行政院國家科學委員會補助專題研究計畫成果報告

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※Cardioprotective Effect of Troglitazone in the Ischemic-Reperfused Canine Heart※
※ Troglitazone 對狗心臟在發生缺血-再灌流時之保護 ※
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計畫類別：□個別型計畫□整合型計畫
計畫編號：NSC89－2314－B－002－117－
執行期間：88 年 08 月 01 日至 89 年 07 月 31 日

計畫主持人：李聰明
共同主持人：蔡長和

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□國際合作研究計畫國外研究報告書一份

執行單位：台大醫院內科

中　華　民　國 89 年 10 月 31 日

行政院國家科學委員會專題研究計畫成果報告

Cardioprotective Effect of Troglitazone in the Ischemic-Reperfused Canine Heart
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一、中文摘要

先前研究顯示 **Troglitazone** 有去除自由基之功能。但 **Troglitazone** 對心肌保護效果則未得而知。本實驗在探究 **Troglitazone** 對狗之心肌梗塞模型之保護效果。對左前降枝之冠狀動脈綁住 60 分鐘，而後再放開 2 小時。分別在綁住冠狀動脈前 15 分鐘，給予安慰劑(對照組)或 **Troglitazone**。在灌流 2 小時結束時，缺血和梗塞面積分別以電腦精確地算出。而側枝循環則以都卜勒方式測量。結果顯示此二組在血壓、心跳和缺血面積皆相似。但在 **Troglitazone** 中，其梗塞面積則名顯地比對照組少(16 ± 11%，VS 37±9%，p=0.005)。血液缺血之後的再灌流會引起自由基之大量上升。而 **Troglitazone** 則可明顯地下降此上升。結論：**Troglitazone** 對再灌流之心肌具有保護效果，而此保護效果係源於對自由基之抑制。

二、計畫成果摘要:(約二百字)

Abstract

This study was to determine the cardioprotective effects of troglitazone in ischemia/reperfusion canine model. Previous studies suggested that troglitazone, a newly developed antidiabetic thiazolidinedione that enhance insulin sensitivity, has a scavenging effect on reactive oxygen species. Dietary supplementation with antioxidant might reduce fatal ventricular arrhythmias and infarct size. However, the cardioprotective effects of troglitazone remain unknown. Myocardial infarction was produced by occlusion of the left anterior descending artery for 60 minutes followed by 2 h of reperfusion. Vehicle or troglitazone were given intravenously 15 min before the coronary artery occlusion. At the end of the reperfusion period, area at risk and infarct size were quantified by computerized planimetry of slices of myocardium previously masked with methyl blue and triphenytetrazolium chloride. An index for collateral flow was determined by positioning the Doppler flow wire in the collateral-dependent vessel distal to the ligation and measuring the flow velocity. Among survivors, infarct size was larger in control (n = 6, 37 ± 9 % of area at risk) than in supplemented (n = 6, 16 ± 11 %, p = 0.005). Reperfusion after 60 minutes of ischemia causes a significant elevation in free radicals. Although the changes of concentrations of thiobarbituric acid-reactive material were similar between the 2 groups, diene conjugates were significantly higher in control compared with in supplemented. Troglitazone limits myocardial necrosis resulting from coronary artery occlusion and reperfusion. The cardioprotective effect of troglitazone may result from inhibition of free radical rise. Our study may point to an additional mechanism for the cardioprotective effect of troglitazone that can not be explained by its glucose-lowering effects.

關鍵詞: Dogs; Intracoronary Doppler flowire; Ischemia; Reperfusion.

三、計畫簡介（Introduction）

During the past few years, myocardial reperfusion therapy, such as primary percutaneous transluminal coronary angioplasty or thrombolytic therapy, has been widely performed in the management of acute myocardial infarction. Although restoration of blood flow arrests the progression of necrosis, paradoxically it is accompanied by functional derangement, including the production of various arrhythmia (1,2). Much evidence now indicates that oxygen free radicals have been implicated as a cause of deleterious effects in the setting of coronary reperfusion. Free radicals are thought to form lipid peroxides that deplete phospholipids from cell membrane, inhibit membrane–associated enzymes, and interacts with proteins to cause tissue damage (3,4). Pretreatment with the oxygen radical scavenger superoxide dismutase had a protective effect, suggesting that oxygen radicals were the cause of this ultrastructural injury. Given their deleterious effects of cells, it is not surprising that reperfusion-related free radicals eventually extent the myocardial infarction.

