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L-Arginine Supplementation Attenuates Oxidative Stress Mediated Gastrointestinal Dysfunction in Experimental Diabetic Rat

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ABSTRACT

Gastrointestinal dysfunction in diabetes is an important microvascular complication. In the present study, we examined the effect of an anti-oxidant L-arginine on gastrointestinal function and oxidative stress in alloxan-induced diabetic rats. Adult SD rats were injected with alloxan to produce experimental oxidative stress characteristics of diabetes mellitus and maintained in this state for 8 weeks to produce gastrointestinal complication. Rats were treated with L-arginine (0.5mg/ml) through drinking water. Body weights, plasma glucose and glycosylated hemoglobin levels were measured at 0,12,21, and 57 days. At the termination of the experiments, gastric emptying, intestinal transit, and tissue motility were measured. Oxidative stress marker malonaldehyde, glutathione, superoxide dismutase and catalase were measured. Alloxan-injected rats showed significant increase in blood glucose, glycosylated hemoglobin, and decreased bodyweight. After 8 weeks, diabetic rats exhibited gastrointestinal dysfunction, evidenced by significant delay in gastric emptying, intestinal transit, and decreased contractile response of fundus and ileum to acetylcholine along with a marked increase in oxidative stress. L-arginine treatment significantly attenuated gastrointestinal dysfunction and oxidative stress in diabetic rats which confirm the role of oxidative stress in diabetic gastrointestinal complication and point to the possible anti-oxidative mechanism being responsible for the gastro protective action of L-arginine.

Keywords: Diabetic complications, Gastric emptying, Intestinal transit, Glycosylated hemoglobin, Nitric oxide, Diabetic gastropathy, Gastroparesis

1. INTRODUCTION

Diabetes and its related complications is one of the leading healthcare problems for humankind. A rough estimate suggests that 150 million people are suffering from the disease and perhaps several millions have not yet been diagnosed. In India above 33 million people are suffering from diabetes. As per WHO estimates, the number would cross 300 million by 2025, with India being the worst affected. Most of the diabetic patients often exhibit variety of gastrointestinal (GI) disorders such as delayed gastric emptying, dysphagia, early satiety, reflux, constipation, abdominal pain, nausea, vomiting, and diarrhea[1]. The severity of these disorders varies in nature and intensity, depending upon the extent of dysregulation in the GI tract. The evidence suggest that GI dysfunction in diabetics can be consequent to autonomic neuropathy leading to abnormalities in intestinal motility, sensation, secretion, and absorption [2]. The mechanisms through which such complications arise are not very clear. Many in vivo and in vitro studies have indicated that oxidative stress is one of the major pathophysiological mechanisms involved in the development of diabetic complications [3-5].

One of the latest entries in the antioxidant arsenal for diabetics is L-arginine, first identified in extracts of etiolated lupine seedlings by Schultz and Steiger in 1886. It has been reported that arginine concentration decreases in plasma of diabetic patients [6] and in plasma and vascular tissue of diabetic rats [7]. In fact how the diabetic state causes the arginine deficiency is not clear. It has been shown that L-arginine ameliorates oxidative stress in liver and brain in 7 days old diabetes mellitus [8]. The researchers have also shown that L-arginine reduced superoxide radical release by isolated aortic rings to control levels, which was unaffected by vitamin E treatment [9]. In our previous studies, it has been shown that daily supplementation of L-arginine for 8 weeks ameliorated oxidative stress in gastrointestinal tissues in diabetic rats [10]. It is interesting to note that supplementation of L-arginine attenuates some of the diabetic complications such as cardiomyopathy [11], nephropathy[12], vascular [13] and endothelial dysfunctions [7]. To our knowledge, however, there is no data concerning the effects of long-term L-arginine supplementation particularly in the state of gastrointestinal dysfunction in alloxan-induced diabetic rats. The present study was conducted to describe these effects of L-arginine. In view of these evidences, it is thought that L-arginine supplementation in diabetic animals may reduce lipid peroxidation and improve the antioxidant enzyme levels; thus provide useful therapeutic tool to attenuate the diabetes-induced GI dysfunctions. Hence a preliminary experimental study had been carried out to support the above possibility. Diabetic gastrointestinal complications were induced by administration of alloxan and were assessed by measuring gastric emptying, intestinal transit and tissue contractility. We had determined the levels of lipid peroxidation (LPO) expressed as thiobarbituric acid reactive substances (malondialdehyde [MDA]) and enzymatic and nonenzymatic antioxidant defense systems, which include reduced glutathione (GSH), and the enzymes superoxide dismutase (SOD) and catalase. Blood glucose level and formation of advanced glycation end products as a marker of oxidative stress were also determined.

