

ANTIPLATELET EFFECTS OF SOME CYCLOOXYGENASE-2 INHIBITORS IN EXPERIMENTAL HYPERCHOLESTEROLEMIA IN RABBITS

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ABSTRACT

Objective: Hypercholesterolemia is an inflammatory disease and a leading cause of atherosclerosis. Platelet hyper-aggregation is observed in hypercholesterolemia and may be mediated by cyclooxygenase (COX) which catalyzes the production of strong inflammatory mediators including thromboxane A₂. To understand better the role of COX we measured the antiplatelet effects of the COX-2 inhibitors nimesulide and celecoxib in hypercholesterolemic rabbits.

Material and Method: We studied rabbits that consumed a normal diet and three groups maintained on a high cholesterol diet that received saline, nimesulide (25 mg/kg) or celecoxib (25 mg/kg) for 20 weeks. Blood was obtained from each rabbit every two weeks for measurement of lipid profile by spectrophotometric assay and platelet

aggregation-induced by arachidonic acid (AA) and platelet activating factor (PAF), was measured in a Lumi aggregometer.

Results: Blood obtained from rabbits fed the high cholesterol diet and receiving only saline treatment showed hyper-aggregation to both AA and PAF, suggesting that hypercholesterolemia induced pro-aggregatory environment in the blood. Nimesulide showed more than 75% and celecoxib more than 37% inhibition of platelet aggregation induced by both AA and PAF in the two test groups of hypercholesterolemic rabbits.

Conclusion: We conclude that the COX inhibitors nimesulide and celecoxib decreased platelet aggregation in hypercholesterolemic rabbits.

Keywords: Platelets, hypercholesterolemia, cyclooxygenase-2, platelet activating factor, arachidonic acid, inflammation. Nobel Med 2017; 13(1): 48-53

DENEYSSEL HİPERKOLESTEROLEMİK TAVŞANLARDA BAZI SİKLOOKSİJENAZ İNHİBİTÖRLERİNİN ANTİTROMBOTİK ETKİLERİ

ÖZET

Amaç: İnflamatuar bir hastalık olan hiperkolesterolemi, aterosklerozun önde gelen nedenidir. Hiperkolesterolemide trombosit agregasyonunda artış gözlenir ve bunda tromboksan A₂'de dahil olmak üzere güçlü inflammatuar mediatörlerinin üretimini katalize eden siklooksijenaz (COX) rol oynayabilir. COX'un rolünü daha iyi anlayabilmek için hiperkolesterolemik tavşanlarda COX-2 inhibitörleri nimesulid ve selekoksibin anti-trombotik etkilerini ölçtük.

Materyal ve Metot: 20 hafta boyunca normal diyet verilen ve tuz, nimesulid (25 mg/kg) veya selekoksib (25 mg/kg) uygulanan 3 grup tavşanla çalışma yaptık. Her tavşandan iki haftada bir kan örneği alınarak spektrofotometrik olarak lipid profiline bakıldı

ve Lumi agregometrede araşidonik asit (AA) ve trombosit aktive edici faktör (PAF) kaynaklı trombosit agregasyonu ölçüldü.

Bulgular: Yüksek kolestrol diyeti ve tuz diyeti yapılan tavşanlardan alınan kanda hem AA hem de PAF'de hiper-agregasyon görülmüştür ki bu hiperkolesteroleminin kanda pro-agregasyonu indüklediğini düşündürmektedir. Bu iki nimesulid ve selekoksib uygulanan hiperkolesterolemik test grubu tavşanlarda, nimesulidin % 75'den fazla, selekoksibin % 37'den fazla AA ve PAF'ın neden olduğu trombosit agregasyonu inhibisyonunu artırdığı gösterilmiştir.

Sonuç: COX inhibitörleri nimesulid ve selekoksibin hiperkolesterolemik tavşanlarda trombosit agregasyonunu azalttığı sonucuna varılmıştır.