Troglitazone, a newly developed antidiabetic thiazolidinedione that enhance insulin sensitivity (5) has a scavenging effect on reactive oxygen species (6). It possesses structural similarity to certain antioxidants, including α-tocopherol and probucol. Cominacini et al (7) showed that under the same stress, troglitazone was much more potent as a radical scavenger than vitamin E. Negasaka et al (8) showed that troglitazone has an inhibitory effect on peroxidation of human LDL. Inoue et al (6) demonstrated that troglitazone can dose-dependently transfer electrons directly to cytochrome *c* and compete for electrons from superoxide with cytochrome *c*.

The purpose of the present study was to determine whether troglitazone can exert cardioprotective effects in normoglycemic animals in infarcts with nearing completion.

四、材料及方法(Subjects and Methods)

Preparation.

The study was conducted on mongrel dogs of either sex, weighing 10-15 Kg. The dogs were intubated and mechanically ventilated (Bennett MA-2) by using room air, delivered at a rate of 20-25 strokes per minutes and a tidal volume of 300 ml. Because of epicardial temperature is a major of myocardial infarct size (9), body temperature was maintained at 38 ± 0.5^0C by means of a temperature probe thermometer inserted into the pericardial cradle so that its sensor surface was adjacent to the posterior surface of the heart. A standard limb-lead electrocardiogram was recorded. Arterial blood samples were taken periodically to determine Po_2, Pco_2, pH, oxygen saturation, hemoglobin concentration, sodium, potassium, chloride and calcium concentration. Plasma glucose concentration was frequently measured with a one-touch glucose analyzer (Lifescan INC, Milpitas, CA). Left ventricular cavity and systemic blood pressures were measured with catheter-tip micromanometers inserted via the left ventricular apex and right femoral artery, respectively. Hydration was maintained by a slow 2.5% glucose infusion. Because operation-related catecholamine release has been implicated in the mechanism of ischemic preconditioning (10), each dog had the same time (15 minutes) for stabilization before the study. Then hemodynamic parameters, including heart rate, arterial blood pressure, left ventricular end-systolic/end-diastolic pressure, and pericardial temperature were continuously recorded on a 4-channel polygraph recorder (Hewlett-Packward model M1962A).

The chest was opened through the left fourth intercostal space, the pericardium was opened, and the heart was exposed. Near the base of the heart, the left anterior descending artery proximal to first diagonal branch was encircled with a 4-0 silk suture. The two ends of the suture were threaded through a piece of tubing, forming a snare that could be tightened to occlude the artery. Coronary occlusion in this region usually resulted in a large ischemic area of the anterolateral and apical regions of the left ventricular wall. Myocardial ischemia was confirmed by

regional cyanosis, acute electrocardiographic ST segment elevation and flow velocity decrease detected by Doppler flow wire. Reperfusion was effected by releasing the snare and was confirmed by visible hyperemia over the surface and hyperemic Doppler shift.

To measure collateral blood flow at baseline and during ischemia, coronary blood flow was detected by intracoronary Doppler flowire, instead of the universally recognized microsphere techniques which were proved to be insensitive to detect collateral flow (11). A 0.014" Doppler wire (FloWire, Cardiometrics, Inc, Mountain View, CA) was introduced through a standard angioplasty-type Y-connector attached to the angiographic catheter into the site distal to ligation. Digitized spectral peak velocity waveforms were averaged to compute the average peak velocity (APV) (cm/sec). The monitor display was continuously recorded on Super VHS videotape for off-line analysis. To determine cross-section area of the artery, a 5-mm segment was measured immediately distal to the tip of the Doppler catheter. Volumetric coronary blood flow (CBF) was calculated as CBF (ml/min) = CSA (mm^2) xAPV (cm/sec) x0.5x0.6. as validated by Doucette et al (12), where CBF is coronary blood flow (ml/min), CSA is cross sectional area (mm^2), and APV is averaged peak velocity (cm/sec).

All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Subjects Committee of the National Taiwan University.

Experimental protocol.

The dogs were assigned randomly to one of two groups. All animals were subjected to a 60-minute coronary occlusion followed by 120 minutes of reperfusion. *Group I* was the control group and only vehicle (5 ml saline) was administrated prior to the 60-minute occlusion. In *Group II*, animals were treated with intravenous 5 mg/kg of troglitazone (Sankyo, Tokyo, Japan). The dose was used as suggestion of Izumi et al (13).

