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Contemporary uses of old folks: the immunomodulatory and toxic potential of fenbufen

Muhammad Furqan Akhtar[1*], Mehr-un-Nisa[1], Ammara Saleem[2*], Irfan Hamid[3], Khalaf F. Alsharif[4], Mohamed M. Abdel-Daim[5,6], Md. Habibur Rahman[7,8], Muddaser Shah[9], Kashif Sohail[10], Zeeshan Javaid[11]

[1]*Riphah Institute of Pharmaceutical Sciences, Riphah International University, Lahore Campus, Lahore, Pakistan*
[2]*Department of Pharmacology, Faculty of Pharmaceutical Sciences, Government College University Faisalabad, Faisalabad, Pakistan*
[3]*Cadson College of Pharmacy, University of the Punjab, Kharian, Pakistan*
[4]*Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia*
[5]*Department of Pharmaceutical Sciences, Pharmacy Program, Batterjee Medical College, P.O. Box 6231 Jeddah 21442, Saudi Arabia*
[6]*Pharmacology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia 41522, Egypt*
[7]*Department of Pharmacy, Southeast University, Banani, Dhaka-1213, Bangladesh*
[8]*Department of Global Medical Science, Yonsei University Wonju College of Medicine, Yonsei University, Wonju 26426, Gangwon-do, South Korea*
[9]*Department of Botany, Abdul Wali Khan University Mardan, Mardan 23200, Pakistan*
[10]*Akson College of Health Sciences, Mirpur University of Science and Technology, Mirpur, AJK, Pakistan*
[11]*Department of Pharmacy, Mirpur University of Science and Technology, Mirpur, AJK, Pakistan*

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ABSTRACT

Fenbfen is used for pain, pyrexia and in the management of osteoarthritis, rheumatoid arthritis and other musculoskeletal disorders. The present research was planned to examine the immunomodulatory activity of fenbufen in different models of cell-mediated immunity (CMI) and humoral immunity (HI). The CMI was evaluated by delayed-type hypersensitivity (DTH) and cyclophosphamide-induced neutropenia assays while HI was appraised by hemagglutination (HA) assay by administering fenbufen at 2, 6 and 10 $mg.kg^{-1}$ and azathioprine 40 $mg.kg^{-1}$ (as standard therapy) to albino mice by intraperitoneal route. The *ex vivo* immunomodulatory action was determined by red blood cell (RBC) membrane stabilization and protein denaturation assays. The results showed that fenbufen treatment had significantly ($p<0.05$-$p<0.001$) reduced white blood cells, hemoglobin content, and red blood cells in the healthy and neutropenic mice. A significant ($p<0.001$) reduction in activities of superoxide dismutase and catalase and glutathione contents, and enhancement of malondialdehyde level were observed in neutropenic mice that were restored by fenbufen treatment. It suppressed DTH reaction after 24, 48 and 72 h post topical application of 2, 4-dinitrofluorobenzene (DNFB). Fenbufen or azathioprine treated groups also showed a significant reduction in the antibody titer against human RBCs induced immune activation in mice as compared to the disease control mice. Fenbufen showed IC_{50} of 14.0, 50.5 and 66.2 $\mu g.ml^{-1}$ whereas, diclofenac sodium showed IC_{50} of 61.0, 126 and 50.5 µg/ml in RBCs membrane stabilization, egg albumin and bovine serum albumin denaturation assays respectively. The current study shows that fenbufen might have potential immunomodulatory activity against CMI and HI. It can be utilized to treat immune system disorders.

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Introduction

Mammals activate a complex biological defense response against noxious injurious agents. This response of the immune system is referred as an acute inflammatory response that is a signaling cascade generated in the body upon experiencing the foreign as well as altered self bodily molecules (1). This response is mediated by four different molecules namely i.e., inducers, sensors, mediators and effectors. The inducers include exogenous and microbial agents and virulent factors. The endogenous agents initiate

* Corresponding author. Email: furqan.pharmacist@gmail.com and amarafurqan786@hotmail.com

the process of acute inflammatory response. The mediator molecules (cytokines) released from the resident cells or present in plasma are the result of vascular and cellular events leading to an inflammatory response (2). The cytokines are produced from macrophages, helper T cells, endothelial cells and mast cells during pathophysiological processes. The inflammatory cytokines for an instance, interleukin (IL)-6, IL-1β, and TNF-α are principally generated from activated macrophages and actively participated in the regulation of inflammatory reactions during cell damage, disease, invasion and inflammation (3). Prostaglandins (PGs) and cyclooxygenase (COX) isoforms, also play a vital role in the inflammatory process (4). The effectors such as neutrophils accumulate at the injury site and are essential in the activation of an acute inflammatory response (5). Non-steroidal anti-inflammatory drugs (NSAIDs) are used for several decades to overcome the inflammatory events in multiple problems of pain such as backache, headache, toothache and arthritis (3).

