EFFECT OF DICLOFENAC SODIUM ON ASPIRIN'S ANTITHROMBOTIC ROLE

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ABSTRACT

Objective: To assess whether diclofenac sodium interferes with the anti-platelet effect of low dose aspirin.
Study Design: Quasi- experimental study.
Place and Duration of Study: Department of Pharmacology and Therapeutics, Army Medical College and Armed Forces Institute of Pathology Rawalpindi.
Material and Methods: Eighteen healthy volunteers, divided into three groups, between the ages of 22-50 years, after written informed consent were selected according to a set criterion. They were given aspirin (150mg) once a day and diclofenac sodium 50mg three times a day for six consecutive days while use of any other drug was prohibited. Blood samples were taken from the study subjects on two occasions, before starting drugs and then on the seventh day. Blood samples were analyzed for platelet aggregation (ADP and collagen induced) and serum thromboxane B2 levels.
Results: When a single daily dose of 150mg aspirin is taken with three daily doses of diclofenac sodium (50mg), results show that the anti-platelet effect of aspirin still remains. The mean platelet aggregation with ADP was reduced to 55.83 ± 5.38 percent from a baseline value of 71.67 ± 5.27 percent. Similarly if collagen was used as a reagent the aggregation of platelets was markedly reduced to 40.83 ± 6.63 from a baseline of 66.67 ± 6.54 percent. Results showed a prominent inhibition of aggregation of 22.10% for ADP and 38.75% for collagen. Also, mean thromboxane B2 levels reduced markedly from 971.11 ± 128.91 pg/ml to 702.99 ± 101.59 pg/ml.
Conclusion: It is safe to use diclofenac sodium with aspirin, as the anti-platelet effect of the latter is not attenuated.

Keywords: Anti platelet, Aspirin, Diclofenac.

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INTRODUCTION

Cardiovascular diseases cause the death of nearly 17.3 million people each year[1]. A key factor in its pathogenesis is thrombosis[2]. Platelets play the vital role of mediators of arterial thrombus propagation by releasing thromboxane A2, which in turn, not only amplifies the signal for thrombus formation but also acts as a vasoconstrictor[3]. Aspirin and related drugs all act by inhibition of the generation of prostaglandins[4]. Membrane phospholipids release arachidonic acid, which under the influence of the enzyme cyclo oxygenase (COX) is converted to different types of prostaglandins, thromboxanes and prostacyclins[5]. These lipid compounds play a keypart in inflammation, pain and fever and because aspirin and the NSAIDs inhibit their biosynthesis, these drugs are effective pain relievers and anti pyretics[6]. Aspirin irreversibly inhibits the enzyme COX by selectively acetylating the hydroxyl group of one serine residue (Ser 530) located near its C terminus[7]. Irreversible inhibition therefore requires the formation of new enzyme for prostaglandin synthesis. Complete blockage of the activity of COX 1 accounts for the ability of aspirin to halt platelet aggregation. It is by virtue of the failure of production of thromboxane A2, a key stimulator of platelet aggregation itself and a potent vasoconstrictor that a platelet plug fails to form[8]. This anti thrombotic role of aspirin is achieved even at low doses, in contrast to the inhibition of COX 2, which only occurs at higher doses. Daily consumption of aspirin ensures that all platelets, both existing in the circulation and

