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Toxicological Study of Diclofenac on Mice Skeletal Muscle; A Drug That Posed Threat To Vultures During The Last Decade

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Abstract

Diclofenac sodium is a non- steroidal anti- inflammatory drug. This drug is an easily available 'over the counter drug' which can be purchased with or without medical prescription. The said drug first came into limelight when extinction of vultures due to consumption of cattle corpse treated by diclofenac was reported in Indian sub-continent. This was followed by banning of the said drug for veterinary purpose. The drug has anti-inflammatory and analgesic effects and is taken for skeletomuscular aches particularly by athletes. The anti-inflammatory action of diclofenac is brought about by inhibiting the formation of prostaglandins which are essential mediators of inflammation. This action is achieved by inhibition of COX-1 and COX-2 enzymes. Keeping the said facts in mind, a study was designed to find out possible toxicity of diclofenac on gastrocnemius muscle. Balb/c male mice were exposed to diclofenac sodium at dose rate of 10 mg/ kg/ body weight from 10-30 days. Body weight and gastrocnemius weight to body weight ratio was measured. Further, protein concentration of gastrocnemius muscle and serum acid phosphatase was analyzed biochemically. Serum alkaline phosphatase isoenzymes were separated by PAGE. Statistical analysis (Student's two tailed t- test) was applied. A noticeable decrease in body mass was reported in experimental animals after 20 and 30 days. Gastrocnemius weight to body weight ratio increased significantly after 20 days whereas a noticeable decrease was observed after 30 days. There was a significant increase in protein content of gastrocnemius after 10 days. Serum acid phosphatase activity was down regulated considerably. Similarly, isoenzymes of alkaline phosphatase in serum showed variations from 10-30 days. Present inquiry gives an insight into probable mechanism of toxicity caused by diclofenac.

Keywords : Diclofenac, Gastrocnemius, Serum, Acid Phosphatase, Alkaline Phosphatase.

Introduction

Nonsteroidal anti-inflammatory drugs, usually abbreviated as NSAIDs, are drugs with analgesic, antipyretic and, in higher doses, with anti-inflammatory effects. As analgesics, NSAIDs are non-narcotic. Physicians try to limit their patients' narcotic use to guard against dependency and abuse, continue an anti-inflammatory medication for the many weeks of fracture healing (Herbenick *et al.*, 2008). Although, the prefix 'non-steroidal' is used to distinguish these drugs from steroids, however both have an eicosanoid-depressing, anti-inflammatory action in common. There are four families of eicosanoids: prostaglandins, prostacyclins, thromboxanes and leukotrienes. These are either derived from ω-3 or ω-6 essential fatty acids (EFAs). Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) taken to reduce inflammation and as an analgesic reducing pain in conditions such as arthritis or acute injury. Diclofenac was reported to cause population declines in *Gyps* vultures of Pakistan, India, and Nepal. These reports raised an alarm all over the Indian sub-continent asit was the first time a pharmaceutical compound had placed wild populations at risk of extinction (Oaks et al., 2004). The first veterinary diclofenac product was registered in 1998 in Pakistan whereas its probable veterinary use in India started in 1994 and vulture mortality due to visceral gout

was already evident in 2000 (Green *et al.,* 2007).

All this reporting was ultimately followed by phasing out diclofenac for veterinary purpose in India in year 2006.The loss of millions of vultures over the last decade has had one of the major ecological consequences across the Indian subcontinent that posed a potential threat to human health.Diclofenac is a widely used drug for treatment of a variety of inflammatory conditions such as rheumatoid arthritis, osteoarthritis and acute muscle aches. Diclofenac inhibits both isoenzymes, COX-1 and COX-2, by blocking prostaglandin (PG) synthesis resulting in analgesic, antipyretic and anti-inflammatory effects (Mc Cormack and Brune, 1991). NSAIDs including diclofenac are administered to athletes after acute soft tissue injuries to minimize pain and inflammation so that athlete can return back to the field after a quick recovery. However, workers reported that short term benefits of these drugs may adversely affect long term healing of injured soft tissues (Friedman, 1996).