Fenbufen belongs to NSAIDs and is prodrug metabolized to biphenylacetic acid (BPAA) which strongly and reversibly inhibits COX-I and COX-II involved in prostaglandin synthesis and thus relieves pain and pyrexia. Fenbufen has also been used in the management of osteoarthritis, rheumatoid arthritis and other musculoskeletal disorders. The usual oral daily dose of fenbufen is 900 mg for an adult. It is quickly absorbed in the blood and the highest level is gained within 70 min with 99% protein binding capacity. The plasma half-life of fenbufen and its metabolites is almost 10 to 17 h and is mostly eliminated as urinary conjugates (6). The unique pharmacological profile of fenbufen is responsible for its lower risk to gastrointestinal side effects in patients as compared to other NSAIDs (7). Additionally, the uncontrolled bombardment of reactive moieties in the body is responsible for illnesses, for instance, cancer, diabetes, pancreatitis and rheumatoid arthritis. Indeed, NSAIDs can scavenge the reactive oxygen species (ROS) to act as antioxidants. Oxidative stress occurs due to an increase in oxidative parameters or a decline in antioxidant defense mechanisms. The oxidative indicators like malondialdehyde (MDA) and antioxidant indicators like superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) are principal biomarkers for oxidative organ damage (8, 9). The SOD and CAT are responsible for the dismutation of superoxide anion into hydrogen peroxide (H_2O_2) and transformation of H_2O_2 into H_2O and O_2 respectively (10).

Immunomodulation therapeutically improves the host auto-regulating processes of the defense system mainly by drug usage. The drugs activating the immune reactions stimulate various substances of the immune system i.e. macrophages, granulocytes, complements, certain T-lymphocytes and effectors. These are also used to combat immunosuppression elicited by certain medications or ecological components. Immunosuppression is the reduction in activation of the immune system and regulates the overactivity of the immune system as evident in autoimmune diseases (11). The immune system can be exploited by immunomodulators through blockage of prostaglandins that influenced both the cell and humoral immunities. Therefore, in this work, the immunomodulatory activity of fenbufen was investigated by utilizing immunological experimental models including delayed-type hypersensitivity (DTH), cyclophosphamide-induced neutropenia and hemagglutination (HA) assays. Furthermore, the drug was evaluated for immunomodulatory potential by using *in vitro* assays.

Materials and methods

Rodent housing and husbandry

Swiss albino mice (♂/♀) of weight ranges from 25-30 g were utilized in the current study. Mice were placed in stainless steel cages in the Animal House, RIPS, Lahore. The standard environmental conditions were provided to the animals including room temperature (25 ± 3ºC), moisture (60-70%) and 12 h light-dark conditions. They were served with a chow pellet diet and tap water with generosity. The earlier approval for the study protocol was obtained from Research Ethical Committee, RIPS, Lahore campus (REC/RIPS-LHR/2019-022). Attempts were made to reduce the number of animals throughout the study. The guidelines of the National Institute of Health (NIH publication no. 85-23) were followed for care and working with animals.

Delayed type hypersensitivity assay

To perform the cyclophosphamide-induced neutropenia or DTH assay, the mice were divided into 6 groups (n=6). Control groups (G-1; normal control) and (G-2; diseased control) in cyclophosphamide-induced neutropenia received 0.2 ml of 5% dimethyl sulfoxide (DMSO) only as a vehicle. The G-3, G-4 and G-5 were served with fenbufen (Shandong Xinhua Pharmaceutical, China) dissolved in 5%v/v DMSO at respective doses of 2, 6 and 10 $mg.kg^{-1}$ intraperitoneally (i.p). Group 6 designated as the reference group (G-6) received a 40 $mg.kg^{-1}$ dose of azathioprine (Mass Pharma, Pakistan) (12).

