ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2012; 3(3):525-529

ORIGINAL RESEARCH ARTICLE

Assessment of Nitric oxide Linked Atherosclerosis in Surgically Induced Type I and Streptozocin Induced Type II Diabetes Model Goats

Bijay Aryal, Mamata Sharma Neupane, Kalpana Dhakal Sharma, Subodh Kumar Yadav, Sanjay Yadav, Pratik Adhikary, Dinesh Thapa and Harish Chandra Neupane*

College of Medicine, Chitwan School of Medical Sciences P.Ltd, Bharatpur-10, Chitwan, Nepal

Received 04 Mar 2012; Revised 22 May 2012; Accepted 30 May 2012

ABSTRACT

Diabetes mellitus is associated with development of cardiovascular complications such as atherosclerosis. In diabetes mellitus, impaired nitric oxide (NO) activity may be present. Hence, the present study was undertaken to compare NO activity between surgically induced Type I and streptozocin induced Type II diabetes in goat. The NO activity was determined in terms of nitrate (NO_3^-).The NO_3^- and peroxynitrite levels in abdominal aortas of goats were determined using validated HPLC method. The levels of NO_3^- were found to be 25.96 ± 8.25 ng/gm and 78.32±10.23 ng/gm in Type I and Type II diabetes model goats which is significantly difference with control group (96.53±11.23ng/gm,p=0.0012). Similarly, peroxynitrite levels were found to be 475.23±13.45 ng/gm and 325.42±9.62 ng/gm in Type I and Type II diabetes model goats respectively, which is significantly difference with control group (52.13±9.36ng/gm,p=0.0001) The result showed that NO_3^- levels were decreased with increased in peroxynitrite levels. Decreased NO activity can be caused by impaired production of NO, due to uncoupling of receptor-mediated signal transduction, a deficiency of the NO synthase (NOS) substrate l-arginine, or a decreased availability of one or more cofactors essential for optimal functioning of NOS. Hyperglycaemia also stimulates the production of advanced glycosylated end products, enhances the polyol pathway and activates protein kinase C. These conditions may lead to increased oxidative stress. Reactive oxygen species rapidly inactivate NO leading to the formation of peroxynitrite. In conclusion, the chances of atherosclerosis and peroxynitrite formation is maximum in patients with type I diabetes than type II.

Key words: Diabetes mellitus, atherosclerosis, oxidative stress and reactive oxygen species.

INTRODUCTION

Vascular diseases are the leading cause of death in modern era. Atherosclerosis usually occurs over years or decades [1]. Many factors are associated with the acceleration of atherosclerosis, regardless of the underlying primary pathogenic change. The well recognized risk factors include elevated plasma total and low-density lipoprotein (LDL) cholesterol level, high blood pressure, reduced high-density lipoprotein (HDL) cholesterol level, high blood level of L-homocysteine and diabetes mellitus (DM) [2,3].

DM is associated with early development of cardiovascular complications. Under physiological conditions the endothelium protects against the development of atherosclerosis. Endothelial cells produce, e.g., nitric oxide (NO), a substance which is capable of keeping vascular tone, coagulation and inflammation well balanced. However, in pathological conditions, such as in DM, impaired NO activity may be present [4]. Endothelial dysfunction, which is impaired ability of vascular endothelium for vasodilation, can be detected at very early stages of atherosclerosis.

It is therefore possible that deficiency in local NO availability could be a final common pathway that accelerates atherogenesis in humans. There are two general mechanisms by which endothelial NO deficiency might cause endothelial dysfunction. First, the synthesis of endothelial NO can be diminished due to a reduced expression or activity of eNOS. Second, the breakdown of eNOS-derived NO may be increased due to oxidative stress. The inactivation of eNOS enzyme may be related to a relative deficiency of NOS substrate, L-arginine, or its cofactor, BH4, or to a decreased clearance of eNOS endogenous competitive

