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Anti-inflammatory Effects of Cyclooxygenase-2 Inhibitors in Rabbits

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Abstract: Hypercholesterolemia is a major risk factor for atherosclerosis while homocysteine and C reactive protein are systemic inflammatory markers elevated in hypercholesterolemia and are by themselves, strong and independent risk factors for developing atherosclerosis. The main objective of this investigation was to determine the effects of cyclooxygenase-2 inhibitors, nimesulide and celecoxib on homocysteine and C reactive protein during experimentally induced hypercholesterolemia in rabbits. Rabbits were divided into four groups; group 1 served as control and consist of normal rabbits that received saline, group 2 consisted of rabbits that were fed high cholesterol diet and received saline, group 3 consisted of rabbits fed with high cholesterol diet that received nimesulide (25 mg/kg) while group 4 consisted of rabbits fed with high cholesterol diet and received celecoxib (25 mg/kg). Rabbits were fed with high cholesterol diet for twenty (20) weeks while their blood was withdrawn every two week for measuring lipid profile and inflammatory biomarkers. Our study indicates that hypercholesterolemia results in elevation of homocysteine and C reactive protein while pretreatment with nimesulide was more effective than celecoxib in bringing down their concentrations to the baseline. Further investigation shows that these effects of cyclooxygenase 2 inhibitors may be mediated through changes in the activity of paraoxonase 1 and concentration of high density lipoprotein. We conclude that use of cyclooxygenase 2 inhibitors, nimesulide and celecoxib can lower C reactive protein and homocysteine levels in diet-induced hypercholesterolemia in rabbits.

Key words: C reactive protein, celecoxib, homocysteine, hypercholesterolemia, nimesulide, paraoxonase 1.

INTRODUCTION

Atherosclerosis, leading to myocardial infarction and stroke, is one of the most important causes of death due to cardiovascular diseases (Modelli *et al.*, 2011). The association of hypercholesterolemia with atherosclerosis is well known. Since atherosclerosis is an inflammatory disease, two of the best known inflammatory markers associated with this disease are homocysteine and C reactive protein (CRP) (Yilmaz *et al.*, 2006). Elevated plasma concentration of CRP and homocysteine are also found to be significantly common in participants categorized as at high 10-year risk for coronary artery disease (Park *et al.*, 2010).

There is significant evidence that both homocysteine and CRP contribute to the atherosclerotic process. Monocytes are found to present in the intima of arteries after increase in the serum CRP levels. Enhanced serum CRP levels increase risk of cardiovascular events and are associated with plaque instabilit. Increased circulatory levels of CRP also enhance the induction of other inflammatory mediators including chemokines and adhesion molecules (Pasceri *et al.*, 2000, 2001). Elevation in the CRP also causes an increase in the uptake of low density protein (LDL) by macrophages and monocytes. Similar findings are also observed with homocysteine on cardiovascular health. Homocysteine enhances the affinity of inflammatory mediators to fibrin, weakens the antioxidant defence by reducing glutathione peroxidase activity and increases the oxidation of lipoproteins (Lentz, 1997; Suliman *et al.*, 2005; Riaz *et al*., 2013).

Cyclooxygenase (COX) which produces prostaglandins (PGs) is one of the most important enzymes implicated in inflammation and exists in two isoforms a house keeping enzyme COX1 and an inflammation-inducible COX-2 enzyme. These PGs are some of the most important mediators of

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inflammation. There is also evidence that PGs can affect homocysteine and CRP (Sanchez-Moreno *et al.,* 2003). Therefore, in the present study we investigated the effects of COX-2 inhibitors, nimesulide and celecoxib on homocysteine and CRP. Since hypercholesterolemia modulates serum PON1 activity (Beer *et al*., 2006), we further investigated the effect of inhibitors on PON1 activity and its relationship with homocysteine concentration.

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits with an average age of 12 weeks and average weight of 1.25-1.75 kg were selected for the study. These animals were divided into 4 groups of 12 rabbits each and were kept in the animal house for a minimum of 10 days to acclimatize before starting any experimentation. Three of the four groups were maintained on a high cholesterol diet while fourth group was fed on normal rabbit diet (control group). One of the three rabbits groups fed on high cholesterol diet received saline, second group received nimesulide and third group received celecoxib to make saline, nimesulide and celecoxib groups respectively.