At the end of the protocol, the coronary artery was clamped, and 4 ml of a solution of methylene blue dye (Sigma Chemical Co., St. Louis, MO.) was injected into the left ventricular apex, defining the ischemic region at risk of necrosis. After a 10-minute incubation in a 1% solution of buffered triphenyltetrazolium chloride (TTC), the slices were again photographed to record the necrotic regions (unstained by TTC) and the noninfarcted regions (stained red by TTC). Later the photographic slides were projected and traced. The areas of ischemic and normally perfused regions and the areas of necrotic and nonnecrotic regions were measured on the tracings by computerized planimetry (Image Pro Plus, Media Cybernetics, Silver Spring, MD). Three areas were multiplied by the weight of each slice, and the results were summed to obtain the mass of risk and infarction.

Histology analysis.

Extensive histological samples were taken from each transverse section, processed by conventional methods, and stained with hematoxylin and eosin, and Masson for contraction band. A pathologist (CCT) who was unaware of the treatment protocol examined the samples for microscopic evidence of contraction band necrosis on random fields at a magnification of 400X. The results from the section in each case with the highest number of bands was used. The histologic severity of contraction band necrosis was used to grade injury on a scale of 0 to 3 for the number of contraction bands: 0 (absent), 1 (mild), 2 (moderate) and 3 (severe) which represented 1, 1 to 5, 6 to 10, and 11 or more cells with contraction bands per sq mm of tissue, respectively (14).

Free radical assays.

Serial serum samples for free radical measurements were withdrawn at baseline (15 minutes after stabilization), 15 minutes after troglitazone injection, immediately before coronary reperfusion, 2, 5, 15 and 60 minutes after reperfusion, and at the termination of reperfusion (2 hours). Samples were centifuged at 2000 *g* at 4^0C for 20 minutes. Plasma specimens were stored at -70^0C in plastic tubes until assay.

Thiobarbituric acid (TBA) reactive material (malondialdehyde). Plasma samples were dilated in phosphate buffer and heat together with a TBA solution (325 mg/ml) in a boiling water bath for 15 minutes (15). The tubes were then collected and absorbances measured at 535 nm. The coefficient of variation between assays in our laboratory has been 12 %.

Diene conjugates. Lipids were extracted from the plasma samples by chloroform-methanol (2:1), dried under nitrogen, then redissolved in cyclohexane, and analyzed spectrophotometrially at 323 nm (16). The coefficient of variation between assays in our laboratory has been 17 %.

Statistics.

Values are expressed as mean ± SD. Differences between groups in hemodynamics, coronary blood flow, blood glucose concentrations, infarct size, and area at risk were compared using unpaired *t* test. Changes along time were assessed by multiple ANOVA analysis. For the comparisons of the severity of contraction band necrosis Gehan's generalized Wilcoxon test was applied. Linear regression analysis was used to determined the relationship between area at risk and infarct size as a percent of the left ventricle. A value of $p < 0.05$ was considered to be significant.

五、結果(Results)

Hemodynamics.

Preocclusion heart rate, arterial pressure, and left ventricular end-diastolic pressure were similar in the two groups (Table 1). The pressure rate index, an index of myocardial oxygen demand, was comparable for the 2 groups before coronary artery ligation and decreased to a similar extent following the study.

Coronary blood flow.

Baseline coronary blood flow measured with intracoronary Doppler flow wire was nonsignificantly different between the two groups. Five minutes after occlusion, collateral blood flow in the center of the ischemic region was very low in both groups, and increased only slightly with time. The amount of decrease after coronary occlusion was within the same range in the two groups. The severity of reperfusion hyperemia between the two groups was similar.

Blood glucose. The blood glucose concentrations in the two groups measured at baseline, 15 min after troglitazone, 30 min after occlusion ,and at 60 and 120 min of reperfusion. At 15 min after troglitazone, blood glucose was significantly decreased compared with that at baseline (76 ± 6 mg/dl vs. 92 ± 5 mg/dl baseline, $p < 0.05$). However, blood glucose concentrations were corrected in the treated group by glucose infusion.

Free radicals.

During the period of cardiac ischemia there was no significant changes in either TBA-reactive material or in diene conjugates. During the period after reperfusion, however, there were significant changes: the level of diene conjugates increased significantly in group 1 compared with those in group 2, especially in the first minutes after reperfusion. The levels of TBA-reactive material were similar in the 2 groups throughout the experiment (Fig. 1).

Infarct size and area at risk.

