IJPSR (2019), Volume 10, Issue 8 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 22 November 2018; received in revised form, 03 March 2019; accepted, 08 March 2019; published 01 August 2019

INFLUENCE OF PIOGLITAZONE ON IMMUNOMODULATORY ACTIVITY OF AZATHIOPRINE IN RODENT MODELS

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Keywords:

Azathioprine, Pioglitazone, Immunomodulation, Antioxidant, Synergistic interaction

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ABSTRACT: The present study was designed to investigate the antioxidant and immunomodulatory activity of Azathioprine-Pioglitazone combination therapy to check whether it has added on benefit over monotherapy with Azathioprine or Pioglitazone in rodent models. The test drugs in combination showed better inhibition of free radicals in both H_2O_2 radical scavenging assay and nitric oxide scavenging assay than individual counterparts revealing its potential antioxidant activity. The combination showed its significant effect on both cell-mediated and humoral immunity to suppress stimulated immune responses in delayed-type hypersensitivity test, carbon clearance test, and nitro blue tetrazolium test. The study proved that Pioglitazone enhances the antioxidant and immunomodulatory activity of Azathioprine indicating the possible synergistic interaction between them.

INTRODUCTION: There is a generation of reactive oxygen and nitrogen species (ROS and RNS) in our body during a normal biochemical reaction. This gets augmented during pathophysio-logical conditions resulting in 'oxidative stress.' During this phenomenon, there is an alteration of cellular constituents leading to various diseased states. This may be effectively neutralized by antioxidants through enhancement of the cellular defenses. Reactive species are also generated during 'phagocytosis' which is the important manifestation of innate immunity. The migration of leukocytes at an inflammatory site results in phagocytosis by both macrophages and neutrophils with the release of enzymes and cytokines.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(8).3843-49

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(8).3843-49

These ROS destroy microorganisms or other foreign matter by acting either of the two oxygen-dependent mechanisms. H_2O_2 and NO are the major free radicals which stimulate the immune system. Several cytokines are produced during chronic inflammatory disorders such as rheumatoid arthritis, asthma, psoriasis, and inflammatory bowel disease. These can recruit activated inflammatory and immune cells to the involved site which amplifies the inflammatory process. The immune system is highly vulnerable to free radical-induced oxidative stress.

The cellular and humoral components of the immune system are particularly sensitive to increased levels of ROS, which may cause premature immunosenescence. It is essential to enhance the immunity of the body system by counteracting this oxidative stress. Immuno-modulatory drugs alter the response of the immune system either by increasing (immunostimulators) or decreasing (immunosuppressive) the production of serum antibodies [1].

Immunostimulators are used to enhance the immune response against infectious diseases, tumors, primary or secondary immunodeficiency, and alterations in antibody transfer, among others [2]. Immunosuppressive drugs are prescribed to reduce the immune response against transplanted organs and to treat autoimmune diseases such as rheumatoid arthritis, lupus, or allergies [3, 4]. However, lack of optimum efficacy and potential safety concerns limit their long-term use as monotherapy in immune modulation, and therefore, combination therapy is recommended.

Azathioprine is the most widely used drug in immune-related disorders. It shows its effect by following mechanisms i) inhibition of clonal proliferation during the induction phase of the immune response, ii) suppression of cell-mediated and antibody-mediated immune reactions [5]. Pioglitazone, a ligand for peroxisome proliferator-gamma (PPAR-γ) acts as an oral anti-diabetic agent. It inhibits the production of inflammatory cytokines and matrix metalloproteinases [6] and causes suppression of pro-inflammatory gene expression [7]. Further, it causes induction of apoptosis in macrophages and T-lymphocytes [8, 9].

Since both Azathioprine and Pioglitazone exert their effects through different mechanisms; it was assumed that Azathioprine and Pioglitazone combination therapy might have synergistic efficacy in immune-compromised patients. In the above context, the present study was conducted to explore add on the benefit of Azathioprine-Pioglitazone combination therapy over mono-therapy with Azathioprine or Pioglitazone in animal models.

