

VASCULAR

Pioglitazone Prevents the Endothelial Dysfunction Induced by Ischemia and Reperfusion in Healthy Subjects

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ABSTRACT

Background: No study has investigated whether pioglitazone (an agonist of peroxisome proliferator-activated receptor gamma) protects against ischemia and reperfusion (IR)-induced endothelial dysfunction in humans.

Methods and Results: In the first crossover study, 20 volunteers were randomized to 1 week of pioglitazone (30 mg/day, p.o.) or control (no treatment). In the second single-arm study, 15 volunteers received pioglitazone and the cyclooxygenase-2 inhibitor meloxicam for 1 week. On day 7, endothelium-dependent flow-mediated dilation (FMD) of the distal brachial artery was measured before and after IR (15 min of ischemia followed by 15 min of reperfusion in the proximal upper arm). Pre-IR brachial-artery diameter and FMD were similar across the two sessions (control, pioglitazone) in protocol 1 and between the two protocols. IR significantly blunted FMD after no treatment (pre-IR FMD: $10.2 \pm 2.6\%$; post-IR FMD: $3.5 \pm 1.9\%$, $P < 0.01$) but not after pioglitazone administration (pre-IR FMD: $9.7 \pm 2.5\%$; post-IR FMD: $8.8 \pm 2.9\%$, $P = 0.11$). This protective effect was accompanied by an increase in serum levels of the antioxidant enzyme extracellular superoxide dismutase and was not affected by concomitant administration of the cyclooxygenase-2 inhibitor meloxicam ($P = 0.10$).

Conclusions: In humans, pioglitazone provides potent protection against IR-induced endothelial dysfunction.

Key words: endothelium, ischemia and reperfusion, pioglitazone, flow-mediated dilation, cyclooxygenase-2 pathway, brachial artery

INTRODUCTION

Multiple lines of evidence have demonstrated that early restoration of coronary perfusion is indispensable in the management of myocardial infarction.(1) However, complete salvage of infarct cannot be achieved because reperfusion itself can contribute to myocardial damage , a phenomenon called ischemia and reperfusion (IR) injury.(2) The vascular endothelium has an important role in the pathophysiology of tissue injury induced by IR.(3, 4) Endothelial cells are susceptible to IR injury and, during ischemia, endothelial dysfunction contributes to IR-induced tissue damage.(5) Therefore, protecting the endothelium from IR injury is of great clinical interest. In humans, an *in vivo* model of IR-induced endothelial dysfunction has been reported and pharmacological protection of IR-induced endothelial dysfunction shown.(6-9)

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily that bind to specific response elements (“PPAR-responsive elements”) in target gene promoters.(10) Synthetic antidiabetic thiazolidinediones such as pioglitazone and rosiglitazone are ligands for PPARs. Pioglitazone has a cardioprotective role in animal models of myocardial IR injury.(11, 12) **Ye et al. reported that the protective effect of pioglitazone on IR injury is dependent on downstream activation of cyclooxygenase (COX)-2.**(12) In humans, a retrospective study showed a potential beneficial effect of pioglitazone on electrocardiographic findings associated with myocardial reperfusion in diabetic patients with acute myocardial infarction.(13) However, there is little evidence of the effect of pioglitazone on the endothelium (especially in IR-induced endothelial dysfunction).

We used a human *in vivo* model of IR-induced endothelial dysfunction. **First, a crossover study was performed** to ascertain if pioglitazone administration prevents IR-induced impairment in endothelium-dependent vasodilatation. **Second, to evaluate the involvement of COX-2 in its effect, we performed a single-arm study which ascertains if meloxicam, a**

COX-2 inhibitor, can diminish the protective effect of pioglitazone on IR-induced endothelial dysfunction.

METHODS

The present study was approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences (Okayama, Japan). Written informed consent was obtained from all subjects before study commencement.