There were no differences in body weight or heart weight between the groups. There was no significant difference in areas at risk (AAR) expressed as a percentage of left ventricle between the 2 groups (Fig. 2), indicating that a comparable degree of ischemic risk. After 1 hour of coronary artery occlusion followed by 2 hours of reperfusion, the necrotic area, expressed as a percentage of the AAR or a percentage of left ventricle, was 37 ± 9 and 16 ± 11 % in the control and 18 ± 5 and 6 ± 4 % in the treated group ($p = 0.005$ and 0.0008), respectively.

The correlation coefficient between infarct size and area at risk was 0.72 ($p < 0.0001$) in the control animals (Fig.3). Troglitazone treatment resulted in a significant downward shift in the regression line, with all infarct sizes smaller than predicted from the area at risk. The slopes of the regression lines in control and treated groups were statistically different ($p =$

0.001).

Histology analysis

. Histological analysis in both groups revealed infarcts composed almost exclusively of contraction band necrosis. The severity of contraction band necrosis was significantly higher in controls compared with the treated group (2.7 ± 0.5 vs. 1.2 ± 0.4, $p = 0.0002$). There was a good correlation between the extent of infarct size normalized by AAR and the severity of contraction band necrosis ($r = 0.88$, $p < 0.0001$).

六、討論(Discussion)

This study demonstrated that pretreatment (before myocardial ischemia) with troglitazone reduces by 62% the extent of myocardial necrosis and fatal ventricular fibrillation after regional ischemia for 60 minutes and reperfusion for 2 hours. Such beneficial effects appear to be mediated, at least in part, through the antioxidant activity of troglitazone and less tissue injury as assessed by myocardial histology.

Troglitazone exerts infarct-limited cardioprotective effects in ischemia/reperfusion canine models. Although causes for the cardioprotective effects of troglitazone remain unclear, the antioxidant effect of troglitazone may play a role. Antioxidants can exert their cardioprotective effects by reducing production of contraction band necrosis. Some myocytes which are reversibly injured at the time that coronary artery occlusion is terminated undergo irreversible cell damage during reperfusion (17,18). Tissue areas that can be salvaged by agents after acute myocardial infarction may be areas with contraction band necrosis. Calcium overloading during reperfusion was supposed to play an important role in the formation of contraction band necrosis (19). Free radical scavengers inhibit a marked increase in cytosolic calcium concentrations on reperfusion of ischemic myocardium (20). Widman et al (21) demonstrated that free radical scavenger can modify the severity of contraction band necrosis, which was consistent with our finding. Further, troglitazone directly inhibits cardiac Ca^{2+} current (22) which could lead to a decreased influx of calcium concentration during reperfusion.

In conclusion, our data support the idea that myocardial infarction after ischemia and reperfusion is mediated via free radicals. Troglitazone acts as an antioxidant may be clinically useful for reducing infarct size, since troglitazone is mainly used for type 2 diabetic patients with insulin resistance, which is associated with increased risk for coronary artery disease and acute myocardial infarction.

七、自評

由於保育動物協會遷就，而前想以狗做時驗也愈發困難，常是有錢買不到狗，雖然台灣到處是流浪狗，但對於醫學研究用之狗，卻常一隻難求，不知可否有解決辦法？

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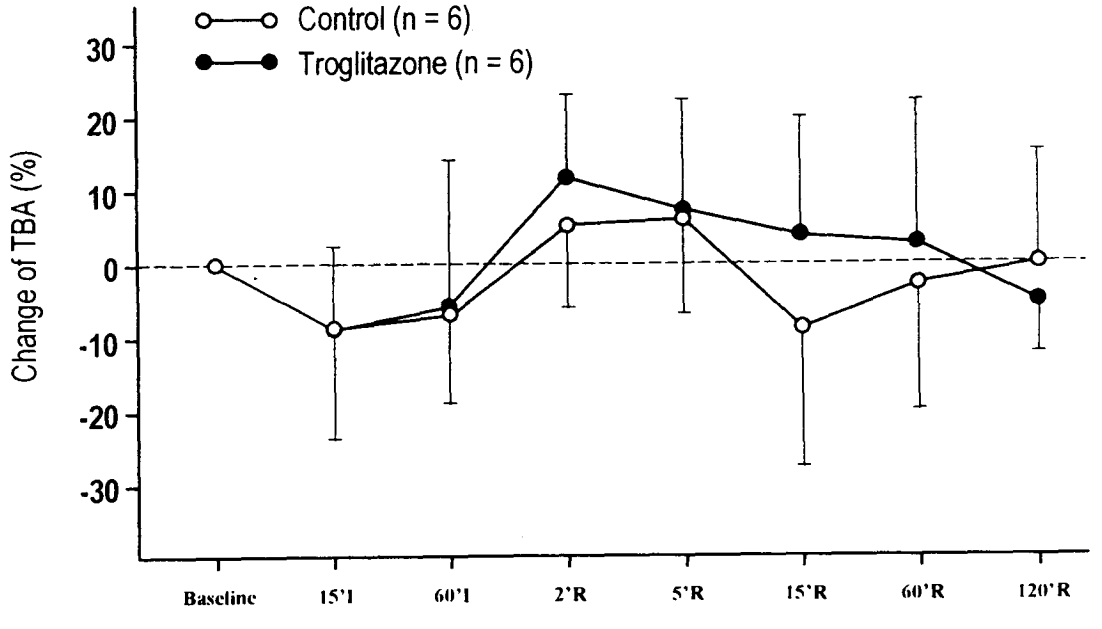
Table 1. Hemodynamics and blood glucose concentrations in troglitazone-treated dogs and controls

