Original Article

Synergistic Protection of L-Arginine and Vitamin E On Lipid Peroxidation of Asthenospermic Patients

Sudha Srivastava, Ph.D.*

National Facility for High Field NMR, Tata Institute of Fundamental Research, Mumbai, India

Abstract

Background: Lipid peroxidation is known to cause various impairments to sperm cells and may play a major role in the etiology of male infertility. Asthenospermia is the main factor of male infertility and has significantly higher level of peroxidation than in normozoospermic males.

Materials and Methods: Using thiobarbituric acid (TBA) assay procedure, we have determined the level of lipid peroxidation as indicated by malondialdehyde (MDA) in the spermatozoa obtained from asthenospermic male semen.

Results: An inverse correlation of MDA concentration with sperm motility is observed. Treatment of cells with L-arginine and vitamin E significantly decreases the MDA concentration and improves the sperm motility as compared to that in case of control samples. A combination of L-arginine and vitamin E shows synergistic effect on sperm motility and prevention of lipid peroxidation.

Conclusion: L-arginine and vitamin E protect the cells against the loss of sperm motility by lipid peroxidation. Therefore, supplementation of both L-arginine and vitamin E may improve sperm motility and increase the possibility of fertilization in asthenospermic subjects.

Keywords: Asthenozoospermia, Lipid-Peroxidation, Vitamin E, L-Arginine, Human Spermatozoa

Introduction

Atmospheric oxygen (O_2) provides the oxidizer that makes the multicellular life possible through aerobic metabolism of foods containing Carbon and Hydrogen. However, the reactivity of O_2 is equally potentially toxic. Phospholipids of cell membrane undergo spontaneous oxidation in presence of atmospheric O_2 resulting membrane damage and cell death. Aerobic metabolism of human sperm cell produces various reactive oxygen species (ROS), which are potentially harmful to the sperm plasma membrane with its high content of polyunsaturated fatty acids (1-3). Under normal physiological conditions oxygen radicals are produced by sperm, through the leakage of electrons onto molecular oxygen from the mitochondrial electron transfer chain (2, 4). It has been long known that these radicals lead to peroxidation of phospholipids in the mitochondria of the sperm and thus to their ultimate immotility (5). An extensive study of lipid peroxidation in mammalian spermatozoa has shown that these cells are highly susceptible to this process (6) and the peroxidation products are potent spermicides (7-11). In normal cells there are a number of antioxidants such as superoxide dismutase (12), glutathione peroxidase (13), vitamin E to detoxify the harmful reactive oxygen radicals and prevent cell damage. Vitamin E is very well known antioxidant present in cell membrane and interrupts the chain reactions associated with lipid peroxidation and scavenges free radicals generated during the normal activity of oxidative enzymes (14). Unlike vitamin E, although amino acid L-arginine does not act as antioxidant, its role in enhancing the motility, metabolic activity and in preventing peroxidation is through generation of nitric oxide where enzyme nitric oxide synthase (NOS) plays an important role (15). L-arginine has been shown to prevent the lipid peroxidation in goat spermatozoa (16). Moreover, L-arginine is known to improve the sperm count and motility in oligospermic and asthenospermic patients without any side effects (17, 18). A deficiency in L-arginine causes derangement of sperm metabolism leading to decrease in the motility and loss of spermatogenesis (19). It plays an important role in stimulating spermatozoa in human, rabbit and goat under *in-vitro* conditions (17, 20, 21).

Asthenospermia is the main factor of male infertility among patients. Malondiald ehyde (MDA) concentration in the spermatozoa of asthensper-

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*** Corresponding Address: National Facility for High Field NMR, Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai-400005, India**
Email: sudha@tifr.res.in

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mic is significantly higher than in normozoospermic males (22, 23). In view of the extensive use of malondialdehyde production as an index of lipid peroxidation in mammalian spermatozoa (24), this index is still the most useful one available for use in assessing damage to sperm by formation and breakdown of lipid peroxides.

