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RESEARCH ARTICLE

Evaluation of the Anti-Inflammatory Activities of Diclofenac Sodium, Prednisolone and Atorvastatin in Combination with Ascorbic Acid

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Abstract: ***Objectives*:** Inflammation is our body's normal defense mechanism, but in some cases, it may be responsible for causing different kinds of disorders. Several anti-inflammatory drugs are present for the treatment of these disorders; however, the conventional anti-inflammatory drugs cause side effects when used in the long term and therefore, it is better to use them in a low dose for a shorter duration of time. This study was designed to find out whether there is an augmentation of the therapeutic effectiveness of the anti-inflammatory drugs like diclofenac sodium (NSAID), prednisolone (steroid) and atorvastatin (statin) when used in combination with ascorbic acid (antioxidant).

***Methods*:** Wistar Rats (n=144) were selected and divided into 24 groups of 6 rats in each. Carrageenan and formalin were used to induce local inflammation and neuropsychiatric effects, respectively. The inhibitions of such responses were measured after administering a drug alone and in combination with ascorbic acid.

***Results*:** In case of carrageenan mediated inflammation, the combination of 5 mg/kg diclofenac and 200 mg/kg ascorbic acid gave the highest inhibition of 74.19% compared to other groups of drugs. The combination of 5 mg/kg diclofenac and 200 mg/kg ascorbic acid gave 97.25% inhibition for formalin-mediated inflammation group. In both cases, combination therapy showed statistically significant anti-inflammatory activities compared to monotherapy (p values <0.05).

***Conclusion*:** All the data clearly indicate new combinations of drug therapy comprising diclofenac sodium, prednisolone, atorvastatin with ascorbic acid, which may be more effective against both local edema and the neuropsychiatric effect caused due to inflammation.

Keywords: Ascorbic acid, atorvastatin, diclofenac sodium, inflammation, prednisolone, immunity.

1. INTRODUCTION

Inflammation is an integral part of body's immune system and plays a protective role against tissue injury, microbial infection, foreign invaders and other harmful conditions. Inflammatory responses are crucial for maintaining normal tissue homeostasis and signs of inflammation can be observed within a few minutes after injuries [1]. Acute inflammation is considered as an essential part of innate immunity of our body which removes noxious stimuli as well as assists to heal affected tissues [1]. Swelling, redness, pain and heat are the classical symptoms of inflammation

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[2] and different pro-inflammatory cytokines and chemokines secreted from different immune cells help to initiate the fight against injury. Later, this system gets suppressed due to the release of anti-inflammatory cytokines and the tissue gets back to its homeostatic condition. However, this protective function can turn into a destructive pathological condition, if the suppressive mechanism loses its control, thus causing tissue damages. Sometimes, severe uncontrolled inflammation can lead to chronic inflammation and may cause different disease conditions like cancer and diabetes [3].

The molecular mechanism of inflammation is a complex process mediated by different key regulators which are involved in the expression of pro-inflammatory molecules [1]. The activation of WBC results in the activation of T lymphocytes which play a significant role in cell-mediated immunity. Activated T lymphocytes activate and stimulate the monocytes and macrophages which release pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1 [4]. Tissue injury stimulates the release of phospholipase A2 which produces arachidonic acid from cell membrane phospholipids. The arachidonic acid acts as a substrate of two major enzymes called cyclooxygenase or COX and 5-lipoxygenase. The COX enzymes are mainly of two types: COX-1 and COX-2. COX-1 promotes prostaglandin synthesis in most cells to promote tissue homeostasis. On the other hand, COX-2, induced by inflammatory stimuli, promotes thromboxane and prostaglandin formation during inflammation [5]. Another contributor to inflammation is oxidative stress which results due to the imbalance of excessive reactive oxygen species (ROS) production and their neutralization by endogenous ascorbic acid. Oxidative stress can induce inflammation by activating different transcription factors which lead to differential gene expressions [6].

There are several classes of drugs available for treating inflammation. Non-steroidal anti-inflammatory drugs or NSAIDs make up the largest quota of drugs administered for inflammation which work on the COX-2 and impede prostaglandins synthesis, specifically PGE2. Steroids are extensively employed to cure inflammation by decreasing the expression of cytokine-induced genes. Also, statins have been administered to cure inflammation, subdue immune cell activation, or seize degenerative processes [7].