Protocol 1

Twenty healthy, nonsmoking volunteers (25–44 years; 19 men and one woman) were enrolled in a randomized, crossover study (Figure 1A). Participants were randomized to receive pioglitazone (30 mg/day) for 1 week or no treatment with a washout period of 1 month. On day 7, venous blood samples were drawn, and flow-mediated dilatation (FMD) in the distal brachial artery measured before and after IR. **Any medications except for pioglitazone were not allowed during this protocol.**

Protocol 2

In a separate single-arm study, 15 healthy volunteers received pioglitazone (30 mg/day) and the cyclooxygenase (COX)-2 inhibitor meloxicam (10 mg/day) for 1 week (Figure 1B). On day 7, FMD was measured before and after IR. **Any medications except for pioglitazone and meloxicam were not allowed during this protocol.**

IR procedure

First, FMD measurement before IR was undertaken (Figure 2). Then, a blood-pressure cuff placed on the proximal portion of the upper arm was inflated to suprasystolic pressure (50 mmHg above systolic blood pressure) for 15 min to induce ischemia. The cuff was then deflated and 15 min of reperfusion was allowed, resulting in a brief episode of IR. FMD after IR was measured again.

FMD measurement before and after IR

FMD of the distal brachial artery was assessed as described previously.⁽¹⁴⁾ A 10-MHz linear array transducer probe (Unex Co. Ltd., Nagoya, Japan) was used to obtain longitudinal

images of the brachial artery at baseline after rest in the supine position for ≥ 5 min. The diameter of the brachial artery was measured from clear anterior and posterior interfaces. A blood-pressure cuff at the right forearm was inflated to suprasystolic blood pressure for 5 min. Measurements of the diameter of the brachial artery were made continuously until ≥ 2 min after release of the blood-pressure cuff. FMD was expressed as percentage dilatation from baseline diameter to maximum diameter (%FMD). To quantify inter- and intra-observer reproducibility, the baseline diameter of the brachial artery and FMD were measured by three individuals. Inter- and intra-observer coefficients were high ($r > 0.90$).

Tests carried out on blood samples

In the protocol 1, the following parameters were measured from blood samples at day 7: plasma glucose; insulin; hemoglobin A1c; serum total cholesterol; triglyceride; high-density lipoprotein-cholesterol; low-density lipoprotein-cholesterol; high-sensitivity C-reactive protein; soluble vascular cell adhesion molecule 1 (VCAM-1); extracellular superoxide dismutase (ecSOD); asymmetric dimethylarginine (ADMA). The serum level of ecSOD was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science Inc. Wuhan, China). Intra- and inter-assay coefficients of variation were $< 10\%$ and $< 12\%$, respectively. The plasma level of ADMA was measured with an ELISA kit (ALPCO Diagnostics, Salem, MA, USA). Intra- and inter-assay coefficients of variation were $< 8\%$ and 9% , respectively. Other markers were measured at SRL Co., Ltd. (Tokyo, Japan).

Statistical analysis

Estimates of sample size for the present study assumed $1 - \beta = 0.8$ and a two-sided α of 0.05, and were made based on previous studies.(7, 8, 15) We assumed that IR decreased FMD from 8% to 2% with a standard deviation of 3%. Calculations of sample size suggested that detection of 50% prevention of this impairment by pioglitazone would require a sample size of 10 subjects in each crossover group.

The normality of the distribution of the variables was assessed by the Shapiro–Wilk test, which demonstrated that apoB-48, insulin, Homeostasis Model Assessment of Insulin Resistance, and pentraxin 3 had a skewed distribution. We used parametric methods and tests (e.g., mean, standard deviation, paired *t*-test) to analyze normally distributed data. We employed nonparametric methods and tests (e.g., median, inter-quartile range, Wilcoxon signed-rank test) to analyze data that deviated from a normal distribution. Carryover and period effects were calculated using the independent-sample *t*-test.(16) Within-group comparisons were made with a paired *t*-test. In protocol 1, the interaction between IR and the randomization group was studied by two-way ANOVA. *Post hoc* comparisons were made using the Bonferroni correction. $P < 0.05$ was set as the threshold for significance. All analyses were carried out using SPSS v17.0 (SPSS, Chicago, IL, USA).

RESULTS

Pre-IR values of the diameter of the brachial artery and reactive hyperemia were similar in the control group and pioglitazone group in protocol 1, and similar between subjects in protocol 1 and protocol 2 (Table 1).

Protocol 1

Protocol 1 was a crossover study (Figure 1). Table 2 showed patients' characteristics and biochemical parameters one week after administration. The serum level of ecSOD was higher after 1 week of pioglitazone administration than in the control. However, the lipid profile and levels of glucose, insulin, soluble VCAM-1, and ADMA were similar after pioglitazone administration and in the control (Table 2).

