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ANTIOXIDANT ACTIVITY AND FREE RADICAL SCAVENGING CAPACITY OF L-ARGININE AND NAHS: A COMPARATIVE *IN VITRO* STUDY

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Abstract: In the family of gaseous transmitters, hydrogen sulfide (H_2S) is considered as third member beside nitric oxide (NO) and carbon monoxide (CO), which can play physiological role in different organs. The present study was designed to elucidate the antioxidant and free radical scavenging potentials of L-arginnine (a source for endogenous production of NO *in vivo*) and NaHS (a source H_2S) individually and in combination. Different assays like 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, percent inhibition of linoleic acid peroxidation and reducing power assays were used to evaluate the free radical scavenging capacity and antioxidant activity of L-arginine and NaHS. Furthermore, study was aimed to know the antioxidant potential of both compounds at their effective doses in human body, which is 56 μM for H_2S and 1.2 g/mL for L-arginine. The study also aimed to clear whether either NaHS, L-arginine or the mixture of NaHS and L-arginine *in vitro* (in the form of new compounds) is responsible for their therapeutic action. Results showed that NaHS, L-arginine and combination of NaHS + L-arginine showed good radical scavenging activity i.e., 55.60%, 52.10% and 52.32%, respectively. Moreover, NaHS was found to have ability to inhibit linoleic acid peroxidation by 53.98% at effective dose while L-arginine did not show inhibition of linoleic acid peroxidation. Combination of NaHS + L-arginine showed 54.15% inhibition of linoleic acid peroxidation, which is similar to that of H_2S. Reducing power of NaHS was 0.073 and L-arginine showed 0.037, combination of NaHS + L-arginine showed 0.063. It can be concluded that NaHS showed better antioxidant potential *in vitro* as compared to L-arginine and the antioxidant activity of the mixture of NaHS + L-arginine is closed to the antioxidant activity of NaHS, which reflects that NaHS is a dominant factor in combination mixture that is responsible for antioxidant activity.

Keywords: H_2S, NO, DPPH, linoleic acid peroxidation

Imbalance between prooxidant and antioxidant activity in human body results in the development of oxidative stress, which is considered as a major route causing cardiovascular disease, cancer (1), hypertension (2), acute respiratory distress syndrome (3), chronic inflammatory diseases (4), ischemia/reperfusion injury (I/R) (5), Parkinson's disease and Alzheimer's disease (6) as well as aging (7). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) start accelerate prooxidant processes and produce toxic results in the body. Hence, there is a need of antioxidants which scavenge these free radicals and makes them inactivate.

Medical gases including hydrogen sulfide (H_2S) and nitric oxide (NO) and carbon monoxide (CO) have captured the interest of researchers by their number of applications in human body. Endogenous H_2S is produced from two sulfur containing amino acids - L-cysteine and L-methionine, by the two enzymes cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) (8). The endogenous concentration of circulating H_2S is 50–160 μM in rat, bovine and human (9). NaHS have been used previously as a donor of H_2S (10, 11). H_2S is found to have many therapeutic applications like anti-inflammatory activity (12) and is involved in regu-

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lation of liver and gastrointestinal functions (13). H_2S can effectively prevent hypertension in rats if it is induced by N^G-nitro-L-arginine methyl ester (L-NAME) (14). Nitric oxide produces vasodilatation by activating cyclic guanosine monophosphate (cGMP) on blood vessels. NO, which is produced from endothelial NOS, reduces the contractility of heart by inhibiting the influx of Ca^{2+} (15). So, systemic or endogenous production of NO in heart failure case can provide protection by its vasodilator and negative ionotropic response. In support of this statement, it is evident that low dose of β blockers (especially carvedilol) have been found to have protective role in case of heart failure by decreasing heart rate (16). L-arginine has been used as precursor of NO (17, 18). L-arginine undergoes enzymatic reaction resulting in endogenous production of NO with the help of endothelial nitric oxide synthase (eNOS). It is evident that L-arginine enhances the antioxidant activity of garlic (19). NaHS has sulfur content like garlic so it is expected from L-arginine to enhance the antioxidant activity of NaHS.

