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Research Article

L-Arginine ameliorates insulin resistance in sickle cell anaemia in the steady state

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Sickle cell anaemia; insulin resistance; L-arginine; oxidative stress

ABSTRACT

There is scanty information on the effect of L-arginine supplementation on glucose metabolism in HbSS patients. The effect of six weeks L-arginine supplementation (1g/day) on oral glucose tolerance test (OGTT) and insulin resistance (HOMA-IR was investigated in 40 adult HbSS and 40 adult HbAA subjects. After a 12-hour overnight fast, 3 mL of blood was withdrawn from an ante-cubital vein of each subject for the estimation of fasting blood glucose (FBG), insulin, L-arginine concentration [R], catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and MDA. OGTT was performed on each subject and then placed on L-arginine for 6 weeks. Parameters were re-measured at the end of the period. Before supplementation, FBG, fasting insulin, HOMA-IR, MDA were higher in HbSS subjects. CAT, SOD, GPX were higher in HbAA subjects. In response to OGTT, glucose and insulin peaked at 30 minutes in HbAA subjects and at 60 minutes in HbSS subjects. Supplementation did not affect these patterns of responses to OGTT. In HbSS subjects, L-arginine reduced FBG, insulin, HOMA-IR and MDA but increased insulin and HOMA-IR in HbAA subjects. Δ[R] correlated negatively with Δglucose, Δinsulin and ΔHOMA-IR respectively in HbSS subjects but positively in HbAA subjects. In HbSS subjects, ΔHOMA-IR correlated negatively with ΔCAT, ΔSOD and ΔGPx respectively but positively in HbAA subjects. Study thus showed impaired glucose and insulin responses to OGTT in HbSS subjects that were not affected by arginine. Supplementation ameliorated insulin resistance and oxidative stress burden in these subjects.

INTRODUCTION

Sickle cell haemoglobinopathy is common among black populations of the world especially Africa and arises from a replacement on the beta chain of haemoglobin of gluthamic acid by valine at the 6th position (Ingram, 1956). Sickle cell anaemia (SCA) is characterized by abnormal haemoglobin production, chronic haemolytic anaemia and periodic, painful vaso-occlusive events which result in acute or chronic tissue damage, organ dysfunction as well as organ damage (Raghupathy and Billett, 2009). Abnormal erythrocyte membrane and subsequent chronic hemolysis had been reported as the common triggers of inflammation in SCA (Kaul and Hebbel, 2000). It had been suggested that chronic inflammation in SCA sufferers led to damage of the β-cell of the pancreas, causing decreased insulin production and defective glycaemic control (Fung *et al.*, 2006). Al-Sultan *et al.* (2010) showed that the average levels of fasting blood glucose, insulin and homeostasis model assessment of insulin resistance (HOMA-IR) were significantly elevated in sickle cell disease (SCD) patients as compared to the controls. In addition, oxidative products (expressed as malondialdehyde and carbonyl levels) were significantly correlated with blood glucose, insulin level, and HOMA-IR in SCD. However, Akinlade *et al.* (2014) observed similar insulin level but lower level of fasting glucose in SCA subjects compared to HbAA subjects. They also showed that patients with SCA had similar insulin sensitivity status as HbAA individuals. Furthermore, Yavroupaulou *et al.* (2017) showed that normoglycemic patients with SCD demonstrated impaired β-cell function with reduced insulin secretion even before OGTT was impaired.

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Other studies demonstrated an association between SCA, inflammation, altered basal metabolic rate and impaired glucose metabolism (Okafor and Osamo, 1982; Borel *et al*., 1998). Inspite of these findings, diabetes mellitus is a rare finding among SCD sufferers (Reid *et al*., 1998, Akinlade *et al*., 2018).

L-Arginine, an amino acid, is an anti-oxidant and its supplementation had shown some promise as a therapeutic agent in the management of SCD. Its usefulness in the treatment of SCD had been supported by several human studies. In sickle cell anaemia subjects, arginine increased antioxidant enzymes levels (Little *et al*., 2009; Kehinde *et al*., 2015), nitric oxide availability [Morris *et al*., 2000], and enhanced blood trace metals levels (Ogungbemi *et al*., 2018). Futhermore, attenuated pressor and heart rate responses to change in posture (Ogungbemi *et al*., 2013) and reduced elevated liver enzymes (ALT, AST and ALP) levels (Jaja *et al.,* 2016) were observed in HbSS subjects supplemented with arginine. In addition, arginine caused a significant reduction in total opioid use and pain scores in children that had acute pain crises (Morris *et al.,* 2013].