MATERIALS AND METHODS:

Materials: Azathioprine was purchased from Rakshit Drugs Private Limited, Hyderabad. Pioglitazone was procured from Hetero Drugs Limited, Hyderabad. Chemicals and reagents used were of analytical grade.

Animals: Wistar albino rats (Approx. 150 to 200 g) and Swiss albino mice (20 to 25 g) were procured from Albino research, Hyderabad. The care and maintenance of the animals were carried out as per the approved guidelines of the Committee for Control and Supervision of Experiments on Animals (Registration number: 1175/PO/Re/S/ 08/CPCSEA).

Methods:

***In-vitro* Anti-Oxidant Assays:**

Hydrogen Peroxide Radical Scavenging Assay: A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Drugs at the concentration of 10 mg/10µL were added to 0.6 ml of H_2O_2 solution, the total volume was made up to 3 ml with phosphate buffer. The absorbance of the reaction mixture was recorded at 230 nm. The blank solution contained phosphate buffer without H_2O_2. [10]

Hydrogen peroxide activity (%) = Abs control - Abs sample/ Abs control × 100

Nitric Oxide Scavenging Activity: Nitric oxide scavenging activity can be estimated using Griessillosovy reaction. Nitric oxide scavenging activity was measured using a spectrophotometer. The drug was prepared in DMSO, was added to different test tubes in varying concentrations (20, 40, 60, 80, 100 µg/mL). Sodium nitroprusside (5 mM) in phosphate buffer was added to each test tube to make volume up to 1.5 ml. Solutions were incubated at 25 ºC for 30 min. After that, 1.5 ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid) was added to each test tube. The absorbance was measured, immediately, at 546 nm and the percentage of scavenging activity was measured concerning ascorbic acid as standard [11].

Nitric oxide Scavenging activity (%) =Abs control - Abs sample/ Abs control × 100

***In-vivo* Immunomodulatory Activity:**

Delayed Hypersensitivity Reaction:

Group I: Normal control.

Group II: Disease control (0.2 ml SRBC administered on day 0, i.p + 0.05 ml administered sub planarly on day 7th.

Group III: SRBC induced animals treated with Azathioprine (4.5 mg/kg, bd.wt, p.o) for 4 days.

Group IV: SRBC induced animals treated with Pioglitazone group (1.35 mg/kg, bd.wt, p.o) for 4 days.

Group V: SRBC induced animals treated with Azathioprine and Pioglitazone (combination) in the doses mentioned above for 4 days.

The Albino rats of body wt. 200-250 g were selected and injected intraperitoneally with a suspension containing 1×10^6 SRBC in 0.2 ml of phosphate buffered saline (PBS) on day zero sensitization. Seven days later (day +7), the same animals were injected subcutaneously with 1×10^6 SRBC suspended in 50 µl of PBS into the right hind footpad for elicitation of the DTH reaction. The left hind footpad will be injected with 50 µl of PBS as a control. Footpad swelling was measured on day +8 with a caliper. The difference between the means of right and left hind footpad thickness gives a degree of footpad swelling which was used for group comparisons. To establish the effect of co-treatment on this immune response, a daily dose of the combination treatment was administered at the induction phase (+4 to +7 days). Simultaneously, another group of animals (controls) was inoculated in the same condition with 0.1 ml of PBS. Each experimental group contains 6 animals [12].

Carbon Clearance Assay:

Group I: Normal control.

Group II: Disease control (0.1 ml Indian ink administered on day 6).

Group III: Azathioprine (4.5 mg/kg, bd.wt, p.o) treated for 5 days.

Group IV: Pioglitazone (1.35 mg/kg, bd.wt, p.o) treated for 5 days.

Group V: Azathioprine and Pioglitazone (combination) in doses mentioned above for 5 days.