Anahtar kelimeler: Trombositler, hiperkolesterolemi, siklooksijenaz-2, trombosit aktive edici faktör, araşidonik asit, inflamasyon. Nobel Med 2017; 13(1): 48-53

INTRODUCTION

Atherosclerosis is an inflammatory disease and leading cause of death from cardiovascular diseases.¹ Development of atherosclerosis is frequently associated with hypercholesterolemia and persistent activation of platelets.^{2,3} Formation of lesions in blood vessels during atherosclerosis is also thought to be initiated by adhesion of platelets and promoted by the migration of monocytes, exposed collagen and denuded or modified endothelium.⁴ Once platelets adhere to the modified endothelium, they become activated and release chemotactic factors, which in association with thrombin cause further migration of monocytes and proliferation of smooth muscle cells.⁵ The thrombosis and unstable plaque, as a result of advanced atherosclerotic lesions, can lead to myocardial infarction and coronary artery diseases.^{4,6,7}

Platelets not only protect against spontaneous hemorrhage, but are also crucial in maintaining vascular integrity, however when activated, they recruit more platelets in the vessel wall and establishing a vicious circle. After activation, platelets express glycoprotein IIb/IIIa receptors whose important role in platelet aggregation is indicated by the protection provided by glycoprotein IIb/IIIa receptors among others and play important role in platelet aggregation as indicated by the fact that glycoprotein IIb/IIIa receptors antagonists protect from platelet aggregation during myocardial infarction.⁸ More importantly activated platelets release free arachidonic acid (AA) which plays a key role in inflammatory reactions; Cyclooxygenases (COX-1 and COX-2) catalyze the conversion of AA into prostanoids,

leading to the formation of prostaglandin and thromboxane mediators.⁹ One of the prostaglandins-thromboxane A (TXA2) is a potent platelet agonist.¹⁰

Under normal physiological conditions COX-2 is not expressed in platelets. However, it is expressed in various inflammatory cells that constitute initial atherosclerotic lesions and in plaques.^{11,12} This makes it likely that platelets activated by hypercholesterolemia express COX-2 and that inhibition of this enzyme would be important is minimizing the risk of thrombus formation and platelet aggregation. Recent studies have in fact shown the beneficial effects of COX-2 inhibitors in myocardial infarction and hypercholesterolemia.¹³⁻¹⁵ Therefore, in the present study we used two COX-2 inhibitors nimesulide and celecoxib to investigate if they can inhibit platelet aggregation during diet-induced hypercholesterolemia in rabbits.

MATERIAL AND METHOD

Experimental hypercholesterolemia was developed in rabbits using high-cholesterol diet.¹⁴ Animals selected for this study were New Zealand white rabbits with a mean weight of 2.150±0.335 kg and a mean age of 12 ±2 weeks. All the animals used in this study were kept in the animal unit of Kohat University of Science and Technology for one week without any experimentation to acclimatize. Four randomly selected groups of rabbits were used with 10 rabbits in each group. These groups were, 1) control group having normal healthy rabbits and received saline pre-treatment, 2) saline group having fed high cholesterol diet and received saline pre-treatment, 3) nimesulide group having fed high cholesterol diet and

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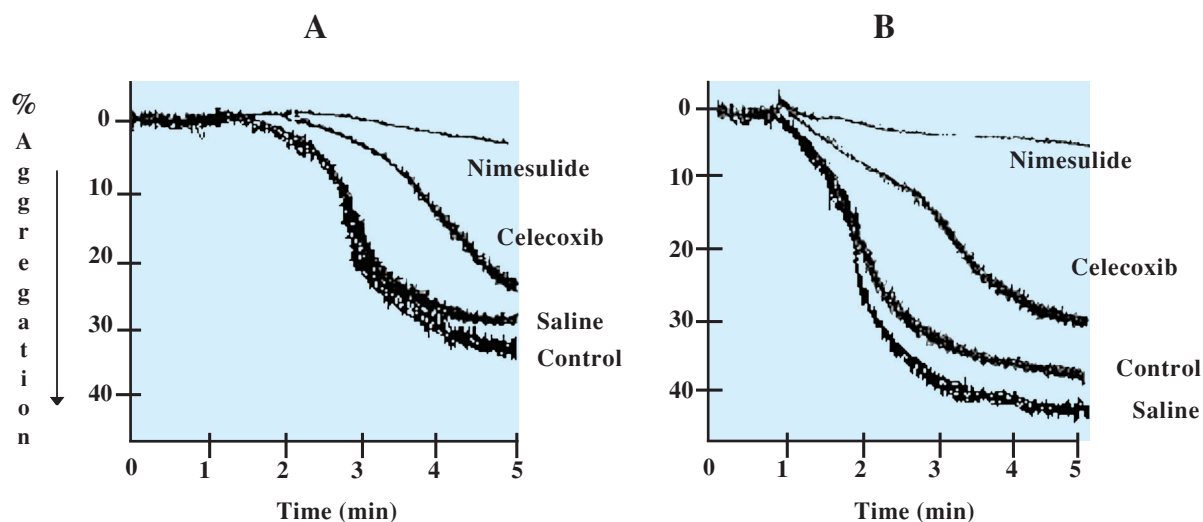


