The Effects Of Metformin And Pioglitazone Versus Their Low Dose Combination On Progression Of Atherosclerosis.

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الخلاصة

أجريت هذه الدراسة لتقييم تأثير عقار المتفورمين والبايوكليتازون على تصلب الشرايين.وأن خمسة وثلاثون ذكرا من الأرانب البيض (نيوزيلند)استخدمت في هذه الدراسة. هذه الحيوانات قسمت بشكل عشوائي الى خمسة مجاميع المجموعة الاولى أعطيت غذاء طبيعي قياسي طوال التجربة لمدة 10 أسابيع،واعتبرت مجموعة سيطرة (الغذاء الطبيعي). تم تغذية الشرايين في جميع المجاميع الأخرى بواسطة أعطائها غذاء عالي الدهون إعطيت الحيوانات في المجموعة الثانية غذاء عالي الدهون فقط واعتبرت بمثابة مجموعة سيطرة عالي الدهون)بالإضافة إلى الغذاء عالي الدهون كل من المجموعات الثلاثة الأخرى أعطيت واحدا من العقاقير التالية :متفورمين (المجموعة الثالثه) أوبايوكليتازون (المجموعة الرابعة) أومتفورمين وبايوكليتازون(المجموعة الخامسة)بت الراتب في النهاية وتم قياس صورة الدهن ، مؤشرات التخثر ، و مؤشرات الاكسدة إلفحص النسيجي للأبهر الرأسي . وتم التوصل إلى النتائج التالية مجموعة متفورمين وبايوكليتازون تسببت في انخفاض كبير في مؤشرات تصور الدم والتخثر قراءة كون بدن باقي المجاميع.

Abstract

Background: **Atherosclerotic diseases remain the leading cause of death in men and women world wide. In addition to lipid lowering, reduction of atherosclerotic disease will require further pharmacological approaches capable of modifying the disease.**

***Materials and methods*:** **35 rabbits were used in this study. Atherosclerosis was carried out by feeding the rabbits an atherogenic diet (AD). The rabbits were randomly assigned into five groups, 7 rabbits each. Group 1 (normal control) received standard chow diet. In addition to AD, the rabbits in group 2 (atherogenic control), group 3 (Met), group 4 (Pio) and group 5 (Met+Pio) received no treatment, metformin, pioglitazone and combination of low doses of metformin and pioglitazone respectively for 10 weeks. At the end of 10th week, all rabbits were sacrificed; serum lipids, MDA, GSH, hsCRP, plasma fibrinogen and aortic histological examination were determined.**

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Results: **Metformin and pioglitazone resulted in significant decrease ($p<0.01$) in AI. Further they caused significant ($p<0.01$) reduction in serum MDA, hsCRP and plasma fibrinogen.**
Serum GSH level was significantly ($p<0.05$) elevated by metformin. Combination treatment decreased AI, serum MDA, plasma fibrinogen and hsCRP, increased GSH and possessed more favorable histological findings better than either drug alone.
Conclusion: **low doses combination of Met+Pio has more favorable atheroprotective effect than either drug alone possibly due to the combined anti-inflammatory and antioxidant effects.**
Key words: **metformin, pioglitazone, atherosclerosis**

Introduction

Atherosclerotic diseases remain the leading cause of death in men and women world wide. Despite the consistent effects of lipoprotein lowering medications in reducing cardiovascular morbidity and mortality, the reductions in clinical events remain modest, ranging from 25% to 35% [(1)].
Thiazolidinediones (TZDs), such as troglitazone, rosiglitazone and pioglitazone, are a novel class of insulin-sensitizing agents. TZDs are pharmacological peroxisome proliferator-activated receptor gamma (PPAR-γ) ligands that are used in the treatment of type II diabetes [(2)]. PPAR-γ is widely expressed in the cardiovascular system [(3)]. A number of studies have demonstrated that TZDs have several beneficial applications (apart from the anti-diabetic action) such as antioxidant, anticancer, anti-inflammatory, antiprocoagulant, antihypertensive and improvement in the endocrine state of women with androgen excess [(4-7)]. In experimental atherogenesis, researchers have investigated a variety of cardioprotective effects of TZDs that are independent of their metabolic benefits [(8)]. It is of particular interest to note that pioglitazone has recently been reported to possess pleiotropic cardioprotective effects [(9)]. Metformin (a biguanide) is an insulin sensitizer, the predominant effect which is to reduce hepatic glucose production and to increase the sensitivity of liver and peripheral tissues to insulin [(10)]. Furthermore, administration of this drug has recently been shown to be associated with decreased all-cause and cardiovascular risk (CV) of mortality. Such improvements in the CV outcomes seen with metformin do not seem to be related to glycaemic control, but rather to specific vasculoprotective, antioxidant and anti-inflammatory, effects of this drug [(11)].