The skeletal muscle is an important seat of metabolism. NS-398, a COX-2 specific inhibitor has been reported to interfere with the healing of injured skeletal muscle (Shen *et al.*, 2005). PGs are synthesized in skeletal muscle and have been shown to have profound effects on skeletal muscle protein turnover (Vandenburgh *et al.*, 1995). Further, these researchers also revealed that PGF2α and PGE2 increase skeletal muscle protein synthesis and degradation, respectively. Decrease in PGE2α in human exercised muscle in response to dose dependent acetaminophen and ibuprofen treatment has been documented (Trappe *et al.*, 2001). COX-2 activity is essential for efficient repair after muscle injury (Shen *et al.*, 2005), as well as recovery from atrophy (Bondesen *et al.,* 2006). Inhibition of COX-2 during skeletal muscle regeneration results in increased fibrosis, elevated expression of transforming growth factor- β and myostatin, and reduced inflammatory cell accumulation (Shen *et al*., 2005). In another study, protein content in NS-398 treated mice skeletal muscle was reported to diminish along with reduced compensatory hypertrophy induced by synergist ablation (Novak *et al*., 2009).

There are certain enzymes which are known as ‘markers’. The measurement of serum levels of the markers is a diagnostic index as it provides information about tissue or cellular damage which results in release of these enzymes. Alkaline phosphatase and acid phosphatase are such marker enzymes used for diagnostic purposes. 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 carboxy terminus of the protein (Harris, 1989). Alkaline phosphatase is a membrane bound enzyme located in the basal membrane of various tissues and serum. Serum alkaline phosphatase is a dimer whereas membrane-bound forms are probably tetramers (Safadi *et al*., 1991). The elevation in the serum level of alkaline phosphatase is used as diagnostic index for skeletal muscle disorders (Kim *et al*., 1984). Alkaline phosphatase shows an association with a number of muscle diseases including neuromuscular disorders, myopathies or other wasting conditions (Kar and Pearson, 1972).Alkaline phosphatase exhibits three different types of activities from different sources serving as hydrolytic enzyme, phosphotransferase and pyrophosphatase. The role of alkaline phosphatase as hydrolytic enzyme functioning at alkaline pH optima has been elaborated earlier (Chakraborty *et al*., 1980). Acid phosphatase is an equally important enzyme functioning altogether at different pH optima than alkaline phosphatase. Tartrate resistant acid phosphatase (TRACP), also known as purple acid phosphatase, is an iron containing enzyme. This enzyme cleaves terminal phosphate group from substrates and is localized in lysosomes. An experimental muscle wasting induced in rats as a result of adenocarcinoma demonstrated direct relationship between myofibre necrosis and acid phosphatase levels (Asotra *et al.*, 1985).

Objective of the Study

Keeping these above stated facts in mind, a study was designed to find out any adverse effects of diclofenac sodium on gastrocnemius muscle of balb/c mice with respect to its physiological status. The goal of this project was to evaluate the toxicity of diclofenac on mouse gastrocnemius in relation to organ weight to body weight ratio and protein content of the said muscle. Further, the biochemical analyses of specific activity of gastrocnemius muscle and isoenzymes’ profile of alkaline phosphatase in serum (through PAGE) were also

the objectives of this study as both are the marker enzymes for muscular degeneration (Kar and Pearson, 1972; Asotra *et al*., 1985).

Drug and Animals

Adult male swiss albino mice of Balb-c strain, weighing 22–24 g were procured from the Central Research Institute, Kasauli (H.P.), India. All the experimental procedures were conducted strictly under the guidelines of the Institutional Animal Ethics Committee (IAEC approval no.: IAEC/Bio/ 2009/6-HPU). The mice were maintained hygienically in the well ventilated room of animal house of the Department of Biosciences (H.P. University) with 12 h day light and a temperature of 25 ± 2 _C. The animals were kept in wire mesh type steel cages floored with paddy husk having six mice per cage. They were given commercial feed (Hindustan Lever Ltd.) and water *ad libitium*. The mice were acclimatized for 2 weeks prior to the investigation. They were checked daily to observe any sign of illness or morbidity. The drug, diclofenac sodium was purchased from Sigma Aldrich Co., USA and stock solution was made in distilled water. Further; dilutions were done according to the body weight records of the animals. The drug solution was hazy white in appearance. The drug was shaken well over a vortex to ensure homogeneity prior to administration. For each analytical procedure, 36 mice were used which were divided into two broad groups: control group and experimental group. Each group was further divided into three subgroups containing six animals per subgroup: day 10,day 20 and day 30 groups. Animals in the experimental group were administered diclofenac sodium intramuscularly at a dose rate of 10 mg/kg body wt/day for 10, 20 and30 days while that of the control group received normal saline. The drug dose chosen was slightly higher than the recommended therapeutic dose (8 mg/kg) for mice. Body weights of control and treated animals were recorded after each stage of investigation. The animals were sacrificed by cervical dislocation at the end of each stage. Both the gastrocnemii were removed, cleared of fats and weighed.