Procedures

Nimesulide and celecoxib groups were subcutaneously injected with nimesulide and celecoxib respectively daily at doses (25 mg/kg) corresponding to those recommended for human while saline and control groups were injected with saline throughout 20 week period. During that period blood was regularly collected every two weeks for measuring homocysteine, CRP, PON1 and lipid profile. Ethical approval for the study was obtained from the Animal Committee of Kohat University of Science & Technology, Pakistan. Al animals were treated with utmost care in line with recommendations made by National Institute of Health USA, for the care of such animals.

Lipid profile

Lipid profile including low density lipoproteins (LDL), high density lipoproteins (HDL) and total cholesterol concentrations were assayed as mentioned in methods described previously (Ahmed *et al.,* 2014; Aslam *et al.,* 2008) utilizing ready to use kits and bench top spectrophotometer.

CRP

An enzyme-linked immunosorbent assay (ELISA) was used to measure CRP levels in the blood of the rabbits as described previously (Kim *et al.,* 2004). Test samples and their appropriate dilutions were incubated with the strips that were coated with anti-CRP antibodies. Same was done with standard solutions in place of test plasma. Excess plasma proteins that did not react with anti-CRP antibodies were washed away. The antigen-antibody complex was treated with the secondary antibodies that were designed against the primary anti-CRP antibodies. A specific coloring agent, chromogen solution was used to produce a color change. The change in the absorbance was measured at 450 nm. Plotting the different values of change in absorbance against standards yielded a standard curve. The CRP values were determined in relation to that standard curve.

Homocysteine

Homocysteine levels in the plasma of rabbits were measured using high performance liquid chromatography (HPLC) by following method described previously (Atanassova *et al.,* 2007). Blood samples obtained from the marginal ear vein of the rabbits were collected in EDTA tubes and were preserved for one hour on ice. The plasma was obtained by centrifuging the blood at 4000 rpm. This plasma, after separation from the blood cells, was analyzed within two hours of collection while the remaining plasma was stored at -20ºC. The homocysteine concentrations were measured using fluorescence detector system with the HPLC and expressed as µmol/L.

PON1

Activity of PON1 was determined in the blood of the rabbits by following procedure described previously (Furlong *et al.,* 2006). This method utilizes $CaCl_2$, NaCl, assay buffer, substrate and fresh plasma. The substrate used in this assay was paraoxon and was prepared in acetone at a

concentration of 120 mM. This stock remains stable for three weeks and has to be diluted 20 times for using in the assay. A change in the absorbance at 405 nm is calculated after the addition of substrate to the assay mix to which all other components including the plasma have been added previously. This change in the absorbance is used along with a molar extinction coefficient of 18.05×10^3 to calculate the activity of PON1.

Statistical analysis

Mean values in different groups were compared using one-way analysis of variance (ANOVA) followed by posthoc tests. The difference between the means was considered statistically significant when $p < 0.05$.

RESULTS

Lipid profile

There was no significant change in total cholesterol levels in control group throughout the study while in saline group total cholesterol increased significantly to 67.22±9.83 mM at week 20 (Table I). In nimesulide group, total cholesterol level increased to 41.55±7.61 mM and in celecoxib group to 45.09±10.77 mM at week 20. In saline group, HDL cholesterol decreased to 0.49±0.12mM at week 20, in nimesulide and celecoxib group, it increased to 0.84±0.20mM and 0.83±0.18 mM respectively at week 20. LDL cholesterol increased in the saline group to 53.09±9.67 mM at week 20 and to 3.82±10.10 mM and 28.52±4.62 mM at week 20 in nimesulide and celecoxib groups respectively (table 1). However, this increase in LDL-cholesterol in nimesulide and celecoxib groups was significantly lower ($p<0.05$) compared to the increase in the saline group at week 20. All the results are expressed as mean ± S.D.