2. MATERIALS AND METHODS

2.1 Chemicals

L-arginine, alloxan, Phenylmethane sulfonylfluoride (PMSF), thiobarbituric acid, 5'-5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich, USA. Total protein assay kit and Glucose GOD-POD kit were purchased from Span diagnostics Ltd., Surat, India. All other chemicals were of analytical grade.

2.2 Animals

Young healthy male Sprague-Dawley rats weighing 260-300g (National Institute of Nutrition, Hyderabad) were housed under the controlled conditions of temperature, humidity (25±2□C, 55±2 %) and dark/light (12/12 h) cycle. They received a standard rodent chow (Goldmohar brand, Lipton India Ltd.) and water ad libitum. Institutional Animal Ethical Committee (IAEC), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, approved the experimental protocols. Every care was taken to minimize animal suffering and reduce the number of animals used.

2.3 Treatment Protocol

Animals were divided in two groups, namely control and diabetic. These groups were further divided into two subgroups (n=7) and one subgroup of each group was employed for L-arginine treatment. L-arginine was added to drinking water of rats daily at a dose of 0.5mg/ml up to 8 weeks following the establishment of diabetes with alloxan. Dose was chosen in accordance with the literature [14-17] and was fixed on the basis of daily intake of drinking water by the rats. The experimental and control animals were kept fasting for 18h prior to every determination.

2.4 Induction of diabetes and assessment of blood glucose level (BGL)

The diabetes was induced in rats by alloxan (120 mg/kg/i.p.) freshly prepared in saline. As alloxan-induced hyperglycemia is reversible, it was later maintained in course of study period by administering alloxan (100mg/kg/i.p.) on day 12 and 21. The rats were given 5% glucose ad libitum for 24 hrs on every alloxan administration in order to prevent alloxan-induced transient hypoglycemia. The blood (~0.5 ml) was withdrawn on day 3, 15 and 57 from the retro-orbital plexus by micro capillary method [18] under light ether anesthesia and BGL were measured by GOD-POD method [19] using commercially available GOD-POD kit and expressed as mg/dl.

The rats showing fasting blood glucose more than 170mg/dl after 3 days of first administration of alloxan were considered as diabetic.

2.5 Glycosylated hemoglobin estimation

Glycosylated hemoglobin was measured by a previously described method [20] and expressed in terms of %.

2.6 Body weight

As body weight is a surrogate measure of appropriate gastrointestinal functioning, it was recorded critically during the time period of study.

Table 1: Plasma glucose levels of normal and diabetic rats treated and not treated with L-arginine

Group	Treatment	Glucose (mg/dl)			
		Day of Estimation			
		00	03	15	57
Non-diabetic	Vehicle	92.2± 2.3	91.8± 1.8	92.1± 2.3	88.9± 1.8
	L-Arg	94.5± 2.3	94.1± 2.2	108.0± 2.5	124.4± 3.1
Diabetic	Vehicle	95.9± 1.6	326.4± 11.3 *	344.3± 13.7 *	322.9± 15.6 *
	L-Arg	95.2± 1.5	326.2± 9.6	244.5± 15.2 #	133.1± 8.1#

.Values are mean ± SEM, n = 6

* P <0.05; when compared with non-diabetic group.
# P <0.05; when compared with diabetic group.