Biochemical Studies

Homogenate Preparation: A weighed amount of gastrocnemius was homogenized in ice cold distilled water which was centrifuged at 4000 rpm for 20 minutes at 4°C. The supernatant was used for the enzyme assay.

Serum Separation:Samples of Sera were prepared by keeping the blood undisturbed overnight at 8°C. Serum was separated as a distinct layer after coagulation of blood cells.

Protein Estimation: Protein estimation in gastrocnemius and serum was done as per the method of Lowry *et al.* (1951).

Acid phosphatase activity(E.C.3.1.3.2.): Acid phosphatase activity in serum was accomplished as per methods described earlier (Weil and Russel, 1940; Tausky and Shorr, 1953) with some modifications. The enzyme was assayed in supernatant in the incubation mixture containing sodium-β-glcerophosphate, sodium barbitone and acetic acid having pH 4.9. Briefly, supernatant and incubation mixture were incubated in a test tube at 37°C for one hr. The reaction was stopped by adding 10% trichloroacetic acid (TCA). The resultant mixture was centrifuged for 10 minutes at 3000 rpm so as to get a clear supernatant. To estimate phosphate content in the supernatant, ferrous sulphate ammonium molybdate reagent and ANSA and distilled water was added to supernatant. After incubating this mixture at 100°C for five minutes. The optical density was read at 650 nm in spectrophotometer. Total amount of phosphate released was calculated by plotting a standard curve of different known concentrations of KH_2PO_4 against optical density.

Native Page for Alkaline Phosphatase: Isoenzymes of alkaline phosphatase were separated from sera samples. Protein estimation was done according to Lowry *et al.* (1951). Native PAGE was conducted according to the method of Epstein *et al.* (1967)with some modifications. A known amount of protein was loaded. The running buffer used was 0.07 M Tris-borate, (pH 9.5) containing 10 mM NaCl. The samples were run on 7% separating and 5% stacking gel at 50V for 30 minutes and at 70V for 3 hr and 30 minutes.

Finally, the gels were removed and enzyme was localized by activity staining method previously described (Manchenko, 2002) with certain modifications. Gels were incubated in 0.1 M Tris-HCl buffer (pH 9.5) containing

sodium-β-glycerophosphate and 0.2 M $MgCl_2$ for one hour at 37°C s followed by shifting the pre-incubated gel into a reaction mixture (ferrous sulphate ammonium molybdate reagent and amino-naphthol-sulphonic acid) at 60°C for 30 minutes. The gels were photographed and analyzed in gel densitometer immediately as these bands tend to fade. Gels were stored in distilled water.

Statistical Analyses

Values are represented as mean ± SEM. Results were analysed using Student's '*t*' test.

Results

Decrease in body weight after diclofenac administration was noticed in the present investigation. Body weight in the control mice was registered as 24.16 ± 0.105 g after 10 days which did not change in treated animals (23.50 ± 0.619 g). However, body mass seemed to be trimmed down abruptly after 20 days (34.50 ± 0.547 g to 23.66 ± 0.760 g) as well as after 30 days from 26.5 ± 0.912 g to 21.00 ± 1.095 g (Fig 1).

Organ weight to body weight ratio showed decrease in diclofenac treated gastrocnemii (4.318 ± 0.039 mg/g) after 10 days as compared to control (4.852 ± 0.213 mg/g). After 20 days of diclofenac administration, treated animals recorded significantly higher gastrocnemius weight to body weight ratio (7.150 ± 0.213 mg/g) as that of the normal animals (3.974 ± 0.337 mg/g). A significant decline in the organ to body weight ratio was noticed in diclofenac treated gastrocnemius (3.565 ± 0.287 mg/g) after 30 days of administration in comparison to normal (4.576 ± 0.263 mg/g; Table I).