Data continue to accumulate supporting a potential causal relationship between inflammation and oxidative stress and the development and progression of atherosclerosis [12]. Thus in addition to lipid lowering strategy, reduction of atherosclerotic disease will require further pharmacological approaches capable of modifying the development and progression of atherosclerosis.
Therefore the present study was conducted to evaluate the effect metformin, pioglitazone and compare the individual effects with their low dose combination on progression of atherosclerosis.

Materials and Methods

Animals

A total number of 35 New Zealand White Male Rabbits weighing 2-2.5 kg were used in this study. The animals were housed in 25 C, 45+5% humidity and 12:12 h light: dark cycle. They were supplied with standard laboratory chow and water *ad libitum* and left to acclimatize for 1 week before the experiments.

Drugs

Metformin (Met) was dissolved in distilled water while Pioglitazone (Pio) was suspended in 1% carboxymethyl cellulose. Met (100 or 200 mg/kg/day) [13] and Pio (5 or 10 mg/kg/day) [14] were administered in a single dose orally by gavage.

Experimental atherosclerosis

Induction of hyperlipidemia and subsequent development of atherosclerosis were carried out by feeding the rabbits an atherogenic diet (AD) (4% cholesterol-enriched diet made by addition of cholesterol powder to chow pellets) for 10 weeks [15].

Experimental protocol

Duration of the study was 10 weeks. The rabbits were randomly assigned into five groups, 7 rabbits each. Group 1 (normal control) received standard chow diet. In addition to AD, the rabbits in group 2 (atherogenic control), group 3 (Met), group 4 (Pio) and group 5 (Met+Pio) received no treatment, metformin, pioglitazone and combination of low doses of metformin and pioglitazone respectively throughout the experiment. At the end of (10^{th} week) experiments, all rabbits were fasted for 18 hours before sacrifice.
They were sacrificed under light chloroform anesthesia and thoracotomy was performed. A about 5 ml of blood was collected

directly from the heart. The aortic arches were dissected out and fixed in 10% formalin.

Biochemical procedures: One ml of the blood was placed in a tube that contained sodium citrate, as anticoagulant. The plasma was prepared for determination of fibrinogen (Kit supplied by Diagnostica Stago) [16]. The rest 4 ml of blood was placed in plane tube and centrifuged at 3000 rpm for 15 minutes to obtain serum for estimation of malondialdehyde (MDA), reduced glutathione (GSH) levels [17,18], high sensitive C-Reactive Protein (hsCRP) (quantitative determination by Bio-ELISA kit) [19] and lipid profile [18,20]: total serum cholesterol (TC), triglycerides (TG), high density and low density lipoprotein cholesterol (HDL & LDL) (using colorimetric assay kits, Ranadox Laboratories, UK) by. Atherogenic index (AI) was also measured by the following equation (AI= TC-HDL/HDL) [18].

Aortic histopathological examination: The specimens were processed by standard procedure and embedded in paraffin wax. The blocks were cut into 5 µm thickness sections in microtome. These sections were stained with Hematoxylin-Eosin (H&E) [21]. A scoring system (American Heart Association classification of atherosclerosis) was used to classify histological changes into phases and degrees [22]. The histological sections were evaluated by a pathologist without prior knowledge of the treatment given to the animals.

Statistics: The data are expressed as mean ± SEM unless otherwise stated. Statistical analysis was carried out using paired t-test and ANOVA. Significance differences was set at $\alpha = 0.05$. P value <0.05 level of significance was considered statistically significant.