Table 2: Glycosylated Haemoglobin levels (%) of normal and diabetic rats, treated and not treated with L-arginine

Group	Treatment	Glycosylated Haemoglobin (%)			
		Day of estimation			
		00	03	15	57
Non-diabetic	Vehicle	3.3±0.1	3.1±0.1	3.4±0.1	3.3±0.1
	L-Arg	3.3±0.1	3.3±0.1	3.6±0.1	3.8±0.2
Diabetic	Vehicle	3.3±0.1	4.1±0.2 *	5.2±0.2 *	6.2±0.2 *
	L-Arg	3.2±0.2	4.2±0.2	4.0±0.2 #	3.6±0.2 #

Values are mean ± SEM, n = 6

* P <0.05; when compared with non-diabetic group.
# P <0.05; when compared with diabetic group

2.7 Assessment of Gastric emptying

The gastric emptying of a non-nutrient solution was assessed by previously reported method [21]. 1.5 ml test meal (0.05% phenol red in 1.5% aqueous methylcellulose solution) was orally administered to overnight fasted rat with the help of specially designed orogastric canula on day 57. After thirty minutes the animal was sacrificed. The stomach was clamped with a string above the lower oesophageal sphincter and a string beneath the pylorus to prevent the leakage of test meal. The isolated stomach was resected and homogenized along with its contents in 20.0 ml of 0.1 N NaOH. In 5.0 ml of stomach homogenate, trichloroacetic acid (0.2 ml of 20% w/v) was added to precipitate proteins and centrifuged. The supernatant was mixed with 4.0 ml of 0.5 N NaOH and the absorbance was read at 560 nm on Shimadzu UV-1601 UV-visible spectrophotometer. The phenol red recovered from the stomach of a rat killed immediately after orogastric administration of meal was considered as the amount of phenol red present at the beginning of emptying. The percentage gastric emptying in rat was calculated by the formula:

% Gastic Emptying = 1- Amt Of Phenol Redrecovered From Stomch at the end of 30 mint. /Amt Of Phenol Redrecovered From Stomch at the beginning ×100

2.8 Intestinal transit (IT)

The intestinal transit of charcoal meal was assessed by method of Janseen and Jagenerous [22]. The rats received charcoal meal containing 10% activated charcoal and 5% gum acacia orally (2.0 ml/rat) on day 57. After 15 minutes, the rats were sacrificed by deep ether anaesthesia. The small intestine was removed from the pyloric sphincter to the ileoceacal junction. The maximum distance traveled by the charcoal meal was noted and expressed as percent intestinal transit using the formula. Stretching of intestine was carefully avoided while measuring the distances.

$$\%\,\text{Transit} = \frac{\text{Distance traveled by charcoal meal}}{\text{Total length of small intestine}} \times 100$$

2.9 Fundus and intestinal Motility

After the overnight fasting for 18h, the rat was decapited and fundus and ileum were isolated. Isolated tissues were washed with fresh aerated Krebs solution. The fundus of stomach was cut in a zigzag manner and one cm long piece of fundus and ileum were set in separate organ baths of a specially designed organ bath assembly under standard experimental conditions. After stabilization for 30 min, the contractile responses of the tissues to different concentrations of acetylcholine (ACh) were recorded. The graph for log dose vs. percent response was plotted and average

EC50 values of ACh for each tissue was calculated and compared [23].

2.10 Assessment of Oxidative Stress

2.10.1 Preparation of tissue homogenate

After receiving the treatment for 56 days, the rats were sacrificed with deep ether anesthesia on 57th day. The stomach and intestine were removed and thoroughly washed with ice-cooled 0.1 M phosphate buffered saline (PBS) containing 0.1mmol/L phenylmethane sulfonylfluoride. The individual tissue was blotted dry and homogenized in 0.1 M PBS in ice-bathing so as to prepare a 10 % suspension. This suspension was then centrifuged at 16000×g for 1 h in a cooling centrifuge at 0ºC. The supernatant was subjected to further assessment of oxidative stress after estimating the protein content [24].

Table 3: Body weights (g) of normal and diabetic rats, treated and not treated with L-arginine.