CRP

There was no significant increase in CRP in control group that consisted of rabbits fed with normal diet and received only saline treatment (Table II). In saline group- fed with high cholesterol diet and administered with saline, CRP level started to raise as early as 2nd weak reaching a value of 1.600±0.360 mg/dL at week 12 and remained roughly at that level till week 20 (1.660±0.45 mg/dL). In nimesulide group- rabbits fed with high cholesterol diet and receiving nimesulide, CRP started to increase from 2nd weak reaching peak value at week 14 and remained roughly at that level till week 20. Nevertheless, there was significant difference ($p<0.05$) between CRP levels at week 20 between the saline and nimesulide group. In celecoxib group-rabbits fed with high cholesterol diet and received celecoxib, CRP levels increased very early during the experimental period, just like saline and nimesulide groups. Although CRP level increased to maximum in this group from week 16-20, it was still significantly lower than saline group at the corresponding weeks (Table II).

Homocysteine

There was slight but non-significant increase in homocysteine in control group (Table III). In saline group- fed with high cholesterol diet and administered with saline, homocysteine level started to raise quickly as early as 2nd weak reaching a value of 46±5.2 μmol/L at week 12 and remained roughly at that level till week 20 (47±5.8 μmol/L). In nimesulide group- rabbits fed with high cholesterol diet and receiving nimesulide, increased sharply from weak 4 reaching peak value at week 14 and remained roughly at that level before decreasing slightly at week 20 (29±3.8 μmol/L). However, there was significant difference ($p<0.05$) between homocysteine levels at week 20 between the saline and nimesulide group. In celecoxib group-rabbits fed with high cholesterol diet and received celecoxib, homocysteine levels increased rapidly at week 4, just like nimesulide group. Although homocysteine level increased to maximum in this group at week 12 where it remained till week 20, it was still significantly lower than saline group at the corresponding weeks (Table III).

PON1

There was no significant increase or decrease in PON1 activity observed in control group (Table IV). In saline group- fed with high cholesterol diet and administered with saline, PON1 activity deceased rapidly at week 2 and 4 after which it decreased steadily till week 10. PON1 activity increased slightly in the 2nd half of the study period

Table I.- Lipid profile of rabbits during hypercholesterolemia. All the values are expressed in mM.

Group	n	Week	Total cholesterol	HDL cholesterol	LDL cholesterol
Control	12	0	5.77±1.10	0.69±0.19	5.11±1.02
	11	20	5.95±1.23	0.72±0.15	5.42±0.66
Saline	12	0	5.62± 0.85	0.71±0.19	5.08±0.90
	10	20	67.22±9.83*	0.49±0.12*	53.09±9.67*
Nimesulide	12	0	5.57± 0.72	0.72±0.14	5.32±0.87
	10	20	41.55±7.61#	0.84±0.20#	27.71±3.82#
Celecoxib	12	0	5.92±0.79	0.71±0.12	5.41±0.69)
	11	20	45.09±10.77#	0.83±0.18#	28.52±4.62#

*p< 0.05 compared to control group, #p<0.05 compared to saline group.

Table II.- CRP levels (mg/dL; Mean±SD) of rabbits in normal, saline, nimesulide and celecoxib groups during different weeks of experimental period.

Weeks	n	Control	Saline	Nimesulide	Celecoxib
0	12	0.14±0.02	0.15±0.03	0.15±0.02	0.15±0.02
2	12	0.12±0.03	0.47±0.14	0.24±0.04*	0.30±0.05*
4	12	0.08±0.04	0.80±0.18	0.27±0.05*	0.34±0.04*
6	12	0.15±0.02	0.90±0.25	0.33±0.07*	0.32±0.06*
8	11	0.16±0.04	1.20±0.35	0.27±0.03*	0.35±0.05*
10	11	0.17±0.03	1.40±0.46	0.34±0.06*	0.37±0.06*
12	11	0.20±0.03	1.60±0.36	0.35±0.07*	0.46±0.08*
14	10	0.15±0.03	1.62±0.36	0.36±0.06*	0.48±0.07*
16	10	0.14±0.04	1.63±0.38	0.34±0.06*	0.54±0.07*
18	10	0.16±0.03	1.65±0.47	0.36±0.05*	0.53±0.08*
20	10	0.17±0.02	1.66±0.45	0.35±0.06*	0.54±0.07*

* represents p<0.05 compared to saline group.

finishing at 268±20 μM/Min at week 20. In nimesulide group- rabbits fed with high cholesterol diet and receiving nimesulide, PON1 increased rapidly at 2nd weak remaining roughly at that level throughout the remaining study period. There was significant difference (p<0.05) between PON1 activity at week 20 between the saline and nimesulide group. In celecoxib group-rabbits fed with high cholesterol diet and received celecoxib, PON1 activity increased very early at week 2 during the experimental period, and continued to increase steadily throughout remaining experimental period finishing at 347±14 μM/Min at week 20. As PON1 activity increased in this group, a significant difference was observed between celecoxib group compared to saline group at the corresponding weeks (Table IV).