No carryover and no period effects were observed for FMD in statistical terms. In the control group and pioglitazone group, the diameter of the brachial artery after IR was slightly greater than that before IR (both $P < 0.05$), but peak reactive hyperemia was similar before and after IR (Table 1). FMD was lower after IR than before IR after 1 week in the control group (pre-IR: $10.2 \pm 2.6\%$; post-IR: $3.5 \pm 1.9\%$, $P < 0.01$) but not after 1 week of pioglitazone administration (Figure 3) (pre-IR FMD: $9.7 \pm 2.5\%$; post-IR FMD: $8.8 \pm 2.9\%$, $[P = 0.11]$ vs. FMD pre-IR, $[P < 0.01]$ vs. placebo, $[P < 0.01]$ for the interaction of IR and group).

Protocol 2

Protocol 2, which was a single-arm study (Figure 2), evaluated involvement of the COX-2 pathway in the effect of pioglitazone on IR-induced endothelial dysfunction. After 1 week of administration of pioglitazone + meloxicam, post-IR FMD was not significantly different from pre-IR FMD (pre-IR FMD: $9.9 \pm 2.2\%$; post-IR FMD: $9.4 \pm 2.4\%$; $P = 0.10$; Figure 4).

DISCUSSION

The present study demonstrated, for the first time in humans, that pioglitazone protects against IR-induced endothelial dysfunction at a conduit artery. This effect is independent of the COX-2 pathway and seems to be mediated by activation of an anti-oxidative stress mechanism.

Our results support animal studies that showed pioglitazone can reduce reperfusion injury (11, 17) and infarct size in the myocardium.(18, 19) IR injury is not restricted to cardiomyocytes and can also affect the coronary endothelium.(2) The pathophysiological significance of IR-induced endothelial injury to large coronary arteries may be related (at least in part) to reactive oxygen species. The superoxide anion reacts with endothelium-derived nitric oxide *via* a radical-radical reaction to generate peroxynitrite (ONOO^-), a potent oxidant and mediator of vascular tissue injury.(20) The superoxide anion also reduces the bioavailability of nitric oxide.(21) Superoxide dismutase (SOD) is a key scavenger of superoxide anions and has three isoforms localized at different sites: copper-zinc SOD in the cytosol; manganese SOD in mitochondria, and ecSOD in the extracellular space.(22) In the present study, pioglitazone treatment for 1 week significantly increased serum levels of ecSOD. ecSOD regulates blood pressure and vascular contraction by modulating endothelial function (achieved by controlling extracellular levels of the superoxide anion and bioactivity of nitric oxide in the vasculature).(21, 23, 24) In accordance with our finding, Adachi et al. reported that pioglitazone administration in diabetic patients increased plasma levels of ecSOD.(25) Thus, the protective effects of pioglitazone upon IR injury could be explained (at least in part) by increases in ecSOD levels.

Human studies have shown that IR-induced endothelial dysfunction can be prevented by sildenafil (8) and exenatide.(15) Multiple lines of evidence suggest that stimuli leading to activation and opening of adenosine triphosphate-sensitive potassium (K_{ATP}) channels can

induce potent protective effects against IR injury.(26) In accordance with this theory, the effects of sildenafil and exenatide are abolished by glibenclamide (a blocker of K_{ATP} channels).(8, 15) In the present study, the effect of an opener of K_{ATP} channels (e.g., sulfonylurea) concomitant with pioglitazone was not tested. One study, however, examined the inhibitory effects of several PPARs on vascular K_{ATP} channels.(27) The concentrations for 50% inhibition of K_{ATP} channels were 95 $\mu\text{mol/L}$ for pioglitazone, which were almost threefold higher than the effective concentration of pioglitazone for the treatment of type-2 diabetes mellitus (T2DM). Thus, the protective effect against IR injury could be achieved by factors other than inhibition of K_{ATP} channels. Another human study showed a protective effect of rosuvastatin on IR-induced endothelial dysfunction, and this effect was annulled by the COX-2 inhibitor celecoxib.(7) In the present study, the COX-2 inhibitor meloxicam did not abolish the protective effect of pioglitazone on IR-induced endothelial dysfunction, suggesting that the effect of pioglitazone was independent of the COX-2 pathway. This observation may have further clinical implications because pioglitazone may circumvent the concern of an increase in cardiovascular mortality in patients receiving COX -2 inhibitors.

