

# Effect of L-arginine and L-NAME on coronary angiogenesis in male diabetic rats

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**BACKGROUND:** Diabetes is associated with several vascular abnormalities due to abnormal angiogenesis. Vascular endothelial growth factor (VEGF) and nitric oxide are among the most important angiogenic factors. The aim of this study was to evaluate the effect of L-arginine (L-Arg) and L-NG-Nitroarginine Methyl Ester (L-NAME) on coronary angiogenesis in male diabetic rats. **METHODS:** 24 male rats were randomly divided into four groups: (1) control, (2) diabetic, (3) diabetic + L-Arg (50mg/kg/day, ip) and (4) diabetic + L-NAME (10mg/kg/day, ip). After three weeks, blood samples were taken and the apex of the hearts was stained for immunohistochemistry. **RESULTS:** Coronary angiogenesis expressed as capillary density/mm<sup>2</sup> was lower in diabetic group compared to the control group ( $p < 0.05$ ). L-Arg significantly improved capillary density in myocardial tissue in diabetic animals ( $p < 0.05$ ); however, L-NAME did not alter it ( $p > 0.05$ ). **CONCLUSIONS:** It seems that L-Arg improves coronary angiogenesis in type I diabetic animals through changes of serum angiogenic factors. More studies are needed to clarify the effect of nitric oxide agonists in different experimental models of angiogenesis.

**KEYWORDS:** L-Arginine, L-NAME, Diabetes, Angiogenesis

## BACKGROUND

Diabetes is one of the most important causes of death in the world.<sup>[1]</sup> Among the diabetic complications, cardiovascular disease specially coronary heart disease (CHD) is the leading cause of morbidity and mortality in diabetic subjects.<sup>[2]</sup>

Angiogenesis is the formation of new capillaries from preexisting vessels.<sup>[3,4]</sup> Physiological angiogenesis occurs during wound healing and menstrual cycle and pathological angiogenesis is seen during tumor growth or endometriosis.<sup>[5]</sup> Impaired angiogenesis is associated with some of diabetic complications. For example, enhanced angiogenesis leads to diabetic retinopathy, while impaired angiogenesis is the cause of cardiovascular disease.<sup>[6-8]</sup>

Numerous angiogenic and antiangiogenic factors have been demonstrated.<sup>[9,10]</sup> Vascular endothelial growth factor (VEGF), is a 45kd glycoprotein which induces endothelial cell proliferation and is one the most important angiogenic factors.<sup>[3,11]</sup> Nitric oxide (NO) is an endothelium-derived relaxing factors which has antiatherosclerotic properties such as reduction of platelet aggregation and inhibition of smooth muscle cell proliferation.<sup>[12]</sup> Recently, it has been suggested that NO is involved in angiogenesis process.<sup>[13]</sup> NO is synthesized from L-arginine (L-Arg) through three isoforms of NO synthase.

In this study, we aimed to investigate the effect of L-Arg and L-NG-Nitroarginine Methyl Ester (L-NAME), a non-specific NO synthase inhibitor, on coronary angiogenesis in type I diabetic rats.

## METHODS

### Animal groups

24 male rats (180-220 g, Pasteur Institute of Iran) were randomly divided into four groups: (1) control, (2) diabetic, (3) diabetic + L-Arg and (4) diabetic + L-NAME. The animals were housed three per cage in a 12 hours light/dark cycle, temperature between 20-25° C and humidity around 60-70%. The ethical committee of the Isfahan University of Medical Sciences approved the experimental procedures.

### Induction of diabetes

For induction of diabetes, streptozotocin (sigma Co, 60 mg/kg) was injected intraperitoneally. After 48 hours, blood glucose concentrations were measured and the animals with blood glucose level higher than 300 mg/dl were considered as diabetic.<sup>[14]</sup>

### Experimental design

After induction of diabetes, the animals in groups 3 and 4 were treated by daily intraperitoneal injection of L-Arg (50 mg/kg, Sigma Co)<sup>[15]</sup> and L-NAME

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(10 mg/kg, Sigma Co),<sup>[16]</sup> respectively. Diabetic group received normal saline injection with the same volume and method. After three weeks, blood samples were taken and centrifuged at 3000 rpm/sec for 20 minutes. Serums were collected in separate Eppendorf tubes and maintained at -70° C for further analysis. Then, the animals were sacrificed and the apex of hearts were removed and put in formalin 10% solution.