The treatment with fenbufen and azathioprine was started on day 1 and continued for 8 days. All mice were carefully shaved from the abdomen with a razor (Gillette) for DTH assay. The 2, 4-dinitrofluorobenzene (DNFB) was mixed in acetone-olive oil (4:1) solution to make 0.5% suspension for sensitization. This suspension served as an antigen for the assessment of the DTH test. All mice were sensitized with 25 µl of 0.5% suspension of DNFB by skin painting on the mice abdomen at day 0, except the negative control group which was sham sensitized with 0.1 ml acetone only. Before applying DNFB, the skin thickness of mice was measured with the help of the Vernier caliper. On day 8, previously sensitized mice were painted with 20 µl (0.2%w/v) DNFB on inner and outer areas of the left ear, except negative control group which was falsely challenged with 0.2 ml acetone (12). The skin thickness was measured at 24, 48 and 72 h after the DNFB challenge by using Vernier caliper.

Cyclophosphamide induced neutropenia

The drug therapy was injected for successive 10 days as aforementioned for the DTH assay. Cyclophosphamide (Pharmedic laboratory, Pakistan) solution (20 mg/ml) was made in distilled water (DW). On the 10^{th} day of the trial, 200 mg/kg cyclophosphamide was administered by i.p route. Blood samples were collected from the tail of each mouse before and 72 h after injection of cyclophosphamide. Total white blood cells (WBCs) count, RBC count and hemoglobin (Hb) content were determined using a hematology analyzer (Norma Diagnostika, Austria) and compared with the control groups (13). The percentage reduction was determined in experimental groups by the following formula.

$$\% \text{ Reduction} = \frac{\text{No. of cells before cyclophosphamide} - \text{No. of cells after cyclophosphamide}}{\text{No. of cells before cyclophosphamide}} \times 100$$

Biochemical analysis of liver tissue homogenate

After 3 days of cyclophosphamide administration, mice were anesthetized with diethyl ether for blood sampling and humanely killed. The liver of each mouse was removed, weighed and quickly washed away with ice-cold normal saline and mixed with phosphate buffer saline (PBS) pH 7.4 to prepare tissue homogenate (10% w/v) with a tissue homogenizer (Daihan Scientific, North America). The homogenates were centrifuged (Centurion scientific, UK) at 5000 rpm for 10 min at 4°C. The supernatant was used for estimation of SOD, CAT, GSH and MDA levels in the liver tissue homogenates of cyclophosphamide treated mice (14). Protein contents were determined by following Lowery's method using BSA as a standard and absorption were taken at 660 nm(3).

Estimation of SOD

The reaction mixtures were prepared by pouring tissue homogenate (0.1 ml), 2.8 ml of 0.1M PBS (pH 7.4) and pyrogallol solution (0.1 ml) in test tubes. The absorbance was recorded at 325 nm using a UV-Vis spectrophotometer (Shimadzu UV–1601, Japan)(15). The regression line equation was used for the calculation of SOD given as follows (14).

$Y = 0.0097x + 0.1941$

Where, Y was absorbance.

Estimation of GSH

It was estimated by following an earlier procedure (16). Briefly, 1 ml sample was mixed in 1ml (10%) trichloroacetic acid (Merck, USA) and allowed to stand followed by the addition of 4 ml (0.1M) PBS and 0.5 ml 5,5-dithiobis-2-nitrobenzoic acid (DTNB) reagent (Ark Pharm, USA) in the supernatant. The absorbance was observed at 412 nm.

Estimation of CAT

The CAT activity was estimated by the H_2O_2 decomposition method by following earlier standard

procedures (16). Shortly, 1 ml (30 mM) H_2O_2 solution and 1.95 ml (50 mM) PBS (pH 7.0) were admixed with 0.05 ml sample. Absorbance was determined at 240 nm. The activity of CAT was calculated by following the formula (16).

$$\text{The activity of CAT} = \frac{\delta O.D.}{E \times Vol.of\ Sample\ (ml) \times mg\ of\ protein}$$

Where, $\delta O.D.$ is a change in absorbance/ 1 min; E is extinction coefficient (0.071 mmol.cm^{-1}).