Mice (20-25 g) were divided into five groups of six mice each. Drugs were administered in various groups for five days. On day six, all the groups were given 0.1 ml of carbon ink suspension through the tail vein. Blood was collected from the retro-orbital plexus at 0 and 30 min immediately after the injection of carbon suspension. Blood was lysed with 2 ml of 0.1% sodium carbonate, and the absorbance was measured at 675 nm [13, 14]. The rate of carbon clearance, termed as the phagocytic index, was be calculated by using the following equation [15].

$$\text{Phagocytic Index} = (\text{In } OD_1 - \text{In } OD_2) / t_2 - t_1$$

OD_1: Optical density at a time (t_1) 0 min after blood collection, OD_2: Optical density at a time (t_2) 30 min after blood collection.

Nitro Blue Tetrazolium (NBT) Reduction Test: Fresh sheep red blood cells (SRBCs) collected from the local slaughterhouse were used to immunize the animals. SRBCs were stored in a sterile Alsever's solution and washed thrice with pyrogen-free normal saline. Animals were immunized by intraperitoneal injection of 1 ml of 20% SRBCs suspension. The day of immunization was considered as day '0'. On the 5th day, blood samples were collected from each animal through the retro-orbital puncture to perform NBT reduction test. On the 5th day after challenge, blood was withdrawn from retro-orbital plexus of each animal. Smears of collected blood samples were made on glass slides. They were treated with 0.4 ml of NBT medium and incubated at 37 °C for 30 min. They were then washed with cold saline water after, the slides were stained with safranin solution. The slides were examined under the microscope. The percentage of cells with reduced NBT dye was determined [16].

Statistical Analysis: Values were expressed as mean ± SEM, n=6. All the groups were compared with normal control by using ANOVA followed by Dunnett's test. Significance was expressed at **P< 0.01 and *P<0.05.

RESULTS:

***In-vitro* Anti-Oxidant Assays:**

Hydrogen Peroxide Radical Scavenging Assay: The Hydrogen peroxide scavenging activity was recorded in terms of percentage inhibition. It was observed that Azathioprine & Pioglitazone showed dose-dependent hydrogen peroxide scavenging activity. Pioglitazone produced better anti-oxidant potential than Azathioprine. The combination of Azathioprine and Pioglitazone exhibited prominent inhibition of hydrogen peroxide radicals which indicate the synergistic effect of the test drugs. The effect of the combination was found to be comparable to that of standard ascorbic acid **Fig. 1**.

Nitric Oxide Scavenging Activity: The nitric oxide scavenging activity was recorded in terms of percentage inhibition. It was observed that

Azathioprine and Pioglitazone had shown a dose-dependent inhibition of the free radicals. The antioxidant property of Pioglitazone was found to be better than that of Azathioprine. The percentage inhibition was increased with increase in the concentration of the drug.

The combination of Azathioprine and Pioglitazone exhibited significant inhibition of nitric oxide radicals which indicates a synergistic effect of test drugs. The impact of the combination was comparable to that of standard ascorbic acid **Fig. 2**.