In this study we attempt to determine the level of lipid peroxidation in the spermatozoa from asthenospermic patients' semen and its possible correlation with sperm motility; and to compare the effects of L-arginine and the well known antioxidant vitamin E on the degree of peroxidation and improvement in sperm motility in these subnormal samples.

Materials and Methods

Semen Samples

The study was approved by the Ethic Committee and informed consent statement was obtained from all the patients. Semen samples were collected from patients consulting KEM Hospital Parel, Mumbai. Asthenospermia was indicated by a sperm concentration of $\geq 20 \times 10^6$/ml and motility <40%. The cells were washed, made into a pellet by centrifugation, and then suspended in an appropriate quantity of buffer to attain the desired concentration of cells. The motility of the cells was checked using an optical microscope. Cells were counted with a cytometer and cell concentration was maintained at 1×10^6 cells/ml in all experiments.

Reagents

Thiobarbituric acid (TBA), L-arginine and vitamin E were purchased from Aldrich Chemical Co. (USA). Other chemicals used were of AR grade. Dulbecco's medium (pH=7.2) was used as buffer along with 0.1% glucose (w/v) which served as fuel to maintain cellular metabolism.

Aerobic Incubation of cell samples

Lipid peroxidation was induced by exposure of the spermatozoa to O_2 during aerobic incubation. The cell pellet was dispersed in buffer to give a sperm suspension containing 1×10^6 cells/ml. The appropriate amount of sample was placed in a shaking water bath at 37°C to insure good contact between gas and liquid phases. Incubations with required concentrations of L-arginine and vitamin E were carried out in the similar manner.

Measurement of Lipid Peroxidation

Production of MDA was used as an index of spontaneous lipid peroxidation by cell sample using thiobarbituric acid (TBA) assay (25). Aerobic incubations of sperm suspensions in the chosen media were terminated at fixed time intervals by chilling the incubation bottles in liquid nitrogen. The sperm suspensions were left in frozen state for 15 minutes and then added to 2 ml of TBA reagent (15% w/v trichloroacetic acid and 0.25 N HCI). The mixture was heated in a boiling water bath for 15 minutes. After cooling, the suspension was centrifuged at 1,500 rpm for 10 minutes. The supernatant was then separated, and absorbance was measured at 532 nm. The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5 M^{-1} cm^{-1}).

Measurement of Motility

Sperm motility was estimated under the optical microscope for the number of sperm actively moving forward. The percentage of sperm with forward motility was calculated from the average of the three samples. The aliquots of the sperm suspensions being incubated were taken for motility assay just before freezing the incubation mixture in preparation for determination of lipid peroxidation by the TBA reagent.

Statistical Methods

Statistical analysis was done using a t-test by personal computer. All data are expressed as mean ±SEM (n=5). p value 0.05 were considered significant. Regression equations and correlation factors were calculated using the least squares method. Appropriate controls were run for each set of experiment.

Results

When cell samples are incubated aerobically in Dulbecco medium at 37°C, the amount of MDA produced increases linearly with incubation time over a period of 17 hours (Fig 1), with a net rate of 0.087 nmol/h per 10^6 cells. The maximal accumulation of MDA in the medium was 1.4 nmol/10^6 cells. After a maximum production is achieved at 17 hours, a net production is no more observed and content of MDA decreases slightly and linearly with time. This decrease may be due to the metabolism of MDA by cells. This is supported by the observation that MDA metabolism in cells involves oxidation by mitochondrial aldehyde dehydrogenase followed by decarboxylation to produce CO_2 and acetate (26). The linear increase of MDA in the medium therefore represents a balance between production and consumption. Incubation of samples along with L-arginine reduces the amount of MDA produced to a large extent. In this case time to attain maximum net MDA takes longer i.e.