Group	Treatment	Body weight (g)			
		Day of estimation			
		00	12	21	57
Non-diabetic	Vehicle	262.2± 3.5	275.5± 4.3	286.0± 3.4	313.2± 3.5
	L-Arg	265.3± 4.2	274.0± 4.1	289.0± 4.3	333.6± 5.6
Diabetic	Vehicle	296.7± 5.4	276.5± 4.1	250.2± 4.3	208.5± 3.1 *
	L-Arg	292.8± 6.5	279.0± 5.9	286.8± 4.2	296.3± 5.4 #

Values are mean ± SEM, n = 6
* *P* <0.05; when compared with non-diabetic group.
# *P* <0.05; when compared with diabetic group.

2.10.2 Lipid peroxidation (LPO) in tissue

MDA, an end product of fatty acid peroxidation, was measured in tissue homogenates as previously described [25]. The method is based on the formation of a red chromophore that absorbs at 532nm following the reaction of thiobarbituric acid (TBA) with malonyldialdehyde (MDA) and other break down products of peroxidized lipids collectively called as thiobarbituric acid reactive substances (TBARS). All samples were run in duplicate and peroxidation was expressed as nM MDA/mg protein.

Table 4: Gastric emptying (%) and intestinal transit (%) of normal and diabetic rats, treated and not treated with L-arginine.

Group	Treatment	Gastric Emptying (%)	Intestinal Transit (%)
Non-diabetic	Vehicle	80.33±1.35	57.90±1.40
	L-Arg	69.50±0.76	62.37±1.37
Diabetic	Vehicle	56.67±2.37*	38.35±1.54*
	L-Arg	78.00±2.06#	54.63±2.04#

Values are mean ± SEM, n = 6
* P <0.001; when compared with non-diabetic group.
# P <0.001; when compared with diabetic group.

Table 5: EC50 of Acetylcholine for Fundus and Ileum of normal and diabetic rats, treated and not treated with L-arginine.

Group	Treatment	EC_{50} in ng	
		Fundus	Ileum
Non-diabetic	Vehicle	187.7±10.8	849.5±23.1
	L-Arg	186.5±12.1	829.7±26.5
Diabetic	Vehicle	379.8±27.3 *	1575.0±44.0 *
	L-Arg	239.8±17.2 #	968.7±38.1 #

Values are means ± SEM, n = 6
* P <0.001; when compared with non-diabetic group.
# P <0.001; when compared with diabetic group.

2.10.3 Reduced glutathione (GSH)

Tissue glutathione (GSH) concentrations were determined according to the method of Beutler et al.[26], using metaphosphoric acid for protein precipitation and 5'-5'-dithiobis-2-nitrobenzoic acid (DTNB) for colour development. The results were expressed as µg of conjugated DTNB per mg of protein.

Table 6: Pylorus lipid peroxidation product (nM MDA/mg protein) and glutathione contents (GSH, □g conj. DTNB/mg protein) as well as superoxide dismutase (SOD, U/mg protein) and catalase (CAT, U/mg protein) activities in normal and diabetic rats, treated and not treated with L- arginine.

Group	Treatment	LPO	GSH	SOD	CAT
Non-diabetic	Vehicle	0.45± 0.04	0.29± 0.01	0.78± 0.03	16.29± 0.98
	L-Arg	0.43± 0.03	0.30± 0.01	0.86± 0.03	18.54± 1.24
Diabetic	Vehicle	0.66± 0.04[c]	0.17± 0.01[a]	0.42± 0.03[a]	8.78±0 .36[a]
	L-Arg	0.45± 0.06[d]	0.26± 0.02[b]	0.70± 0.02[b]	15.41± 1.03[b]

Values are mean ± SEM, n = 6
a P <0.001 as compared to control
b P <0.001 as compared to diabetic control
cP <0.01 as compared to control
dP <0.01 as compared to diabetic control

Table 7: Ileum lipid peroxidation product (nM MDA/mg protein) and glutathione contents (GSH, □g conj. DTNB/mg protein) as well as superoxide dismutase (SOD, U/mg protein) and catalase (CAT, U/mg protein) activities in normal and diabetic rats, treated and not treated with L-arginine.