Apart from being able to act against inflammation, these anti-inflammatory drugs possess moderate to severe side effects. For instance, chronic NSAID intake by geriatric patients results in increased susceptibility to many diseases such as peptic ulcer, acute renal failure, cardiovascular and cerebrovascular diseases [8-10]. Adverse effects of statins include musculoskeletal pain, fatigue and weakness, rhabdomyolysis [11] as well as increased risk of diabetes mellitus [12] Osteoporosis, myopathy, avascular necrosis, bone infarction and various psychiatric disorders are some notable side effects of glucocorticoids. Administration of oral glucocorticoids is also linked with adverse systemic effects; such as hypertension, hyperglycemia and obesity that may increase the risk of ischemic heart disease and heart failure as well [13]. Therefore, an alternative anti-inflammatory treatment option with fewer side effects is needed immensely.

Therefore, in this study, we investigated different combinations of therapeutic options for managing inflammatory diseases and compared their efficacy with conventional anti-inflammatory mono-therapeutic approaches. Here, the effects of ascorbic acid (antioxidant) alone and in combination with diclofenac sodium (NSAID), prednisolone (steroid) and atorvastatin (statin), were assessed in experimental animals to study which combination therapy provides the better anti-inflammatory effects with minimum side effects and to infer if atorvastatin could replace diclofenac sodium and prednisolone as an alternative therapy for inflammation.

2. MATERIALS AND METHODS

2.1. Animal Model

A total of 144 Wistar Rats (*Rattus norvegicus*) were collected with an average age of 3 months and an average weight of 230 gm-250 gm, from Jahangirnagar University (Pharmacology Laboratory), Savar, Dhaka, Bangladesh. All animal experiments (creating inflammation using carrageenan and formalin, intraperitoneal injection of drugs, measurement of paw edema with ple-

thysmometer and observation of behavioral changes) were carried out following the ethical guidelines mentioned by the Faculty of Biological Science, University of Dhaka.

2.2. Chemicals

Inflammation inducing agents, carrageenan (Sigma-Aldrich) and formalin (Viola Vitalis), drugs like ascorbic acid, diclofenac sodium, prednisolone and atorvastatin (Incepta Pharmaceuticals Ltd, BD) were used in pure form.

2.3. Selection and Maintenance of Rats

All the Wistar Rats were kept in a suitable temperature of 22°C-25°C and their normal lifestyle was maintained. Standard pellet chows were provided as their food, which were collected from the Animal Resources Facility of ICDDR,B (International Centre for Diarrhoeal Disease Research, Bangladesh).

2.4. Inflammation Creation in Rats

Carrageenan and formalin were used as inflammatory agents. 0.1 ml of freshly prepared 1% suspension of carrageenan was used to induce local inflammation in the rat paw [14]. On the other hand, 20 µl of 5% formalin solution was injected beneath the footpad of the rats to induce inflammation producing neurological effects [15].

2.5. Grouping of Animals and Drug Administration

Drug administration protocol was carried out following the methods described previously [16-19]. The rats were primarily divided into two groups and one group was injected with carrageenan whereas, the other group was injected with formalin to induce inflammation. Afterward, each group was divided into 12 sub-groups (each subgroup contained 6 rats) consisting of one negative control and eleven experimental sub-groups. Each of the experimental sub-group was administered with monotherapy of ascorbic acid (100 mg/kg and 200 mg/kg), diclofenac sodium (5 mg/kg), combination therapy of diclofenac sodium (5 mg/kg) and ascorbic acid (100 mg/kg and 200 mg/kg), prednisolone (5 mg/kg), combination therapy of prednisolone (5 mg/kg) and ascorbic acid (100 mg/kg and 200 mg/kg), atorvastatin (8 mg/kg) and combination therapy of atorvastatin (8 mg/kg) and ascorbic acid (100 mg/kg and 200 mg/kg), respectively. In case of carrageenan, drugs were administered through the intraperitoneal route and for formalin, drugs were given orally.