Results

Effects of atherogenic diet

Compared to normal diet control, rabbits fed on cholesterol-enriched diet only for 8 weeks (atherogenic control) showed significant changes in serum lipid profile, oxidation and inflammatory markers. Significantly ($p<0.001$) high levels of serum TC, TG, VLDL and LDL were noticed in those animals (Table 1). Serum HDL was significantly ($p<0.05$) decreased. Also atherogenic index was significantly ($p<0.001$) increased. In addition atherogenic diet resulted in significant ($p<0.001$) elevation in serum fibrinogen, hsCRP and MDA levels and significant ($p<0.01$) reduction in serum GSH level (Table 1).

Effects of drug treatment:

Compared to atherogenic control, treating hyperlipidemic rabbits with metformin, pioglitazone or low dose combination of metformin and pioglitazone resulted in significant changes in serum lipid profile and atherogenic index, oxidation and inflammatory markers:

Metformin resulted in significant decrease ($p<0.01$) in serum TC, TG and LDL and AI and significant increase ($p<0.05$) in serum HDL. VLDL was not significantly ($p>0.05$) changed (Table 3). Serum MDA level was significantly ($p<0.05$) reduced while serum GSH level was significantly ($p<0.05$) elevated (Table 4). Also metformin treatment caused significant ($p<0.01$) reduction in plasma fibrinogen and hsCRP (Table 4).

Pioglitazone resulted in significant decrease ($p<0.01$) in serum TC, TG, LDL and VLDL and AI. Serum HDL was not significantly changed (Table 3). Serum MDA level was significantly ($p<0.05$) reduced while serum GSH level was not significantly ($p>0.05$) changed (Table 4). Further plasma fibrinogen and hsCRP were significantly ($p<0.01$) reduced by pioglitazone treatment (Table 4).

Combination treatment of Met+Pio significantly ($p<0.01$) decreased serum TC, TG, LDL and VLDL and AI and increased HDL. In addition combination treatment resulted in significant reduction in serum MDA ($p<0.01$), plasma fibrinogen ($p<0.01$) and hsCRP and significant elevation in serum GSH level ($p<0.01$).

Comparisons among treated groups revealed that combination treatment decreased AI better than either drug alone. In addition the largest decrement in serum MDA, plasma fibrinogen and hsCRP were observed in combination treatment. The largest increment in serum GSH level was also noticed in combination treatment.

The median histopathological grade of atherosclerotic changes was significantly different between all the 5 study groups. The median was highest in atherogenic control (advance, phase V) and lowest in the normal diet control (no abnormality)(Figure1). Among the 3 types of treatment, each one was associated with a median aortic change that is significantly lower than the atherogenic control. Among these 3 treatments the combination treatment was associated with the lowest median aortic change (initial) which was significantly lower than that of metformin and pioglitazone groups (median aortic change = intermediate).

Table-1: Changes in serum lipid profile of rabbits fed on atherogenic diet for 10 weeks, (N=7).

Parameter	Group	
	Normal Control	Atherogenic Control
TC	66± 3.1	816± 23.1
TG	52±2	311±19
VLDL-C	10.1±1	62.8±2.6
LDL-C	25±0.8	658.4±22.3
HDL-C	17.2±0.5	13.7±0.2
AI	2.3±0.15	41.3±0.5

Table-2: Changes in oxidation and inflammatory markers of rabbits fed on atherogenic diet for 10 weeks, (N=7).

Parameter	Group	
	Normal Control	Atherogenic Control
MDA	1.86± 0.1	4.2 ± 0.2
GSH	3.9±0.05	2.11 ± 0.03
Fibrinogen	141±5	462 ± 6.2
hsCRP	0.8±0.01	4.2 ± 0.2

Table-3: Effects of metformin (mg/kg), pioglitazone (mg/kg) and low dose combination treatment on serum lipid profile and atherogenic index, (N= 7).