	Troglitazone (n = 6)	*Control (n = 6)*
Baseline		
HR (bpm)	197 ± 33	189 ± 26
MBP (mm Hg)	121 ± 5	124 ± 23
SBP*HR (*10^2)	318 ± 68	302 ± 88
LVEDP (mm Hg)	11 ± 4	9 ± 4
Coronary blood flow (ml/min)	40 ± 6	37 ± 5
Glucose (mg/dl)	92 ± 7	89 ± 6
Occlusion, 30 min		

Occlusion, 30 min		
HR (bpm)	192 ± 27	188 ± 22
MBP (mm Hg)	109 ± 11	118 ± 23
SBP*HR (*10^2)	283 ± 67	296 ± 95
LVEDP (mm Hg)	15 ± 6	15 ± 4
Coronary blood flow (ml/min)	4 ± 5	5 ± 3
Glucose (mg/dl)	94 ± 5	92 ± 7
Reperfusion, 60 min		
HR (bpm)	170 ± 41	190 ± 18
MBP (mm Hg)	107 ± 15	123 ± 19
SBP*HR (*10^2)	245 ± 95	293 ± 59
LVEDP (mm Hg)	16 ± 7	15 ± 6
Coronary blood flow (ml/min)	45 ± 6	42 ± 7
Glucose (mg/dl)	92 ± 6	95 ± 5
Reperfusion, 120 min		
HR (bpm)	176 ± 34	184 ± 19
MBP (mm Hg)	115 ± 14	127 ± 24
SBP*HR (*10^2)	264 ± 86	294 ± 85
LVEDP (mm Hg)	16 ± 9	19 ± 5
Coronary blood flow (ml/min)	42 ± 6	39 ± 6
Glucose (mg/dl)	92 ± 5	89 ± 8

Data are expressed as mean ± SD. HR: heart rate; LVEDP: left ventricular end-diastolic pressure; MBP: mean blood pressure; SBP: systolic blood pressure.

Figure 1. Line graphs showing the relative amount of TBA (upper panel) and diene conjugates (lower panel) in arterial plasma during different phases of the study. Data are expressed as mean ± SD. I: during ischemia; R: during reperfusion. *p < 0.05 vs. controls. †p < 0.05 vs. baseline data.