2.6. Carrageenan-Induced Paw Volume Measurement

This test was performed according to the method described by Winter *et al*., 1962 [14]. Inflammation was induced by injecting carrageenan in the right hind paw of a rat to produce paw edema and the value was considered as the control value. The paw edema volume was measured by plethysmometer, each hour up to 4th hour after injecting carrageenan and compared with the carrageenan mediated paw edema in the groups treated with monotherapy and combination therapy of drugs. The drugs were administered one hour before the carrageenan injection and the percentage of inhibition was measured every hour up to 4 hours [20]. The following formula was used for determining the percentage of inhibition [14].

$$PI = \frac{Paw\ Volume\ of\ Control\ (Vt-V0) - Paw\ volume\ of\ Treated\ (Vt-V0)}{Paw\ Volume\ of\ Control\ (Vt-V0)} \times 100$$

where, V_t = Paw volume at t time, V_o = Paw volume at 0 time.

2.7. Formalin-Induced Paw Licking Measurement

This test was conducted according to the method described by Hunskar and Hole, 1987 [14]. Formalin generally produces a neurological effect like licking, itching, flinching, shaking and jerking, *etc*. like activity. The number of licking was counted for a total period of 30 min. Then, this was compared with the number of licking of the rats in the groups which were treated with the same drug regimen alike carrageenan induced groups, one hour before formalin injection. After that, the percentage of inhibition was measured by the following equation and compared with the standard group [14].

$$PI = \frac{Number\ of\ paw\ licking\ in\ Control - Number\ of\ paw\ licking\ in\ treated}{Number\ of\ paw\ licking\ in\ control} \times 100$$

2.8. Statistical Analysis

Statistical analysis was performed to compare the anti-inflammatory effect of ascorbic acid, diclofenac sodium, prednisolone and atorvastatin alone as well as in combination with ascorbic acid for both carrageenan and formalin-induced rat model. GraphPad Prism 7 software was used for statistical analysis.

3. RESULTS

3.1. Carrageenan-Induced Inflammation

The average value of total paw edema of 6 rats after administration of carrageenan was found to be 4.32% ± 0.77. It has been found that the groups given 100 mg and 200 mg ascorbic acid, showed average inhibition of 18.98% ± 0.27 and 20.95% ± 0.53 respectively, where a higher dose clearly showed elevated inhibition. On the other hand, 5 mg/kg diclofenac sodium monotherapy showed an average inhibition of 40.51% ± 0.42. However, in combination therapy with 100 mg and 200 mg ascorbic acid, the average values were 45.64% ± 0.99 and 74.19% ± 0.39 inhibition, respectively. In case of mono-therapy of 5 mg/kg prednisolone, the average inhibition was found to be 32.48% ± 0.17 whereas, the combination therapy of prednisolone with 200 mg ascorbic acid exhibited 69.71% ± 0.32 inhibition. Lastly, the combination therapy of 8 mg/kg atorvastatin with 200 mg ascorbic acid showed better inhibition with a value of 50.20% ± 0.38 compared to the monotherapy of atorvastatin which showed almost half of the inhibition, 23.42% ± 0.31. Therefore, it can be concluded from Fig. (**1**) that the combination of diclofenac sodium and ascorbic acid demonstrated the highest percentage of inhibition. However, the lowest percentage of inhibition was found in case of 100 mg ascorbic acid monotherapy.

3.2. Formalin-Induced Inflammation

In the control group, the number of licking was observed in rats after administering formalin at 10, 20 and 30 minutes and the average number of licking was found to be 60.67. In this model, 200 mg ascorbic acid monotherapy showed the highest percentage of inhibition, 97.53% ± 0.824. However, diclofenac sodium and 200 mg ascorbic acid combination therapy exhibited the second highest percentage of inhibition of 97.25% ± 1.55. In case of prednisolone (5 mg/kg), the combination therapy with 200 mg ascorbic acid showed much better result which was 74.18% ± 0.78 inhibition compared to monotherapy which is only 53.57% ± 1.76. Moreover, a better result was found when 8 mg/kg atorvastatin was administered with 200 mg of ascorbic acid and the percent inhibition value was 86.26% ± 0.78. The comparative study is shown in Fig. (**2**) and Table **1** shows the summary of the anti-inflammatory effect of the drug for both carrageenan and formalin-induced inflammation.