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those freshly released from the bone marrow do not secrete thromboxane A2. The trouble that lies with lifelong consumption of aspirin is the presence of other ailments that old age brings along with it. Osteoarthritis, rheumatoid arthritis, osteoporosis and Alzheimer's are just a few to name. For an individual who is already taking aspirin, this means he now has a longer prescription to adhere to. Usually the addition in this list is one of the non steroidal anti inflammatory drugs (NSAIDs)[9]. Studies show that nearly 5% of prescriptions in the western world have a common denominator; one of the NSAIDs[10]. A potent inhibitor of both COX 1 and 2, is diclofenac, a safe, effective and commonly used NSAID[11]. Its analgesic effect is attributed to a powerful inhibition of COX 2, exhibiting some selectivity for this enzyme so that inflammatory prostaglandins are no longer produced[12]. Available in oral, topical and injectable formulations, the drug is used both on a short term as well as long term basis for treating a number of ailments both in children and in adults[13]. NSAIDs also block thromboxane A2 synthesis, but inhibition is competitive and reversible. For as long as an NSAID stays in the COX channel, it prevents aspirin from accessing the serine residue and irreversible inactivation of COX 1[14]. If aspirin is metabolized before the NSAID leaves COX1, thromboxane A2 synthesis will resume as soon as the NSAID itself is removed from the circulation. It is in this manner that NSAIDs can interfere with aspirins thromboprophylactic activity[15]. There is therefore an expected probability that if these two drugs are used simultaneously, the beneficial role of aspirin may completely be overpowered by diclofenac. The aim of our study is to find out whether this occurs and if so then to what extent?

MATERIAL AND METHODS

This quasi-experimental study was conducted in the department of Pharmacology and Therapeutics, Army Medical College and Armed Forces Institute of Pathology, Rawalpindi. Study protocol approval was sought from ethics committee of Centre for Research in Experimental and Applied Medicine (CREAM) Army Medical College, Rawalpindi. Sample size was calculated by EPI info calculator. Eighteen healthy human volunteers (six females and twelve males) who were between the ages of 22-50 years, within 30% ideal body weight, with an unremarkable medical history and physical examination were selected for this research by non probiblity convenience sampling. Those who were smokers, below 22 years or above 50 years, had a bleeding disorder or any allergy to a drug and had a history of any gastrointestinal or cerebrovascular disease were not included[14]. No concomitant medication was taken during the course of the study and written informed consent was taken from all participants at the start of the research. Study subjects were assigned to one of the three groups, each group having six members. The drugs used in the study were Tab Aspirin (Highnoon Labs Ltd) and Tab Diclofenac sodium (Sami Pharmaceuticals) and were to be taken orally with plain water for six consecutive days. The division of the groups was done as follows:

- Group-A: (n=6) Control group, received aspirin 150 mg/day[14]
- Group-B: (n=6) Diclofenac sodium 50 mg three times a day[16]
- Group-C: (n=6) Diclofenac sodium 50 mg 8hrly and aspirin 150mg daily[16], as above.

Blood sampling was done on two occasions, once before taking the drug, i.e. on day zero and secondly, 12 hours after the last dose, i.e. on day seven. Using strict aseptic measures approximately 9ml of blood was collected from the antecubial vein of each subject into a test tube already containing 1ml of tri sodium citrate, so that the blood anticoagulant ratio was 1:10[16]. Platelet aggregation was assessed by using light transmission agreggometry in platelet rich plasma (PRP), a method considered as the gold standard for analysis of platelet function[17]. The collected samples were transported to Armed Forces Institute of Pathology (AFIP) where impedence aggregometer (Model 700, Chrono-Log Corporation, Havertown, PA) was

available[18]. First, complete blood count was performed on each sample and platelet count was assessed. These samples were then centrifuged at 1200 rpm for 10 minutes at 37°C to obtain platelet rich plasma (PRP). Platelet count was then reassessed from the PRP of each sample obtained. Any sample with a platelet count of less than 100 x10^9 per liter was considered unsuitable for the test. In order to obtain platelet poor plasma (PPP), blood was centrifuged at 3400rpm for 3 minutes. Five hundred microliters of PRP was pippeted out from each sample, one at a time and emptied into small cuvettes. Small magnetic stirrers were then placed in each cuvette. Chrono Log Aggregometer Model 700 was used for assessing platelet aggregation. Using one sample at a time and considering PPP as control, aggregation was assessed with two agents i.e., first with 1µL collagen and then with 5µL of ADP[19]. Using Aggrego Link 8 on a computer, a graph was plotted showing percentage of aggregation on y-axis and time in seconds on x-axis. Results were then compiled and analyzed.