24 hours and the production rate is 0.029 nmol/h per 10^6 cells, which is three times slower as compared to that in the cells without L-arginine. In this case after attaining a net maxima, the consumption rate of MDA is higher as observed by the faster decaying curve after 24 hours. This could be due to the enhanced metabolic activity of the cells in presence of L-arginine (21, 27). On the other hand incubation with vitamin E, a well known antioxidant reduces the MDA production to a very large extent. It attains a maximum net production at of 0. 38 nmol/10^6 cells in 24 hours at a rate of 0.015 nmol/h per 10^6 cells, which is almost six times lower to the control cells. After 24 hours the net production is more or less constant and attains a plateau. However, when cells are incubated in presence of both, L-arginine and vitamin E in combination, a synergistic effect is observed where the net MDA production is at a very slow rate (0.012 nmol/h per 10^6 cells) and remains constant through out until 30 hours and declines thereafter. Here again the effect of L-arginine in enhancing the metabolic activity assists in bringing the MDA level to almost close to zero.

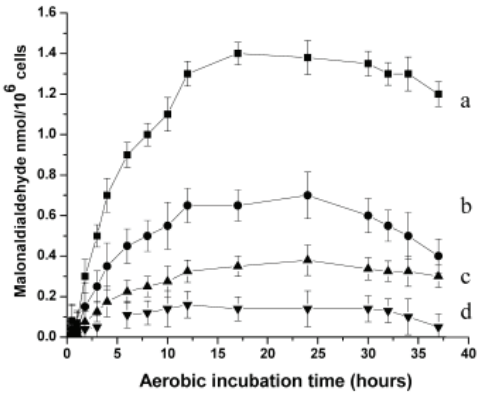

Fig 1: Malondialdehyde production by human asthenospermic cell as a function of aerobic incubation time at 37 °C. (a) cells incubated in the Dulbecco's medium serve as control, (b) cells incubated with L-arginine (5mM), (c) cells incubated with vitamin E (1mM) and (d) cells incubated with L-arginine (5mM) + vitamin E (1mM). Each point represents the mean of 3-5 experiments. Error bars are the standard deviations.

In order to see the change in sperm motility with incubation time, we monitored the cell motility at different intervals of time during aerobic incubation under the four conditions mentioned above. As observed from Fig 2, the motility of the cells decreases drastically with increasing incubation period. In control samples, the percent motility reduces to almost half of the initial value in just 2 hours of incubation period. Within 5 hours of incubation the cells become completely immotile. On the other hand, on incubation in presence of L-arginine, Vitamin E and in combination, it is observed that L-arginine retains/emhances the motility of the cells for a longer time as compared to that in presence of vitamin E. When used in combination, the motility increases and cells remain motile until 23 hours.

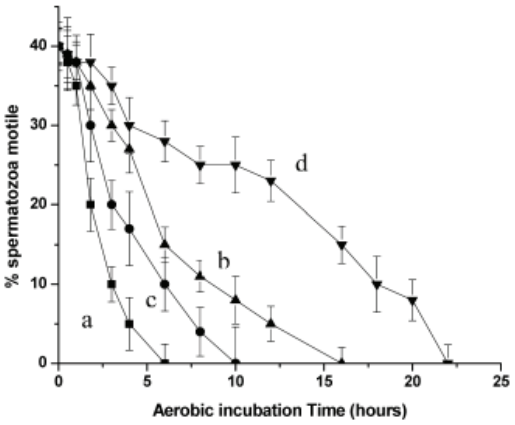

Fig 2: Sperm motility as a function of aerobic incubation time at 37 °C. (a) cells incubated in the Dulbecco's medium serve as control, (b) cells incubated with L-arginine (5mM), (c) cells incubated with vitamin E (1mM) and (d) cells incubated with L-arginine (5mM) + vitamin E (1mM). Each point represents the mean of 8-10 experiments. Error bars are the standard deviations.

A correlation between MDA concentration and sperm motility in asthenospermic samples is indicated in Figure 3. An inverse correlation of MDA concentration with sperm motility is observed in all the cases. There was little change in the correlation of sperm motility with MDA concentration in the control samples ($r=0.39$, $p<0.0001$) (Fig 3A). However, the correlation of sperm motility with MDA concentration in the cells treated with L-arginine, vitamin E and both in combination became highly significant after treatment. In case of L-arginine treated cells, the MDA concentration decreases (~25-60 %) of that of control with simultaneous increase in sperm motility ($r =0.64$, $p<0.0001$) (Fig 3B). Similarly, the treatment with vitamin E gives rise a large change in correlation ($r=0.098$, $p<0.0001$) as compared to that of control samples, with a decrease in MDA concentration (~40-70 %) and a significant increase in motility (Fig 3C) is observed. However, a combined treatment with both L-arginine and vitamin E, gives rise a further decrease in MDA concentration (~ 60-95%) with a significant change in correlation ($r=0.085$, $p<0.0001$), and consequent large enhancement in sperm motility.