3.3. Statistical Analysis

One way ANOVA test showed a significant improvement in the anti-inflammatory activity of the marketed anti-inflammatory drugs in combination with ascorbic acid compared to anti-inflammatory drugs alone ($p < 0.05$) in carrageenan rat models of inflammation. In this model, the highest efficacy was observed for diclofenac sodium 5 mg + ascorbic acid 200 mg treatment group which was significantly higher than diclofenac sodium alone (adjusted $p < 0.0001$). In the case of the formalin model, the anti-inflammatory efficacy of anti-inflammatory drugs was found to be statistically higher in combination with ascorbic acid, compared to the anti-inflammatory drugs alone ($p < 0.05$). Here as well, the diclofenac sodium 5 mg + ascorbic acid 200 mg combination showed the highest efficacy among all the combination groups (adjusted $p < 0.0005$). The comparative results are exhibited in Table **1** and Figs. (**1** and **2**).

4. DISCUSSION

Diclofenac sodium is one of the most common choices of medication for treating acute inflammation and pain which work by inhibiting the cyclooxygenase (COX) pathway and thus preventing the synthesis of prostaglandin and other eicosanoids [21]. Besides, atorvastatin has also been found to reduce circulating C-reactive protein (CRP) levels, pro-inflammatory cytokines, vascular reactive oxygen species (ROS) production as well as the expression of soluble intercellular adhesion molecule-1 and lipopolysaccharide-induced secretion of IL-6 and TNF-α by monocytes and macrophages [22], thereby combating inflammation. Studies have also shown the anti-inflammatory

