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Mechanism of Action of L-arginine on the Vitality of Spermatozoa is Primarily Through Increased Biosynthesis of Nitric Oxide

Sudha Srivastava,[1,3] Prashant Desai,[2,4] Evans Coutinho,[4] and Girjesh Govil[3]

Tata Institute of Fundamental Research,[3] Colaba, Mumbai-5, India
Department of Pharmaceutical Chemistry,[4] Bombay College of Pharmacy, Kalina, Mumbai-98, India

ABSTRACT

The ability of sperm to fertilize the egg is primarily dependent on sperm motility and membrane integrity. Nitric oxide (NO) plays a decisive role in regulating multiple functions within the male reproductive system. The aim of the present study is to determine the mechanism by which L-arginine confers a protective action on spermatozoa obtained from the goat epididymis. NO is synthesized from L-arginine by the enzyme nitric oxide-synthase (NOS) present in spermatozoa. A possible participation of NO and NOS in arginine action has been suggested.

Nitric oxide, sperm, sperm capacitation, sperm maturation, sperm motility and transport

INTRODUCTION

L-arginine plays a key role in modulating host defenses and cellular immunity. It also actively participates in sperm formation [1]. A deficiency in L-arginine causes derangement of sperm metabolism leading to decrease in motility and loss of spermatogenesis [2]. Administration of L-arginine to oligospermic and asthenospermic patients results in an improvement in both the sperm count and motility without any side effects [3, 4]. L-arginine plays an important role in stimulating sperm motility in humans, rabbits, and goats under in vitro conditions [3,5,6]. In earlier publications, we have shown that L-arginine enhances the rate of glycolysis, resulting in higher rates of ATP and lactate generation in spermatozoa [6]. The influence of arginine in reversing impairment caused by glycolytic inhibitors (potential contraceptives) [7] has also been studied. The reversal effect of arginine involves an eight-fold higher metabolic activity compared to inhibited cells. Structurally related amino acids such as nitro-arginine, homo-arginine, lysine, and ornithine are ineffective [6,7].

The membrane lipids of spermatozoa (which are mainly phospholipids) are highly susceptible to the action of peroxidizing agents, which may be natural or inadvertently present due to extraneous factors. Natural lipid peroxidation is commonly observed in all living cells including spermatozoa. Moreover, during preservation, spermatozoa are likely to be exposed to radiation, cold shock, or various preservatives. Such exposure may lead to peroxidation, which forms lipid peroxides or their degradation products such as fatty acid peroxides, all of which are strongly spermicidal. The peroxides constitute a potential hazard to the structural and functional integrity of spermatozoa, lessening the motility and metabolic activity of cells that are aging either within the reproductive tract in vivo or during storage in vitro [8, 9]. L-arginine prevents membrane lipid peroxidation in spermatozoa under different peroxidation conditions [10, 11]. It has been proposed that the beneficial effects of L-arginine are linked to nitric oxide (NO) [12]. NO is a short-lived free radical, synthesized in many mammalian cell types by a class of NADPH dependent enzymes called nitric oxide synthases (NOS). These enzymes catalyze the conversion of L-arginine to L-citrulline and NO [13]. The reaction is inhibited by L-arginine analogs. In this paper, we present results of our experiments conducted with the aim to elucidate the mechanism by which L-arginine enhances the metabolism and motility and prevents the oxidative damage of spermatozoa.

MATERIALS AND METHODS

Chemicals

L-arginine, D-arginine, N^G-Nitro-L-arginine methyl ester (L-NAME), D-NAME, N^G-Monomethyl-L-arginine acetate (L-NMMA), and N^G-Nitro-L-arginine (L-NNA) were purchased from Sigma Chemicals Co. (St. Louis, MO). Other chemicals used were of AR grade. Dulbecco medium (pH 7.2) with glucose (0.1% w/v) was used as buffer and fuel for cellular metabolism.