Both medical gases have captured the interest of researchers since last decades but still many therapeutic applications needs clarity like interdependable production of H_2S and NO or an intermediate molecule formation (11, 20, 21). Present study aimed to solve this mystery by using *in vitro* study first, without enzymatic involvement. After these experiments, will be conducted *in vivo* studies for enzymatic action and outcome correlation with *in vitro* study. This study was conducted to evaluate the *in vitro* antioxidant potential of NaHS and L-arginine alone and in combined form. Furthermore, this study was extended to answer the dispute between different schools of thought whether an intermediate product is formed by combining NaHS and L-arginine or anyone of these two is dominant in this mixture. This factor will be partially studied in presented *in vitro* studies.

MATERIALS AND METHODS

Chemicals

L-arginine as a source of nitric oxide (NO) and NaHS as a source of H_2S, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Other chemicals of analytical grade like anhydrous sodium carbonate, ammonium thiocyanate, ferrous chloride ($FeCl_2$), methanol and chloroform were purchased from Merck (Darmstadt, Germany).

Preparation of solutions

Solutions were prepared on the basis of ED_{50} values already reported in previous studies. First of all, individual solutions of NaHS (3.125–100 µM) and L-arginine (0.075–2.4 mg/mL) were prepared as shown in Table 1. Combined solution of NaHS and L-arginine was prepared in such a way that effective doses (ED_{50}) or therapeutically active concentrations of NaHS and L-arginine were mixed together as shown in Table 1.

Antioxidant activity

Antioxidant activities of abovementioned 3 forms of drugs were investigated using the following methods.

DPPH radical-scavenging activity

Antioxidant activities of NaHS, L-arginine and combination of NaHS + L-arginine were assessed by their ability to scavenge free stable radicals like 2,2-diphenyl-1-picrylhydrazyl (DPPH). The assay was performed as reported before (22). Different concentrations of sodium hydrogen sulfide (NaHS) (3.125–100 µM) and L-arginine (0.075–2.4 mg/mL) were prepared on the basis of their doses used for different studies. A third solution was prepared by combining the serial dilution

Table 1. Solutions of NaHS, L-arginine and their mixture used in the study.

Conc.	NaHS (µM)	L-arginine (mg/mL)	NaHS + L-arginine (µM + mg/mL)
F	100	2.4	100 + 2.4
E	50	1.2	50 + 1.2
D	25	0.6	25 + 0.6
C	12.5	0.3	12.5 + 0.3
B	6.25	0.15	6.25 + 0.15
A	3.125	0.075	3.125 + 0.075

in such a manner that the effective doses of NaHS + L-arginine (50 µM + 1.2 mg/mL) were mixed together as shown in Table 1.

Samples (125 µL) were mixed with 125 µL of 90 µM solution of DPPH in methanol. BHT was taken as a positive control. The samples were incubated at room temperature for 30 min and the absorbance was recorded at 515 nm using spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). Percentage (%) scavenging of DPPH free radical was calculated by the following formula:

$$RS\ (\%) = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control whereas A_{sample} is the absorbance of the tested samples.

Percentage inhibition of linoleic acid peroxidation for NAHS, L-arginine and combination of NaHS + L-arginine

Linoleic acid peroxidation inhibition was done by using method described in (23). Different concentrations of NaHS, L-arginine and combination of NaHS + L-arginine were used as shown in Table 1. Samples (5 mg) of NaHS, L-arginine and combination of NaHS + L-arginine were mixed with 0.13 mL of linoleic acid, 10 mL of 99.8% ethanol and 10 mL of 0.2 M sodium phosphate buffer (pH = 7). The mixture was diluted with water up to 25 mL and the solution was incubated for 175 h at 40°C in an incubator. The extent of oxidation was measured by colorimetric method (24). BHT was taken as a positive control and sample without antioxidant is taken as blank.

Percentage inhibition of linoleic acid peroxidation was calculated by using the following formula: 100 – [(Abs. increase of sample after incubation / Abs. increase of control after incubation) × 100].

Reducing power assay for NaHS, L-arginine and combination of NaHS + L-arginine

Reducing power assay was performed as described in (23). Various concentrations of NaHS, L-arginine and NaHS + L-arginine were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide. These mixtures were incubated at 50°C for 20 min. After this, 2.5 mL of 10% trichloroacetic acid were added and the mixture was centrifuged at 650 rpm for 10 min. The upper layer was mixed with 5 mL of deionized water and 1 mL of 0.1% ferric chloride and absorbance was measured at 700 nm. High absorbance indicates high reducing power. BHA was used as a positive control.