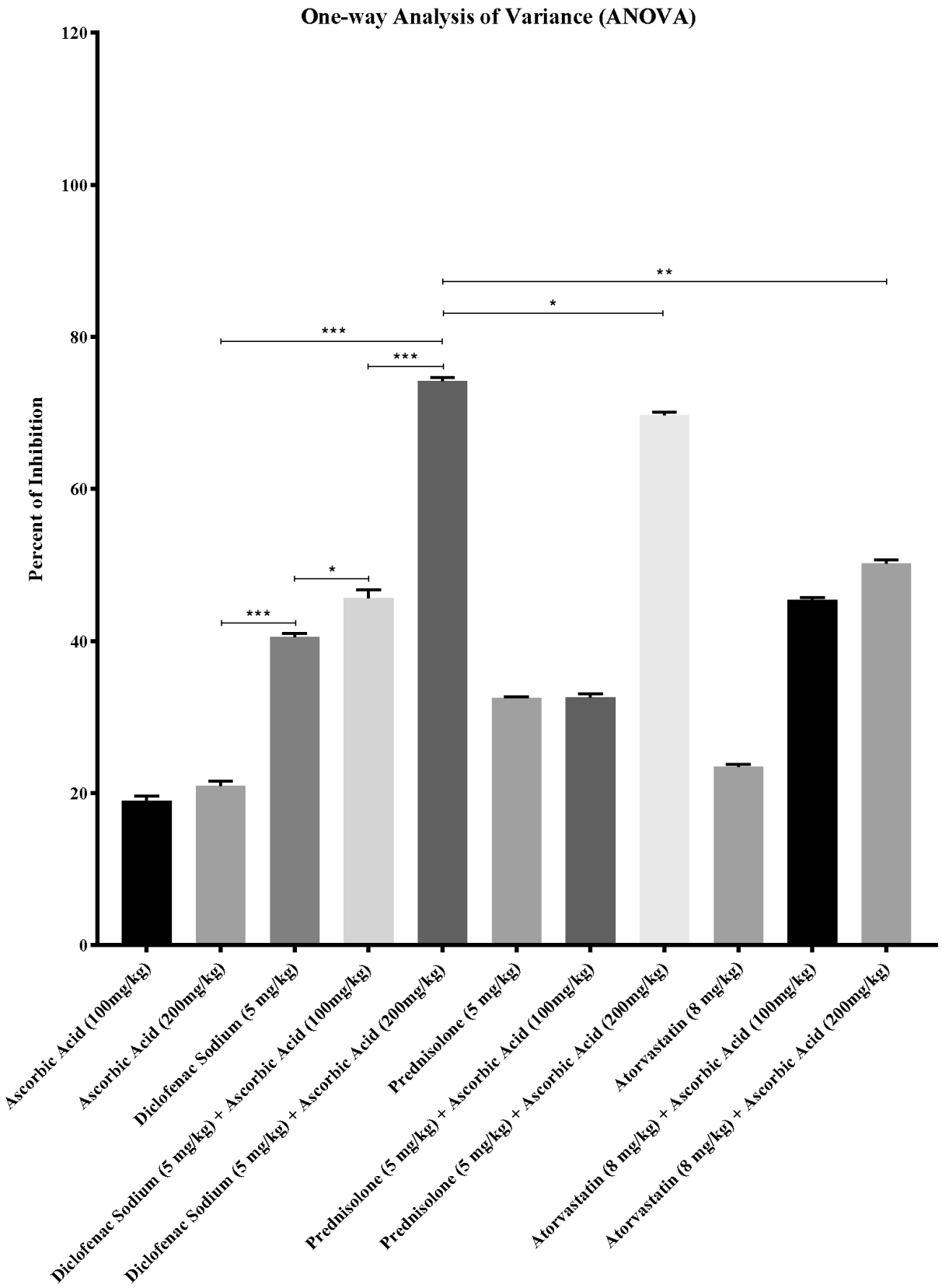

Fig. (1). Anti-inflammatory activities in different groups of carrageenan induced rat models. The drugs were administered prior to the carrageenan injection. The highest anti-inflammatory activity (adjusted $p < 0.0001$) was observed in case of combination of diclofenac sodium (5 mg/kg) and ascorbic acid (200 mg/ kg) compared to others.