Repeated administration of diclofenac showed increase in protein content of treated gastrocnemius (3.995 ± 0.141) in comparison to control (2.125 ± 0.083 µg / mg of fresh tissue weight) after 10 days. After 20 days of diclofenac therapy slight but insignificant decline in gastrocnemius protein content in treated group (3.951 ± 0.166) was observed as compared to the control animals (4.252 ± 0.183 µg/ mg of fresh tissue weight). Similarly, diclofenac treatment failed to bring any significant difference after 30 days in gastrocnemius muscle protein with mean protein content recorded as 9.367 ± 0.426 and 9.695 ± 0.138 µg / mg of fresh tissue weight for control and treated animals respectively (Fig. 2).

The activity of acid phosphatase was also estimated in the sera after diclofenac therapy where wide-ranging results were recorded in all the stages of investigation. Serum ACPase activity was down-regulated significantly ($P<0.01$) from 0.733 ± 0.067 µM Pi/mg of serum proteins (control) to 0.596 ± 0.052 µM Pi/mg of serum proteins (treated) in diclofenac treated animals for 10 days as compared to non-treated animals. After 20 days of diclofenac administration, trivial rise in the enzyme level was evident in the treated group (0.345 ± 0.024 µM Pi/mg) in comparison to the normal (0.299 ± 0.017 µM Pi/mg; $P>0.05$). After one month drug therapy, ample increase was documented in the serum ACPase activity in drug administered animals from 0.427 ± 0.028 µM Pi/mg to 0.523 ± 0.012 µM Pi/mg comparison to the control group which was significant at $P< 0.05$ (Fig. 3).

Alkaline phosphatase activity in serum and its different isozymes were studied after diclofenac treatment for different durations. Image analysis depicted two major bands as band I towards cathode and band II towards anodal end. A noteworthy increase in band I in 10 days diclofenac administered group (61.23% ± 2.099) in comparison to normal (38.76% ± 2.099) was reported whereas band II decreased significantly from 63.51% ± 0.947 to 36.53% ± 0.991 in densitometric analysis. Diclofenac treatment for 20 days increased band I in treated group (70.06% ± 2.667) from normal (29.74% ± 2.737). Band II also showed a significant rise from 36.38% ± 2.042 to 62.78% ± 2.713 in drug treated serum after 20 days. After 30 days of diclofenac therapy, band I decreased from 62.36% ± 1.523 (control) to 37.68% ± 2.236 in treated group. Similar trend in case of band II was observed as a cut off from 53.16% ± 0.84 to 46.84% ± 0.84 after diclofenac administration for 30 days (Table II & Fig.4a – c).

Discussion

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most popular classes of drugs with anti-inflammatory, analgesic, and antipyretic effects. All these effects are mediated by inhibition of the biosynthesis of prostaglandins. Prostaglandins are also potent modulators of inflammation, as evidenced by the ability of inhibitors of PG synthesis known as NSAIDs like diclofenac to ameliorate pain and inflammation following muscle injury and other types of tissue damage.PGs are known to regulate protein synthesis and degradation in muscle, both *in vivo* and *in vitro* (Vandenburgh, et al., 1990). Diclofenac is a well-known member of acetic acid family of NSAIDs which is traditionally used as an anti-inflammatory agent and pain reliever for the pains associated with arthritis, osteoarthritis and ankylosing spondylitis.

Veterinary use of diclofenac has been reported to be a major cause of the catastrophic collapse of *Gyps* vulture populations in the Indian subcontinent (Oaks *et al.*, 2004). Diclofenac is a widely available drug in the Indian subcontinent, where it is used for the symptomatic treatment and management of inflammation, fever, and/or pain associated with disease or injury in domestic livestock. Athletes have relatively unrestricted access to NSAIDs, as they are readily available over-the-counter preparations and not considered as performance enhancing drugs by the World Anti- Doping Agency (World Anti-Doping Agency, 2008). Athletes have been reported to be the frequent users of NSAIDS, with approximately a quarter of athletes competing at the Sydney 2000 Olympic Games taking NSAIDs (Alaranta *et al*., 2008) in the 3 days before random drug testing. Further, it has been reported earlier by workers that these drugs including diclofenac are taken by the players as a prophylactic measure one day before as a prophylactic measure. The objective of the present study was to investigate toxicological effects of diclofenac on a healthy gastrocnemius muscle in terms of its mass and protein content along with activity of acid and alkaline phosphatase enzyme in serum.