Amino acids such as L-arginine are insulin secretagogues (Gannon and Nuttall, 2010). By this property, L-arginine could elevate insulin levels in SCD sufferers precipitating unwanted or unanticipated complications in this group of subjects that have a rare occurrence of diabetes mellitus (Reid *et al.,* 1998; Akinlade *et al.,* 2018). Inspite of its many potential benefits to SCA patients, the effect of L-arginine on glucose tolerance and insulin resistance in these subjects has not been reported.

This study investigated the effect of L-arginine supplementation on glucose and insulin responses to OGTT in SCA using non SCA with HbAA as controls. It also examined the effect of arginine supplementation on the relationship between insulin resistance and antioxidant enzymes levels in both groups of participants.

METHODS

Forty non SCA subjects (20 males and 20 females) and forty SCA subjects (20 males and 20 female) were studied after due ethical approval had been obtained from the Lagos University Teaching Hospital (LUTH) Health Research Ethics Committee (ADM/DCST/HREC/APP/1359). The HbAA subjects (electrophoretically determined) were students of tertiary institutions in Lagos, Nigeria. The HbSS subjects were patients in the steady state (electrophoretically determined) attending the Out-Patients' Sickle Cell Clinic of LUTH, Lagos, Nigeria. Drug history, family history of diabetes mellitus and/or hypertension, and frequency of blood transfusion were obtained using a short-structured questionnaire. Excluded from the study were subjects with other forms of genotype apart from HbSS (this was confirmed through haemoglobin electrophoresis), diabetes mellitus, hypertension, human immunodeficiency virus (HIV), hepatitis, cancer and established endocrine dysfunctions.

All subjects gave informed consent before participating in the study. On entering the laboratory after a 12-hour overnight fast, the age (years) height (centimeter) and weight (kilogram) of each subject were measured and recorded. Three milliliter of blood was withdrawn from an ante-cubital vein of each subject: 1 mL was put into EDTA bottle for the estimation of blood cell indices including red blood cell (RBC) count, packed cell volume (PCV), haemoglobin concentration (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV)) and 2 mL into plain sample bottle for the estimation of fasting blood glucose, plasma insulin, L-arginine (R) and antioxidant enzymes activities (CAT, SOD and GPx) and malondialdehyde (MDA). Serum was obtained by centrifuging blood at 3000 rpm for 15 minutes. Each subject was then placed on L-arginine supplement at a dose of 1 g/day for six weeks (Kehinde *et al.*, 2015). L-arginine powder, 100% pure-free form, (NOW FOODS, Dallas, USA) was capsulated at the Herbal Clinic, Department of Pharmacognosy, University of Lagos, Lagos, Nigeria. After the six weeks of L-arginine supplementation, the aforementioned procedure and measurements were taken again.

Estimation of some haematological parameters

Red blood cell count (RBC, million/mm^3), packed cell volume (PCV, %) haemoglobin concentration [Hb, g/dL], mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, pg) and mean corpuscular haemoglobin concentration (MCHC, g/dL), were determined using an automated counter (Mindray BC 2800 Haematology Automated System, China).

Determination of plasma L-arginine concentration (R, µmol/L).

L-Arginine concentration was determined using an assay kit, (Oxford Biomedical Research, U. S. A.).

Assay of insulin.

Insulin was assayed using commercial assay kit and following manufacturer's (DiaMetra, Italy) instructions.

Determination of blood glucose
Fasting blood glucose was determined by using the ONETOUCH Ultra 2 Blood glucose monitoring system (LIFESCAN, CHINA).

Estimation of malondialdehyde
Malondialdehyde, (MDA), level in blood was determined using the thiobarbituric acid method. Malondialdehyde was estimated using the spectrophotometer at 535 nm based on the principle that lipidperoxides condense with 1-methyl-2-phenyl indole under acidic conditions (Titus *et al.,* 2004).

Determination of serum levels of antioxidant enzymes.
Serum CAT and SOD levels were assayed as described by Rukkumani *et al,* (2004) while serum GPx level was measured as described by Ellman (1959).