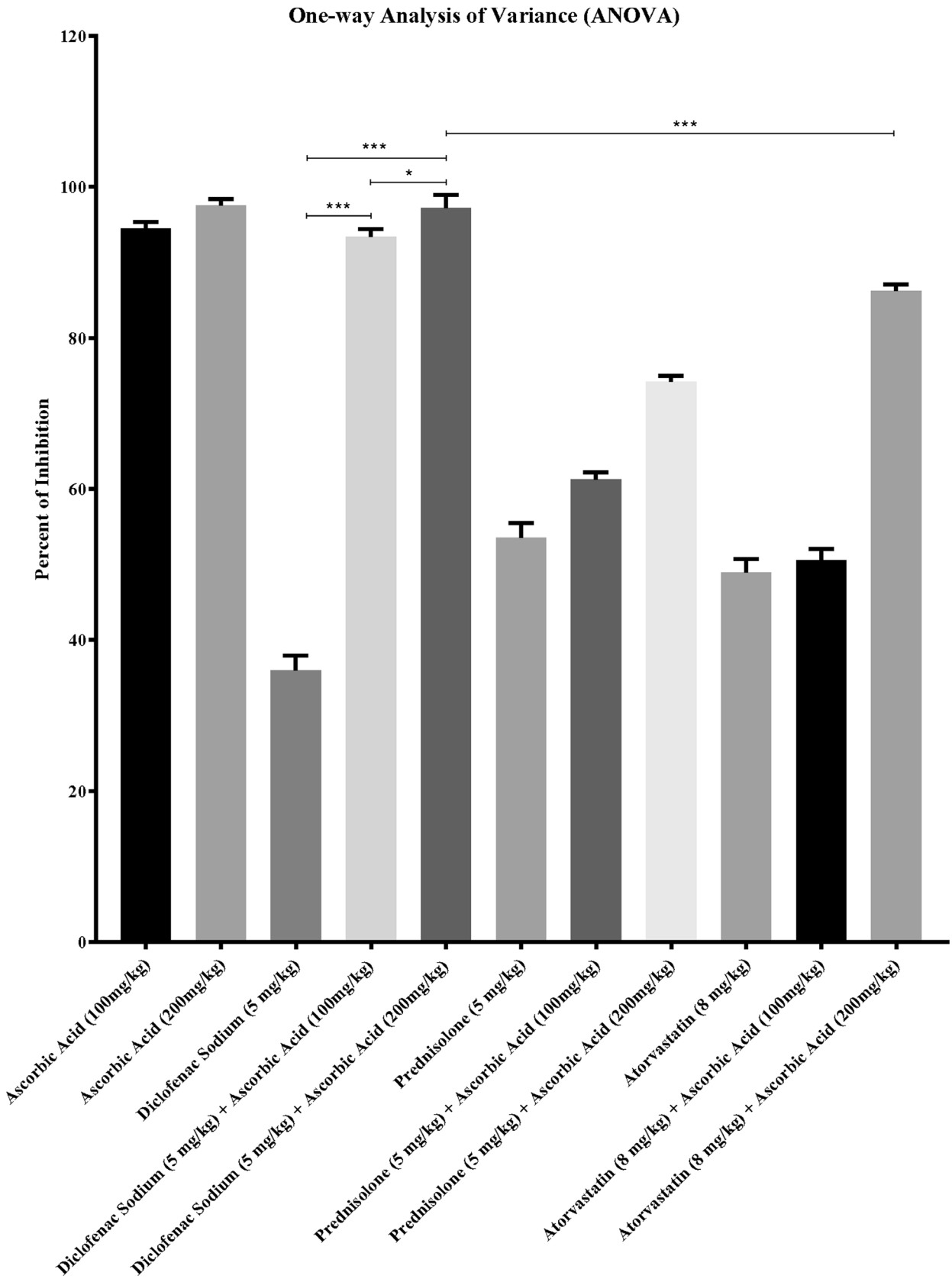

Fig. (2). Anti-inflammatory activities in different groups of formalin induced rat models. The drugs were administered one hour prior to formalin injection. The highest anti-inflammatory activity was observed in case of combination of diclofenac sodium (5 mg/kg) and ascorbic acid (200 mg/ kg) among all the combination groups (adjusted p <0.0005).

Table 1. Summary of anti-inflammatory effects (percent inhibition with standard deviation) of drugs for both carrageenan and formalin-induced inflammation.

Therapy	Carrageenan	Formalin
Ascorbic acid 100 mg	18.98% ± 0.53	94.51% ± 0.78
Ascorbic acid 200 mg	20.95% ± 0.53	97.53% ± 0.824
Diclofenac sodium	40.51% ± 0.42	35.99% ± 1.76
Diclofenac sodium+ Ascorbic acid 100 mg	45.64% ± 0.99	93.41% ± 0.95
Diclofenac sodium+ Ascorbic acid 200 mg	74.19% ± 0.39	97.25% ± 1.55
Prednisolone	32.48% ± 0.17	53.57% ± 1.76
Prednisolone+ Ascorbic acid 100 mg	32.57% ± 0.45	61.27% ± 0.82
Prednisolone+ Ascorbic acid 200 mg	69.71% ± 0.32	74.18% ± 0.78
Atorvastatin	23.42% ± 0.31	48.90% ± 1.65
Atorvastatin+ Ascorbic acid 100 mg	45.37% ± 0.27	50.55% ± 1.35
Atorvastatin+ Ascorbic acid 200 mg	50.20% ± 0.38	86.26% ± 0.78

effects of prednisolone due to its inhibitory effect on prostaglandin synthesis and leukocyte migration to inflamed cells. In addition to that, glucocorticoids repress the transcription of many genes encoding pro-inflammatory cytokines and chemokines, cell adhesion molecules and key enzymes involved in the initiation and/or maintenance of the host inflammatory response [23]. Apart from these conventional anti-inflammatory options, exogenous ascorbic acid protects our body against oxidative stress by neutralizing excessive reactive species and retaining the balance and also acts as a scavenger for free radicals, thereby playing a contributing role in modulating inflammation [6].