The specific molecular mechanism of action of pioglitazone in the present study is not clear. Clinical studies have shown that pioglitazone administration for >3 months improves endothelial function in patients with T2DM and impaired glucose tolerance.(28-30) In the present study, 1-week treatment with pioglitazone showed a protective effect. Fujisawa et al. reported that pioglitazone treatment for 24 h attenuated hyperglycemia-induced production of reactive oxygen species in human umbilical vein endothelial cells.(31) Thus, the direct effect of pioglitazone after relatively short-term treatment could explain our findings. However, the systemic effects on endothelium-dependent vasodilatation, and the effect of single administration of pioglitazone on IR-induced endothelial dysfunction, need to be evaluated further.

The benefit of lowering glucose levels using several antidiabetic drugs on cardiovascular mortality in patients T2DM remains uncertain. Studies have shown that intensive glycemic control does not reduce cardiovascular mortality and increases the risk of severe hypoglycemic attacks.(32-35) Two recent studies using the dipeptidyl peptidase-4 inhibitors alogliptin (36) and saxagliptin (37) also did not show superiority of an additional dipeptidyl peptidase-4 inhibitor in terms of cardiovascular mortality. However, the Prospective Pioglitazone Clinical Trial In Macrovascular Events (PROactive) showed that pioglitazone reduced the prevalence of all-cause mortality, non-fatal myocardial infarction, and stroke.(38) Taken together with our data, those results suggest that pioglitazone is a beneficial drug for the prevention of cardiovascular events in T2DM.

Our study had limitations. First, data were obtained from the blood circulation in the forearms of healthy volunteers, so potential differences between forearm circulation and coronary circulation, as well as between healthy and diabetic individuals, need to be considered. Second, even though protocol 1 had a cross-sectional design, baseline data in individual groups were not available. Statistical analyses showed no carryover effects or period effects between the two treatment periods with regard to FMD values, but we cannot exclude the possibility they influenced the results. Third, this study included only one woman, so the observed effects may not be applicable to women.

Conclusion

We demonstrated that pioglitazone administration could provide potent endothelial protection *via* an anti-oxidative stress effect. These findings represent the first human evidence for the effects of pioglitazone on reperfusion injury in the endothelium. Additional studies are necessary to investigate the mechanisms and their potential clinical implications in greater detail.

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FIGURE LEGENDS

Figure 1. Flowchart of the crossover trial. In protocol 1. Participants were randomized to receive pioglitazone (30 mg/day) for 1 week or no treatment with a washout period of 1 month. In protocol 2, participants received pioglitazone (30 mg/day) and the cyclooxygenase (COX)-2 inhibitor meloxicam (10 mg/day) for 1 week. On day 7, venous blood samples were drawn, and flow-mediated dilation (FMD) in the distal brachial artery measured. IR: ischemia and reperfusion.

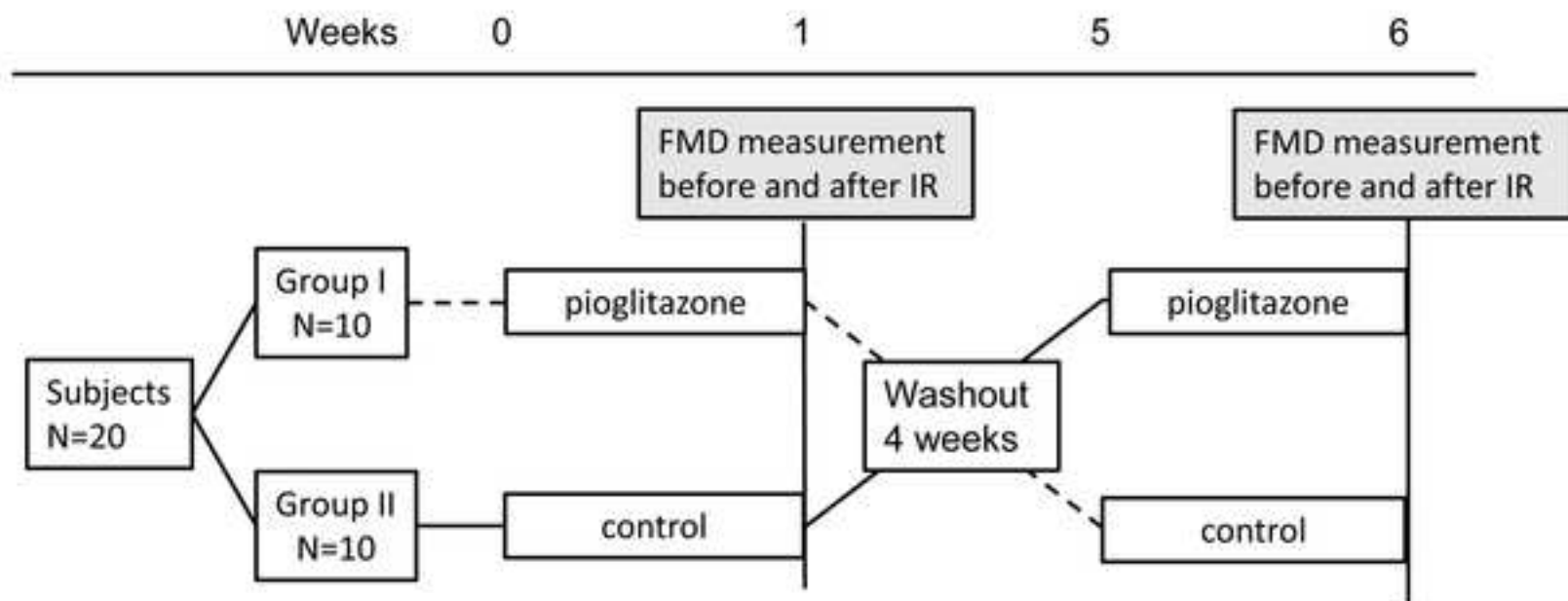
Figure 2. Flow-mediated dilation (FMD) was measured before and after ischemia and reperfusion (IR) procedure.