RESULTS AND DISCUSSION

The endogenous concentration of circulating H_2S is 50–160 µM in rat, bovine and human (22, 23).Therapeutic dose of H_2S considered for animal is 56 µM as the vasorelaxant effects of H_2S has been proved by (24), which shows that H_2S relaxes the isolated aorta at concentration as low as 18 µM and 60 µM pretreated with 20 mM KCl or PHE, respectively. Antioxidant role of sodium hydrogen sulfide has been previously reported partially (11) as scavenger (21) protecting neurons against oxidative stress (25). In one study (24) it was demonstrated that plasma level of H_2S was 50 µM. Tissue level of H_2S is thought to be higher than plasma level. So, therapeutic concentration of H_2S is considered to be 56 µM.

DPPH radical-scavenging activity

NaHS is used in different concentrations ranging from 3.125 to 100 µM as shown in Figure 1.

Free radical scavenging activity of NaHS, L-arginine and equimolar concentrations of both NaHS and NO were evaluated by their ability to scavenge DPPH free radical. Abovementioned figure shows that the lowest concentration of NaHS, which is 3.125 µM, has 47.04% scavenging ability whereas 50 µM has 55.60% and maximal dose (100 µM) has maximum scavenging 75.84%. So, maximum concentration of NaHS has the highest antioxidant potential. Therapeutic dose (56 µM) gives 61.39% scavenging of DPPH and is showing good antioxidant potential which is ideal from safety and efficacy point of view. These results show that NaHS has antioxidant activity by scavenging free radicals and these *in vitro* results justify the use of NaHS *in vivo* as well.

L-arginine is a precursor of nitric oxide (NO). NO is generally known as prooxidant and inflammatory mediator (26, 27). L-arginine is used in different concentrations ranging from 0.075 mg/dL to 2.4 mg/dL. In order to make comparison with NaHS, L-arginine solution has been used in therapeutic range which is 1.2 mg/dL (27). Radical scavenging potential of different concentration of L-arginine is shown in Figure 2. Therapeutic dose (1.2 mg/dL) has shown 52.10% free radical scavenging, which is higher than for minimum concentration of L-arginine (0.075 mg/dL) with 46.83% free radical scavenging and less than that of maximum concentration (2.4 mg/dL) with 63.08% free radical scavenging. Scavenging potential of L-arginine has been proved previously (27) but no other mechanisms were elucidated to prove it as antioxidant.

Proposed mechanism for NO may be as below:

$$\text{Lipid} \xrightarrow{O_2} \text{LOO}^{\bullet} \xrightarrow{NO^{\bullet}} \text{LOONO} \begin{cases} \xrightarrow{NO^{\bullet}} \text{LONOO} \\ \text{LONO/LONOO} \\ \text{LOOH} + NO_2 \end{cases}$$

L = lipid

Above highlighted reaction is rate limiting step and calculation suggested that in *in vivo* settings, NO is better scavenger of LOO as compared to tocopherol. This reaction also showed that 2 molecules of NO are consumed for each LOO molecules but rate of reaction is faster than tocopherol potential antioxidant (27).

Scavenging potential of NO depicts its antioxidant potential by scavenging free radicals.

When NO and H_2S are produced inside the body by using their precursors, an intermediate compound is formed that may be nitroxyl or nitrosothiol (11, 20, 21, 24, 26). Present study was aimed to know the difference in antioxidant potential of donors of H_2S and NO alone and in combination. These finding may serve baseline studies in *in vivo* models of study. In this *in vitro* assay 3 solutions were prepared NaHS, L-arginine and combination of NaHS and L-arginine as shown in Table 1. Combined solution was prepared in such a way that therapeutic concentrations of NaHS and L-arginine were in the same concentration in mixture (that is E).

Results of DPPH free radical scavenging assay showed that NAHS, L-arginine and combination of NaHS and L-arginine at doses (50 µM, 1.2 mg/mL and 50 µM + 1.2 mg/mL) showed 55.61%, 52.1% and 52.32 % scavenging of free radicals at therapeutic doses, respectively, as shown in Figure 3. At maximum doses of NAHS, L-arginine and combination of NaHS and L-arginine (100 µM, 2.4 mg/mL