Cells

Goat testes were procured from a slaughterhouse and used within 1 hour of the animals' death. The epididymis was separated from the testes. One set of experiments was performed on cells taken from two testes of the same goat. Cells were collected from the caudal region by gentle mincing and tweezing in Dulbecco buffer. Tissue pieces were removed by allowing the cell suspension to settle for 5 minutes. The cells were washed, made into a pellet by centrifugation, and then suspended in an appropriate quantity of buffer to attain the desired concentration. Cells were counted and their motility checked with a cytometer. Cells exhibiting motility greater than 50% were used for experiments. Sample concentration was maintained at $1x10^6$ cells/ml in all experiments.

Metabolism

The ^{13}C NMR experiments were carried out on a Brüker AMX 500 FT-NMR spectrometer at 125.7 MHz using 25,000 Hz spectral width, 60° flip angle, 2 s relaxation delay with power gated broadband proton decoupling, and 32 transients were accumulated for each experiment. ^{13}C chemical shifts have been reported with respect to sodium 3-trimethylsilyl-(2,2,3,3-D)-propionate (TSP). A 10 Hz exponential multiplication factor was applied prior to the Fourier transformation. Buffer with 10% D_2O was used for NMR field-frequency locking. NMR measurements were conducted in real time on viable sperm under anaerobic conditions. 1-^{13}C glucose was used as a substrate for glycolytic reaction. Cells were incubated with the requisite concentration of L-arginine and incubation was allowed for half an hour, which is the optimum time for maximum arginine intake [5]. Spectra (32 transients) were recorded with the time and mid-time for each experiment taken as the corresponding

[1]Correspondence: Sudha Srivastava, National Facility for High Field NMR, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai-5, India. FAX: + 91 22 22804682;
e-mail: sudha@tifr.res.in
[2]Current address: Department of Medical Chemistry, University of Mississippi, University, MS 38677.

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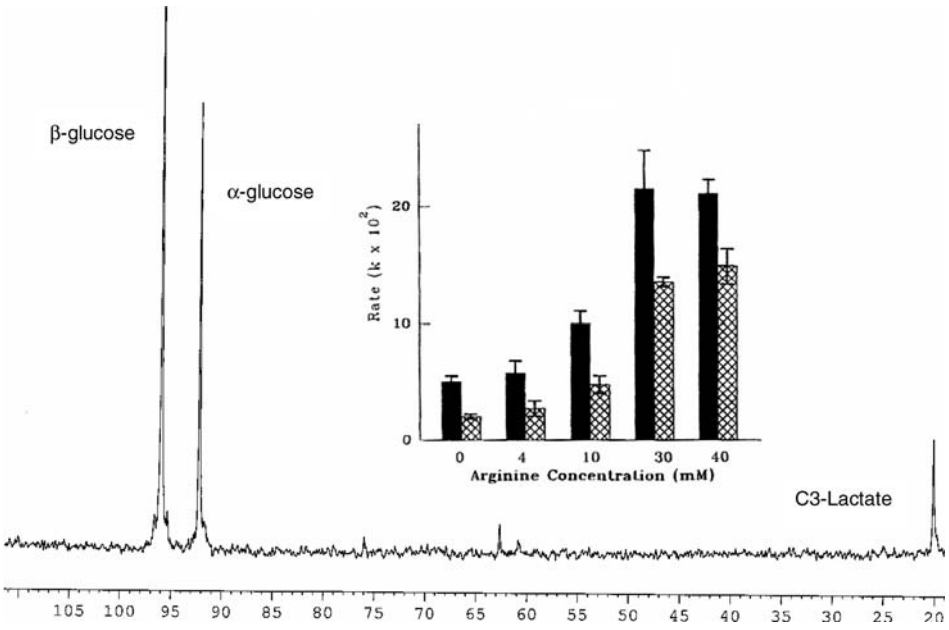