Figure 3. Flow-mediated dilatation (FMD) before and after ischemia and reperfusion (IR) after pioglitazone administration for 1 week and no treatment. In the control group, FMD was significantly lower after IR than before IR. This effect was prevented by pioglitazone administration. Data are the mean \pm SD.

Figure 4. Flow-mediated dilatation (FMD) before and after ischemia and reperfusion (IR) after administration of pioglitazone + meloxicam for 1 week. FMD was not attenuated after IR. Data are the mean \pm SD.

Figure 1

A



B

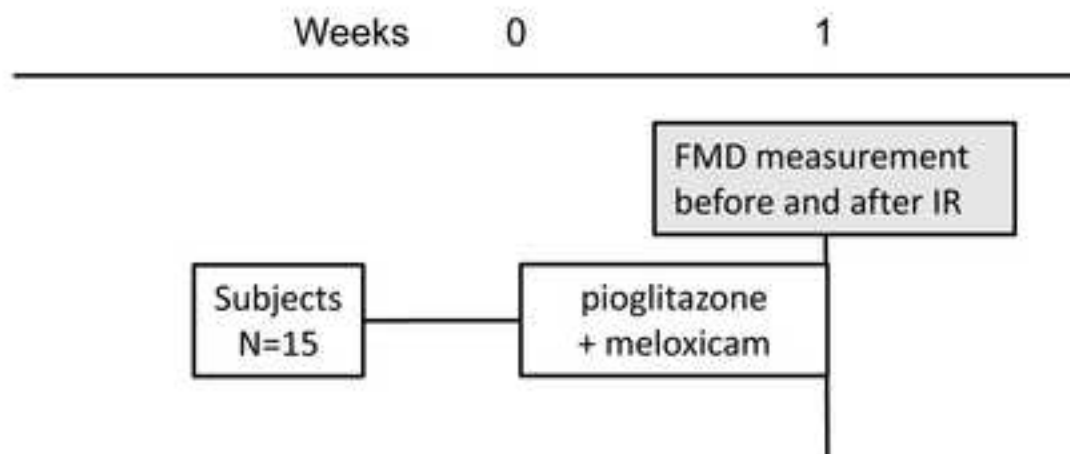


Figure 2

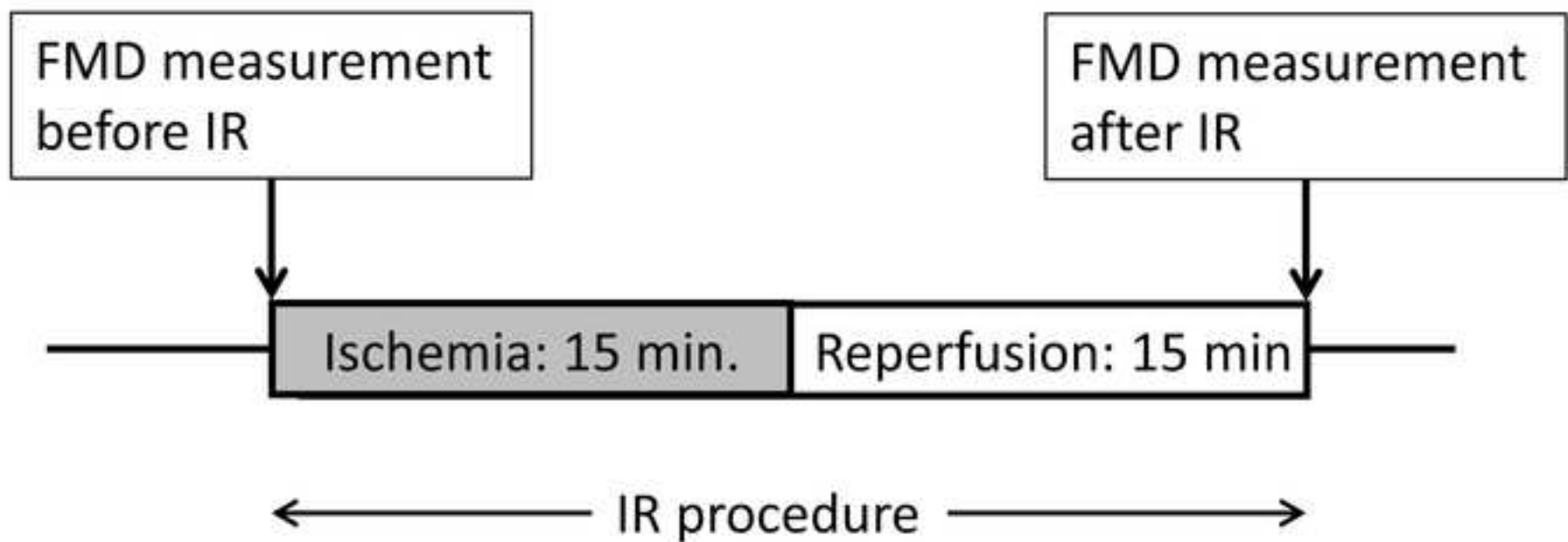


Figure 3

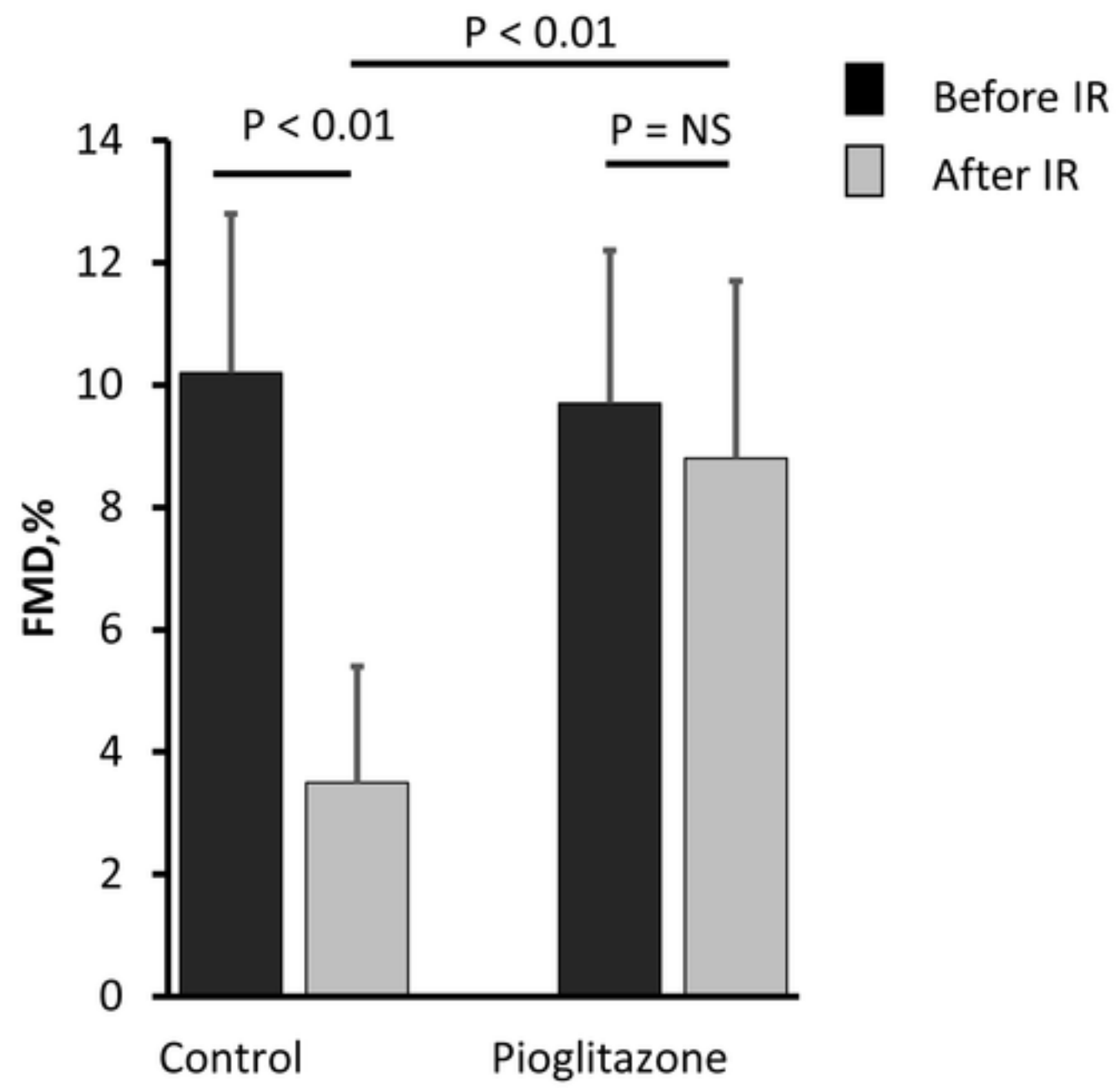


Figure 4

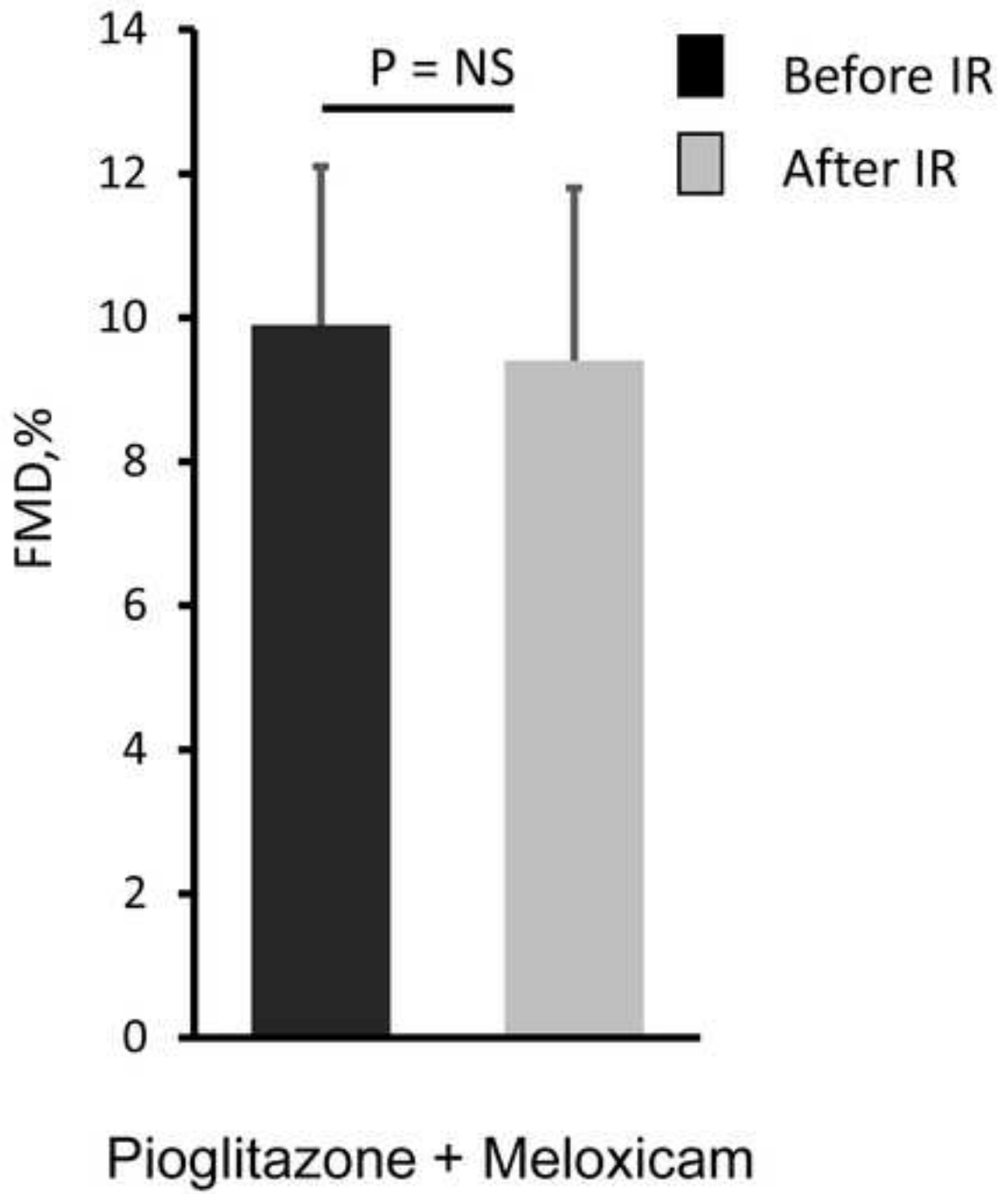