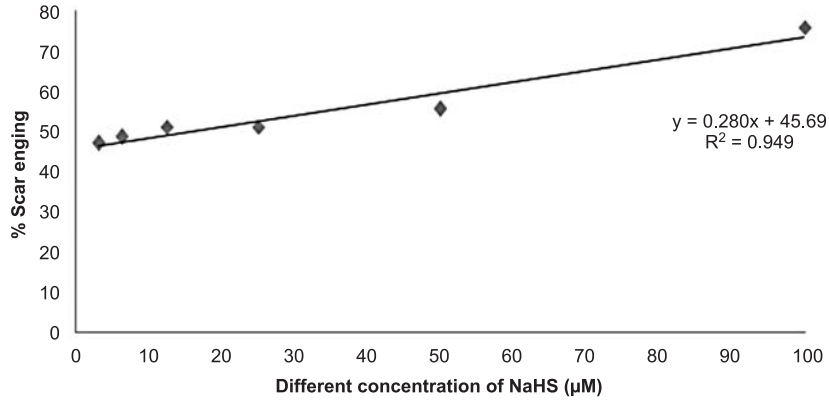

Figure 1. Antioxidant activity of different concentrations of NaHS

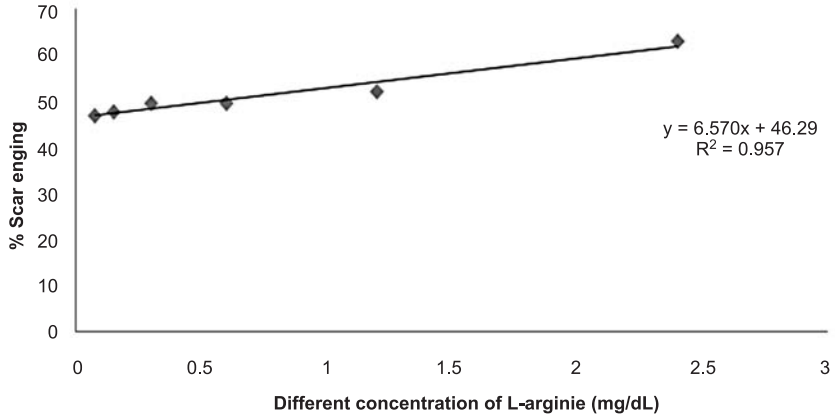

Figure 2. Antioxidant potential of different concentrations of L-arginine

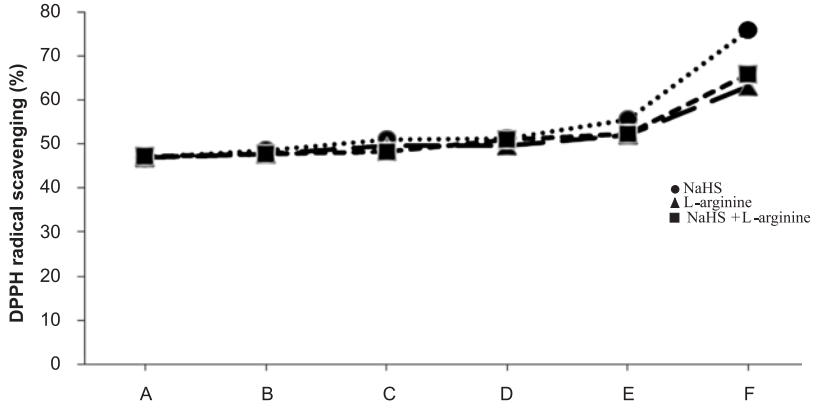

Figure 3. Antioxidant potential comparison of NaHS, L-arginine and combination of equimolar concentration of (NaHS + L-arginine) showing scavenging ability of different concentrations of NaHS (cf. Table 1) in DPPH assay

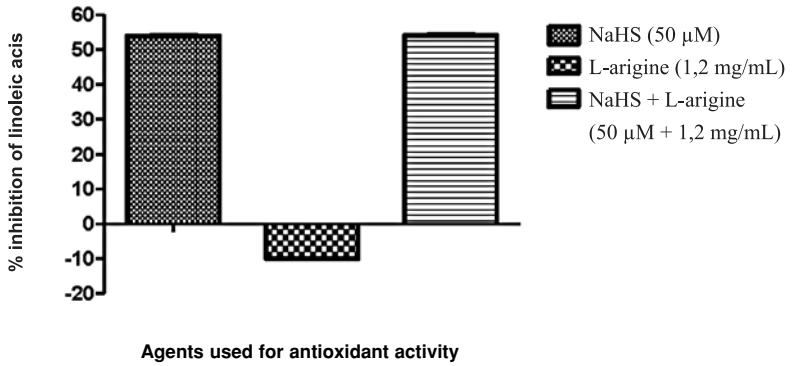

Figure 4. Comparison between NaHS, L-arginine and NaHS + L-arginine in linoleic acid peroxidation inhibition