***Corresponding Author:** Dr.Harish Chandra Neupane**, Email:** harish.neupane@cmc.edu.np

inhibitor, asymmetric *N*G *N*G-dimethyl-L-arginine (ADMA) [5,6].The accelerated NO decomposition occurs probably due to enhanced formation of free radicals such as superoxide anion. The superoxide anion reacts rapidly with endothelium-derived NO, forming a powerful oxidant, peroxynitrite, which accelerates atherosclerosis by initiating lipid peroxidation in LDL and also by inactivating eNOS by directly attacking the endothelium [7, 8]. The superoxide anion levels are normally maintained low by superoxide dismutase, which is the major antioxidant enzyme in the blood vessel wall. In patients with cardiovascular superoxide dismutase activity is down regulated [5, 8, 9]. Hence, the present study was undertaken to compare NO and peroxynitrite levels in abdominal aortas of surgically induced Type I, streptozocin induced Type II and sham control goats. The NO activity was determined in terms of nitrate (NO_3^-) and peroxynitrite. The levels of NO and peroxynitrite levels in aortas were determined using validated HPLC methods.

MATERIALS AND METHODS

Chemicals and reagents

Streptozocin (Sigma–Aldrich Co, St Louis, MO, USA) was obtained as gift samples from department of pharmacology, college of medicine, Dankook University, Cheonan, South Korea. Methanol and acetonitrile grade were pursed from Triveni Interchem P.Ltd (India). Formic acid AR was pursed from Manav Biochem Impex P.Ltd (India). Reagent grade triple deionized water was pursed from Organo Biotech Laboratories. P.Ltd (India). All other chemicals and reagents used were of analytical grade.

Apparatus and conditions

Levels of NO_3^- and peroxynitrite were determined simultaneously by HPLC-UV using previous reported method [11]. Briefly, the chromatography was performed at room temperature with a mobile phase (pH 8.5) composed of borate buffer/gluconate concentrate, methanol, acetonitrile and deionized water (2:12:12:74, v/v/v/v), with a flow rate of 1.3 mL/min. The borate buffer/gluconate concentrate consisted of 0.07 mol/L sodium gluconate, 0.3 mol/L H_3BO_3, 0.1 mol/L $Na_2B_4O_7$ and 3.8 mol/L glycerol in deionized water (1.6 g sodium gluconate, 1.8 g H_3BO_3, 2.5 g $Na_2B_4O_7$ and 25 mL glycerol were dissolved in deionized water to 100 mL). The borate buffer of the mobile phase served to adjust the pH of the mobile phase to 8.5. Calibration of the pH meter (HI 9321, Hanna Instruments) at pH 7.01 and 10.01 was performed with standard buffers HI 7710P (Hanna Instruments). The mobile phase was filtrated prior to use. Ultraviolet detection was performed at 214 nm. The injected volume of the samples was 5uL. System control and data analysis were carried out using Shimazdu MS software (Version 6.5, Shimazdu Inc.). HPLC columns YMC® C18 (Waters, MO, USA), 50 mm × 2.0 mm, 3 μm particle size and guard column (C18, 4.0 X 2.0mm, phenomenex, CA, USA) were used for analyzing samples. A standard mixture of nitrate and peroxynitrite was used to determine the retention times and separation of the peaks. Nitrate and peroxynitrite concentrations were equal in the mixture solution and were in the range of 0–1000 mmole/L.

Animal handling and surgical procedures

7.0 to 8.0 kg male goats were pursed from Madhuvan Ecovillage Center, Chitwan, Nepal. The goats were acclimated for one week before study. Upon arrival, animals were randomized and housed in strictly controlled environmental condition of 20 to 25^{o}C temperature and 48 to 52% relative humidity (RH). A 12 hour light and dark cycle was used with an intensity of 150 to 300 Lux. All animal procedures were based on a guideline recommended by institutional animal care and experiment committee of Chitwan Medical College. Before conducting the study, goats were categorized into three groups; Control group (n=6), Type I DM group (n=6) and Type II DM group (n=6).Type I and Type II DM in animals were induced using previous reported method [12]. Briefly, Type II DM was induced by administrated streptozocin (30mg/kg, intravenous route) daily up to one month and Type I DM was induced by thermo coagulation and ligation of duct of wirsung (**Fig 1**) and duct of santorini (**Fig 2**) under Xylocaine (80mg/kg) and Ketamine Hydrochloride (10mg/kg) general anesthesia. The DM in both groups were confirmed by measurement of blood sugar (fasting and random) after surgery and drug administration. The animals were sacrificed after obtaining stable fasting glucose level 600mg/dl. Abdominal aorta were collected and stored in 50% methanol solution at -4^{o}C until analysis begins.