However, not many studies have been conducted to assess the anti-inflammatory efficacy of conventional anti-inflammatory drugs in combination with anti-oxidants like ascorbic acid. To the best of our knowledge, this is the first report, where we have studied the anti-inflammatory efficacy of ascorbic acid in combination therapy with conventional anti-inflammatory drugs and have compared their effects with anti-inflammatory mono-therapy. From Table **1**, we can see that, in case of carrageenan-induced inflammation, the best result was shown by the combination therapy of diclofenac sodium and 200 mg ascorbic acid with 74.19% inhibition. The combination therapy of prednisolone and 200 mg ascorbic acid comes at the second place with 69.71% inhibition. The better performance of diclofenac sodium combination therapy than prednisolone combination therapy is probably due to the fact that diclofenac sodium works on the molecular level by inhibiting COX and halting prostaglandin synthesis and in carrageenan-induced inflammation, there is an increased expression of COX 2 and PGE2 [20]. However, prednisolone works on the genetic level by repressing the transcription of several genes which take a longer time to show the expected effect as it is a longer route of action. In both cases, the addition of ascorbic acid potentiated their actions. The poorest results were obtained from ascorbic acid monotherapy as it works against only oxidative stress and cannot suppress other mediators of inflammation. According to Fig. (**3**), combination therapy of diclofenac sodium and 200 mg of ascorbic acid showed a gradual increase in effectiveness and the best results were obtained in the 4^{th} hour and combination therapy of diclofenac sodium and 100 mg ascorbic acid showed best effects in the 1^{st} hour and then in the 4^{th} hour. Diclofenac sodium monotherapy gave peak performance in the 1^{st} hour and then gradually declined. Effect of prednisolone monotherapy gradually increased till the 3^{rd} hour and then declined. Prednisolone