#### *Serum NO and VEGF measurements:*

Serum NO concentrations ( $\mu\text{mol/l}$ ) were measured using Griess reagent system (Promega Corp, USA; Cat#G2930). The limit detection of this kit is 2.5  $\mu\text{mol/l}$ . Serum VEGF concentrations were measured using enzyme-linked immunosorbent assay (R&D systems, USA; Cat#RRV00).

#### *Angiogenesis Assay*

Immunohistochemistry method was used for evaluation of angiogenesis. For this purpose, the paraffin-embedded sections (5 $\mu\text{m}$ ) were prepared and stained by rat monoclonal antibody against murine CD31 as a marker of endothelial cells. Then, ten microscopic fields from each section were selected and number of CD31 positive cells measured by two observers and capillary density was reported as CD31 positive cell per square millimeter.

#### *Statistical analysis*

SPSS 16 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data are reported as mean  $\pm$  SE. One-Way ANOVA was used for comparison of data between groups. Paired t-test was used for paired data. P-value less than 0.05 was considered statistically sig-

nificant.

## RESULTS

#### *Effect of L-Arg and L-NAME on serum NO and VEGF concentration*

Serum NO concentration was similar between groups before experiment ( $p > 0.05$ ). At the end of experiment, serum NO level in diabetic animals was lower than control and pre-experiment ( $p < 0.05$ ) (Figure 1). L-Arg significantly increased serum NO concentration in diabetic group ( $8.57 \pm 1.12$  vs.  $4.89 \pm 0.41$   $\mu\text{mol/l}$ ;  $p < 0.05$ ); however, L-NAME could not reduce serum NO level ( $p > 0.05$ ).

Serum VEGF level was not different between the groups at baseline ( $p > 0.05$ ). At the end of experiment, serum VEGF concentration in diabetic group was significantly higher than control ( $116.8 \pm 5.12$  vs.  $78.22 \pm 5.14$  pg/ml;  $p < 0.05$ ) (Figure 2). It was also significantly higher than pre-experimental level ( $116.8 \pm 5.12$  vs.  $68.22 \pm 2.58$  pg/ml;  $p < 0.05$ ). L-Arg and L-NAME both increased serum VEGF concentration in diabetic animals, although it was not statistically significant.

#### *Coronary angiogenesis*

Coronary angiogenesis which was expressed as capillary density/ $\text{mm}^2$  was lower in diabetic group than control ( $p < 0.05$ ) (Figure 3a). L-Arg significantly improved capillary density in myocardial tissue in diabetic animals ( $p < 0.05$ ); however, L-NAME did not alter it ( $p > 0.05$ ). Samples of section stained immunohistochemically are presented in figure 3b.