Figure 1. Representative tracings showing inhibition of platelet aggregation in blood obtained from rabbits from different groups against AA at week 0 (A) and week 2 (B). Control is aggregation curve obtained by adding AA (1.8mM) and was taken as 100% aggregation. AA: Arachidonic acid

Table 1. Lipid profile of rabbits during hypercholesterolemia. All the values are expressed in mM and as mean (SD). Number of rabbits (n) is 10 for each group.				
Group	Week	Total Cholesterol	HDL Cholesterol	LDL Cholesterol
Control	0	5.11(0.18)	0.73(0.12)	5.13(0.78)
	10	5.39(0.32)	0.76(0.23)	5.10(1.21)
	20	5.52(0.37)	0.75(0.19)	5.19(0.98)
Saline	0	5.07(0.20)	0.72(0.16)	5.02(0.91)
	10	*65.23(15.34)	0.52(0.21)	*63.32(11.65)
	20	*75.36(16.45)	*0.48(0.12)	*70.67(14.32)
Nimesulide	0	5.09(0.24)	0.71(0.15)	4.98(1.01)
	10	#48.12(13.56)	#0.87(0.26)	#42.61(8.61)
	20	#41.35(10.33)	#0.83(0.23)	#26.51(5.67)
Celecoxib	0	5.14(0.35)	0.74(0.14)	5.12(0.87)
	10	*55.83(11.52)	#0.82(0.17)	#40.32(12.66)
	20	#49.82(12.38)	#0.80(0.16)	#24.71(4.17)

*: $p < 0.05$ compared to control group, #: $p < 0.05$ compared to saline group, SD: standard deviation

received nimesulide and 4) celecoxib group having fed high cholesterol diet and received celecoxib. Group 3 was administered nimesulide (25 mg/kg), group 4 was given celecoxib (25 mg/kg) while the group 1 and 2 were given saline throughout week 0 to week 20. The doses of COX-2 inhibitors used in this study were selected based on their use in humans and were interpolated to rabbits.

All rabbits had free access to food and water. All experiments were conducted within 2-3 hours of collection of blood from the marginal ear vein of rabbits. Blood specimen were obtained from rabbits at two-week intervals, starting at week 0 till week 20. All experiments on rabbits were conducted according to ethical guidelines as provided by National Institute of Health, USA. Prior

ethical approval for the study was obtained in this regard from Kohat University of Science & Technology, Kohat, Pakistan. High cholesterol diet was produced in-house and consists of nutritive L, choker, salt, cholic acid butter fat, powdered milk, potassium meta-bisulphate 1, molasses, oil, cholesterol, fish meal.

Total Cholesterol

This assay is based on the production of quinoneimine which acts as indicator and is produced by the action of 4-aminophenazone, hydrogen peroxide, 4-chlorophenol and peroxidase. The assay was conducted as per instructions of the manufacturer and total cholesterol was measured by oxidation and enzymatic hydrolysis method as described previously.¹⁴

HDL-Cholesterol

Determination of HDL cholesterol was based on the precipitation of LDL and VLDL and measuring the remaining fraction of cholesterol and as described previously.¹⁴ The kit was purchased from RANDOX RANDOX (Cat; CH204) and assay was performed according to the manufacturer's instructions. In the presence of magnesium ions, phosphotungstic acid was used to quantitatively precipitate VLDL and LDL as well as chylomicrons. HDL fraction was found in the supernatant after centrifugation and determined quantitatively.