FIG 1. A ^{13}C NMR spectrum (125.76 MHz) of spermatozoa obtained from the cauda region of goat epididymis. Spermatozoa were incubated with 1-^{13}C-labeled glucose, and the spectrum was recorded after 2 hours of glucose addition. Inset figure shows rate of glucose consumption and that of lactate production for different concentrations of L-arginine. The glucose ($\alpha+\beta$ anomers) intensity data with time has been fitted to a first-order rate equation (decaying) to determine the rate constant for substrate consumption, whereas for lactate generation a rising exponential equation has been used for the calculation of the rate constant. Glucose consumption rate, black bars; Lactate production rate, patterned bars. All data are expressed as mean SEM (n = 3). Values were considered significantly different if $P < 0.05$ following a Student *t*-test.

experimental time. Appropriate controls were run for each set of experiments. The intensity of the signal was measured by peak integration (area under the curve) using inbuilt Brüker software. The NMR signals of glucose (substrate consumption) and of lactate (buildup) were monitored over an initial 3-hour period.

Lipid peroxidation

Lipid peroxidation can be induced using various stimuli such as exposure to UV radiation, cold shock, or a catalytic amount of Fe^{+2}-ascorbate [8, 10, 14]. We have earlier published a detailed study on the effect of arginine [11] on lipid peroxidation in spermatozoa. In the current study, peroxidation has been induced in the cells by application of a cold shock. Cells were frozen in liquid nitrogen for 30 minutes followed by thawing at 25°C. Cells were pre-incubated at 25°C for 30 minutes with the respective agent under study (L-arginine, D-arginine, L-NAME, D-NAME, L-NMMA, L-NNA, or sodium nitroprusside) before exposure to cold shock.

Assessment of lipid peroxidation

Lipid peroxidation was assessed by the formation of thiobarbituric acid reactive substances (TBARS) [15]. TBA reagent (2 ml composed of 0.375% w/v thiobarbituric acid and 15% w/v trichloroacetic acid in 0.25 N hydrochloric acid) was added to the samples followed by incubation for 10 minutes at 100°C. This was cooled and centrifuged and the absorbance of the supernatant was recorded at 532 nm. The amount of TBARS formed was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Statistical analysis

All data are expressed as mean ± SEM (n=3). Values were considered significantly different if $P<0.05$ following a Student *t*-test.

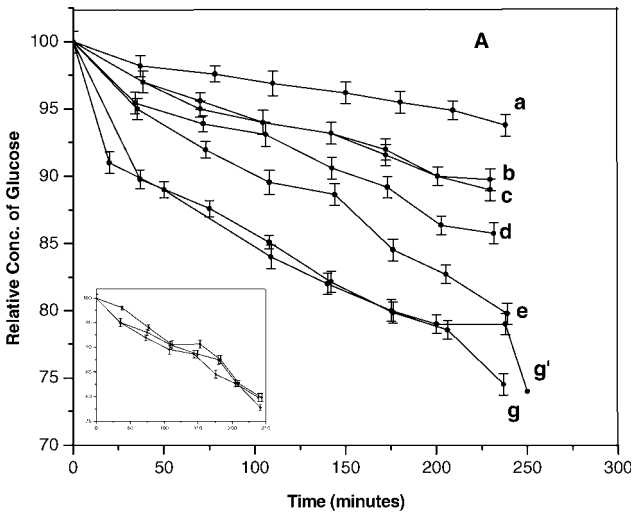
RESULTS

Cell Metabolism

In cellular NMR, signals from macromolecules such as proteins, nucleic acids, lipid assemblies, and carbohydrates are broad and do not provide useful information. However, ^{1}H, ^{13}C, and ^{31}P signals from the low molecular weight compounds are sharp and can be used as probes of cell metabolism. There is great advantage in using ^{13}C labeled substrates for the simple reason that, when cells are fed with labeled substrate, the label appears at the corresponding carbon atom in the various metabolites (Figure 1). For example, in glycolysis a decrease in the substrate signal, and build-up of signals from metabolite can be monitored [6] using ^{13}C NMR. The progress of glycolysis can thus be monitored by directly measuring the change in the concentration of metabolites with time. In this way, the effect of various external factors on the metabolism can easily be determined.