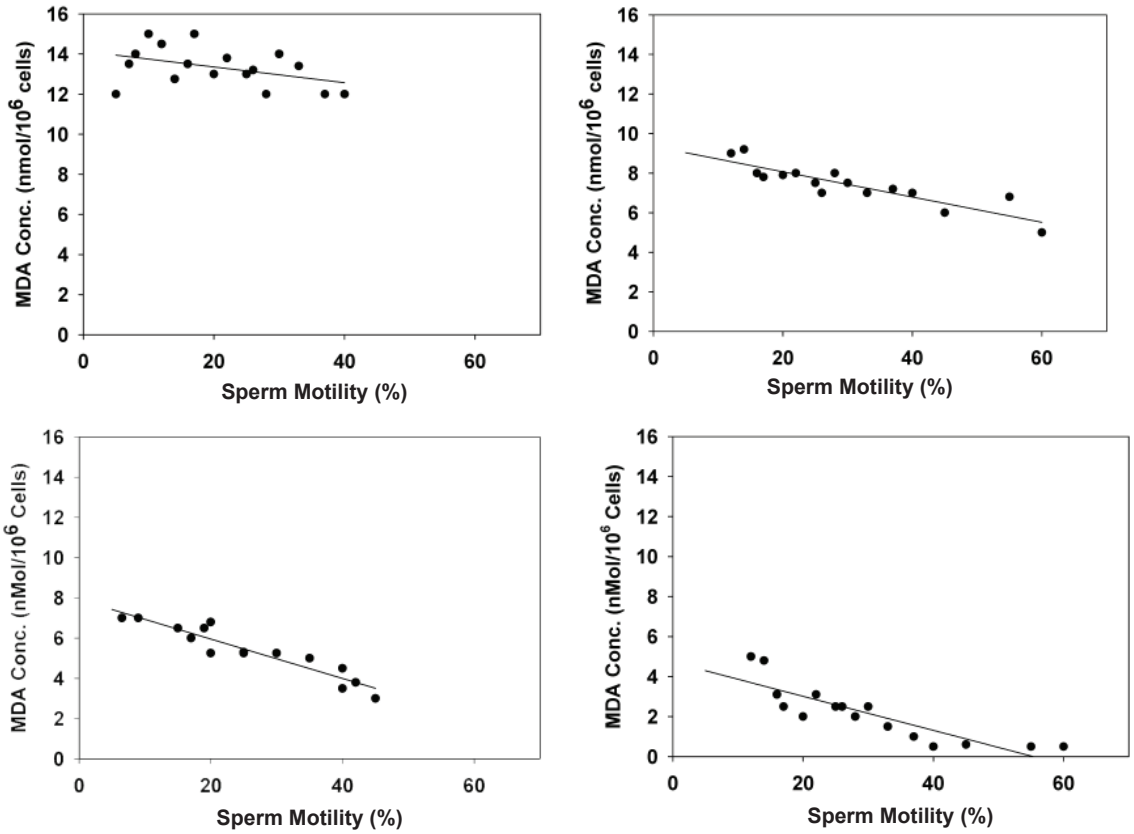

Figure 3. Correlation between MDA concentration and sperm motility in spermatozoa from asthenospermic patients. A:Cells incubated in the Dulbecco's medium serve as control, B: Cells incubated with L-arginine (5mM), C: Cells incubated with vitamin E (1mM) and D: Cells incubated with L-arginine (5mM) + vitamin E (1mM).