and 100 µM + 2.4 mg/mL) free radical scavenging was 75.84, 63.08 and 65.82%, respectively. At minimum doses, 3.125 µM, 0.075 mg/mL and 3.125 µM + 0.075 mg/mL of NaHS, L-arginine and NaHS + L-arginine, they showed 47.04, 46.83 and 47.15% of free radical scavenging activity, respectively. Free radical scavenging abilities of NaHS, L-arginine and combination of both showed similar results. These *in vitro* results confirm and justify the *in vivo* use of these drugs to validate antioxidant potential.

Linoleic acid peroxidation inhibition by NAHS, L-arginine and combination of NAHS + L-arginine

Inhibition of linoleic acid peroxidation is another role of any antioxidant to play, so NAHS, L-arginine and NAHS + L-arginine were tested for their ability to inhibit linoleic acid peroxidation.

NaHS showed linoleic acid peroxidation inhibition by 53.98% at therapeutic concentration (50 µM) and L-arginine showed no inhibition of linoleic acid peroxidation even at therapeutic dose (1.2 mg/mL). However, combination of both doses of NaHS and L-arginine showed linoleic acid peroxidation inhibition by 54.15%, which is similar to that by H_2S. It appears from the data that L-arginine does not inhibit linoleic acid peroxidation, so NaHS is playing predominant role in this mechanism. In combined solution, linoleic acid peroxidation inhibition may be due to NaHS as percentage of inhibition is very close to that of NaHS or may be due to some intermediate compound that is formed by mixing both solutions.