Estimation of MDA

The lipid peroxidation was guesstimated from MDA concentration. To estimate the amount of MDA, the tissue homogenate (1 ml) and thiobarbituric acid reagent (3 ml) were mixed, shaken and incubated on an ice bath for 15 min. After cooling, the resultant solutions were centrifuged for 10 min at 3500 rpm. The upper layer was decanted to measure absorbance at 532 nm. The MDA concentration was estimated by the following formula (17).

$$\text{MDA level} = \frac{Abs532 \times 100 \times VT}{(1.56 \times 10^5)\, WT \times Vu}$$

Where, Abs_{532} was absorbance, 1.56×10^5 was molar extinction of co-efficient, *VT* was the total volume of the mixture (4ml), *WT* was the weight of detached liver, *Vu* was aliquot volume.

Hemagglutination assay

The RBC was separated from a fresh blood sample of a human who was not taking any kind of NSAIDs for the last 2 weeks. For HA, blood (5 ml) was collected and centrifuged at 5000 rpm for 10 min at 4°C. The RBC pellets were isolated and washed thrice with pyrogen-free PBS (5 ml) (18). The concentration of RBCs was adjusted to 0.5×10^9 cells/ml and volume was made up to 15 ml with PBS for immunization (19).

To experiment, all mice groups including disease control, experimental and reference groups were immunized with 0.5×10^9 cells/ml of RBCs at day 0. For HA assay, a study designed was the same as mentioned above except there were 5 groups of mice (n=6). Therapy was injected i.p. for seven consecutive days. On the 7th day, blood was withdrawn by cardiac puncture from the heart and was allowed to clot in plain tubes (19). Serum was separated from clotted blood in a tube which was centrifuged for 5 min at 4ºC and 2000 rpm. Supernatants containing the serum of mice blood were kept refrigerated till HA was carried out (20).

The antibody titer against 10% suspension of RBCs was determined using the HA titer method in round bottom 96 well plates. A 25 µl of PBS was poured into all wells of the plate. A 25 µl of serum was then poured into the first column of a microtiter plate. Then 25 µl of liquid was taken from the first well and added to the next well. A two-fold serial dilution process was continued to the 11th well. In the end, 25 µl from the 11th well was discarded. The last well was denoted as control where no serum was placed (20). Then 25 µl of 10% RBCs prepared in PBS were pooled as antigens to the serum containing antibodies in a microtiter plate followed by incubation at 37ºC for 1 h. Values of HA titer were determined by visually examining the greatest dilution of serum showing HA.

RBC membrane stabilization assay

The blood suspension was prepared by collecting the blood sample in a test tube from a healthy human who was not taking any NSAIDs for the previous 2 weeks. Saline solutions of two different concentrations were prepared (Isosaline NaCl w/v 0.85% and hyposaline NaCl 0.25% w/v). The blood was centrifuged for 10 min at 3000 rpm and washed thrice with enough volume of isosaline. The volume of blood was then reconstituted as 10% v/v suspension with normal saline. The assay mixture (4–5 ml) was comprised of 2 ml of hyposaline (0.25% w/v NaCl), 1 ml of PBS (0.15 M, pH 7.4), 0.5 ml of 10% RBC suspension and 1 ml of different concentrations i.e. 32, 64, 128, 256, 512 µg/ml of fenbufen. Diclofenac sodium (DS) at the same concentration range was evaluated as a standard drug (21). In the control solution, 1 ml of isosaline was used in place of the test solution. The assay mixtures were placed in an incubator (Shanghai MedEco, China) for 30 min at 37°C and centrifuged at 3000 rpm for 20 min. The absorbance was taken at 560 nm after cooling(21). The percentage of RBC membrane stabilization was calculated by employing the following formula.

$$\%\ \text{of membrane stabilization} = \frac{Abs.\ of\ Control - Abs.\ of\ Test}{Abs.of\ Control} \times 100$$

Egg albumin denaturation assay

A 5 ml of reaction mixture comprised of 0.2 ml of eggs albumin (from hens' egg), 2 ml of different concentrations of 32, 64, 128, 256, 512 µg/ml of fenbufen and 2.8 ml of PBS (pH 6.4). The DW in place of the test compound was used as negative control while DS (Siza International, Pakistan) as standard. Then the solutions were placed in the incubator at 37°C for 15 min and warmed at 70ºC for 5 min. The absorbance was taken at 660 nm after cooling. The test was performed in triplicate and percentage inhibition of protein denaturation was calculated (21).