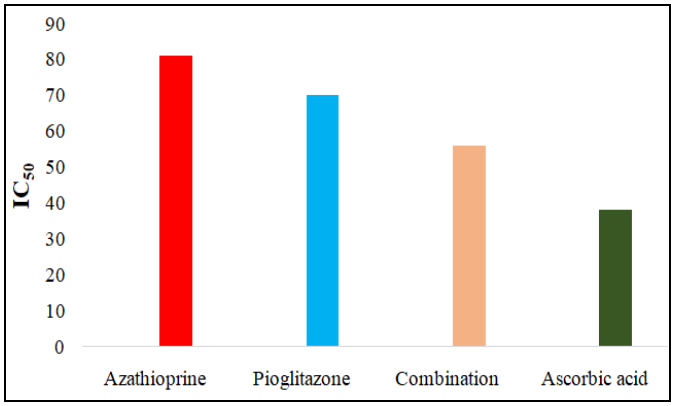

FIG. 1: HYDROGEN PEROXIDE RADICAL SCAVENGING ASSAY OF AZATHIOPRINE, PIOGLITAZONE AND THEIR COMBINATION

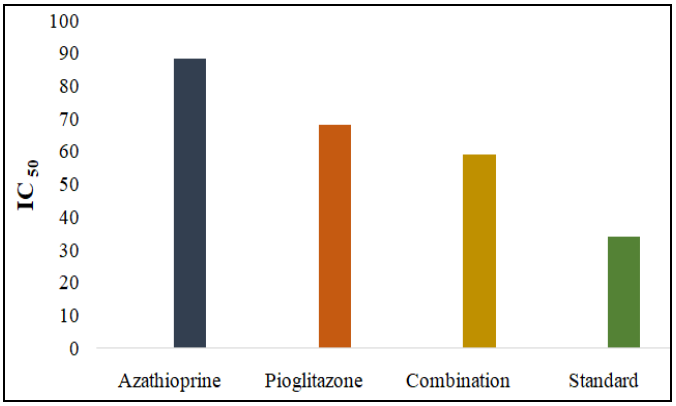

FIG. 2: NITRIC OXIDE SCAVENGING ACTIVITY OF AZATHIOPRINE, PIOGLITAZONE AND THEIR COMBINATION

***In-vivo* Immunomodulatory Activity:**

Delayed Hypersensitivity Reaction: The DTH response against SRBC revealed a decrease in footpad thickness due to treatment with Azathioprine, Pioglitazone, and their combination. Azathioprine showed better immunomodulatory activity compared to Pioglitazone indicated by less paw thickness whereas the combination of two drugs produced a better effect compared to the effect of individual drugs, indicating synergistic effect **Fig. 3**.

Carbon Clearance Assay: Administration of Azathioprine and Pioglitazone in the dose of 4.5 & 1.35mg/kg respectively, caused increased clearance of carbon particles from blood which was evident by a significant increase in the phagocytic index ($P<0.01$). Azathioprine produced a better effect when compared to Pioglitazone, and the combination of two drugs exhibited a more significant effect than that of individual drugs ($P<0.01$) indicating synergistic interaction between selected drugs **Fig. 4**.