Data Analyses
The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as:
HOMA-IR = [fasting insulin (U/mL) × fasting glucose (mmol/L)]/22.5 (Matthews et al., 1985).
Results are presented as mean ± SEM. Statistical analyses were made using Graph pad prism 5. Statistical comparisons were made using paired Student's t-test and significance level was adopted at $p < 0.05$ for each analysis.

RESULTS.

Table 1 shows a comparison of the physical characteristics of the subjects. Before arginine supplementation, plasma arginine (R) in both groups of subjects was similar (Table 2; a Vs b). The table also shows that plasma NO_X, CAT and GP_X were higher in HbAA than in HbSS subjects ($p<0.05$, $p<0.001$, $p<0.001$ respectively).

Table1. Comparison of Physical and some haematological parameters of subjects.

Parameters	HbAA (n=40) (± SEM)	HbSS (n=40) (± SEM)	p level
Age (years)	30.7 ± 1.0	27.5 ±1.2	NS
Height (cm)	172.5 ± 2.0	167.5 ± 2.0	NS
Weight (kg)	69.2 ± 1.0	59.8 ± 1.1	< 0.01
BMI	23.1 ± 0.6	21.8 ± 0.6	< 0.05
Hct (%)	40.3 ± 1.3	26.1 ± 1.1	< 0.001
RBC (10^6/µL)	4.8 ± 0.2	3.2 ± 0.2	< 0.001
Hb (g/dL)	13.7 ± 0.4	8.9 ± 0.3	< 0.001

Table 2: Comparison of measured parameters between HbAA and HbSS subjects

Parameters	Before Supplementation		After Supplementation		p Values			
	HbAA (a)	HbSS (b)	HbAA (c)	HbSS (d)	(a) Vs (b)	(a) Vs (c)	(b) Vs (d)	(c) Vs (d)
Hct (%)	40.3 ± 1.3	26.1 ± 1.1	41.9 ± 1.0	27.7 ± 1.1	< 0.001	NS	NS	<0.001
RBC (10^6/µL)	4.8 ± 0.2	3.2 ± 0.2	4.8 ± 0.1	3.3 ± 0.2	< 0.001	NS	NS	<0.001
Hb (g/dL)	13.7 ± 0.4	8.9 ± 0.3	14.0 ± 0.3	9.1 ± 0.4	< 0.001	NS	NS	<0.001
CAT (U/mg prot)	3.6 ± 0.6	0.9 ± 0.3	6.9 ± 0.9	2.1 ± 0.6	< 0.001	< 0.01	< 0.01	<0.05
SOD (U/mg prot)	2.4 ± 0.1	2.7 ± 0.1	4.0 ± 0.4	4.3 ± 0.4	< 0.5	NS	< 0.01	< 0.05
GPx (U/mg prot)	2.5 ± 0.3	1.0 ± 0.1	3.5 ± 0.4	1.7 ± 0.3	< 0.01	< 0.01	< 0.05	<0.05
MDA (µmol/mg prot)	28.7 ± 2.8	42.0 ± 3.9	14.7 ± 1.4	10.4 ± 0.9	< 0.01	< 0.05	< 0.01	<0.001
[R] (µmol/L)	65.1 ± 2.0	63.7 ± 1.5	72.6 ± 1.9	86.3 ± 6.2	NS	< 0.01	< 0.01	<0.05
FBG (mg/dL)	88.3 ± 2.9	113.3 ± 2.3	89.3 ± 2.2	98.4 ± 3.7	< 0.05	NS	< 0.05	<0.05
Fasting Insulin (µU/mL)	.2 ± 0.3	16.2 ± 3.6	5.8 ± 1.2	1.9 ± 0.3	< 0.001	< 0.01	< 0.001	<0.01
HOMA-IR (AU)	0.3 ± 0.1	4.1 ± 0.5	1.9 ± 0.5	0.5 ± 0.1	< 0.001	< 0.01	< 0.001	<0.001

Conversely, SOD, MDA, fasting glucose, fasting insulin and HOMA-IR were significantly higher in HbSS subjects than in the HbAA subjects. (Table 2; a Vs b).
In HbAA subjects, arginine supplementation had little or no effect on fasting glucose concentration, decreased MDA ($p<0.01$) and significantly elevated the other measured parameters (Table 2; a Vs c). In HbSS subjects, supplementation decreased MDA ($p<0.001$), fasting glucose ($p<0.05$), fasting insulin and HOMA-IR ($p<0.001$ in each case). All other measured parameters were significantly elevated (See Table 2; b Vs d).