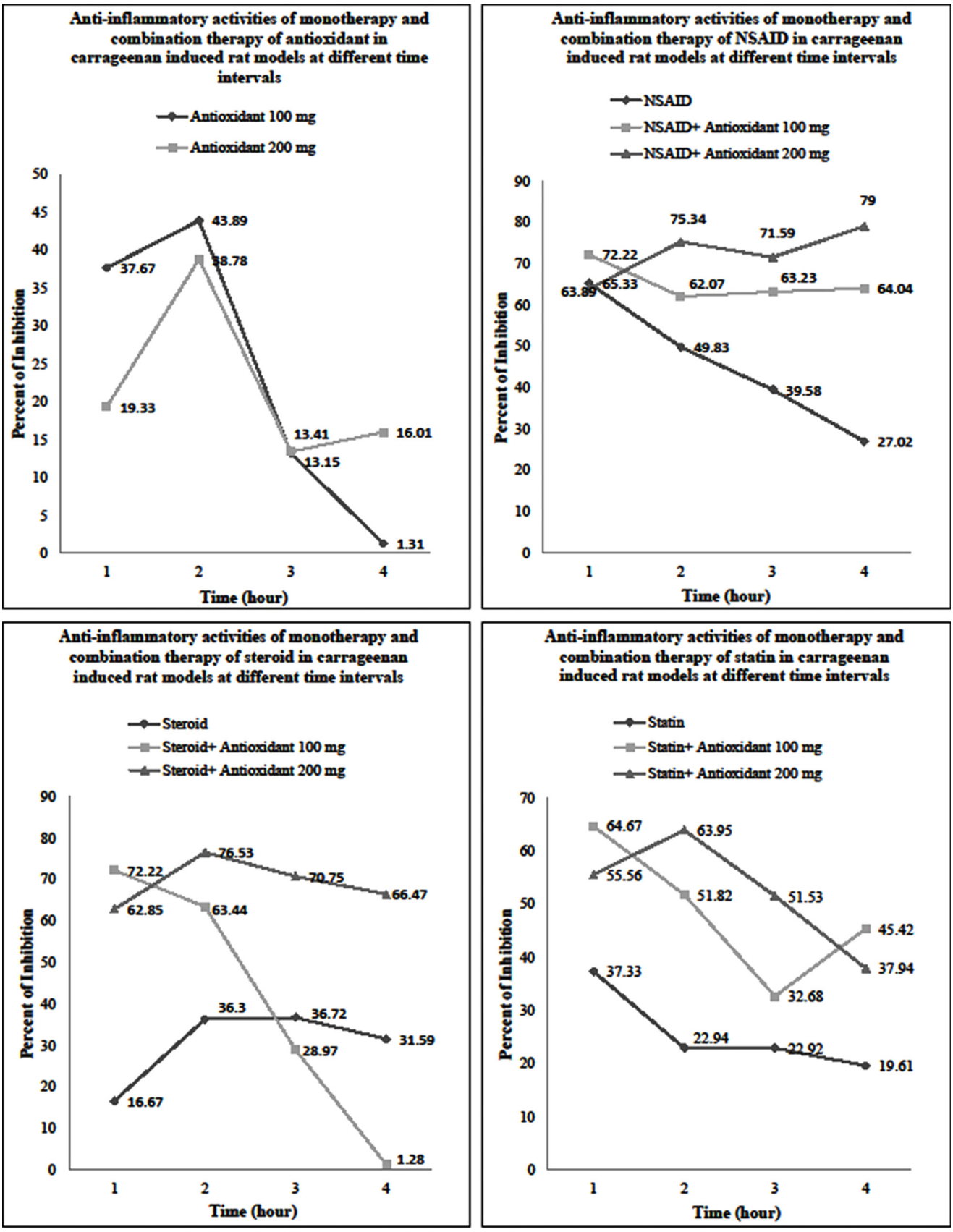

Fig. (3). Percent of inhibition of paw edema in rats after administering monotherapy of ascorbic acid, diclofenac sodium, prednisolone and atorvastatin as well as combination therapy with ascorbic acid at different time intervals.

combined with 100 mg ascorbic acid gave peak performance in the 1st hour and then kept on declining. However, in combination with 200 mg ascorbic acid, prednisolone's effect was the highest in the 2nd hour and then it kept on declining. For atorvastatin monotherapy and combination therapy with ascorbic acid, the best effects were in the 1st hour which then gradually declined. Ascorbic acid monotherapy gave the best results in the first two hours and the peak performance in the second hour and then the efficacy declined.

As for formalin-induced inflammation, the monotherapy of ascorbic acids showed the best results. The combination therapy of diclofenac also showed excellent results, however, the monotherapy of diclofenac showed the poorest result. Atorvastatin, an FDA approved lipid-lowering drug [24] and prednisolone both showed moderate results in both cases of monotherapy and combination therapy, however, the results were better in case of combination therapy. The better performance of diclofenac sodium is because of their inhibitory effect on the COX pathway as it is a major component of the inflammatory response [21].

Between carrageenan and formalin-induced inflammation, better inhibition was obtained in case of formalin. This is probably because carrageenan is a stronger inflammatory agent than formalin, as there are several inflammatory mediators involved in carrageenan-induced inflammation such as prostaglandins, serotonin, and histamine [25]. However, in case of formalin, the main contributors are bradykinin and reactive oxygen species and due to this reason, the results were much better regarding formalin-induced inflammation as there are fewer mediators to suppress [26]. Clearly, in both the cases of carrageenan and formalin-induced inflammation, the monotherapy and the combination therapy with a higher dose of ascorbic acid showed much better results for diclofenac sodium. Thus, we cannot replace diclofenac sodium either by atorvastatin or by ascorbic acid monotherapy as their effects were not that prominent.

CONCLUSION

This study demonstrates the effectiveness of alternative drugs for the treatment of inflammatory diseases. The conventional anti-inflammatory agents have many side effects and the long term use of such drugs is very harmful for the patients. Therefore, to compensate for these problems, establishing a new drug regimen has become necessary. From the study, we can infer that all the drugs combined with ascorbic acid exhibited satisfactory anti-inflammatory activity compared to monotherapy, however, the combination of diclofenac sodium and 200 mg dose of ascorbic acid showed higher anti-inflammatory actions in both cases of local and neuropsychiatric inflammation. Ascorbic acid has actually potentiated the anti-inflammatory effects of the conventional anti-inflammatory drugs. Our results have shown that a combination therapy of conventional anti-inflammatory and anti-oxidants like ascorbic acid could be a better choice rather than anti-inflammatory drugs alone for treating both local as well as systemic inflammation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the institutional ethical committee of Faculty of Biological Science, University of Dhaka, Bangladesh, (FBS/09/2018).

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are basis of this research. The experimental procedures on animals were conducted in accordance to the ethical guidelines mentioned by the Faculty of Biological Science, University of Dhaka (FBS/09/2018), Bangladesh.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available in the [BracU Institutional Repository] at [http://hdl.handle.net/10361/11067], reference number [13146021].

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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