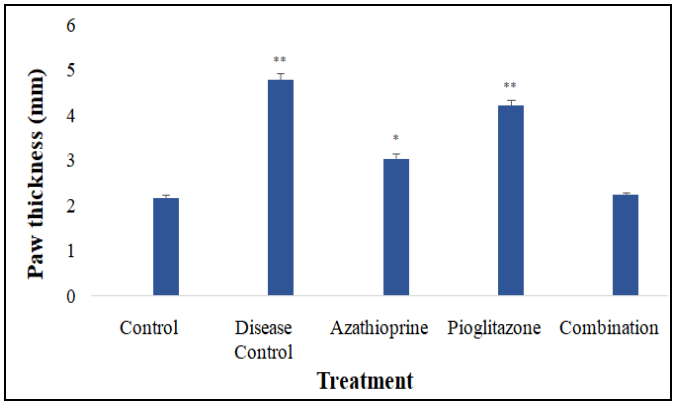

FIG. 3: EFFECT OF AZATHIOPRINE, PIOGLITAZONE AND THEIR COMBINATION IN DELAYED TYPE HYPERSENSITIVITY REACTION

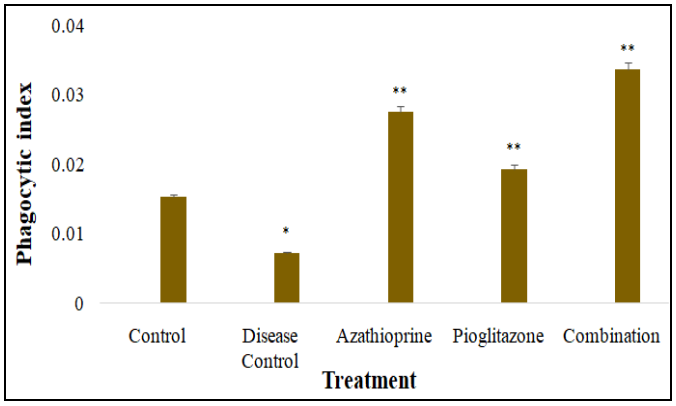

FIG. 4: EFFECT OF AZATHIOPRINE, PIOGLITAZONE AND THEIR COMBINATION BY CARBON CLEARANCE TEST

Nitro Blue Tetrazolium (NBT) Reduction Test: Azathioprine and Pioglitazone stimulated the phagocytic activity of macrophage to the extent of 45% and 36% at 4.5 & 1.35 mg/kg dose respectively. The combination of the above two drugs has produced better augmentation of phagocytic activity to the extent of 58% indicating synergistic interaction between Azathioprine and Pioglitazone **Fig. 5**.

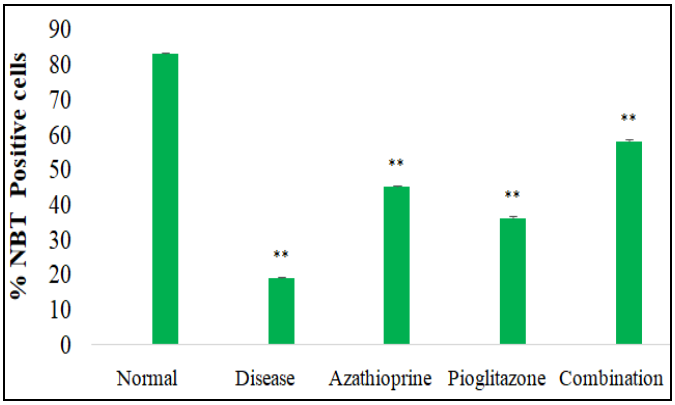

FIG. 5: EFFECT AZATHIOPRINE, PIOGLITAZONE AND THEIR COMBINATION BY NITRO BLUE TETRAZOLIUM (NBT) REDUCTION TEST

DISCUSSION: Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly and gain access inside the cell. H_2O_2 probably reacts with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical which may be the origin of many of its effects [17]. Azathioprine, Pioglitazone and their combination showed prominent hydrogen peroxide scavenging activity. The H_2O_2 scavenging activity of Azathioprine and Pioglitazone might be attributed to the electron donating nature of mercaptopurine and 2, 4-thiazolidinedione respectively, which neutralizes hydrogen peroxide into water. The synergistic interaction between Azathioprine and Pioglitazone might be due to the combination of their electron donating nature.

The anti-oxidant activity of test drugs was further confirmed by nitric oxide radical scavenging assay. NO radical plays multiple roles in a variety of biological processes which serves as an effector molecule, neuronal messenger, vasodilator, anti-microbial agent, *etc.* [18] It has been reported to react with $\cdot O_2$ radical to form peroxynitrite radicals ($ONOO^-$) that cause toxicity to biomolecules such as proteins, lipids, and nucleic acids [19]. Azathioprine and Pioglitazone significantly inhibit generation of NO· and HO· radicals in a dose-dependent manner. This effect of the test drugs might be due to their electron donating nature.

The synergistic interaction observed with the combination of drugs might be due to the combined electron donating nature of the two drugs. These observations further highlight the importance of Azathioprine and Pioglitazone in preventing physiological disturbance caused by NO· and $\cdot O_2$ radicals.