Almost immediately after formation, thromboxane A2 decomposes to form thromboxane B2 (t½ 5-7mins). Thus in order to analyze platelet COX activity, thromboxane B2 levels in plasma or urine are measured. In order to estimate serum thromboxane levels, Human Thromboxane A2 (TX-A2) ELISA Kit was purchased from CUSABIO. From each sample taken under strict aseptic conditions, about 1ml of serum was separated and stored at-80°C for the analysis of thromboxane B2 levels[20]. Serum was obtained by

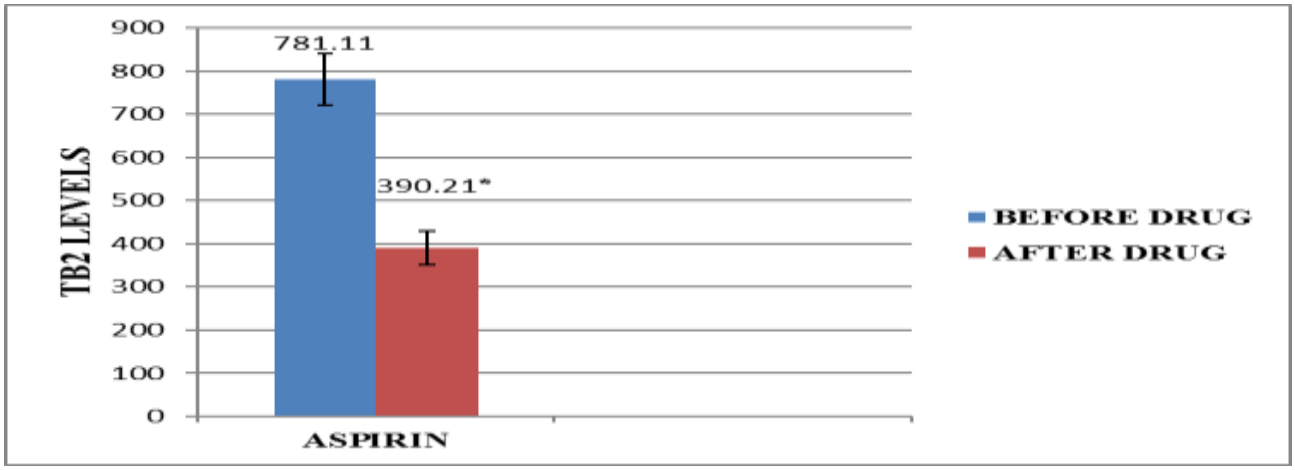

Figure-1: Results of Thromboxane B2 levels for group A (aspirin 150 mg/day).