LDL-Cholesterol

Basically the principles of centrifugation and precipitation at the isoelectric point were used to determine LDL concentrations in the blood and as described previously.¹⁵ The assay was performed according to the manufacturer's instruction (RANDOX, UK, Cat: CH9702). In brief, heparin was used to precipitate LDL

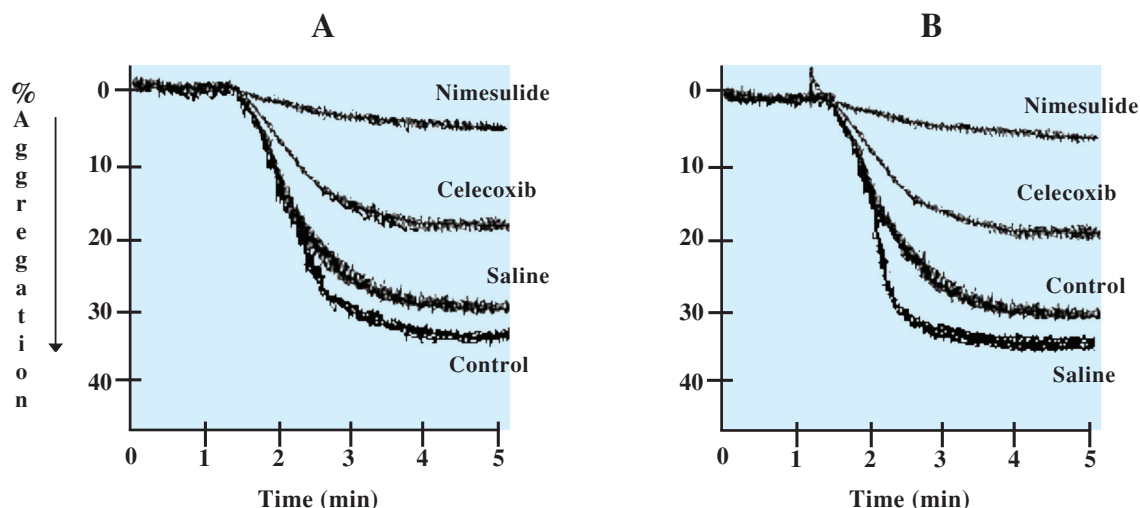


Figure 2. Representative tracings showing inhibition of platelet aggregation in blood obtained from rabbits from different groups against PAF at week 0 (A) and week 2 (B). Control is aggregation curve obtained by adding PAF (0.8µM) and was taken as 100% aggregation.

PAF: Platelet activating factor

at their isoelectric pH (5.04). Centrifugation (Fisher Scientific, Loughborough, UK) was done to separate various fractions. LDL was determined by subtracting cholesterol in the supernatant from the total cholesterol.

Platelet Aggregation

Blood from rabbits of various groups was collected from the marginal ear vein and mixed with anticoagulant sodium citrate in a ratio of 9 to 1.¹⁶ Platelet rich plasma (PRP) was obtained by centrifugation of blood at 20°C for 15 minutes at 260 g. Platelet poor plasma (PPP) was obtained by centrifuging the remaining blood for 10 minutes at 1200 g. Phase contrast microscopy was used to measure the platelet count. All experimentation with platelets was done at 37°C with platelet count was within 2.5 and 3.0 x 10⁸ mL⁻¹ of plasma.¹⁷⁻¹⁹

Platelet aggregation was measured with 450 µL of PRP in a dual channel Lumi aggregometer (Model 400, Chronolog Corporations Chicago, USA). Aggregating agent was added in enough volume to make up the total to 500 µL. The concentration of AA and PAF used to produce aggregation was 1.8 mM and 0.8 µM respectively.²⁰⁻²² After addition of AA or PAF, inhibitory effect of nimesulide and saline pretreatment was monitored. The effect of nimesulide on AA and PAF-induced platelet aggregation in the blood of normal rabbits was also observed. The aggregation or lack of it was measured as a function of time as indicated by a change in transmission of light, for 5 minutes.

Statistical Calculations

All the experiments were carried out in triplicate and data were statistically analysed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. $p < 0.05$ was considered significant.