Immune system of our body plays crucial role, as an overactive immune system may lead to certain fatal disease because of various hypersensitive or allergic reactions which may cause numerous derangement, loss of normal capacity to differentiate self from non-self-resulting in immune reactions against our cells and tissues called autoimmune diseases like myasthenia gravis, rheumatoid arthritis, serum sickness, pernicious anemia, *etc*., are the severe issues for medical and pharmaceutical community because of unknown etiology [20]. The immune system is involved in the etiology as well as pathophysiologic mechanisms of many diseases. Modulation of the immune functions either by stimulation or suppression may help to maintain a disease-free state.

A delayed hypersensitivity reaction is an essential feature of chronic inflammation. Such an event can be observed in pathological conditions like rheumatoid arthritis. It is a T- cell-mediated event whereby T cells first infiltrate into tissues and are further stimulated by antigen presenting cells leading to the production of cytokines that mediate local inflammation [21]. Overexpression of INF-γ, IL-2, IL-3, IL-17 are observed in this event [22, 23]. This type of hypersensitivity can be induced by SRBC represents 'tuberculin type reaction'[24]. The reaction is characterized by swelling and inflammation at the site of SRBC. Initially, the event of antigen-antibody interaction causes redness, edema and local inflammation which persist till mediators of the immune system destroy the antigen.

In the present study, both Azathioprine and Pioglitazone attenuated foot paw thickness by inhibiting pro-inflammatory cytokines and subsequent activation of matrix metalloproteinases and erosion of cartilage and bone. The drugs showed synergistic interactions when administered together which might be due to their combined inhibition of inflammatory cytokines and its consequences. The aim of phagocytosis is the elimination of microorganisms and foreign substances along with the removal of injured/dead cells. Cells of the reticuloendothelial system (RES) comprise of the diffuse system which consists of phagocytic cells.

Cells of RES play an important role in the removal of particles from the bloodstream. In the present study, the carbon clearance test was done to assess the effect of Azathioprine, Pioglitazone and their combination on 'reticuloendothelial system.' The rate of clearance of carbon ink particles is directly associated with the functioning of macrophages [25]. The phagocytic index of Azathioprine and Pioglitazone was found to be significant which might be due to their activation of macrophages through T helper cells.

The combination of two drugs produced a significantly better effect than that of individual drugs which might be due to the similarity in their mechanism of action resulting in synergistic interaction. The immunomodulatory activity of test drugs was evaluated by NBT reduction test. The NBT reduction test gives information about the phagocytic and intracellular killing potential of phagocytes.

In this test, the NBT dye is readily ingested by phagocytes and consequently reduced by intracellular superoxide anion radicals (O_2^-.) to form formazan crystals. The higher amount of NBT reduction represented the higher activity of oxidizing enzyme reflecting the stimulation of phagocytes in proportion to intracellular killing [26]. The suppression of the intracellular killing potential of phagocytes by Azathioprine and Pioglitazone was evidenced by a decrease in the intracellular reduction of the NBT dye (formazan crystals) and exhibited dose-dependent suppressive effect on NBT reduction. This might be due to the activation of phagocytes *i.e.,* neutrophils by active metabolites mercaptopurine and 2, 4-thio-zolidinediones of the test drugs. The combination of drugs produced synergistic interaction because of the overlap of the action of one drug on another drug.

CONCLUSION: The combination of Azathioprine and Pioglitazone exhibited significant immuno-modulatory activity than their counterparts due to their effect on phagocytic components of the immune system. This study highlights the benefit of combination therapy over monotherapy in the management of immune-related disorders. It should be further evaluated clinically to bring into human use.

ACKNOWLEDGEMENT: Authors are grateful to the principal and management of Gokaraju Rangaraju College of Pharmacy for providing all the facilities required to carry out this work.

CONFLICT OF INTEREST: There are no conflicts of interest.

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How to cite this article:
Babu PV, Soundarya V and Raju MG: Influence of Pioglitazone on immunomodulatory activity of Pzathioprine in rodent models. Int J Pharm Sci & Res 2019; 10(8): 3843-49. doi: 10.13040/IJPSR.0975-8232.10(8).3843-49.