Protein denaturation assay

The reaction solution (0.5 ml) contained of 0.45 ml of 5% BSA solution and 0.05 ml of test compound at 32-512 µg/ml. The pH was adjusted to 6.3 using 1N HCl. For control, 0.05 ml DW was used. The samples were placed in an incubator for 20 min at 37°C and then heated to 51ºC for 20 min. The turbidity was determined at 660 nm after cooling. The mean of three tests was used to determine the percentage inhibition of BSA denaturation (21).

Statistical analysis

All the data were presented as Mean ± SEM. For *in vivo* data, one-way Analysis of Variance (ANOVA) was applied followed by Bonferroni's multiple comparison test however, for *in vitro* tests, two-way ANOVA followed by Bonferroni's multiple comparison test was applied. The level of significance was considered at *p < 0.05, **p < 0.01 and ***p < 0.001. The data were analyzed by GraphPad Prism 6 version (USA).

Results and discussion

Effect of fenbufen on the DTH

The effect of fenbufen on DTH was determined by measuring the changes in thicknesses of the ear in the mice of all groups at 24, 48 and 72 h after applying DNFB. After 24 h, a significant increase in the thickness of ear in disease control group (G-2; 0.52 ± 0.009 mm), 2 mg.kg^{-1} fenbufen treated group (G-3; 0.48 ± 0.02 mm), and reference (Azathioprine; 40 mg.kg^{-1}) treated group (G-6; 0.47 ± 0.019 mm) was recorded in comparison to normal control group (G-1; 0.28 ± 0.01 mm) whereas, group of mice treated with fenbufen 6 mg.kg^{-1} (0.40 ± 0.028 mm) and 10 mg.kg^{-1} (0.37 ± 0.014 mm) inhibited the increase in ear thickness significantly in comparison to disease control group as shown in Figure 1A. Similarly, the thickness of the ear in the animals was recorded 48 h after applying DNFB. The data showed a significant increase in ear thickness in groups (G-2; 0.40 ± 0.031 mm), (G-3; 0.39 ± 0.009 mm) and (G-6; 0.38 ± 0.01 mm) in contrary to normal control group (G-1; 0.25 ± 0.025 mm), while G-4 (0.347 ± 0.026 mm) and G-5 (0.30 ± 0.02 mm) exhibited a significant reduction in the ear thickness in comparison to disease control mice (G-2; 0.40 ± 0.031 mm) as shown in Figure 1B.

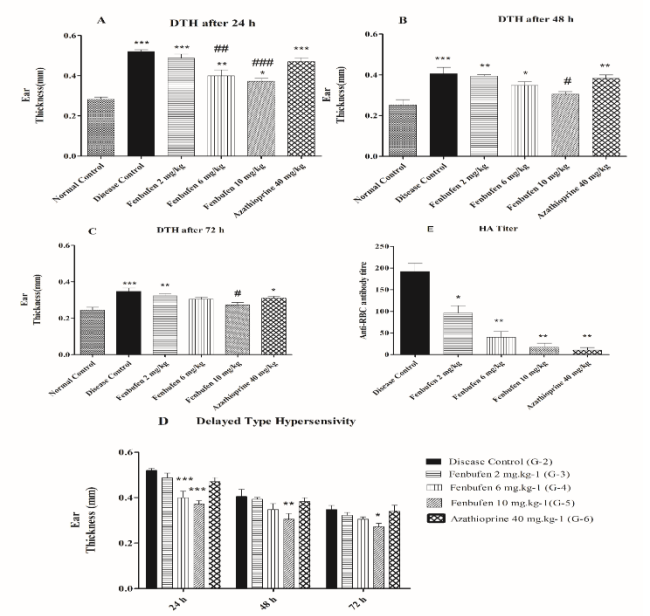

Figure 1. Effect of fenbufen on delayed-type hypersensitivity reaction (DTH) and hemagglutination assay (HA) (A) after 24 h (B) after 48 h and (C) after 72 h (D) comparison of effect at different time interval (E) Effect on HA titer.

Furthermore, the ear thickness of mice in all groups was measured 72 h after DNFB. The G-2 (0.34 ± 0.018 mm), G-3 (0.30 ± 0.03 mm) and G-6 (0.34 ± 0.27 mm) showed a significant increase in thickness of ear in comparison to G-1 (0.24 ± 0.01 mm). Moreover, G-4 (0.305 ± 0.010 mm) and G-5 (0.27 ± 0.01 mm) still caused a significant reduction in the thickness of the ear in comparison to G-2 (0.34 ± 0.018 mm) as shown in Figure 1C. The effect of fenbufen therapy on ear edema at different intervals was summarized in Figure1D.