Group	Treatment	LPO	GSH	SOD	CAT
Non-diabetic	Vehicle	0.32± 0.02	0.20± 0.01	1.59± 0.09	10.07 ±0.43
	L-Arg	0.30± 0.04	0.21± 0.01	1.80± 0.04	10.89 ±0.76
Diabetic	Vehicle	0.58± 0.03[a]	0.13± 0.01[a]	1.08± 0.06[a]	5.51± 0.81[a]
	L-Arg	0.31± 0.04[b]	0.22± 0.01[b]	1.5±0 .08[d]	9.66± 0.59[b]

Values are mean ± SEM, n = 6
a P <0.001 as compared to control
b P <0.001 as compared to diabetic control
dP <0.01 as compared to diabetic control

2.10.4 Superoxide dismutase (SOD) activity

Tissue SOD activities were determined by the method of Marklund et al [27]. The ability of the enzyme to inhibit the autooxidation of pyrogallol in presence of EDTA was used as a measure of SOD activity.

One unit of the enzyme activity was the 50% inhibition of the rate of the autooxidation of pyrogallol as determined by the change in absorbance/min at 420nm. The activity of SOD is expressed as units/mg protein. The assay was performed in duplicate in two-fold concentration range.

2.10.5 Catalase (CAT) activity

Catalase activity was measured by the breakdown of hydrogen peroxide catalyzed by catalase enzyme. The results were expressed as CAT activity U/mg of protein [28].

2.10.6 Statistical Analysis

The data were analyzed with one-way ANOVA followed by Newman-Keuls comparison test for multiple comparisons using Graph pad prism. For blood glucose, body weight and glycosylated haemoglobin, the significance was calculated by Two-way ANOVA followed by Bonferroni posttests. The intergroup difference was considered significant when $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Glucose levels

The diabetic group which received time spaced multiple doses of alloxan on day 1, 12 and 21 exhibited significantly higher levels of blood glucose when estimated on day 3, 15 and 57. The BGL in L- arginine supplemented diabetic group were significantly lesser than vehicle treated diabetic group on day 15 and almost normal on day 57.

3.2 Glycosylated haemoglobin

The HbA1C, was twice increased in diabetic compared to non-diabetic vehicle treated rats ($P<0.001$). This increase was attenuated with L-arginine supplementation ($P<0.001$).

3.3 Body weight

The non-diabetic vehicle treated rats exhibited gradual increase in body weight over a period of 8 weeks. However, vehicle treated diabetic rats exhibited significant weight loss ($P<0.001$). The daily supplementation of diabetic rats with L-arginine significantly prevented this weight loss.

3.4 Gastric emptying and Intestinal transit

The vehicle treated non-diabetic rats exhibited 80.33±1.35% gastric emptying of the phenol red and the charcoal meal traveled up to 57.9±1.4% of the total length of the intestine. In vehicle treated diabetic group, gastric emptying and intestinal transit

were significantly reduced to 56.67±2.37% and 38.35±1.54% respectively (P<0.001). L-arginine supplementation did not produce any per se effect on these parameters in vehicle treated non-diabetic rats however, in diabetic group, it has significantly improved gastric emptying to 78±2.06% and intestinal transit to 54±2.04% (P<0.001).

3.5 Fundus and intestinal Motility

It is observed that the contractile responses of fundus and ileum to ACh were significantly low in vehicle treated diabetic group (P<0.001). These responses were almost normal in L-arginine supplemented diabetic group (P<0.001). L-arginine per se did not produce any effect on contractility and responsiveness of tissues in vehicle treated non-diabetic rats.

3.6 Oxidative stress parameters

Lipid peroxidation levels as assessed by MDA in tissue were found to be significantly higher in (P<0.001) vehicle treated diabetic group in comparison to vehicle treated non-diabetic group. However L-arginine had reversed this hike in tissue MDA level in diabetic rats. L-arginine had no per se effect on MDA level in vehicle treated non-diabetic rats.

Tissue antioxidant components (SOD, CAT and GSH) in vehicle treated diabetic group were lower than in vehicle treated non-diabetic group (P<0.001). However this decrease in antioxidants level was restored by concurrent supplementation with L-arginine.

