45% ± 1.041 to 55% ± 1.041; as compared to control group was seen. In the similar trend as that of first stage of investigation, appreciable decline in the enzyme activity after 30 days of drug administration (57.74 ± 1.171 to 42.25% ± 1.171) was noticed (Fig. 5).

With regard to the biochemical analysis of APase in serum, insignificant decrease in the serum APase activity was noticed after 10 days treatment from 0.0458 ± 0.0062 µM Pi/µg of protein/h to 0.0372 ± 0.0022 µM Pi/µg of protein/h which amplified after 20 days from 0.0181 ± 0.0012 µM Pi/µg of protein/h to 0.0239 ± 0.001 µM Pi/µg of protein/h and again showed a significant hike after 30 days from 0.0308 ± 0.0011 µM Pi/µg of protein/h to 0.0336 ± 0.0011 µM Pi/µg of protein/h (Fig. 6).

Discussion

Many nonsteroidal anti-inflammatory drugs have been reported to cause alterations in different tissues. Diclofenac associated hepatitis and histopathological lesions in liver and kidney have been reported in earlier studies²⁵-²⁶. The present investigation revealed remarkable alterations at morphological as well as biochemical levels in mice gastrocnemius. A number of deviations in the muscle under investigation were noticed in terms of degenerating sarcolemma, fibrolysis, atrophying fibers, abnormally hypertrophied fibers, merging of fibers and delineated arrangement of nuclei etc. which seemed to be irreversible during this stretch of study. With fibrolysis taking place in the fiber, tension generated within the fiber is altered, thus, causing bilateral compression which results in reduction of sarcoplasm.

Fig. 2—(A)-T.S. Gastrocnemius control: Intact fibers (IC) showing intense APase activity around sarcolemma shown with (↑); (B)-T.S. of Gastrocnemius of diclofenac treated group for 10 days exhibiting intense APase activity (↑), atrophied cells (↑↑), degenerating cells with noticeable degenerating Foci (↑↑); (C)-Section of gastrocnemius from diclofenac treated group for 20 days showing overall lesser enzyme activity around the fibers; higher enzyme activity (↑) around intact cells (IC), reduced activity around degenerating cells (↓), few merged fibers(*) also seen; (D)-Section of gastrocnemius from diclofenac treated group for 30 days revealing lighter staining for APase, merging fibers(↓), fibrolysis fibers (↓↓), APase activity positive around intact cells(↑).
and the myofibrils leading to atrophy. COX-2 activity is essential for efficient repair after muscle injury as well as recovery from atrophy. NS-398, a specific COX-2 inhibitor was documented to inhibit the proliferation and differentiation of myogenic cells in vitro. Furthermore, it decreased the regeneration of injured muscle by delaying the maturation of regenerating myofibers, promoted fibrosis by upregulating transforming growth factor (TGF-β1) expression in mice gastrocnemius. It is speculative that the observed histopathological changes in gastrocnemius might be due to diclofenac administration which is also a partial COX-2 inhibitor. Rows of nuclei in interfibrillar region and their displacement from peripheral positions to centre of the muscle fiber were indicative of some alterations. Such changes in the nuclear morphology are apparently on account of loss of nucleoplasmic substances. The administration of different classes of COX inhibitors like aspirin, nimesulide and celecoxib when used in animal studies at the clinically safer doses have also caused alterations in the biochemical and biophysical state of the intestinal brush border membrane while diclofenac was reported to have negative effect on colon anastomotic healing in rats. Tubular epithelial cell degeneration and necrosis in kidney and acute hepatitis has been earlier reported in rats on administration of the high dose of diclofenac that may be reversible or irreversible.