In the current study, cells were incubated using glucose labeled with ^{13}C at the C-1 position, as substrate. ^{13}C NMR spectra monitored as a function of time show a decrease in glucose (α and β anomers) and an increase in lactate signal (the C-3 methyl) intensities. Experiments performed in presence of arginine indicate that the rates of glucose consumption and lactate generation increase monotonically with an increase in the L-arginine concentration up to an optimum concentration of about 30 mM and drop slightly thereafter (inset Figure 1). The maximum increase is five- to seven-fold higher than that of the control. Thus, it can be inferred that the presence of L-arginine at lower concentrations induces higher glucose metabolism. A positive correlation between the rate of substrate consumption and the degree of motility has also been established and reported. This would suggest that L-arginine might lead to enhanced sperm motility. Specificity of arginine action is strongly suggested, since the structurally similar amino acid L-ornithine, which lacks the guanidine group but which has a similar chain length, is unable to induce significant stimulation of glycolytic activity [6, 16]. It has been shown that both the chain length and the terminal guanidino group play a crucial role in inducing a stimulating action [6]. Another interesting observation is that L-arginine binds to the cell membrane involving the guanidine group end of the amino acid in the binding process. On the other hand, the observed effects in ornithine are negligible [6].

Efforts have been made to detect citrulline, the metabolic product of arginine, using proton NMR [6] and ^{15}N NMR (using ^{15}N labeled arginine (data not shown)). However no signal from citrulline could be detected. It turns out, therefore, that the catalytic quantity of citrulline produced along with NO is lower than quantity that can be detected byNMR. NO is produced in response to a variety of stimuli and displays a wide spectrum of activities such as inhibition of platelet aggregation and adhesion, exhibits cytotoxic functions, causes vasodilation, and plays important roles in neurotransmission and in various male reproductive functions [13]. It enhances c-GMP synthesis



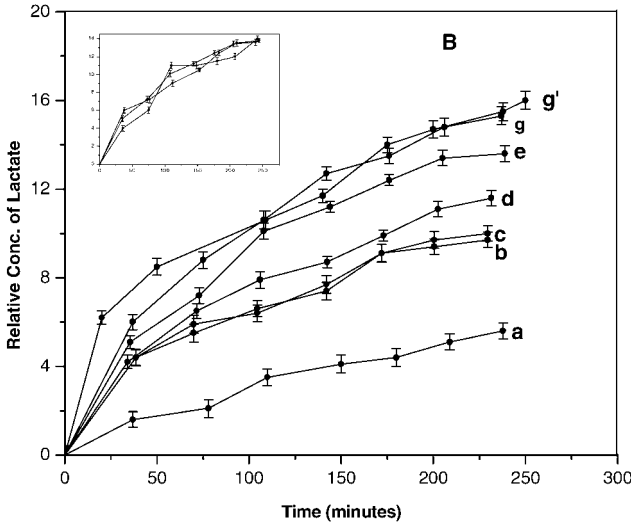


FIG 2. Effect of L-arginine on sperm metabolism in presence of inhibitors. Figure shows variation in ^{13}C signal intensity with time. (A) Decrease in glucose concentration ($\alpha+\beta$ anomers). (B) Increase in lactate concentration. Curve (a) cells metabolizing under normal conditions; (g) cells were incubated at 25°C for 30 min with L-arginine (5 mM); (b) cells incubated with 5 mM arginine + 5 mM L-NAME; (c) cells incubated with 5 mM arginine + 1 mM L-NAME; (d) cells incubated with 5 mM arginine + 50 μM L-NAME; (e) cells incubated with 5 mM arginine +5 μM L-NAME. Curve (g′) cells incubated with 5 mM arginine + 1 mM D-NAME. Glucose and lactate signals of subsequent spectra were integrated and are expressed with respect to the glucose signal of the first spectrum, which is assigned as 100. Inset figure shows comparison of effect of L-NAME, L-NMMA and L-NNA at 1mM conc. All data are expressed as mean SEM (n = 3). Values were considered significantly different if $P < 0.05$ following a Student *t*-test.