The comparison of treatment groups with normal control group (G-1) were denoted by *, ** and ***, which represented $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively whereas, the comparison with disease control group (G-2) was indicated by #, ## and ###, which represented $p<0.05$, $p< 0.01$ and $p < 0.001$ respectively.

Effect on hemagglutination assay (HA)

The anti-RBCs antibody titer was significantly decreased in experimental groups G-3 (96 ± 16.5), G-4 (40 ± 13.7) and G-5 (17 ± 9.5) treated with fenbufen 2 $mg.kg^{-1}$, 6 $mg.kg^{-1}$ and 10 $mg.kg^{-1}$ respectively as compared to disease control group G-2 (192 ± 19.0). Similarly, group G-6 (10 ± 6.0) treated with azathioprine also significantly attenuated the antibody titer as depicted in Figure 1E.

Cyclophosphamide induced neutropenia

The effect of fenbufen on the number of WBC, RBC and Hb levels was determined before and 72 h after administration of cyclophosphamide (200 $mg.kg^{-1}$) in all groups of mice pretreated with different doses of fenbufen or azathioprine for 10 days.

A substantial decrease in WBC count was noticed before cyclophosphamide administration in G-3 (8.15 ± 0.48 × $10^3/mm^3$), G-4 (7.27 ± 0.34 × $10^3/mm^3$), G-5 (6.75 ± 0.17 × $10^3/mm^3$) and G-6 (4.82 ± 0.23 × $10^3/mm^3$) in comparison to normal control group G-1 (10.92 ± 0.72 × $10^3/mm^3$). Correspondingly, the numbers of WBCs were counted after 72 h of post-administration, a noteworthy reduction in WBCs counts in G-3 (3.62 ± 0.18 × $10^3/mm^3$), G-4 (3.07 ± 0.08 × $10^3/mm^3$), G-5 (2.62 ± 0.17 × $10^3/mm^3$) and G-6 (1.60 ± 0.17 × $10^3/mm^3$) were noticed as compared to disease control group G-2 (4.97 ± 0.34 × $10^3/mm^3$) as shown in Table 1. The RBCs counts, prior to administration of the neutropenic agent, were notably reduced in G-5 (7.96 ± 0.28 × $10^6/mm^3$) and G-6 (7.22 ± 0.11 × $10^6/mm^3$) in comparison to normal control group G-1 (9.18 ± 0.58 × $10^6/mm^3$) whereas, RBCs count was insignificant in G-3 (8.88 ± 0.02 × $10^6/mm^3$) and G-4 (8.40 ± 0.01 × $10^6/mm^3$) as compared to G-1. Moreover, RBCs count after 72 h of cyclophosphamide administration was considerably reduced in G-6 (6.34 ± 0.39 × $10^6/mm^3$) and non-significantly fall in fenbufen treated groups in contrast to the disease control group (8.31 ± 0.34 × $10^6/mm^3$) as shown in Figure 2.

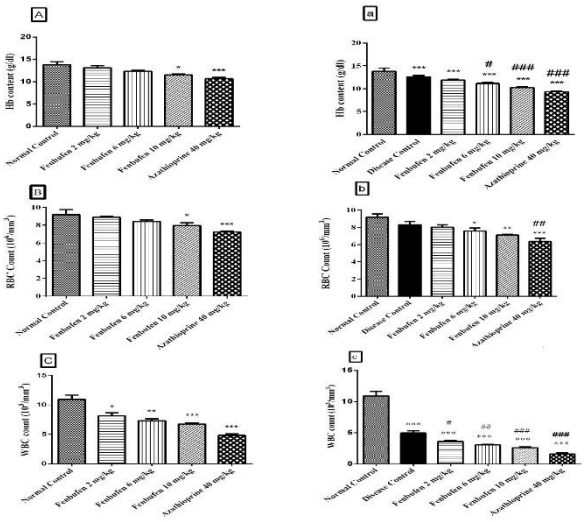

Figure 2. Effect of fenbufen on WBC, RBC and hemoglobin in mice before and after administering cyclophosphamide. Values as Mean ± SEM, n=6. One-way ANOVA followed by Tukey's test. *p<0.05,**p<0.01,***p<0.001. Capital alphabets exhibited before and lower alphabets indicated after cyclophosphamide administration.