Sample Preparation

Sample preparation involved a protein precipitation method with acetronitrile. An aliquot of grinded tissue sample 100μL was spiked 20 μl sodium gluconate 1 μg/ml, prepared in methanol/water 50/50, v/v and extracted with 400 μl acetronitrile solution. The organic layer was

dried under the gentle stream of nitrogen 40°C. The dried extract was reconstituted with 800 µl of 5 0 %methanol and 5 µl was injected to HPLC system.

Data Analysis

The student's *t-test* for independent data was used to compared the NO parameters between DM groups and control group, with significance assigned at P <0.05. All other data were expressed as mean± standard deviation (SD).

RESULTS AND DISCUSSION

Analytical condition and Validation

In order to develop an analytical method with desired LLOD (10ng/gm), it was necessary to use HPLC-UV detection, because HPLC analytical methods provide the very low limits of detection (LOD) required for trace mixture analysis.[12] Chromatographic conditions, especially the composition of the mobile phase, were optimized to achieve good resolution and symmetrical peak shapes for NO_3^- and the peroxynitrite, acceptable retention factors (k'≥2), and a short run time. The isocratic mobile phase consisting of 0.07 mol/L sodium gluconate, 0.3 mol/L H_3BO_3, 0.1 mol/L $Na_2B_4O_7$ and 3.8 mol/L glycerol in deionized water (1.6 g sodium gluconate, 1.8 g H_3BO_3, 2.5 g $Na_2B_4O_7$ and 25 mL glycerol were dissolved in deionized water to 100 mL) was found to be suitable. A flow rate of 1.3 ml/min was required to elute the $NO_3^-$and the peroxynitrite at retention times of 1.51 and 1.43 min, respectively. The sodium gluconate was found to be necessary in order to increase the pH and protonate peroxynitrite to produce a symmetrical peak shape at a satisfactory retention factor. The percentage of sodium gluconate was also optimized to achieve a symmetrical peak shape.

The calibration curve drawn for nitrate in tissue sample was linear over the concentration range 10 to 500 ng/gm. The best linear fit and least squares residuals of the calibration curve were achieved using a $1/x^2$ weighing factor, giving a mean linear regression equation for the calibration curve of *y=0.75214x + 0.001162* . The correlation coefficient (r^2) for nitrate was 0.99991. The inter- and intra-day precisions were expressed as CV % and were below 15% (maximum 12.41% and minimum 8.12% for LLOD sample), and the accuracy was between 82.14% and 101.94%, which complies with the FDA regulations. The recovery percentages of QC samples were between 98.45% and 105.71%. The extraction procedure showed good sensitivity, specificity, precision, accuracy, recovery, and linearity, and hence the method was successfully implemented for the analysis of tissue samples (**Table 1**).

Similarly, the calibration curve drawn for peroxynitrite in tissue sample was linear over the concentration range 10 to 500 ng/gm. The best linear fit and least squares residuals of the calibration curve were achieved using a $1/x^2$ weighing factor, giving a mean linear regression equation for the calibration curve of *y=0.2415x + 0.006935* . The correlation coefficient (r^2) for nitrate was 0.9981. The inter-and intra-day precisions were expressed as CV % and were below 15% (maximum 9.68% and minimum 9.63% for LLOD sample), and the accuracy was between 98.52% and 112.36%, which complies with the FDA regulations. The recovery percentages of QC samples were between 96.32% and 100.96%. The extraction procedure showed good sensitivity, specificity, precision, accuracy, recovery, and linearity, and hence the method was successfully implemented for the analysis of tissue samples (Table 1).Peaks corresponding to nitrate and peroxynitrite, observed in tissue samples using the HPLC-UV conditions were described in (**Fig 3**).