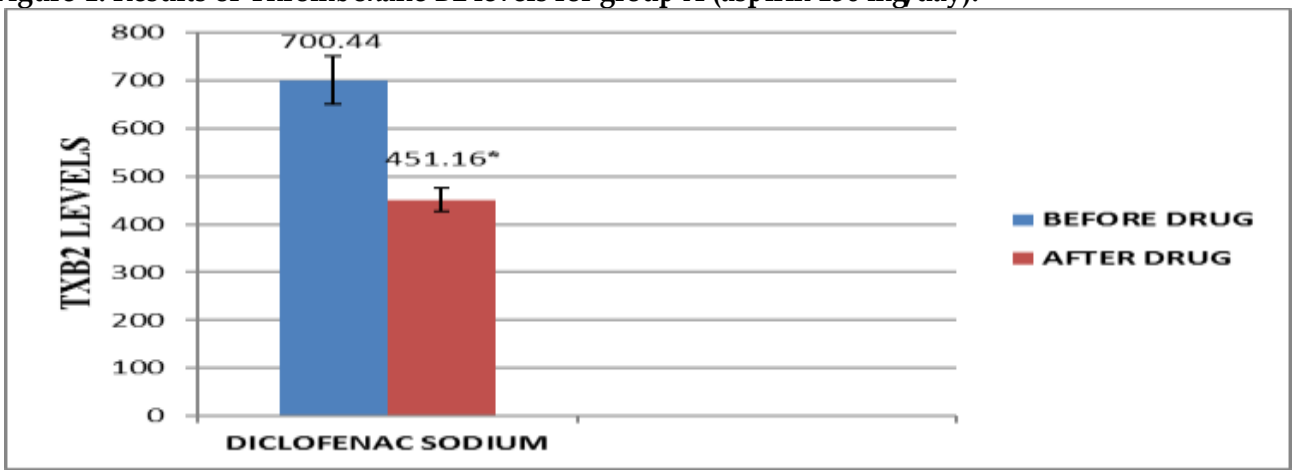

Figure-2: Results of Thromboxane B2 levels for group B (diclofenac sodium 50 mg three times a day).

centrifuging the samples at 3200 rpm for 15 mins[16].

All reagents (HRP-avidin, TMB substrate and stop solution) were freshly prepared according to the instruction manual. Hundred µL of each sample were added to each well of the coated assay plate. After two hours of incubation at 37°C, the liquid was removed without washing. Hundred µL of Biotin-antibody was then pipetted into each well and the plate was incubated for one hour at the same temperature. After removal, the wells were each washed three times with wash buffer (pH 7.3). Then, 100 µL of HRP-avidin (horseradish peroxidaseavidin) was added to the wells and incubated in the same manner as the previous step. This was then followed by aspirating and washing the wells five times each. Ninety µL of TMB (3,3′,5,5′-Tetramethylbenzidine) Substrate was then added to each well and incubated this time for about 30 minutes, ensuring complete protection from light. Lastly, 50 µL of stop solution (sulphuric acid solution) was pippeted into the wells and gently tapped. Then, using an auto mated ELISA reader the optical density of each well was measured. Readings at 540nm were subtracted from readings at 450nm and results were calculated. Values obtained were plotted on a computer spreadsheet. A standard curve was plotted and the concentration of each sample was calculated from the following formula:

- Total TXB2 in sample (pg/ml) = TXB2 (pg) in purified sample
- Volume of sample used for purification (ml)

Statistical Analysis

The results of serum analysis were expressed as means ± standard error of means (SEMs). Arithmetic means and SEMs were calculated on the computer using SPSS Version 20 and applying paired sample t-test. Difference between two observations was considered significant if the *p*-value was found to be less than 0.05.

RESULTS

In the first group, which was given low dose aspirin (150mg) daily, platelet aggregation was inhibited significantly when assessed with both reagents, i.e., ADP as well as with collagen. Mean platelet aggregation of six subjects with collagen before receiving the drug was 84.16 ± 4.16 percent. Six days later it reduced to 25.50 ± 5.96 percent, showing a highly significant inhibition of aggregation, with a *p*–value of 0.001. Similarly, with ADP the levels before giving the drug were 85.83 ± 5.23 percent and were reduced to 73.33 ± 3.07 percent after aspirin intake, again giving a significant *p*-value of 0.03. On analysis of thromboxane B2 levels, aspirin showed a similarly significant effect. Mean plasma levels of 781.11 ± 51.39 pg/ml lowered down to 390.21 ± 34.09 pg/ml after six days of regular aspirin use