thus leading to an enhancement of the metabolic rate. It also enhances the Ca level in the mitochondria, generating a higher ATP level. These two effects lead to increased sperm motility [17–20]. Exogenous NO released by NO donors, such as sodium nitroprusside, is known to play an important role in sperm hyperactivation in vitro and is beneficial for the

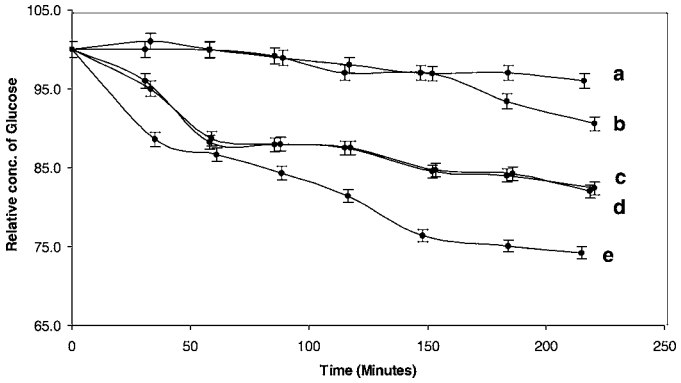


FIG 3. Effect of L-arginine on sperm metabolism compared with sodium nitroprusside. Figure shows variation in ^{13}C signal intensity of glucose ($\alpha+\beta$ anomers) with time. Curve (a) cells metabolizing under normal conditions; (e) cells incubated with 5 mM L-arginine; (b, c and d) cells incubated with 1 μM, 10 μM, and 20 μM of sodium nitroprusside respectively. All data are expressed as mean SEM (n = 3). Values were considered significantly different if $P < 0.05$ following a Student *t*-test.

maintenance of post-thaw sperm motility and viability [21–23]. Based on these observations it may be hypothesized that arginine might lead to enhanced metabolic activity and motility of sperms by inducing an increase in NO production. To test this hypothesis, the effect of L-arginine on glycolysis was studied in the presence of L-NAME, L-NMMA, L-NNA, known inhibitors of NOS [24–26]. The inactive analogue D-NAME was used as a control. Figure 2A shows the results of this study with varying concentrations of the NOS inhibitor L-NAME and its comparison with L-NMMA and L-NNA. The top curve (a) shows moderate glucose consumption for cells metabolizing under normal conditions, and curve (g) shows enhanced consumption in the presence of 5 mM arginine. Curve (g') in presence of D-NAME (inactive analogue of inhibitor) served as a control. It is observed that the effect of L-arginine goes down as the concentration of inhibitor is increased from 5 μM to 5 mM (curves e-b). The inset figure shows a comparative effect of three inhibitors (L-NAME, L-NMMA, L-NNA (1mM), which are more or less same. The inactive analogue D-NAME (curve g') does not alter the arginine action. A similar effect is observed for lactate build-up (Figure 2B). To substantiate that the effect of L-arginine on glycolysis is through NO generation, a similar experiment was performed in the presence of a known nitric oxide generator, sodium nitroprusside (Figure 3). Curve (a) shows the rate of consumption of glucose in cells metabolizing under normal conditions; curve (e) in the presence of L-arginine; and curves (b-d) in the presence of increasing concentrations of sodium nitroprusside. The effect of sodium nitroprusside is evident as it approaches the effect of arginine in enhancing glucose consumption (Figure 3) and lactate production (figure not shown). These observations strongly support the conclusion that arginine action on glucose metabolism in sperm is through NO generation.