Determination of Nitrate and peroxynitrite levels in abdominal aortas of goats

The levels of NO_3^- were found to be 25.96 ± 8.25 ng/gm and 78.32±10.23 ng/gm in Type I and Type II diabetes model goats which is significantly difference with control group (96.53±11.23ng/gm,p=0.0012). Similarly, peroxynitrite levels were found to be 475.23±13.45 ng/gm and 325.42±9.62 ng/gm in Type I and Type II diabetes model goats respectively, which is significantly difference with control group (52.13±9.36ng/gm,p=0.0001). The result showed that NO_3^- levels were decreased with increased in peroxynitrite levels (**Table 2**). Endothelial dysfunction, which is impaired ability of vascular endothelium for vasodilation, can be detected at very early stages of atherosclerosis [8, 9]. It is therefore possible that deficiency in local NO availability could be a final common pathway that accelerates atherogenesis in humans. There are two general mechanisms by which endothelial NO deficiency might cause endothelial dysfunction. First, the synthesis of endothelial NO can be diminished due to a reduced expression or activity of eNOS. Second, the breakdown of eNOS-derived NO may be increased due to oxidative stress. The inactivation of eNOS enzyme may be related to a relative deficiency of NOS substrate, L-arginine, or its cofactor, BH4, or

to a decreased clearance of eNOS endogenous competitive inhibitor, asymmetric *N*G *N*G-dimethyl-L-arginine (ADMA) [5,6]. The accelerated NO decomposition occurs probably due to enhanced formation of free radicals such as superoxide anion. The superoxide anion reacts rapidly with endothelium-derived NO, forming a powerful oxidant, peroxynitrite, which accelerates atherosclerosis by initiating lipid peroxidation in LDL and also by inactivating eNOS by directly attacking the endothelium [7, 8]. Decreased NO activity can be caused by impaired production of NO, due to uncoupling of receptor-mediated signal transduction, a deficiency of the NO synthase (NOS) substrate l-arginine, or a decreased availability of one or more cofactors essential for optimal functioning of NOS [11,12]. Hyperglycaemia also stimulates the production of advanced glycosylated end products, enhances the polyol pathway and activates protein kinase C. These conditions may lead to increased oxidative stress. Reactive oxygen species rapidly inactivate NO leading to the formation of peroxynitrite [5, 7, 11]. In conclusion, the chances of atherosclerosis and peroxynitrite formation is maximum in patients with type I diabetes than type II.

Table 1: Validation of the HPLC method for measuring Nitrate and Peroxynitrite.

Parameters	Obtained Results (Nitrate)	Obtained Results (Peroxynitrite)
Lower limit of detection (ng/gm)	10 ng/gm	10ng/gm
Calibration range (ng/gm)	10-500 ng/gm	10-500 ng/gm
Calibration equation	y = 0.75214x + 0.001162	y = 0.2415x + 0.006935
Coefficient if regression(r^2)	0.99991	0.9981
Interday Precision (CV %,n=5) [a]		
10 ng/gm	8.12.	9.68
100 ng/gm	9.35	8.25
200 ng/gm	11.25	11.78
500 ng/gm	13.25	8.62
Interday Accuracy (%,n=5) [b]		
10 ng/gm	101.94	98.52
100 ng/gm	104.79	112.36
200 ng/gm	103.46	90.25
500 ng/gm	101.95	107.24
Intraday Precision (CV %,n=5) [a]		
10 ng/gm	12.41	9.63
100 ng/gm	6.28	12.45
200 ng/gm	9.58	9.32
500 ng/gm	10.12	8.12
Intraday Accuracy (%,n=5) [b]		
10 ng/gm	82.14	98.56
100 ng/gm	107.19	100.25
200 ng/gm	103.25	104.89
500 ng/gm	100.35	100.28
QC samples recovery (%,n=5)		
10 ng/gm	105.71	100.96
200 ng/gm	98.45	99.25
500ng/gm	99.28	96.32

[a] *%CV=Standard deviation of concentrations determined x 100/ Mean concentration determined*

[b] *Accuracy=Mean concentration determined x100/Concentration expected,*

The intra-and inter-day precisions expressed as coefficient of variations percent (% CV) should not exceed 15% at any concentration level, with the exception of LLOD, QC samples, where should not exceed ±20% (Bioanalytical Method Validation, FDA guidelines, May 2001).