An overall decrease in body mass (31.42 % and 20.75 %) was noticed after 20 and 30 days of diclofenac administration respectively, however, in the beginning of the treatment period viz. after 10 days, no noteworthy change in body mass was observed. These results obtained in the present investigation go parallel with previous reports where significant loss in body weight after diclofenac treatment was documented in rats (Besen *et al.*, 2009). Further, the weight loss in treated animals could be an anorectic effect. It has been shown previously that diclofenac treatment caused decrease in food consumption in rats (Yasmeen *et al.*, 2007). Gastrointestinal toxicity induced by diclofenac as result of cyclooxygenase derived PG inhibition might also contribute to weight changes (Takeuchi *et al.*, 2003). There was recorded increase in body weight after diclofenac and ibuprofen (Koopmans *et al.*, 1987) which could be due to sodium retention because of renal prostaglandin inhibition.

Analysis of organ weight in toxicological study is an important endpoint for identification of potentially harmful effects of chemicals (Bailey *et al.,* 2004). Further, the organ weight to body weight ratio is studied to predict the organ weights. The ratio of gastrocnemius weight to body weight was reported to show a decrease of 11% after 10 days indicating attenuation of protein anabolism thereby leading to lesser muscle mass. About 79.91% increase in gastrocnemius weight/body weight ratio was seen after 20 days of diclofenac treatment. After one month, this ratio declined (22.09%). The increase in muscle mass perhaps is due to suppression of cytokines which in turn caused increase in muscle protein. Pro-inflammatory cytokines increase the synthesis of prostaglandin-E2 (PGE2) in many cells types (Perkins and Kniss, 1997) by inducing the activation of cyclooxygenase 2 (COX2), a rate-limiting enzyme in the synthesis of PGE2 from arachidonic acid. A decrease of PGE2 production has been shown to preserve protein synthesis and to decrease protein degradation in skeletal muscle in highly inflammed rat models (Whitehouse *et al.,* 2001). Furthermore, the blunting of increases in muscle protein synthesis and decreases in proteolysis that occur in the postprandial state in ageing rats and human beings can be restored by the use of NSAIDs (Reiu *et al.*, 2009). Decrease in muscle mass is in agreement with previous reports where decline in $PGF_{2\alpha}$ responsible for muscle protein synthesis (Vandenburgh *et al.*, 1995) was observed after use of acetaminophen and ibuprofen in exercised muscle (Trappe *et al.*, 2001).

Diclofenac administration resulted in innumerable physiological changes in mice

gastrocnemius as well as in sera. Gastrocnemius protein content was found to be increased to 88% after 10 days of diclofenac treatment. Increase in muscle protein can be hypothesized to be due to collagen concentration which has also augmented after diclofenac treatment during the course of study (in press). Collagen forms about 85% of total extracellular matrix. The protein itself is synthesized by fibroblasts and extruded in the extracellular spaces. Preventing proteolysis and promoting protein anabolism in older rat skeletal muscles by Inhibition of low grade inflammation using ibuprofen (Rieu *et al.*, 2009) could be another possible reason for increase in muscle protein. Furthermore, restoration of regulation of protein metabolism in postprandial state was associated with decreased levels of IL_6 and $IL_{1\beta}$. After 20 days of diclofenac therapy, a decrease of 7.07% was noteworthy in this investigation.The transcription factor, NF-kB (nuclear factor- kB) is known to play a role in muscle myogenesis and muscle growth/ atrophy (Guttridge *et al.*, 2000) and it can be inhibited by several NSAIDs (Lille *et al.*, 2001). Further, indomethacin inhibits prostaglandins which may inhibit myogenesis and protein turn over in muscle (Horsley and Pavlath, 2003).An inhibition of protein synthesis in isolated rat skeletal muscle with different cyclooxygenase inhibitors was also reported previously. The increased rate of muscle protein synthesis normally seen 24 h after high-intensity eccentric resistance exercise was attenuated by consumption of ibuprofen and acetaminophen at over-the counter levels in another study (Trappe *et al.*, 2001).