Furthermore, the Hb content before cyclophosphamide administration in G-3 (13.10 ± 0.47 g/dl) and G-4 (12.32 ± 0.19 g/dl) was insignificant whereas in G-5 (11.50 ± 0.20 g/dl) and G-6 (10.67 ± 0.31 g/dl) was significantly reduced in comparison to normal control group G-1 (13.80 ± 0.67 g/dl). Similarly, the Hb content after 72 h of cyclophosphamide administration was significantly reduced in G-4 (11.10 ± 0.32 g/dl), G-5 (10.22 ± 0.16 g/dl) and G-6 (9.32 ± 0.21 g/dl) as compared to disease control group G-2 (12.62 ± 0.27 g/dl) as shown in Figure 2. The percentage reduction of WBC, RBCs and Hb after administering cyclophosphamide was the highest with fenbufen 10 mg/kg and azathioprine as presented in Table 1.

Analysis of oxidative stress biomarkers in Cyclophosphamide induced neutropenia

The SOD, CAT and GSH levels were significantly reduced in diseased control group ($p<0.001$) in contrast to normal mice group. However, the experimental groups treated with fenbufen 10 $mg.kg^{-1}$

(8.02 ± 0.15 µg.mg^{-1}) and reference group treated with azathioprine 40 mg.kg^{-1} (7.27 ± 0.37 µg.mg^{-1}) showed an elevated level ($p<0.05$) of SOD in comparison to the disease control group (5.78 ± 0.15 µg.mg^{-1}) while the reduced CAT level was slightly ($P < 0.05$) restored in G-5 (1.17 ± 0.04 µg.mg^{-1}) while, G-6 (0.55 ± 0.07 µg.mg^{-1}) showed insignificant effect in comparison to G-2 (0.68 ± 0.07 µg.mg^{-1}). Likewise, the reduced level of GSH was significantly reversed with fenbufen 6 mg.kg^{-1} (0.77 ± 0.05 µg.mg^{-1}) and 10 mg.kg^{-1}(0.86 ± 0.04 µg.mg^{-1}) treated groups. The MDA level was substantially ($p<0.001$) augmented in G-2 (211.0 ± 4.35 nmol.mg^{-1}) and G-6 (215.6 ± 2.60 nmol.mg^{-1}) in contrast with the normal mice (142.3 ± 3.48 nmol.mg^{-1}). The increased MDA concentration was expressively ($P < 0.001$) restored in fenbufen 6 mg.kg^{-1} (175.3 ± 3.93 nmol.mg^{-1}) and 10 mg.kg^{-1} (155.6 ± 4.80 nmol.mg^{-1}) treated mice (Table 2).

Table 1. Effect of fenbufen on WBC, RBC and hemoglobin after administering cyclophosphamide expressed as a percentage reduction

Treatments	Hemoglobin (g/dl)	White blood cells (10^3/mm^3)	Red blood cells (10^6/mm^3)
Disease Control (G-2)	8.6	54.4	9.4
Fenbufen 2 mg.kg^{-1} (G-3	8.3	55.5	9.8
Fenbufen 6 mg.kg^{-1} (G-4)	10.7	57.7	10
Fenbufen 10 mg.kg^{-1} (G-5)	11.3	61.1	10.8
Azathioprine40 mg.kg^{-1}(G-6)	12.2	66.8	12

Table 2. Effect of fenbufen on oxidative stress biomarkers in Cyclophosphamide induced neutropenia; Treatment groups (A), Superoxide dismutase (µg/mg of protein) (B), Catalase (µg/mg of protein) (C), Malondialdehyde (nmol/mg of protein) (D), GSH (µg/mg of protein) (E)

A	B	C	D	E
Normal Control G-1	9.07 ± 0.31	1.40 ± 0.05	142.3 ± 3.48	0.89 ± 0.05
Disease Control G-2	5.78 ± 0.15***	0.68 ± 0.07***	211 ± 4.35***	0.52 ± 0.02***
Fenbufen 2 mg.kg^{-1} G-3	6.37 ± 0.16***	0.71 ± 0.07***	194.6 ± 4.6***	0.69 ± 0.01
Fenbufen 6 mg.kg^{-1} G-4	6.95 ± 0.14***	0.90 ± 0.02**	175.3 ± 3.93**	0.77 ± 0.05
Fenbufen 10 mg.kg^{-1} G-5	8.02 ± 0.15	1.17 ± 0.04	155.6 ± 4.80	0.86 ± 0.04
Azathioprine 40 mg.kg^{-1}G-7	7.27 ± 0.37**	0.55 ± 0.07***	215.6 ± 2.6***	0.62 ± 0.04**