The results of the present study demonstrate that treatment of L-arginine for the period of 8 weeks attenuates gastrointestinal dysfunction in experimental diabetic rats. In this study, we hypothesized that L-arginine treatment in vivo could prevent the abnormalities in gastrointestinal function brought about by diabetes and found that long-term treatment of diabetic rats with the precursor of endothelium-derived relaxing factor/nitric oxide (EDRF/NO) prevented impairment in gastrointestinal function caused by experimental diabetes. In the present study, the beneficial effects of chronic L-arginine treatment on gastrointestinal dysfunction was specific for diabetic condition because L-arginine treatment did not show any effect in control rats. It has been reported that arginine concentrations are decreased in plasma of diabetic patients [29] and STZ-diabetic rats [13]. Although we have not measured the levels of plasma or tissue arginine, lack of amino acid levels in diabetic rats may cause a decrease in EDRF/NO synthesis and consequently may cause gastrointestinal dysfunction in diabetes. Our results in the present study and previous findings confirm the beneficial effect of L-arginine treatment on gastrointestinal dysfunction in diabetic rats.

Muscle wasting in diabetics is another consequence of diabetic condition due to non-availability of glucose for energy purposes. Improved body weight on arginine supplementation indicates the favored utilization of conventional metabolic substrate and thereby might have prevented the muscle wasting.

Gastrointestinal dysmotility is a common clinical feature of diabetes mellitus that involves autonomic neuropathy or an impaired cholinergic neurotransmission and reduced smooth muscle response to neurotransmitters. The present investigation revealed that the isolated GI tissues like fundus, and ileum from the diabetic group responded less to the dose of acetylcholine and appeared restored in arginine supplemented diabetic group. Thus, the improvement in the response probably, may be due to prevention of damages to contractile mechanism of tissues due to improved BGL, which ameliorates the oxidative stress.

In the diabetic state, it is well known that oxidative stress is increased due to excessive production of oxygen free-radicals and decreased antioxidant defense systems. The oxidative stress may cause development of long-term diabetic complications as a result of glycoxidation and lipid peroxidation [4]. It has been reported that oxidative stress exists in the gastrointestinal tissues of diabetic rats and is attenuated by daily supplementation of L-arginine [10]. Therefore, the oxidative stress in diabetic animals might be responsible for gastrointestinal dysfunction in chronic diabetes. In addition, it has been reported that L-arginine supplementation could prevent number of diabetic complications due to its antioxidant property. Therefore, a possible mechanism by which L-arginine supplementation improves gastrointestinal dysfunction in diabetes may depend on inhibiting the oxidative stress. Indeed, in our study, there is a positive correlation between improved gastrointestinal function and normalized lipid peroxidation along with restoration of antioxidant enzyme levels in L-arginine-treated diabetic rats. Consistent with present findings, studies published showed that treatment orally with L-arginine reduced lipid peroxidation products in patients with diabetes [29]. However, whether the lipid peroxidation-lowering effect of L-arginine results from its direct superoxide scavenging properties or increasing NO synthesis indirectly is controversial because NO also has antioxidant activity. On the other hand, advanced glycation end products accumulate in diabetes and can inactivate NO. As it has been reported that L-arginine can directly inhibit AGEs production in vitro, it is possible that L-arginine improves gastrointestinal functions in diabetes by preventing NO quenching induced by AGEs. Taken together, these findings suggest that the beneficial effect of L-arginine treatment in diabetic rats is attributable to inhibition of the oxidative stress caused by the oxygen free radicals.

In conclusion, the findings obtained in this study show that chronic treatment of diabetic rats with L-arginine in vivo prevents

the gastrointestinal dysfunction observed in untreated diabetic rats and that the beneficial effect of L-arginine on gastrointestinal functions of diabetic rats may result from its lipid peroxidation lowering effect together with increasing the antioxidant enzyme levels. Therefore, it is suggested that L-arginine treatment in addition to hypoglycemic agents, e.g. insulin and oral antidiabetics, may be applied as a therapeutic regimen for diabetic patients.

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