Fig. 1 shows glucose and insulin responses to OGTT before supplementation with L-Arginine in both groups of subjects. In HbAA subjects, blood glucose peaked at

30 minutes and thereafter declined while in HbSS subjects, blood glucose peaked at 60 minutes and thereafter declined. Insulin responses to OGTT in both groups of subjects followed the same pattern as glucose responses in HbAA and HbSS groups respectively.

Table 3: Correlation coefficient (r) between measured variables.

Parameters	HbAA	HbSS
Δ[R] Vs Δglucose	0.66*	- 0.82***
Δ[R] Vs ΔInsulin	0.79**	- 0.88***
Δ[R] Vs ΔHOMA-IR	0.80**	- 0.88***
Δ[R] Vs ΔCAT	0.70**	0.82***
Δ[R] Vs ΔSOD	0.68*	0.84***
Δ[R] Vs ΔGP_X	0.80***	0.72**
Δ[R] Vs ΔMDA	- 0.78**	- 0.80**
ΔHOMA-IR Vs ΔCAT	0.83***	- 0.86***
ΔHOMA-IR Vs ΔSOD	0.73**	- 0.90***
ΔHOMA-IR Vs ΔGP_X	0.82**	- 0.85***
ΔHOMA-IR Vs ΔMDA	- 0.88***	- 0.82***
ΔInsulin Vs ΔCAT	0.90***	0.85***
ΔInsulin Vs ΔSOD	0.86***	0.82***
ΔInsulin Vs ΔGP_X	0.74**	0.90***
ΔInsulin Vs ΔMDA	- 0.82***	0.70**
Δglucose Vs ΔCAT	0.86***	0.83***
Δglucose Vs ΔSOD	0.84***	0.72**
Δglucose Vs ΔGP_X	0.78**	0.72**
Δglucose Vs ΔMDA	- 0.74**	- 0.82***

*=p< 0.05; **=p<0.01; ***=p<0.001

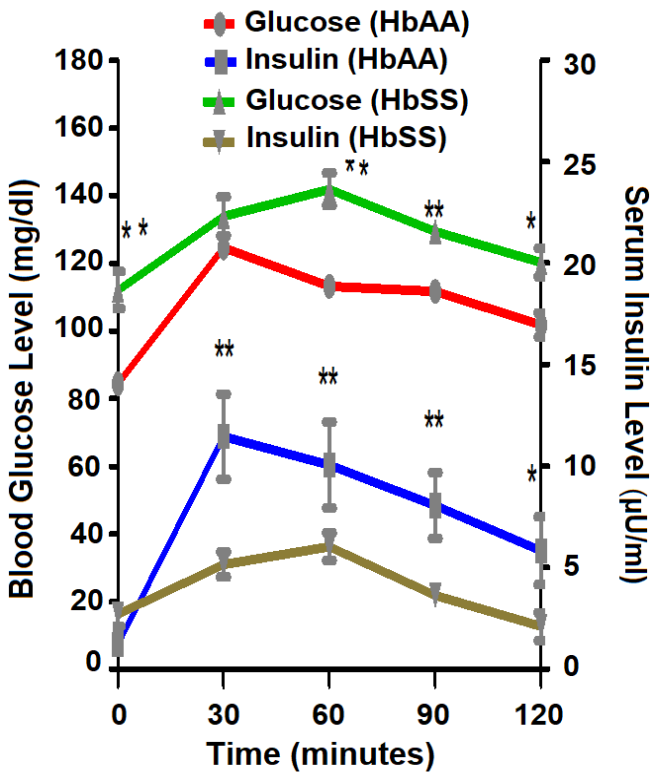

Fig.1: Glucose and insulin responses to glucose loading before L-arginine supplementation in HbAA and HbSS subjects.