Table 2. Percent inhibition of platelet aggregation induced by AA and PAF in blood obtained from rabbits in different groups (nimesulide, celecoxib, saline) at week 0-20. All values are expressed as percentage inhibition and as mean±SD. Number of rabbits (n) is 10 for each group.

Week	AA			PAF		
	Nimesulide	Celecoxib	Saline	Nimesulide	Celecoxib	Saline
0	*81±8	*21±4	10±3	*76±7	*25±4	12±3
2	*85±9	*38±3	2±2	*75±8	*40±6	3±2
4	*88±7	*37±4	-3±2	*77±6	*41±5	5±2
6	*90±8	*40±5	-4±3	*78±6	*39±6	-5±3
8	*92±9	*39±4	-2±2	*80±9	*39±4	-3±2
10	*91±6	*36±4	-5±3	*78±10	*40±7	-4±2
12	*90±8	*37±6	-6±2	*77±8	*38±4	-4±2
14	*86±7	*36±5	-2±2	*80±7	*39±5	-2±2
16	*85±9	*38±4	-2±2	*80±9	*38±6	-4±3
18	*84±8	*39±3	-4±3	*80±9	*41±6	-2±1
20	*85±8	*40±4	-3±2	*81±7	*40±7	-3±2

PAF: Platelet activating factor, AA: arachidonic acid, *: $p < 0.05$ compared to saline group, SD: standard deviation

RESULTS

Lipid profile of the four groups is shown in the Table 1. Table shows increase in the total cholesterol and LDL while a decrease in the HDL was observed in all groups after feeding them on high cholesterol diet. The hypolipidemic effects of nimesulide and celecoxib observed in our study were consistent with previous studies.^{14,15}

Nimesulide showed more than 75% and celecoxib more than 20% inhibition of platelet aggregation induced by both AA and PAF when aggregation was monitored in the blood of normal rabbits without hypercholesterolemia (Figure 1 and 2). However, at week 2 after high cholesterol

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feeding, nimesulide group showed 86% inhibition while celecoxib group showed only 38% inhibition against AA-induced platelet aggregation (Figure 1). Platelet aggregation prompted by the PAF was potently inhibited by nimesulide (75%) while celecoxib was able to show only partial block (Figure 2). This indicates that while both nimesulide and celecoxib were effective against AA and PAF-induced aggregation, the effect of nimesulide was more potent than celecoxib. However, compared to the effect of celecoxib on normal rabbits, their inhibitory effect on platelet aggregation induced by both AA and PAF had increased significantly after the induction of hypercholesterolemia. While there was no difference between the effects of celecoxib against AA or PAF at week 2, nimesulide displayed slightly more inhibition against AA-induced aggregation compared to PAF.

The potency of antiplatelet effect of nimesulide against AA-induced platelet aggregation increased as rabbits were continued to fed on high cholesterol diet and the inhibition reached more than 90% at week 8 and 10 after which the antiplatelet effect of nimesulide came down to 85% at week 20 (Table 2). The antiplatelet effect of celecoxib against AA-induced platelet aggregation remained stable at around 40% throughout week 2-20. Similarly, antiplatelet effect of nimesulide against PAF-induced platelet aggregation reached to 80% at week 8 and then remained roughly at that level throughout the remaining experimental period. The antiplatelet effect of celecoxib against PAF-induced platelet aggregation, just like its effect against AA-induced platelet aggregation, remained stable at roughly 40% throughout the study (Table 2 and Figure 2).

Rabbits fed with high cholesterol diet receiving only saline did not show inhibition against either AA or PAF-induced platelet aggregation. Instead they displayed negative inhibition which indicates that their blood appeared to be more susceptible to the platelet agonists (Table 2). This means that barring first 4 weeks, the blood obtained from hypercholesterolemic rabbits was more pro-aggregatory to both AA and PAF compared to the blood of the normal rabbits.