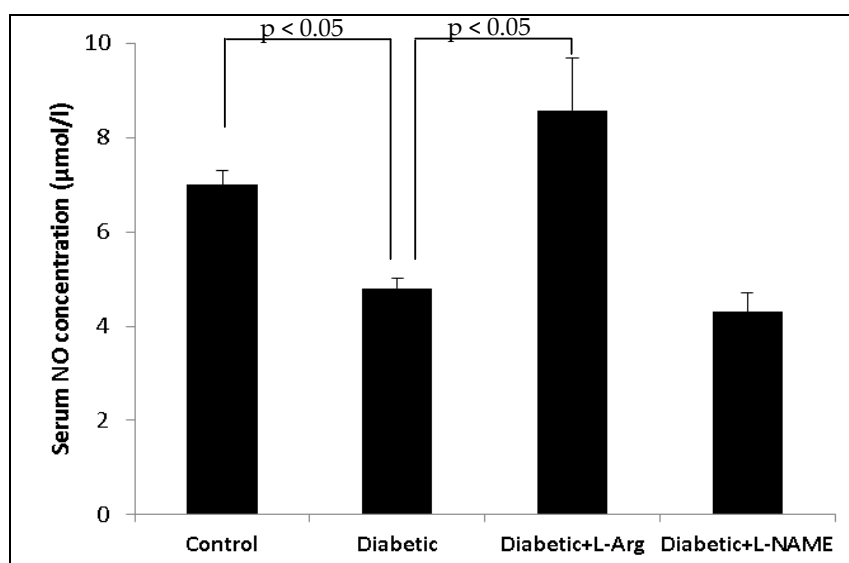


Figure 1. Serum nitric oxide concentration ( $\mu\text{mol/l}$ ) at the end of experiment (L-Arg: L-arginine)

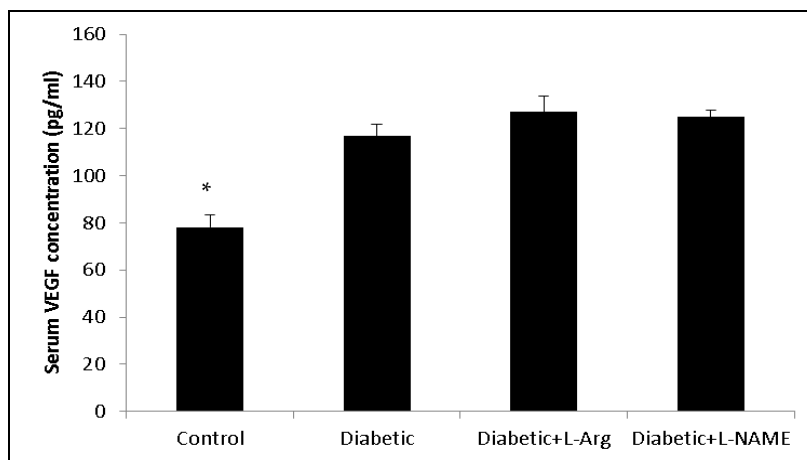


Figure 2. Post-experiment serum VEGF concentration (pg/ml) in all experimental groups (\* p < 0.05 compare to other groups. L-Arg: L-arginine)

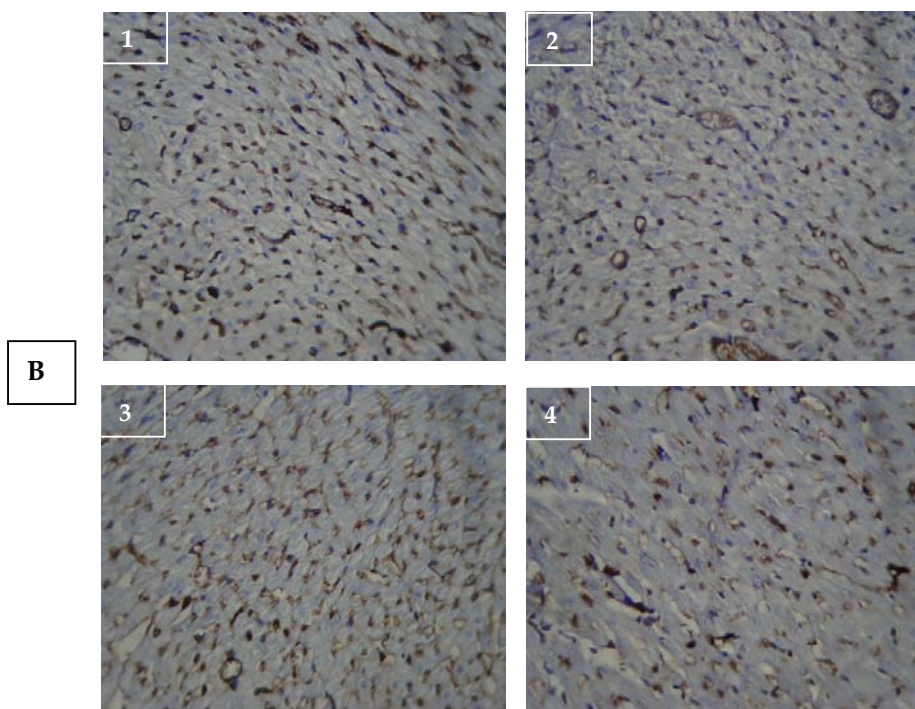
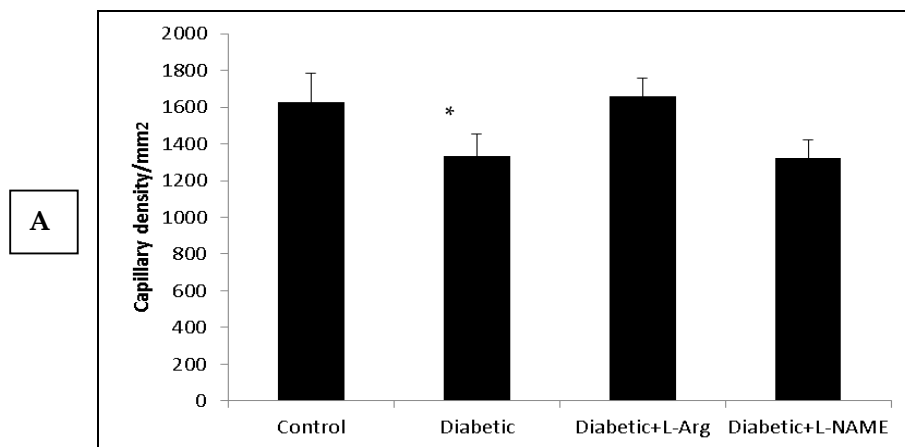