Fig 1: Thermo coagulation and ligation of duct of wirsung

Fig 2: Thermo coagulation and ligation of duct of santorini

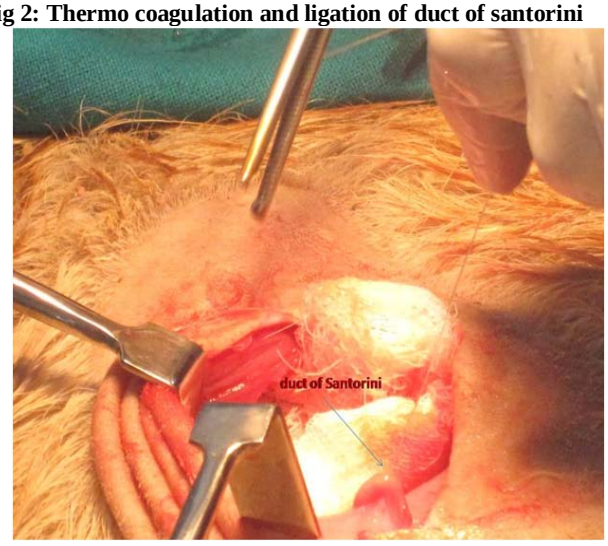

Fig 3: HPLC -UV peaks corresponding to nitrate and peroxynitrite observed in tissue samples

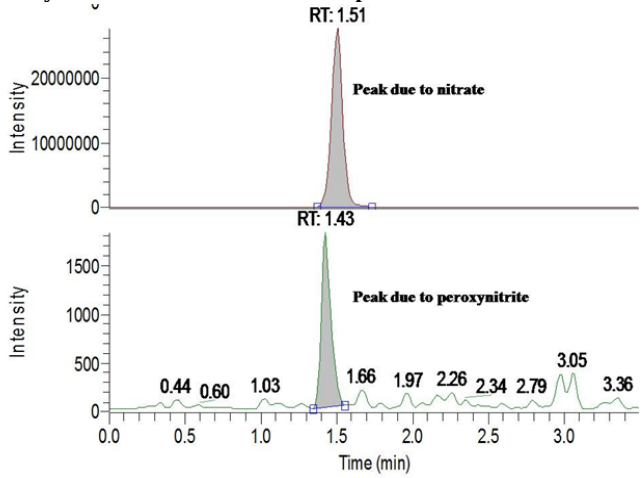

Table 2: Determination of Nitrate and Peroxynitrite levels in abdominal aortas of goats

Parameters	Mean±SD (ng/gm)
Control Group (n=6)	
Nitrate	96.53
Peroxynitrite	52.13
Surgical induced group (Type I DM,n=6)	
Nitrate	25.96
Peroxynitrite	475.23
Streptozocin induced group (Type II DM,n=6)	
Nitrate	78.32
Peroxynitrite	325.42

ACKNOWLEDGEMNT

The authors would like to thank Chitwan School of Medical Sciences P.Ltd for providing research facility.

REFERENCES

1. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol.1996; 271(5 Pt 1):C1424-37.
2. Jia, L.; Bonaventura, C.; Bona ventura, J.; Staml er, J.S. Nitric oxide, superoxide, and Peroxynitrite. Nature, 1996, 380:221.
3. Kelm M.Nitric oxide metabolism and breakdown. Biochim Biophys Acta. 1999; 1411(2-3):273-89.
4. Benzuly KH, Padgett RC, Kaul S, Piegors DJ, Armstrong ML,Heistad DD. Functional improvement prece des structural regression of atherosclerosis. Circulation 1994; 89(4):1810-8.
5. John S, Schmieder RE.Impaired endothelial function in arterial hypertension and hype rcholesterolemia: potential mechanisms and differences. J Hypertens. 2000; 18(4):363-74.
6. Landmesser, U.; Merten, R.; Spiekermann, S.; Buttner, K.;Drexler, H.; Hornig, B.Atherosclerosis. Circulation, 2000; 101:2264.
7. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999; 340(2):115-26.
8. Li H, Forstermann U. Nitric oxide in the pathogenesis of vascular disease. J Pathol. 2000; 190(3):244-54.
9. Drexler H, Zeiher AM, Meinzer K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. Lancet. 1991 Dec 21-28; 338(8782-8783):1546-50.
10. Stroes ES, Koomans HA, de Brui n TW, Rabelink TJ.Vascular function in the forearm of hypercholesterolaemic patients off and on lipid-lowering medication. Lancet. 1995; 346(8973):467-71.
11. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. J Clin Invest. 1993; 91(6):2546-51.
12. Mugge A, Brandes RP, Boger RH, Dwenger A, Bode-Boger S, Kienke S, Frolich JC, Licht len PR. Vascular release of superoxide radicals is enhanced in hypercholesterolemic rabbits. J Cardiovasc Pharmacol. 1994; 24(6):994-8.