DISCUSSION

Inflammation in the ischemic heart is significantly contributed by PGs following the expression of COX-2 in ischemic myocardium.²³ Other pro-inflammatory molecules including various oxygen radicals and cytokines also induce COX-2, and both human and animal model show the presence of COX-2 in atherosclerosis.^{11,24,25} In patients with coronary artery disease, administration of celecoxib was associated with reduction in oxidative stress and inflammation as well as improving endothelial function.²⁶ Human atherosclerotic lesions are found to express COX-2 as well as the major cells involved in the atherogenesis including smooth muscle cells, endothelial

cells and macrophages.¹¹ These findings lead us to hypothesize that anti-inflammatory and antioxidant properties of selective COX-2 inhibitors may be helpful in preventing atherogenesis. Since hypercholesterolemia is a strong instigator of atherosclerosis, we monitored the platelet aggregation in the cholesterol rich environment of hypercholesterolemia. Working on this hypothesis, our present study investigated the effects of nimesulide and celecoxib on platelet aggregation by physiologically relevant platelet agonists, PAF and AA in the blood of the rabbits on diet-induced hypercholesterolemia. The results indicate that nimesulide is a potent inhibitor of AA and PAF-induced platelet aggregation while celecoxib showed only partial inhibition to both AA and PAF in the plasma of these rabbits. This is the first study which demonstrates the inhibitory potential of nimesulide against platelet aggregation in rabbits with diet-induced hypercholesterolemia.

Although pretreatment with both nimesulide and celecoxib resulted in the inhibition of AA as well as PAF-induced platelet aggregation, the magnitude of the response was different for two COX-2 inhibitors. Nimesulide was more potent, achieving more than 90% inhibition compared to celecoxib which could show a maximum of 40% inhibition against AA-induced platelet aggregation. Similarly, nimesulide pretreatment was more effective than celecoxib pretreatment against PAF-induced platelet aggregation. This pattern of effects was similar to that observed in some previous studies.^{14,15} While the effect of celecoxib against both platelet agonists was stable (about 40%) throughout the experimental period, the magnitude of nimesulide effect against AA reached at maximum at week 8 and 10 and then tapered off to the level of week 2. The effect of nimesulide against PAF reached maximum level at week 8 and 10 and then it remained roughly at that level for the remainder of the study.

There is considerable literature showing adverse effects of NSAIDs including COX-2 inhibitors. A recent review indicates that most of the NSAIDs are associated with adverse cardiovascular events.²⁷ Some of the NSAIDs cause adverse effects independent of the dose while for others, their cardiovascular adverse effects increase with increasing the dose. Furthermore, most of the cardiovascular events are associated with long term use of these drugs but when used for shorter durations and with lower doses, these NSAIDs have significantly low adverse effects.²⁷ When used in healthy individuals, COX-2 inhibitors do not affect endothelial function negatively.²⁸ It is reported that celecoxib is associated with increasing coronary vasodilation in cardiovascular patients.²⁶ Although, the drugs we used in this study (nimesulide and celecoxib) are relatively safer, one should be still careful when using these COX-2 inhibitors. On the other hand, COX-2 itself is shown to mediate the

cardio-protection through ischemic preconditioning in rabbits.²⁹ This, however, seems to be mediated by COX-2 products, PGE₂ or PGI₂ as revealed by the analysis of that study.²⁹ Although, platelets do produce PGE₂, production of PGI₂ is not reported in platelets.³⁰ Therefore, it is possible that TXA₂ produced through COX-2 cause platelet aggregation while PGI₂ is involved in late phase of ischemic preconditioning of rabbit heart.

Although the mechanism of observed antiplatelet function of COX-2 inhibitors especially that of nimesulide, was not investigated in the current study, previously reported COX dependent and independent properties of nimesulide may explain their antiplatelet actions. Previous studies suggest that nimesulide decreases PKC activity and Ca⁺⁺

influx, inhibits PAF and TXA₂, while enhances the levels of cytosolic cAMP.^{13,31,32} All these effects, independent of the COX-2 inhibitory actions of nimesulide, have the ability to inhibit platelet aggregation. Furthermore, at low substrate concentrations, nimesulide is also shown to possess significant COX-1 inhibitory potential.³³ In conclusion, nimesulide and celecoxib albeit less potently inhibits platelet aggregation in experimentally-induced hypercholesterolemia in rabbits and this effect may be dependent or independent of its COX-2 inhibitory activity.

*The authors declare that there are no conflicts of interest.



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