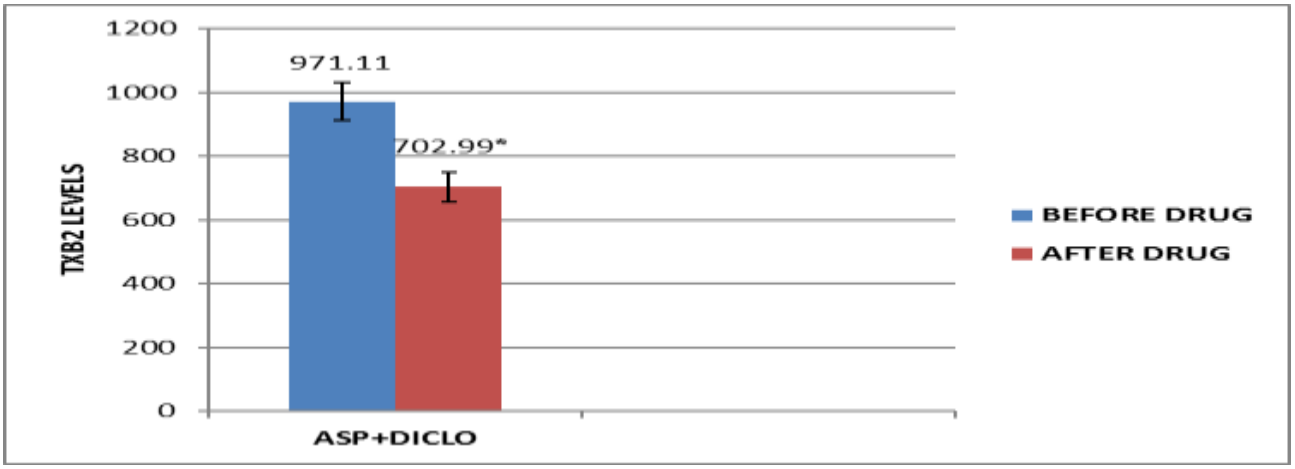

Figure-3: Results of Thromboxane B2 levels for group C (aspirin 150 mg/day and diclofenac sodium 50 mg three times a day).

and is depicted in fig-1. A *p*-value for this parameter also came out to be significant, i.e. <0.001. In our second group, results show that when used continuously for six days, 50mg of diclofenac sodium thrice a day doesn't significantly inhibit platelet aggregation neither with ADP nor with collagen. For six study subjects the mean platelet aggregation of 72.50 ± 4.95 percent with ADP was reduced to 69.17 ± 3.96 percent, giving a *p*-value of 0.50. With collagen as the reagent the results didn't differ much. A mean platelet aggregation of 75.83 ± 3.74 percent before the drug remained nearly the same after taking it regularly, i.e. 75.83 ± 3.00 percent. The *p*- value with collagen thus was insignificant at 1.00. The serum thromboxane B2 levels were markedly reduced by diclofenac sodium, i.e. from a baseline level of 700.44 ± 61.51 pg/ml thromboxane B2 was reduced down to 451.16 ± 31.82 pg/ml, as evident in fig-2. This significant depletion however was not reflected by a decrease in platelet aggregation, whether ADP induced or collagen induced. When a single daily dose of 150mg aspirin is taken with three daily doses of diclofenac sodium (50mg), results of group C show that the antiplatelet effect of aspirin still remains. With such a dosing regimen mean platelet aggregation with ADP was reduced to 55.83 ± 5.38 percent from a baseline value of 71.67 ± 5.27 percent, thus giving a significant *p*-value 0.03. Similarly if collagen was used as a reagent the aggregation of platelets before drug intake was 66.67 ± 6.54 percent and it was markedly reduced to 40.83 ± 6.63 percent afterwards. A *p*-value was again calculated to be immensely significant, i.e. 0.00. These results were further consolidated when thromboxane B2 levels were measured. Mean thromboxane B2 levels reduced markedly from a baseline of 971.11 ± 128.91pg/ml to 702.99 ± 101.59 pg/ml, as shown in fig-3. A *p*-value was again significant at 0.02.

The results of platelet aggregation both collagen induced and ADP induced alongwith serum thromboxane A2 levels of group A, B and C are shown in the table.