Membrane Peroxidation

The in vitro preservation conditions—in particular, freezing, exposure to ionizing radiation, and addition of a variety of preservatives—are likely to alter the phospholipids in cell membrane resulting in destabilization of plasma and acrosomal membranes [8, 9]. The result is a free radical chain reaction, forming cytotoxic malondialdehyde, which can be estimated using standard procedures [15]. However, when such cells are pre-incubated with arginine, the peroxidation level decreases

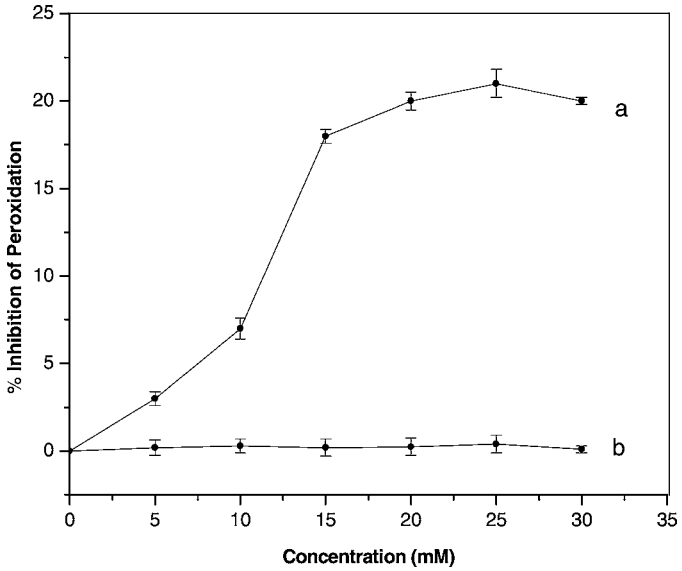


FIG 4. Inhibitory effect of (a) L-arginine and (b) D-arginine on lipid peroxidation induced by freezing. Cells were frozen with different concentrations of L-arginine. Percent inhibition is calculated in terms of reduction in amount of TBARS produced as compared to control. All data are expressed as mean SEM (n = 3). Values were considered significantly different if $P < 0.05$ following a Student *t*-test.

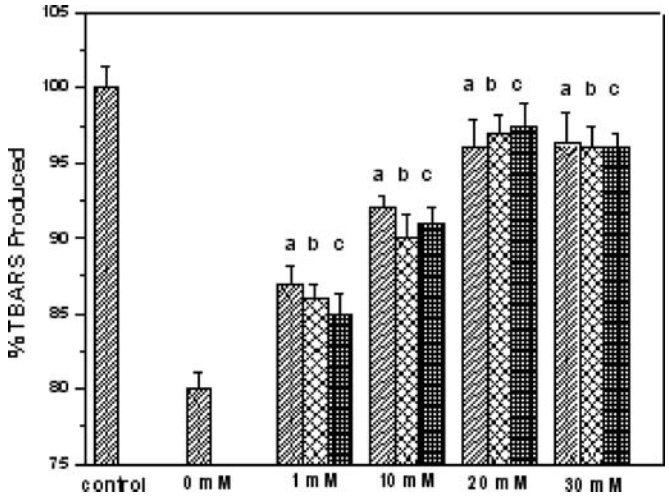


FIG 5. Effect of (a) L-NAME, (b) L-NMMA, and (c) L-NNA on protective effect of L-arginine against lipid peroxidation induced by freezing. Prior to the freezing, cells were incubated at 25°C with different concentrations of inhibitors along with 5 mM of L-arginine. Amount of TBARS produced for the control is taken as 100%. All data are expressed as mean SEM (n = 3). Values were considered significantly different if $P < 0.05$ following a Student *t*-test.