Parameter	Group			
	Atherogenic Control	Metformin	Pioglitazone	Combination
TC	816± 23.1	632 ± 22	601 ± 24.7	465.12 ± 17
TG	311±19	237±25.6	231±.6	209±25
VLDL-C	62.8±2.6	58.4±2.2	54.7±2	45.5±2.3
LDL-C	658.4±22.3	572±20	528.3±19	410.3±26
HDL-C	13.7±0.2	20±0.2	15.5±0.6	22.2±0.3
AI	41.3±0.5	25±0.1	26±0.6	19±0.3

Table-4: Effects of metformin (mg/kg), pioglitazone (mg/kg) and low dose combination treatment on oxidative stress and inflammation, (N=7).

Parameter	Group			
	Athergenic Control	Metformin	Pioglitazone	Combination
MDA	4.2 ± 0.2	2.61 ± 0.3	2.91 ± 0.3	2 ± 0.22
GSH	2.11 ± 0.03	2.72 ± 0.01	2.42 ± 0.06	3.1 ± 0.03
Fibrinogen	462 ± 3.2	292 ± 8	261±2	211±6
hsCRP	4±0.19	2.7±0.4	2.1±0.2	1.3±0.12

Table -5: Aortic atherosclerotic lesions (degrees) of different groups at the end of 10 weeks of the study.

Atherosclerotic Lesion	Group									
	Control		AC		Met		Pio		Met+Pio	
	No.	%	No.	%	No.	%	No.	%	No.	%
Normal	7	100	0	0	0	0	0	0	2	28.6
Initial	0	0	0	0	2	28.6	3	42.9	4	57.1
Intermediate	0	0	1	14.3	5	71.4	3	42.9	1	14.3
Advance	0	0	6	85.7	0	0	1	14.3	0	0
Complicated	0	0	0	0	0	0	0	0	0	0
Total	7	100	7	100	7	100	7	100	7	100
Median	Normal		Advance		Intermediate		Intermediate		Initial	

Table-6: Aortic atherosclerotic phases of different groups at the end of 10 weeks of the study.

Atherosclerotic Phase	Group									
	Control		AC		Met		Pio		Met+Pio	
	No.	%	No.	%	No.	%	No.	%	No.	%
Normal	7	100	0	0	0	0	0	0	2	28.6
Phase 1	0	0	0	0	0	0	1	14.3	4	57.1
Phase 2	0	0	0	0	1	14.3	2	28.6	1	14.3
Phase 3	0	0	0	0	4	57.1	3	42.9	0	0
Phase 4	0	0	2	28.6	1	14.3	1	14.3	0	0
Phase 5	0	0	4	57.1	0	0	0	0	0	0
Phase 6	0	0	0	0	0	0	0	0	0	0
Total	7	100	7	100	7	100	7	100	7	100
Median	Normal		Phase 5		Phase 3		Phase 3		Phase 1	

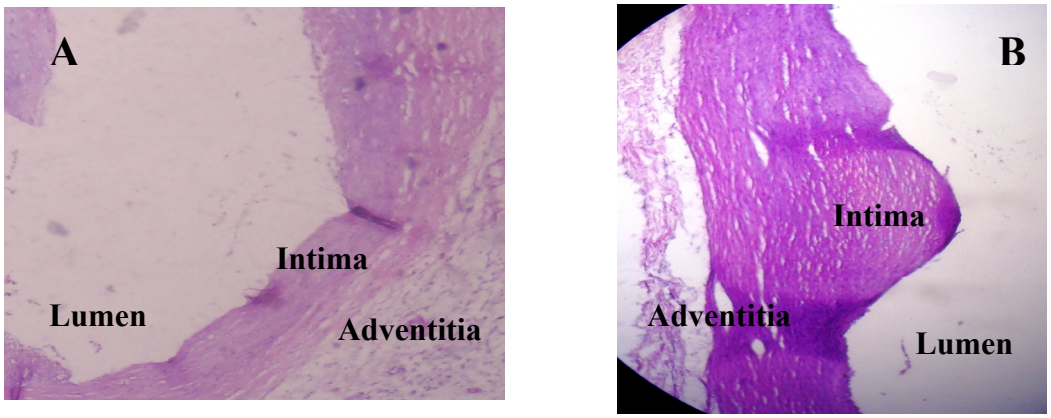

Figure (1): Photomicrograph of histological section in rabbits' aortic arch. Section (A) shows normal intimal thickness and intact continuous endothelium, no abnormality. Section (B) shows diffuse intimal thickening with lipid and collagen collection within the intima, advance atherosclerotic changes. Stained with haematoxylin and Eosin (x100).