Table: The comparison of platelet aggregation parameters of group a, group b and group C.

	Group A (n=6)		*p*-value	Group B (n=6)		*p*-value	Group C (n=6)		*p*-value
Parameter	**Day 0**	**Day 7**		**Day 0**	**Day 7**		**Day 0**	**Day 7**	
Platelet aggregation with ADP (%) Mean ± SEM	85.83 ± 5.23	73.33 ± 3.07	0.037	72.50 ± 4.95	69.17 ± 3.96	0.50	71.67 ± 5.27	55.83 ± 5.38	0.03
Platelet aggregation with collagen (%) Mean ± SEM	84.16 ± 4.16	25.5 ± 5.96	0.001	75.83 ± 3.74	75.83 ± 3.00	1.00	66.67 ± 6.54	40.83 ± 6.63	<0.001
Thromboxane B2 Levels (pg/ml) Mean ± SEM	781.11 ± 51.39	390.21 ± 34.09	<0.001	700.44 ± 61.51	451.16 ± 31.82	0.02	971.11 ± 128.19	702.99 ± 101.59	0.02

DISCUSSION

Many individuals are long term consumers of low dose aspirin with the hope that an unfortunate cardiovascular event will not strike them. They also usually are prescribed NSAIDs for minor aches and pains. The current study was designed to evaluate if, in the Pakistani population, this clinical combination causes a pharmacodynamic interaction that can totally or partially negate the well-established and well documented antithrombotic role of low dose aspirin[1,14]. In our study aspirin not only inhibited aggregation of platelets with collagen (69.70%) and ADP (14.07%), it also strongly lowered the thromboxane B2 levels (*p*-value 0.00). Aspirin inhibits the aggregation of platelets, most strongly arachidonic acid (AA) induced, while collagen and ADP induced aggregation are also blocked by the drug. We used the latter two reagents in our study. The anti-platelet properties of aspirin are largely attributed to COX1

inhibition. Studies show that collagen induced aggregation only partially depends upon cyclo oxygenase and that, maybe, aspirin can block platelet function through certain non COX1 pathways[20]. Individuals in the second group were given tab diclofenac. Our results reflect a negligible inhibition of platelet aggregation both with ADP (4.59%) and collagen (1%). However thromboxane B levels were reduced significantly (700.44pg/ml reduced to 451.16pg/ml). In congruence with our results, Niemi and his fellows also showed a lack of any influence on ADP induced aggregation by diclofenac sodium[21]. Tyutyulkova and his colleagues drew similar conclusions with ADP but demonstrated an inhibition of aggregation with collagen[22]. In 2003 Munsterhjelm and colleagues performed a similar study in which intravenous diclofenac sodium was used. They concluded that 5 mins after an infusion of diclofenac sodium, aggregation with collagen, ADP and epinephrine are all inhibited but within 90 mins the reversibility of this inhibition starts becoming evident, so much so that by 22-24 hrs after the infusion, no such effect remains[23]. We collected blood samples from study subjects at least 12 hrs after the last dose of diclofenac sodium. It thus appears that probably sufficient time had elapsed for any anti platelet effect to remain. When we combined aspirin with diclofenac sodium, the thromboprophylactic role of the former still remained. Platelet aggregation with ADP and collagen fell significantly (*p*-values 0.03 and 0.00 repectively) as did thromboxane B2 levels (*p*-value 0.02). Schuijt *et al* also concluded that if used together, there is no suppression of aspirins cardio-protection by diclofenac sodium[16]. Although classified as a non-selective inhibitor of cyclo oxygenase, studies have shown that diclofenac exhibits some partiality towards cyclo oxygenase 2 which could explain why it has a weak role in blocking the aggregation of platelets.

CONCLUSION

It is safe to use diclofenac sodium with aspirin, as the anti-platelet effect of the latter is not attenuated.

CONFLICT OF INTEREST

This study has no conflict of interest to declare by any author.

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