by about 20%. Moreover, it is observed that the inhibition of lipid peroxidation by L-arginine is dependent on concentration (Figure 4). The percentage inhibition is almost linear up to a concentration of 20 mM of L-arginine. On the other hand, the inactive analogue D-arginine does not show such an effect. To support the above results, activity of NOS, which is responsible for NO generation, was inhibited using L-NAME, L-NMMA, and L-NNA, and the effect of L-arginine on lipid peroxidation was monitored. As shown in Figure 5, the results indicate that in the absence of the NOS inhibitor (0 mM inhibitor), L-arginine provides significant protection against damage caused due to peroxidation (measured in terms of amount of TBARS produced) induced by freezing. However, when cells were preincubated with increasing concentrations (1 mM to 30 mM) of inhibitors, L-arginine appeared to lose its protective effect. This is clearly indicated by amount of TBARS produced in such cases (Figure 5). In another experiment, sodium niroprusside, a nitric oxide generator, mimicked the effect of L-arginine in a dose-dependent manner (Figure 6) by providing protection against membrane peroxidation induced by freezing of sperm. These experiments suggest that the protection against membrane peroxidation in sperm provided by L-arginine is most likely through increased generation of NO. Similar effects have been observed with cells in which peroxidation has been induced by UV radiation or by chemical methods [11].

DISCUSSION

Nitric oxide has been shown to inactivate superoxide (O_2^-) anions [27]. These anions are regularly released by mammalian cells during oxygen consumption and cause peroxidative damage to membrane phospholipids. Sperm are known to be particularly susceptible to such lipid peroxidation [28]. Sperm with damaged membranes are impaired functionally [28], which suggests that the presence of a free radical scavenging system would be beneficial to sperm-producing tissues. Kisa et al. have shown a correlation between sperm motility and the levels of NO and TBARS present in rat testicular tissue [29]. L-arginine, which provides protection against lipid peroxidation, is less likely to act as an antioxidant (like α-tocopherol) since it does not have any directly oxidizable groups. On the other hand, in neutrophils it has been shown that the ratio of NO to superoxide determines the nature of their interaction [30]. When NO predominates, it inactivates superoxide; when superoxide predominates, it inactivates NO. Thus in general, higher NO concentration is expected to reduce lipid peroxidation by inactivating superoxides. As discussed above, L-arginine has been shown to increase generation of NO. Based on this, it can be postulated that L-arginine protects spermatozoa against lipid peroxidation through increased NO production.

Thus, it has been very clearly shown that the effects of L-arginine in inducing sperm metabolism and preventing lipid peroxidation can be reversed by inhibiting the enzyme nitric oxide synthase using inhibitors, such as L-NAME, L-NMMA, and L-NNA. Also, an independent NO generator like sodium nitroprusside appears to mimic the effects of L-arginine in both these cases. These results strongly support the proposal that L-arginine exhibits its effects on spermatozoa through increased biosynthesis of nitric oxide. However, there seems to be

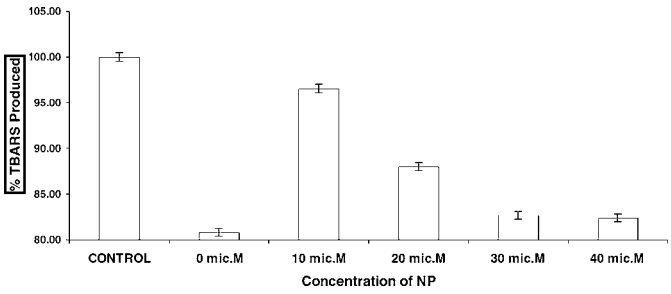


FIG 6. Protective effect of sodium nitroprusside against lipid peroxidation induced by freezing. Prior to the freezing, cells were incubated at 25°C with different concentrations of sodium nitroprusside. Cells incubated with 5 mM L-arginine have been used for comparison. Amount of TBARS produced for the control is taken as 100%. All data are expressed as mean SEM (n = 3). Values were considered significantly different if $P < 0.05$ following a Student *t*-test.

involvement of other minor mechanisms as well, as indicated by the fact that complete reversal of effects of L-arginine by a inhibitor has not been observed. This forms the basis for further investigation.

These results provide a good understanding of the factors affecting fertility, both in vivo and in vitro, and issues related to stability of spermatozoa during storage. This understanding in turn would be useful for developing measures to correct infertility as well as to improve conditions and media for storage of spermatozoa.

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