All values were demonstrated as mean ± SEM, n=6. One-way ANOVA followed by Bonferroni's multiple comparison test. *, ** and *** showed significantly different group as compared to normal control at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

RBC membrane stabilization

Fenbufen stabilized the RBC membrane dose-dependently and the highest protection (57.5 ± 0.55) was revealed by the highest concentration (512 µg.ml^{-1}) that was notably ($p<0.001$) varied from (DS (72.4 ± 0.45) at a respective concentration as mentioned in Table 3. The IC50 value of fenbufen and DS were 14 µg.ml^{-1} and 61 µg.ml^{-1} respectively.

Table 3. Effect of fenbufen on RBC membrane stabilization and protein denaturation

Concentration (µg/ml)	RBC stabilization assay		Egg Albumin denaturation		Serum denaturation	
	Fenbufen (% protection)	Diclofenac sodium (% protection)	Fenbufen (% inhibition)	Diclofenac sodium (% inhibition)	Fenbufen (% inhibition)	Diclofenac sodium (% inhibition)
32	40.8 ± 0.70***	56.4 ± 0.45	8.75 ± 0.45***	25.9 ± 0.55	8.10 ± 0.90***	30.2 ± 1.65
64	47.7 ± 1.50***	60.5 ± 2.55	13.6 ± 0.59***	29.3 ± 0.40	13.6 ± 1.30***	37.2 ± 1.20
128	52.1 ± 0.20***	66.0 ± 1.00	20.5 ± 0.20***	32.4 ± 0.45	18.2 ± 1.20***	42.5 ± 0.50
256	54.5 ± 0.75***	69.4 ± 0.45	25.8 ± 0.15***	35.9 ± 0.10	24.7 ± 1.70***	45.8 ± 0.15
512	57.5 ± 0.55***	72.4 ± 0.45	33.6 ± 0.90***	40.5 ± 0.50	34.4 ± 0.60***	53.2 ± 0.60

Inhibition of egg albumin denaturation

The fenbufen and DS inhibited the egg albumin denaturation in a dependent manner as expressed in Table 3. The maximum % inhibition of protein denaturation was revealed by fenbufen (33.6 ± 0.90%) that was remarkably ($p<0.001$) different from the DS (40.5 ± 0.50%) at concentration 512 $\mu g.ml^{-1}$. The IC_{50} value of fenbufen and DS was recorded as 50.5 and 126 $\mu g.ml^{-1}$ respectively.

Inhibition of BSA denaturation

It was found that the inhibitory activity of fenbufen against protein denaturation was increased with increasing concentration and significantly differed from DS. The fenbufen showed 34.4% inhibition of protein denaturation that was substantially ($p<0.001$) varied from DS (53.2 ± 0.60%) at 512 $\mu g.ml^{-1}$. This indicated that diclofenac sodium was more potently inhibited the protein denaturation as compared to fenbufen. The IC_{50} of fenbufen was 66.2 $\mu g.ml^{-1}$ and DS was 50.5 $\mu g.ml^{-1}$ (Table 3).

Results were presented as mean ± SEM, n=3. One-way ANOVA followed by Bonferroni's multiple comparison test. *** showed statistically different as compared to the diclofenac sodium at the same concentrations at $P < 0.001$ respectively.

Conclusion

The immunomodulatory effect of fenbufen and azathioprine on CMI was confirmed by attenuation of DTH induced by DNFB, and the WBCs, RBCs and Hb contents in a mouse model of cyclophosphamide-induced neutropenia. The immunomodulatory activity of fenbufen and azathioprine on humoral immunity was also evident by HA assay in which different doses significantly reduced the antibody titer. Fenbufen stabilized the RBC membrane and inhibited the protein denaturation in a dose-dependent manner. It is an inference from the present findings that fenbufen might exert an immunomodulatory effect by influencing both the cell-mediated and humoral immunity and thus fenbufen can be employed for treating autoimmune disorders. Further immunomodulatory mechanisms of fenbufen must be sorted out in the future. However, the immunomodulatory use of fenbufen may be ascribed with concomitant monitoring of liver function tests.

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Interest conflict

The authors declare no conflict of interest.

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