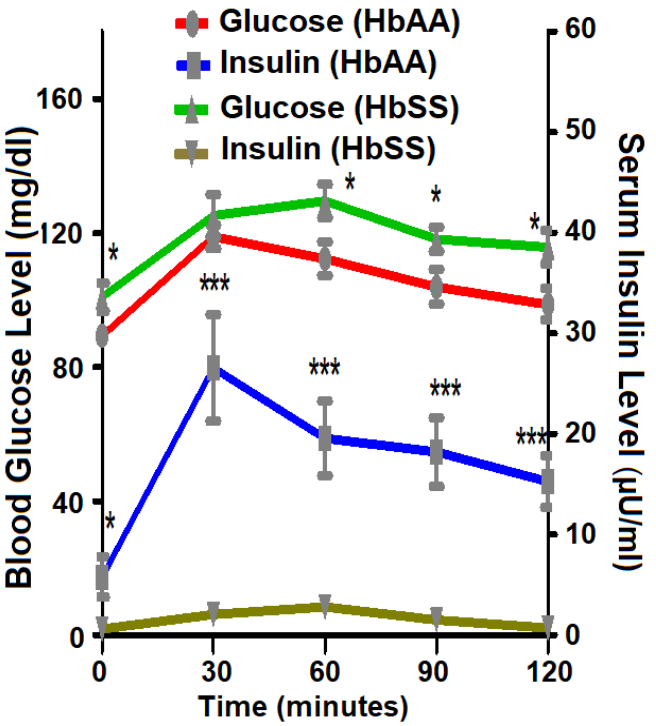

Fig. 2: Glucose and insulin responses to glucose loading after L-arginine supplementation in HbAA and HbSS subjects.

Fig. 2 shows glucose and insulin responses to OGTT after L-arginine supplementation in both groups of subjects. Supplementation did not change the pattern of glucose response in either of the two groups. Thus, glucose response peaked at 30 minutes in HbAA subjects and at 60 minutes in HbSS subjects. The pattern of insulin response in either of the groups was also unaffected by supplementation.

Table 3 shows correlation coefficients (r) calculated between change in serum arginine concentration ([ΔR]) or change in insulin resistance (ΔHOMA-IR) and the other measured parameters in the subjects. In HbAA subjects, [ΔR] correlated positively with Δglucose (0.66; $p<0.05$), Δinsulin 0.79; $p<0.01$), ΔHOMA-IR (0.80; $p<0.001$). In HbSS subjects, [R] correlated negatively with Δglucose (-0.82; $p<0.001$), Δinsulin (-0.88; $p<0.001$), and ΔHOMA-IR (-0.88; $p<0.001$) (Table 3). Table 3 also shows that in HbAA subjects ΔHOMA-IR correlated positively with each of the antioxidant enzymes but correlated negatively with each of the antioxidant enzymes in the HbSS subjects.

DISCUSSION

This study has shown that in HbSS subjects fasting plasma glucose, fasting serum insulin and HOMA-IR levels were elevated above normal values in contrast to HbAA subjects whose values were within the normal ranges. These results agree with those of Al-Sultan *et al.* (2010) but contrast those of Akinlade *et al.* (2014). Thus, HbSS subjects exhibit insulin resistance. In addition, after OGTT, glucose and insulin levels in HbAA subjects peaked at 30 minutes and progressively fell at 60, 90 and 120 minutes showing a normal response to OGTT (Takahashi *et al.,* 2018) while in HbSS subjects, glucose and insulin peaked at 60 minutes and thereafter fell, suggesting an impaired response to OGTT (Takahashi *et al.,* 2018).

Our results also show that there was a higher level of oxidative stress burden in the HbSS subjects as evidenced by lower blood Arginine, CAT, SOD, and GPX and higher MDA in comparison to the HbAA subjects. This agrees with earlier studies (Kehinde *et al.,* 2015).That L-arginine supplementation increased blood arginine, CAT, SOD and GP_X while MDA fell in both groups of subjects agrees with earlier findings (Little *et al.,* 2009; Morris *et al.,* 2000; Ogungbemi *et al.,* 2018). In HbAA subjects, arginine supplementation did not affect fasting glucose but elevated fasting blood insulin and HOMA-IR. In addition, arginine did not affect the pattern of glucose or insulin response to OGTT in these subjects. Pahlavani *et al,* (2017) had however shown that arginine supplementation reduced fasting blood glucose in healthy men. The elevation of blood insulin by arginine supplementation in HbAA subjects may be due to the fact that arginine is an insulin secretagogue. It must be noted, however, that although insulin and HOMA-IR values were elevated by arginine supplementation, the values remained within normal limits.