Figure 3. (A) Coronary angiogenesis expressed as capillary density/mm<sup>2</sup> (\* p < 0.05 compare to control and diabetic+L-Arg; (B) representative images of myocardial tissue (×400) stained by rat monoclonal antibody against CD31 antibody in control (1), diabetic (2), diabetic+L-Arg (3) and diabetic+L-NAME (4) groups

## DISCUSSION

NO is synthesized in endothelial cells by L-Arg as precursor and oxygen molecule using three isoforms of NO synthase. This enzyme converts L-Arg to NO and L-citrulline. Impairment of NO pathway in diabetes has been documented in several studies.<sup>[17]</sup> In this study, we found lower serum NO concentrations in diabetic animals compared to the control group. Several mechanisms have been suggested for reduced NO availability in diabetic subjects. Hyperglycemia causes increased free fatty acid, hyperinsulinemia and lowers NO production.<sup>[12]</sup> It also increases NO degradation by oxidative stress<sup>[18]</sup> and may activates protein kinase C which results in production of end products of glycation.<sup>[19]</sup>

In this study, L-Arg administration significantly increased serum NO concentration in diabetic animals. Administration of L-Arg in diabetic animals improves endothelial function and endothelium-dependent relaxation in coronary arteries and aortae.<sup>[20,21]</sup> NO also has a direct and indirect role on angiogenesis.<sup>[13]</sup> In the present study, coronary angiogenesis in diabetic group was lower than control. The effect of diabetes on angiogenesis is different depends on the tissues. In retina, increased angiogenesis leads to diabetic retinopathy, while, reduced angiogenesis causes impaired wound healing in diabetic subjects. Changes or alteration in function of VEGF and NO may be responsible for this difference. VEGF is a 45kd protein and is known as endothelial cell proliferation factor.<sup>[22]</sup> A clinical study demonstrated that in diabetic patients without atherosclerosis, serum VEGF was similar to control group;<sup>[23]</sup> however, in diabetic patients with peripheral or coronary atherosclerosis, serum VEGF was higher. In another study, plasma VEGF level in uncontrolled diabetic patients was higher than control group<sup>[24]</sup> and treatment of hyperglycemia reduced plasma VEGF level. In the present study, we found that serum VEGF concentration was high in diabetic animals, while, capillary density in myocardial tissue was lower than control. This may be reflected as VEGF resistance which suggested by Waltenberger et al.<sup>[25]</sup> NO is also a key factor during angiogenesis process and we found that L-Arg improved myocardial capillary density. Thus, it seems that there is a defect in function or synthesis of L-Arg in diabetic subjects. More studies need to clarify the function of new capillaries in reduction of cardiovascular mortality and morbidity in long-term use of this drug.

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