Table 1. The effect of IR in the brachial arterial diameter and blood flow data

| | | Before ischemia and reperfusion | | | After ischemia and reperfusion | | |
|------------|--------------------------|---------------------------------|------------------------------------|------------------------|--------------------------------|------------------------------------|------------------------|
| | | Diameter at baseline (mm) | Diameter after cuff deflation (mm) | Reactive hyperemia (%) | Diameter at baseline (mm) | Diameter after cuff deflation (mm) | Reactive hyperemia (%) |
| Protocol 1 | Control | 3.45 ± 0.35 | 3.80 ± 0.38 | 495 ± 131 | 3.54 ± 0.38* | 3.67 ± 0.41* | 533 ± 159 |
| | Pioglitazone | 3.52 ± 0.40 | 3.86 ± 0.40 | 511 ± 160 | 3.59 ± 0.45* | 3.93 ± 0.44 | 556 ± 119 |
| Protocol 2 | Pioglitazone + Meloxicam | 3.49 ± 0.42 | 3.82 ± 0.44 | 515 ± 159 | 3.52 ± 0.41* | 3.81 ± 0.45 | 502 ± 150 |

Data are the mean ± SD.

*P < 0.05 vs. corresponding value before ischemia and reperfusion.

Table 2 Patients' characteristics one week after administration

| | Control (n=20) | Pioglitazone (n=20) | P |
|--------------------------------------|-----------------|---------------------|------|
| Age (years) | 32 ± 7 | | |
| Male (n) | 19 | | |
| Body mass index (kg/m ²) | 23.3 ± 1.3 | 23.3 ± 1.3 | 0.56 |
| Systolic blood pressure (mmHg) | 119 ± 10 | 120 ± 8 | 0.87 |
| Diastolic blood pressure (mmHg) | 73 ± 5 | 73 ± 7 | 0.96 |
| Heart rate (beats/min) | 62 ± 4 | 62 ± 4 | 0.41 |
| LDL-C (mg/dl) | 109 ± 28 | 108 ± 28 | 0.51 |
| HDL-C (mg/dl) | 69 ± 12 | 68 ± 12 | 0.46 |
| TG (mg/dl) | 82 (65 -109) | 77 (69 - 89) | 0.54 |
| Glucose (mg/dl) | 89 ± 14 | 88 ± 14 | 0.94 |
| Insulin (μU/ml) | 2.4 (2.0 - 4.4) | 2.0 (2.0 - 3.8) | 0.79 |
| sVCAM-1 (ng/ml) | 633 (543 -676) | 565 (513 - 730) | 0.72 |
| hsCRP (ng/ml) | 220 (102 - 403) | 113 (75 - 191) | 0.01 |
| ADMA (nmol/ml) | 0.36 ± 0.06 | 0.36 ± 0.05 | 0.39 |
| ecSOD (ng/ml) | 42 ± 12 | 53 ± 15 | 0.03 |

Data are the mean ± SD or median (interquartile range), as appropriate. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; sVCAM-1, soluble vascular cell adhesion molecule 1; hsCRP, high-sensitivity C-reactive protein; ADMA, asymmetric dimethylarginine; ecSOD, extracellular superoxide dismutase.

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