In HbSS subjects, supplementation decreased fasting blood glucose, fasting insulin, HOMA-IR and did not affect the pattern of glucose or insulin response to OGTT. Although these effects may be due to the disease, it is however not clear by what mechanisms L-arginine achieved these effects. The significantly negative correlation coefficients between change (Δ) in blood arginine level and change in blood glucose or insulin or HOMA-IR or MDA confirm an inverse relationship between blood arginine levels and those measured parameters. The significantly positive correlations between change (Δ) in blood arginine and change (Δ) in CAT or SOD or GPx levels confirm that arginine supplementation caused the elevation of the blood antioxidant enzymes levels and agrees with earlier observations ((Little *et al.,* 2009; Kehinde *et al.,* 2015). Reduction in malondialdehyde level with a concomitant increase in antioxidant enzymes levels signifies a reduction in oxidative stress burden in these subjects (Kehinde *et al.,* 2015). Furthermore, the significantly negative correlation coefficients calculated between change in HOMA-IR and change in each of the antioxidant levels suggest that elevated antioxidant enzymes levels possibly brought about by arginine supplementation contributed to the lowering of insulin resistance in the sickle cell disease subjects. In summary, low dose, oral, chronic arginine supplementation may have exerted its effect on insulin resistance through the reduction of oxidative stress burden. Earlier studies had shown that oxidative stress may interfere with insulin signaling by impairing insulin uptake through a direct effect on insulin receptor functions (Houstis *et al.,* 2006; Dokken *et al.,* 2008) which in turn leads to an increase in plasma insulin level. On the other hand, oxidative stress may reduce glucose uptake by inhibiting the translocation of glucose transporter to the plasma membrane leading to increased blood glucose concentration (Garvey *et al.,* 1998) which in turn induces pancreatic secretion of insulin.

Earlier studies had suggested the arginine-nitric oxide pathway as the major pathway for arginine activity in sickle cell anaemia (Morris *et al.,* 2000; Bakshi and Morris, 2016). Some other studies had suggested that arginine may exert its beneficial effects by reducing oxidative stress burden in sickle cell disease sufferers. Co-administering hydroxyurea with L-Arginine

increased the plasma level of glutathione peroxidase in HbSS patients (Little *et al.,* 2009). In addition, arginine supplementation decreased oxidative stress burden by increasing total antioxidant enzymes and decreasing malondialdehyde (MDA) levels in HbSS subjects (Kehinde *et al.,* 2015). Ogungbemi *et al,* (2018) also demonstrated that arginine supplementation elevated trace metals levels and antioxidant enzymes levels in HbSS subjects. Trace metals form an integral part of antioxidant enzymes and may contribute to the integrity and proper functioning of the antioxidant enzymes.

Dosage of L-arginine.

In this study, 100% pure-free form L-arginine powder, (NOW FOODS, Dallas, USA) capsulated at the Herbal Clinic, Department of Pharmacognosy, University of Lagos, Lagos, Nigeria was used. Each subject was placed on L-arginine supplement at a dose of 1 g/day for six weeks. Although low-dose L-arginine had been found to be sub-therapeutic, repeated supplementation with lower doses may restore depleted arginine stores and become therapeutic (Morris et al., 2000). The authors could not find any study that investigated the effect of L-arginine supplementation on glucose metabolism in SCD sufferers even though arginine supplementation may be beneficial in the management of diabetes mellitus (Stancic *et al.,* 2012).

L-arginine had been reported to exert some side effects when supplemented in high, supraphysiological doses and for long periods of time. Higher oral doses of L-arginine-HCl (>9 g/day) had been associated with nausea, gastrointestinal discomfort, and diarrhea (Grimble, 2007; Stancic *et al.,* 2012). Apart from inducing changes in numerous chemicals and electrolytes in the blood, including potassium it could also cause anaphylaxis (Stancic *et al.,* 2012).

There are some limitations to this study. We did not explore the effect of higher doses of L-Arginine. Lifestyle was also not controlled. Subjects were allowed their normal diets and their daily routines. Investigators depended on the subjects to regularly take their daily supplements and only confirmed through telephone calls. This study was not planned as a clinical trial. Therefore, these results will have to be authenticated by a well-structured, randomized, blinded, placebo-controlled study to give it more validity.

In conclusion, this study has shown that in SCA subjects, chronic, low dose, oral supplementation with l-arginine did not affect the impaired glucose and insulin responses to OGTT. However, supplementation reduced blood glucose, blood insulin and insulin resistance in these subjects by probably reducing oxidative stress.

Conflict of interest

The authors declare that there are no conflicts of interests regarding the contents of this study.

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