The present study revealed that diclofenac induces remarkable changes in specific activity of alkaline phosphatase in serum and mice muscle as well. Alkaline phosphatase has been categorized as cell activity biomarker as well as hydrolytic enzyme functioning at alkaline pH optima by a number of workers earlier. Biochemically, it was found that specific activity of APase in gastrocnemius decreased to 48.74% and increased to 132.96% after 10 and 20 days.
days respectively. These altered patterns of the enzyme activity were further confirmed by densitometric analysis of gels where APase activity declined (37.73%) which augmented to 122.2% after 20 days drug administration. The lytic process and stimulation of alkaline phosphatase has been documented in skeletal muscle by some workers earlier. In another study, enhanced fibrolysis was documented with parallel rise in APase activity in sciattectomized chick gastrocnemii.

In the treated spleen, a significant increase in the activity of APase was observed in the foamy cells and macrophages as well after use of diclofenac in rabbit. After 30 days of treatment, activity of APase decreased to 13.97% in gastrocnemius in biochemical assay while electrophoretic analysis also recorded parallel results (26.83% diminution). In previous studies with aspirin and nimuslide on rat intestine, the increase in the alkaline phosphatase enzyme activity was indicative of intestinal dystrophy. The decline observed in APase activity during initial stage could be due to the cytoprotective role of non-steroidals to some extent. However, when the drug was continued for another 10 days, enhanced APase activity was noticed which further diminished after 30 days (13.97% diminution). From these varied trends of APase activity, it is hypothesized that by lowering APase, diclofenac has played role of anti-inflammation protecting the tissue while in 2nd stage of the study, trend of enzyme reversal may be due to an adverse effect of the drug. Higher levels of alkaline phosphatase activity in diseased human muscle has been associated with lytic role of enzyme and also leading to proliferation of non-contractile connective tissues. It is noteworthy that the role of alkaline phosphatase varies differently in different tissues. APase has been proposed to be a function related marker in the renal proximal epithelia having protective effect on proximal tubule damage of the kidney. However, after aspirin treatment, 80% decrease in APase activity in rat heart was documented to play cardiovascular protective role by preventing calcification of the system.

Advanced age and long-term physical exercise cause changes in the activity of APase in rat muscle. The nonspecific 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 as APase being a metaenzyme. In serum, APase specific activity decreased to 18.78% and showed 132.04% increase after 10 and 20 days and again after 30 days of diclofenac treatment, increase (109.09%) was noticed. Previous studies have shown that catalaflam (potassium diclofenac) significantly increased the activity of alkaline phosphatase in rat serum which is indicative of skeletal and muscular disorders.

The trends of APase in gastrocnemius and serum in the present investigation exhibited unexpected similar decline in the enzyme activity after 10 days of drug treatment and hypothesized that decrease in the serum APase could be accounted for its hike in other tissues. However, increase in the serum APase during the last two stages definitely hints towards some deleterious effects of the drug on different tissues including gastrocnemius for which many morphological evidences have been proposed. Increased transmembrane transport of diclofenac has been suggested where APase is involved in the absorption and transportation across these membranes. The involvement of alkaline phosphatase in active transport was reported by a number of workers.

Histochemical studies on gastrocnemius muscle revealed lesser APase activity around sarcolemma initially. It was noticed that atrophied (narrow) fibers showed greater activity as compared to the normal fibers after 10 days of treatment. It has been reported earlier that increased fiber alkaline phosphatase positivity is correlated with an increased incidence of degeneration and regenerative changes, fibrosis and atrophy in skeletal muscles. An association of APase with pathological conditions has also been reported for skeletal muscle of chicks and rats, in which striated muscles revealed increased activity in pathological conditions such as denervation.

Declined levels of APase later on suggested that the system may show adaptive responses towards otherwise cytotoxic effects of the drug. However, the level of serum APase was found to be higher in last two stages of investigation suggesting some kind of deleterious effects caused due to diclofenac treatment. An increase in the activity of serum alkaline phosphatase after aspirin treatment was reported earlier and this increase was linked with the hepatotoxic effects caused by aspirin. Although, there remains a question of future research due to limitation of this study as to look for other possible reasons of raised levels of serum APase taking other vital organs such as liver, kidney, bone and intestine etc. into consideration. It is concluded from...
the present study that altered APase activity after sub-chronic diclofenac treatment can cause histopathological and physiological changes in skeletal muscle and may affect other tissues as well.

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