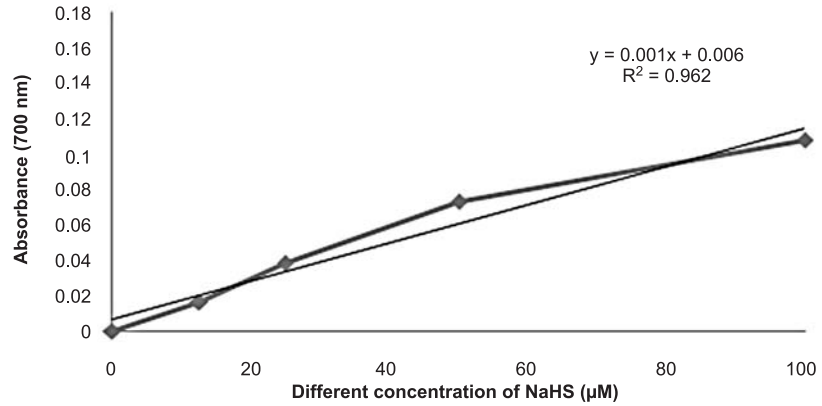

Figure 5. Reducing power potential of NaHS at different concentrations

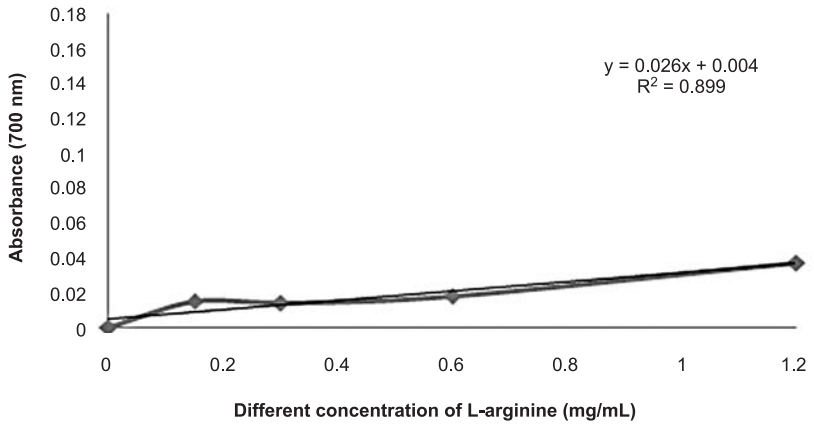

Figure 6. Reducing power potential of L-arginine at different concentrations

Reducing power assay by NaHS, L-arginine and combination of NaHS + L-arginine

Figure 5 is showing the reducing power potential of NaHS at different concentrations. Chemistry of this assay involves the conversion of yellow color to various shades of blue to green color. The presence of antioxidant which is reducing agent in its potential, changes ferric/ferricyanide to ferrous form Fe^{2+}. From the figure it is clear that NaHS has reducing power ability from 0–100 µM concentration. Present study measure the reducing power potential by following procedure which was reported earlier (27). The greater the absorbance the higher will be reducing power ability. Reducing power of NaHS at therapeutic dose is 0.33 at 700 nm while response remains the same when the concentration was enhanced up to 100 µM.

Nitric oxide being prooxidant has shown weak reducing power ability which is one of the factors contributing to its antioxidant potential. Not being potent reducer, L-arginine (precursor of NO) showed its weak reducing ability in the concentration ranging between 0 to 1.2 mg/mL as shown in Figure 6. Therapeutic dose (1.2 mg/mL) showed absorbance 0.030 at 700 nm.

When combined solution of NaHS + L-arginine was tested for reducing ability, the results were more similar with that of NaHS as shown in Figure 7, which suggests that in this mixture either it is NaHS playing dominant role or it is due to an intermediate product that is contributing to its role. Minimum dose A of combined solution showed no activity while dose F has shown significantly the highest absorbance 0.16 as compared to 0.10 of

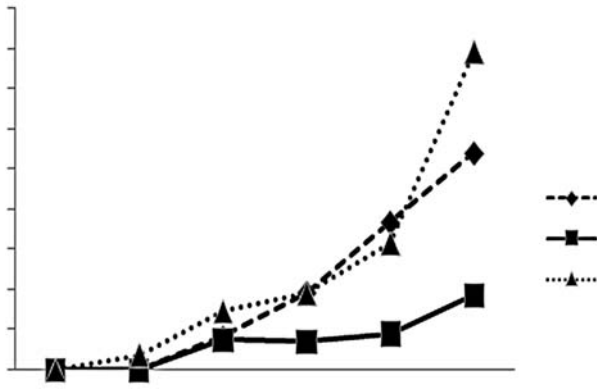

Figure 7. Comparison of reducing power assay between NaHS, L-arginine and NaHS + L-arginine

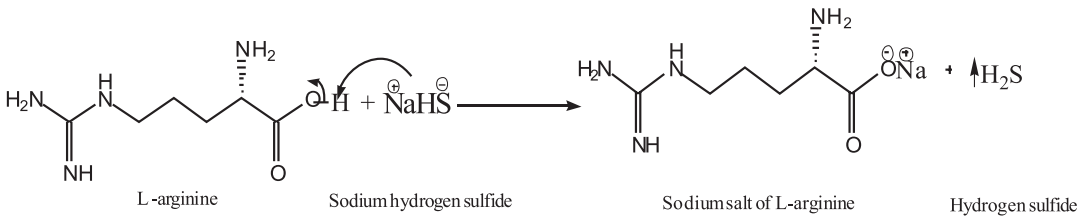

Figure 8. Proposed mechanism of *in vitro* reaction between NaHS and L-arginine showing the release of H_2S.

NAHS and 0.03 of L-arginine. Concentration E (1.2 mg/mL of L-arginine and 50 μM NaHS) showed better response (0.16) as compared to other concentrations.

Proposed mechanism of NaHS + L-arginine

When NaHS is combined with L-arginine, an acid base reaction will take place as shown, as bisulfide is a strong base so it will abstract a proton from L-arginine resulting in the formation of its sodium salt along with evolution of hydrogen sulfide gas. Apparently, it can be concluded that both NaHS and L-arginine in combined solution individually produced their pharmacological responses as shown in the proposed mechanism (Fig. 8). However, *in vivo* study may solve this ambiguity.

CONCLUSION

Present study has demonstrated that sodium hydrogen sulfide has potential antioxidant activity by free radical scavenging, inhibiting linoleic acid peroxidation and as a reducing agent. L-arginine showed weak antioxidant activity by reducing power assay but is good free radical scavenger. In comparison, NaHS is more potent antioxidant than L-arginine, whereas on combination, reaction between NaHS and L-arginine occurs, H_2S and sodium salt of L-arginine is produced which are responsible for individual pharmacological responses, which further needs to be verified in *in vivo* studies.

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