DISCUSSION

Consistent with the previous studies, there was significant increase in CRP and homocysteine levels in rabbits after feeding them with cholesterol rich diet. However, this increase in the CRP and homocysteine is neutralized considerably in rabbits pretreated with nimesulide and celecoxib. A decrease in the levels of these inflammatory markers in both the groups pretreated with COX-2 inhibitors suggests a mitigating effect by these anti-inflammatory drugs. The effect started as early as 2nd week and was observed throughout most of the experimental period. Such effects were not observed in saline-treated hypercholesterolemic animals. Therefore, our results suggest strong attenuating effect of nimesulide and celecoxib on CRP and

Table III.- Homocysteine levels (µmol/L; Mean±SD) of rabbits in normal, saline, nimesulide and celecoxib groups at different weeks of the experimental period.

Weeks	n	Control	Saline	Nimesulide	Celecoxib
0	12	14±2.2	15±3.1	15±2.5	15±2.6
2	12	15±2.7	20±3.2	18±2.6	17±2.4
4	12	16±4.6	34±4.2	23±3.6*	24±3.8*
6	12	17±3.2	38±4.5	25±3.8*	26±4.2*
8	11	18±3.6	40±4.2	27±3.8*	30±3.8*
10	11	18±3.9	42±5.3	28±4.0*	32±5.1*
12	11	20±4.3	46±5.2	27±4.1*	34±5.0*
14	10	21±4.2	46±5.6	31±4.5*	33±4.9*
16	10	20±3.6	46±5.3	31±4.3*	33±5.3*
18	10	21±3.9	47±5.9	30±4.6*	34±5.2*
20	10	22±4.0	47±5.8	29±3.8*	34±5.3*

* represents $p<0.05$ compared to saline group.

Table IV.- PON1 activity (µM/min; Mean±SD) of rabbits in normal, saline, nimesulide and celecoxib groups at different weeks of the experimental period.

Weeks	n	Control	Saline	Nimesulide	Celecoxib
0	12	298±12	290±14	303±18	299±18
2	12	295±13	278±15	319±14*	321±16*
4	12	294±14	266±18	319±21*	320±19*
6	12	297±20	260±14	317±20*	323±13*
8	11	297±18	262±21	321±18*	334±14*
10	11	299±14	259±20	317±12*	337±20*
12	11	296±21	263±18	316±13*	345±12*
14	10	297±15	260±14	310±14*	346±13*
16	10	299±18	263±21	318±20*	344±20*
18	10	297±16	266±15	320±12*	346±18*
20	10	294±19	268±20	317±13*	347±14*

In the table above, PON1 activity measured in µM/Min at the given week; SD is standard deviation while n refers to the number of animals in each group at a given week. * represents $p<0.05$ compared to saline group.

homocysteine in rabbits over a period of 20 weeks.

Previous studies on the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on CRP have produced results that are not consistent. In patients with stable angina, a reduction in CRP level was observed after the administration of Aspirin (Ikonomidis *et al.,* 1999) while in healthy individuals, Aspirin had no effect on CRP levels (Feldman *et al.,* 2001). Administration of celecoxib was found to decrease CRP levels in patients with stable coronary artery disease but rofecoxib administration was associated with no such effect (Chenevard *et al.,* 2003). However, these studies measured CRP levels on single or couple of time points while our study was relatively long and measured CRP levels at multiple time points during a 20 week period. The baseline CRP values were also higher in our studies as hypercholesterolemia produced significant elevation of CRP. Our results which suggest attenuating effects of nimesulide and celecoxib on CRP do not provide mechanistic understanding as how inhibition of COX-2 would affect CRP. However, one mechanism that can be put forward is a reduction in PGs as suggested by a previous study (Sanchez-Moreno *et al.,* 2003). The study shows that reduction in CRP after orange juice consumption is associated with decrease in PGE_2. This may explain why COX-2 inhibitors decreased CRP levels in this study.