Biochemical analyses of acid phosphatase in serum showed varied trends which showed a significant decrease after 10 days whereas afterwards it increased. The hike was ample and significant after 30 days. Possible explanation for this kind of variation is probably that as diclofenac stabilizes the lysosomal membrane and retains the enzymes during earlier stages of investigation, however after continuous treatment, perhaps there is disintegration of plasma membrane that led to leaching of acid phosphatase from muscle into blood. In pathologically changed muscle fibers, both acid phosphatase activity and the occurrence of lysosomal structures are more marked (Engel and Dale, 1968). In previous work, acid phosphatase was found to increase in the blood sample in collagen induced arthritis in monkeys which was suppressed after treatment with sustained-release diclofenac preparations (Takahashi *et al.*, 2002). Diclofenac stabilizes lysosomal membranes and thus inhibits the release of lysosomal enzymes, which are probably responsible for the tissue destruction occurring in inflammatory rheumatic diseases. Furthermore, lysosomal enzymes association to pathological changes in different tissues like liver, kidney and spleen in adjuvant induced arthritic rats has been documented earlier (Subramanian and Ramalingam, 2000).

Isozymes of alkaline phosphatase in serum were also analyzed by native PAGE and two major bands; Band I towards cathodal end indicative of APase isoenzymes from intestine/ kidney and band II representing isoenzymes of alkaline phosphatase from liver/bone/tissue non-specific APase towards anodal end were separated (Horney *et al.*, 1992). During this study, there was a noteworthy hike in APase isozymes band I suggesting a leakage of the same from kidney/ intestine. However, band II showed an initial decrease which increased during last stage of investigation hinting to have escaped from liver/bone and other tissues. The increase in APase activity in serum is a contributory effect of its leakage from different tissues. Increase in serum APase was also reported by Reddy *et al.* (2006) after diclofenac administration in birds as compared to control. Increase in serum APase was reported by Ebong *et al.* (1998) following diclofenac administration in wistar rats.Advanced age and long-term physical exercise cause changes in the activity of APase in rat muscle (Reznick *et al.*, 1989). The elevation in serum APase is used as a diagnostic index for skeletal and muscle disorders (Kim *et al.*, 1984).Sub-chronic administration of diclofenac led to decrease in body mass after 30 days of drug treatment in the present investigation as well as there was an insignificant decline in gastrocnemius protein content after 20 days. Furthermore, a reduction in gastrocnemius mass was also reported during this study which can possibly be due to COX-2 inhibition leading to IGF-1 (insulin like growth factor-1) expression (Granado *et al.,* 2007) and hence could be responsible for the observed reductions in muscle mass and protein content.

Conclusion

Previous reports and present study hints that longer use of diclofenac interferes with normal physiology of gastrocnemius muscle not only in terms of muscle mass and protein content but also leads to the deviation in activity of the biomarkers enzymes. Although much remains to be learnt about toxicological effects and mechanisms of diclofenac yet further studies are considered necessary with regard to other crucial parameters of muscle physiology which would further open up the perspective for investigating the side effects of diclofenac.

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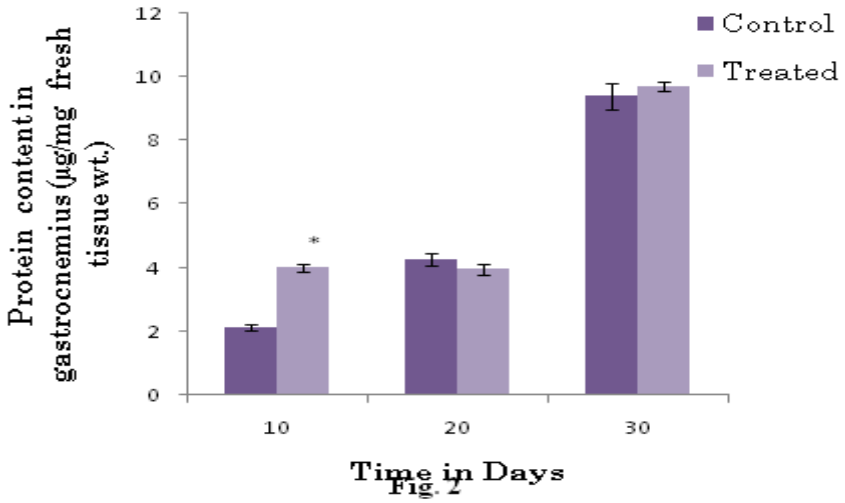

Fig 2: Change in gastrocnemius protein content after repeated administration of diclofenac in µg/ mg of fresh tissue weight. Values are mean ± SEM; *P<0.001, n=6.