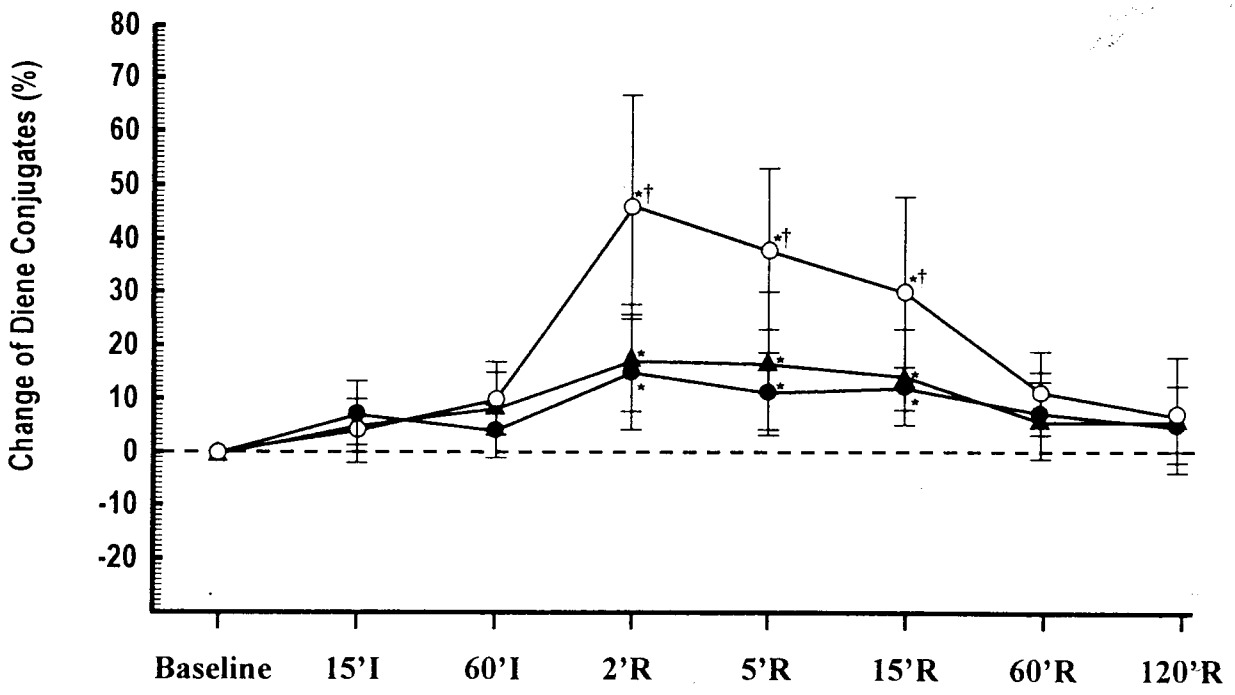


Figure 2. Effects of vehicle or troglitazone on the area at risk (AAR), indexed to left ventricle (LV) and necrosis area indexed to AAR and to the LV. Data are expressed as mean ± SD.

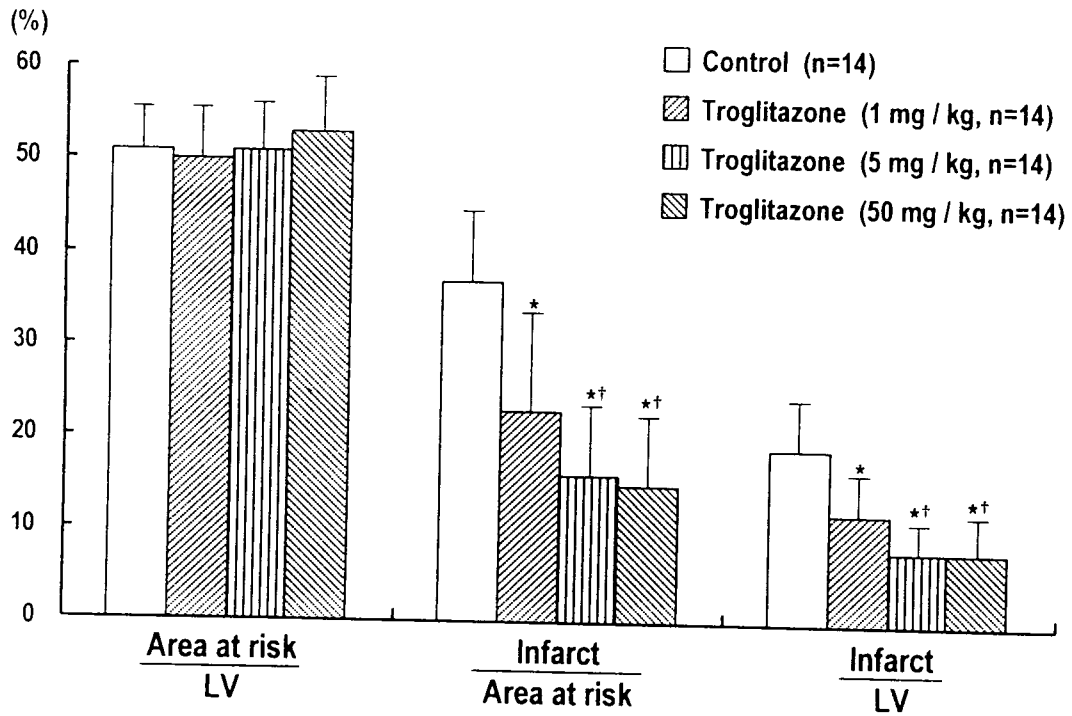


Figure 3. Graph shows correlation between area at risk and infarct size. In the control a close linear relation is observed (r = 0.72). In the troglitazone-treated group, all infarcts were smaller than predicted from area from the area at risk.