Discussion

In the current study, feeding of a high-cholesterol diet to rabbits for 8 weeks caused marked hypercholesterolemia in which serum TC, TG, VLDL and LDL level were found to be increased while HDL decreased. Atherogenic index was further increased. Similar changes in these parameters have been reported by Thakur *et al.* (2001), Yanni (2004) and Pandya *et al.* (2006) [15,23,24]. These remarkable changes in the lipid profile are anticipated and might be attributed to the exogenous cholesterol. In the present study, we found that atherogenic diet promoted oxidative stress as evident by elevation in serum MDA (lipid peroxidation product) and reduction in serum GSH levels. These findings are in agreement with previous studies [24,25]. The observed changes in oxidation markers may be attributed to severe hyperlipidemia that enhances the process of lipid peroxidation and consumption of the antioxidant GSH [24]. In this study we noticed elevation of plasma fibrinogen in hypercholesterolemia. Similar findings were reported by Omran *et al.* (2007) [26]. Hypercholesterolemia may accelerate the rate of prothrombin activation and enhance inflammatory process thereby increase fibrinogen, acute phase reactant. Moreover we observed that hsCRP level was elevated in hypercholesterolemic rabbits. These findings are in agreement with the findings of Ma H *et al* (2008) [27]. Hypercholesterolemia may lead to the elevated CRP secretions through reducing PPARγ expressions in adipocytes [27]. The pathophysiological significance of the elevated plasma fibrinogen and serum level of hsCRP in hyperlipidemic rabbits may reflect an inflammatory state related to atherosclerosis and causally involved in atherogenesis.

In the present study, we demonstrated that metformin induced decreases in TC, TG and LDL and increase in HDL. VLDL was not changed. These changes are consistent with previously reported effects of metformin on lipid levels [11,28]. Another study reported that HDL was unchanged by metformin therapy [29]. Furthermore, we observed that treatment with pioglitazone was found to produce significant decreases in TC, TG, LDL and VLDL and increase in HDL. This is in agreement with previous findings of Deeg *et al* (2007) [30]. Changes in lipid levels were more favorable in the group treated with low dose metformin and pioglitazone combination. Reduction in atherogenic index was greater than that induced by either drug alone.

In the present study, we found that both metformin and pioglitazone treated groups revealed reduction in serum MDA and elevation in

serum GSH levels. These results are in agreement with previous studies [11,28]. Another previous study by Yilmas *et al.* (2005) [31] have shown that treatment with metformin did not exert any significant alteration on oxidation parameters. Levels of fibrinogen and hsCRP were also decreased in both the pioglitazone and metformin treated groups. These findings are consistent with previous reports [11,28,32]. Of note, the decrease in MDA, fibrinogen and hsCRP and increase in GSH induced by low dose metformin/pioglitazone combination was significantly greater than that caused by metformin or pioglitazone alone. Among the three types of treatments, the combination treatment was associated with the lowest median aortic change (initial) which was significantly lower than that of metformin or pioglitazone groups (median aortic change = intermediate).

The favorable observations of metformin might be explained by its antioxidant and anti-inflammatory properties [11,28]. Direct gene regulation, potent anti-inflammatory and antioxidant properties may contribute to the antiatherogenic effect of pioglitazone [32]. However the clinical use of these drugs is limited by potential side effects that are partly dose and host dependent. Taken together, the additive use of low doses and the combined anti-inflammatory and antioxidant effects of the two drugs representing different pharmacological classes (pioglitazone, a thiazolidinedione; and metformin, a biguanide) have more favorable antiatherosclerotic effect and side effect profile than either drug alone (hypoglyemic dose). The current study suggests that combination of low doses of metformin and pioglitazone may have atheroprotective effects therefore could represent further strategy in reducing cardiovascular risk.

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