Discussion

Lipid peroxidation by free oxygen radicals leads to impaired sperm motility (28-30). Moreover, the poorly motile spermatozoa are more susceptible to lipid peroxidation than motile ones. It has been shown that human spermatozoa became immotile within 5 minutes after the addition of lipid peroxide and within 5-30 minutes of addition of reactive oxygen radicals, depending on the concentration (31,32). A close linear correlation exists between the extent of peroxidation and the loss of sperm motility (1, 33). The results presented here show that in asthenospermic samples, the MDA concentration of human spermatozoa correlates inversely with the percent of sperm motility and suggest that lipid peroxidation renders the spermatozoa immotile. The mechanism by which lipid peroxidation renders spermatozoa immotile is due to the damage to the sperm membrane, which results in leakage in intracellular constituents, including adenine and pyridine nucleotides, and enzymes (8). Without intracellular ATP, there can be no motility. It would therefore be expected that the percent immotile sperm produced by this mechanism would correlate with the extent of lipid peroxidation (MDA production) and not with the time of exposure to aerobic incubation as can be correlated from Figures 1 & 2.

Results further indicate that rate of MDA production decreases when cells are treated with vitamin E strongly suggesting that vitamin E plays a major role as a scavenger for reactive oxygen species, leading to a decrease in lipid peroxidation and ultimately to the protection of spermatozoa. The antioxidant activity of Vitamin E is based on the ease with which the hydrogen on the hydroxyl group of the chroman ring can be donated to neutralize a free radical creating tocopheroxyl radical (34). Our results are supported by those from a previous study (35), which showed that most rat tissues, including the testes, are more susceptible to lipid peroxidation in vitamin E-deficient animals compared to control animals fed normal diet thus indicating the involvement of vitamin E in the inhibition of lipid peroxidation. (14). In addition, the decrease in MDA concentration and the increase

in sperm motility in the vitamin E-treated cells were greater than those in the control cells. This might indicate that the MDA concentration needs to be reduced to a certain level to achieve better sperm motility. These results are further supported by the reports where it has been shown that vitamin E supplementation improved sperm motility and increased the possibility of fertilization in asthenospermic subjects (36).

Further, treatment with L-arginine which is known to enhance metabolic activity and motility reduces the MDA concentration to a lesser extent as compared to that with vitamin E treated cells. However, the motility of these cells is higher. This is supported by earlier reports in case of goat spermatozoa where L-arginine enhances the motility 6-8 folds (21) and prevents lipid peroxidation in a concentration dependent manner. Since L-arginine does not have any directly oxidizable groups it is less likely that it acts as and antioxidant (like vitamin E). It has been shown that the presence of the enzyme NOS generates Nitric Oxide (NO) in spermatozoa (37). It is also reported that NO reduces lipid peroxidation in spermatozoa and L-arginine protects spermatozoa against lipid peroxidation through generation of NO (15, 16).

The anti-oxidant properties of vitamin E are said to make sperm more fertile by protecting from damage and increasing quality. It also corrects the functioning of the endocrine glands that produce hormones, which influence growth, development and metabolic activity. In the present study a synergistic effect is observed when L-arginine is used in combination with vitamin E towards the enhancement of motility and prevention of lipid peroxidation. It also seems that the reduction in MDA concentration that resulted from treatment with vitamin E and L-arginine produced a great increase in sperm motility that improved the correlation between these two parameters. Therefore a combined administration of vitamin E and L-arginine has a synergistic, protective effect on sperm function that may be mediated by enhanced NO production.

Conclusion

Lipid peroxidation has a deleterious effect on the semen quality and MDA is an index of lipid peroxidation which may be a diagnostic tool for the analysis of infertility in the asthenozoospermic patients. Moreover, since the MDA concentration of the spermatozoa in asthenospermic patients is measured higher in comparison to the MDA concentration in normospermic patients (22), measurement of MDA concentration could possibly be used in the assessment of male infertility.

In conclusion, L-arginine and vitamin E both protect the cells against the loss of sperm motility due to the lipid peroxidation. Therefore, supplementation of both L-arginine and vitamin E may improve sperm motility and increase the possibility of fertilization in asthenospermic subjects.

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