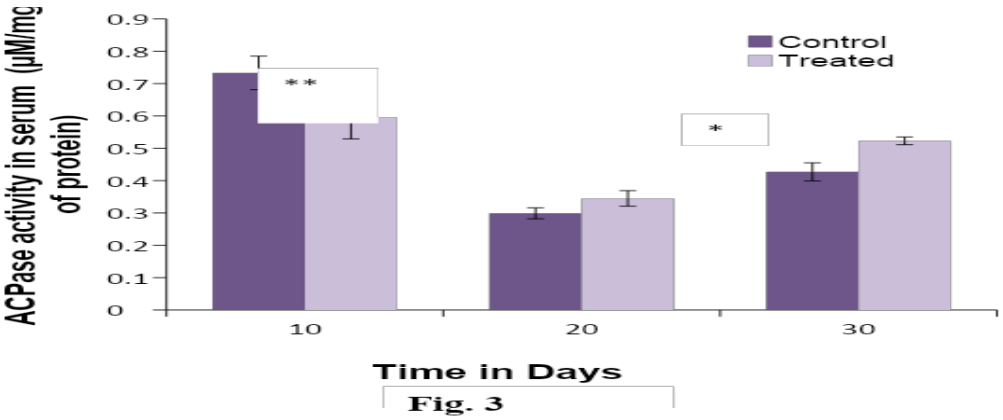

Fig 3: Acid phosphatase activity (µM Pi/ mg protein) in serum of control and diclofenac treated mice. Values are mean ± SEM; **P<0.01, *P<0.05, n=6.

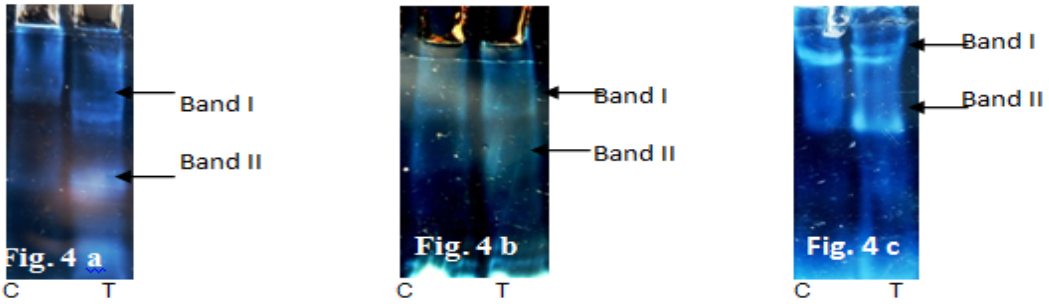

Fig. 4 (a-c)

Fig 4 (a, b and c):7% native PAGE to resolve isoenzymes of alkaline phosphatase in mice serum after 10, 20 and 30 days of diclofenac treatment respectively. C- control and T- diclofenac treated.

Days	10	20	30
Control	4.852 ± 0.213	3.974 ± 0.337	4.576 ± 0.263
Treated	4.318 ± 0.039	7.15 ± 0.213**	3.565 ± 0.287*

Table I: Change in organ weight to body weight ratio (mg/g) after 10, 20 and 30 days of diclofenac treatment in mice gastrocnemius. Values are mean ± SEM; **P<0.02, *P<0.01(n=6).

Groups	Day 10		Day 20		Day 30	
Isoenzymes	Control	Treated	Control	Treated	Control	Treated
Band I	38.76% ± 2.099	61.23% ± 2.099*	29.74% ± 2.737	70.06% ± 2.667***	62.36% ± 1.523	37.68% ± 2.236**
Band II	63.51% ± 0.947	36.53% ±0.991***	36.38% ± 2.042	62.78% ± 2.713*	53.16% ± 0.84	46.84%±0.84^{+}

Table II: % activity of alkaline phosphatase isoenzymes (band I and band II) in mice serum after 10, 20 and 30 days of diclofenac administration. Values are mean ± SEM; ***P<0.001, **P<0.002, *P<0.01, $^{+}$P<0.02 (n= 6).