NSAIDs (aspirin and salicylic acid), and statins such as atorvastatin has shown inhibitory effects on plasma homocysteine levels but compared

to the effects observed with nimesulide and celecoxib in the present study, their doses were much lower. The doses required by nimesulide and celecoxib to down regulate plasma homocysteine levels were about 100 times higher than the doses required by statins and reseveratrol (in micromolar range) to show comparable effects (Schroecksnadel *et al.,* 2005). It is likely that antioxidant nature of these compounds have added to their potency. The studies showing the effect of wine consumption on plasma homocysteine levels have been discordant just like the effects of Aspirin on CRP levels. There are studies which suggest lowering of homocysteine levels after wine consumption (Dixon *et al.,* 2002), others show increase in homocysteine concentrations (Mennen *et al.,* 2003), while some observe no substantial increase or decrease (Ganji and Kafai, 2003.). These effects of COX-2 inhibitors and other compounds on plasma homocysteine levels can reduce the potential harmful effects of hyperhomocysteinemia.

The effects of COX-2 inhibitors in reducing homocysteine levels in our study may be explained by enhanced activity of PON1-an enzyme which can detoxify homocysteine. PON1 is an antioxidant enzyme primarily secreted by the liver and is found in the blood attached to HDL (Deakin *et al.,* 2002). Association studies also link PON1 deficiency with the vulnerability to cardiovascular diseases (Costa *et al.,*2003). Antioxidant effects of HDL mediated through PON1 include detoxification of oxidized phospholipids and thus protecting against cardiovascular diseases (Aviram *et al.,* 1998). Mice which have PON1 gene knocked in show a decrease in the lesions associated with atherosclerosis (Tward *et al.,*2002). However, mice were found to be more vulnerable to atherosclerosis and oxidation when PON1 was knocked out (Shih *et al.,*1998). Therefore, an increase in the activity of PON1 observed in the present study may be responsible for the low plasma homocysteine concentrations in rabbits. Celecoxib being more potent in enhancing PON1 activity but slightly less potent in lowering homocysteine concentration in the present study also suggests that mechanisms other than PON1 activity are also be responsible for decreasing homocysteine concentrations.

Increase in HDL levels may also account for elevation of PON1 activity observed in the study. Consumption of vitamins, pomegranate juice and red wine each is found associated with increased PON1 activity. A negative correlation between PON1 activity and plasma homocysteine is observed in age-related macular degeneration (Baskol *et al.,* 2006; Ikeda *et al.,* 2001), in Behcet disease patients (Mungan *et al*., 2006) and in children with autism (Pascal *et al.,* 2005). Previous studies have shown that COX-2 inhibitors can directly enhance antioxidant defense system during hypercholesterolemia by enhancing the activities of GPx and SOD or may act by increasing HDL concentrations. Therefore, it is likely that PON1 activity is increased in the present study in response to both increases in HDL as well as increase in antioxidant enzymes.

A recent review has summarized the clinical data related to the cardiovascular adverse effects of NSAIDs including COX-2 inhibitors. The review highlights that except for naproxen, long term use of NSAIDs is associated with cardiovascular adverse effects. These adverse effects are mostly observed with high doses and long term use of NSAIDs, however, with some NSAIDs, their cardiovascular adverse effects were found to be dose-independent (Varas-Lorenzo *et al.,* 2013). In healthy volunteers, though, endothelium dependent or independent vascular functions are not compromised (Verma *et al.,* 2001). Although nimesulide and celecoxib are relatively safer drugs at the doses used in this study, we are cautious in generalizing our findings to other COX-2 inhibitors.

CONCLUSIONS

We conclude that both nimesulide and celecoxib can lower CRP and homocysteine levels, though, it does not show a direct causal effect of COX-2 inhibitors on the inflammatory markers-homocysteine and CRP. It is, however, possible to have indirect effects through PON1 and HDL as indicated by our results. Prospective clinical studies may reveal whether nimesulide or celecoxib or both have positive effects on these inflammatory markers in patients with hypercholesterolemia. This would help such patients as they have enhanced profile of circulatory inflammatory markers and at the same

time deficient in both HDL and PON1. COX-2 inhibition seems to affect both of them.

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