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Effect of Sodium Nitrate ($NaNO_3$) on Sperm Motility and Abnormality: An *In vitro* Approach

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Abstract

Nitrates (NO_3) are the naturally occurring inorganic ions with beneficial effects in both plants and animals. However, NO_3 in excess has adverse effect on male and female reproductive systems. The study was undertaken to investigate the effect of 10mg/ml and 100mg/ml of sodium nitrate ($NaNO_3$) on sperm motility and abnormality at different time intervals (5,10,15,20,25 and 30 min). Treatment of cauda epididymal sperm suspension with 10mg/ml of $NaNO_3$ significantly reduced the sperm motility only at 25 and 30 min time intervals compared to controls. However, 100mg/ml of $NaNO_3$ resulted in significant reduction of sperm motility from 5 min to 30 min time interval in time dependent manner and complete loss of sperm motility was observed at 15 min of incubation. In addition, the number of abnormal spermatozoa was significantly high in 100mg/ml of $NaNO_3$ treated groups compared to controls and 10 mg/ml of $NaNO_3$. These results clearly reflected the toxic effect of NO_3 on spermatozoa and its capability in fertilization.

Keywords: Abnormality; *In Vitro*; Motility; Sodium nitrate; Spermatozoa

Introduction

Nitrate (NO_3) and nitrite (NO_2) are the naturally occurring inorganic ions involved in nitrogen cycle. There are numerous sources of nitrates which can be classified into exogenous sources and endogenous sources. Leafy vegetables are the exogenous sources of nitrates which account for more than 70% of nitrates ingested in human diet. Nitrites are produced endogenously through the oxidation of nitric oxide (NO) and through a reduction of NO_3 by commensal bacteria in mouth and gastrointestinal tract [1]. In biological system, NO_3 gets converted to NO_2 and NO and are interchangeable [2].

Besides having beneficial effect, NO_3 has adverse effects on the body. The connection between human health and harmful effect of NO_3 was first reported by Comly in 1945 after observing cyanosis in residents of Iowa (USA). The primary effect of NO_3 is the formation of methemoglobinemia in infants which reduces capacity to release oxygen to tissues due to oxidation of iron group of haem group [3,4]. Further, NO_3 causes improper thyroid functioning via inhibition of sodium iodide symporter [5]. Increase in NO_3 concentration causes myocardial infraction and hypotension [6]. And also affects liver by increasing bilirubin and transaminase levels [7].

Numerous studies have reported the toxic effect of NO_3 on reproductive system [8-12]. It is evident that NO_3 is a potential endocrine disruptor of reproductive endocrinology [13]. The adverse effects of NO_3 on reproductive system viz, mummified foetuses, lesions on the cervix, uterus and placenta and maternal death were observed in NO_3

exposed guinea pigs [14]. Further, higher risk of ovarian cancers in women drinking NO_3 contaminated water [15] and incidences of abortions in potassium nitrate (KNO_3) administered pregnant cows [16,17] were reported. In male rat and mice models, treatment of different doses of sodium nitrate ($NaNO_3$), sodium nitrite ($NaNO_2$) and KNO_3 caused a significant reduction in weight of testis and epididymis, sperm count and sperm motility and increased sperm abnormality [9-12]. In addition NO_3 causes lesions in spermatocytes and spermatids of germ layers, degenerations of leydig cells and arrest of spermatogenesis [9,10]. Further, NO_3 affects steroidogenesis of male reproductive system by decreasing the activities of 3βHSD (3-beta-hydroxysteroid dehydrogenase) and 17βHSD (17-beta-hydrosteroid dehydrogenase) and serum concentration of testosterone [10,11,18].

Besides *in vivo* studies, few *in vitro* studies have focused on the toxic effect of NO on human spermatozoa. For instance, treatment of NO declined forward progressive movement of spermatozoa in *in vitro* [19]. Studies have reported that the reduced sperm DNA integrity with the association and high concentration of NO in seminal plasma [20]. In addition, high concentration of NO was reported in semen samples of patients suffering from asthenozoospermia than that of normozoospermic individuals [21]. Further, higher concentration of NO caused decreased zona binding capacity of sperm and embryonic development [22]. These results clearly indicate that NO has adverse effect on sperm functions. Though there are enough reports on effect of NO on human spermatozoa, the studies related to the action of NO_3 on spermatozoa in *in vitro* is lacking. Hence, there is a need for investigation to assess the toxic effect of $NaNO_3$ (source of NO_3) on motility and abnormality of spermatozoa at different time intervals. with this background, the present study was conducted to investigate the dose and time dependent effect of two different concentrations of $NaNO_3$ (10mg/ml and 100mg/ml) on sperm motility and sperm abnormality in *in vitro.*

Materials and Methods

Animals

The study included adult male wistar rats weighing 180-200g procured from central animal facility of University of Mysore, Mysore. Polypropylene cages were used to maintain the animals under standard laboratory conditions and were provided with rat chow and water *ad libitum* and relative 12h light/dark cycle. The ethical acceptance to conduct the experiment was obtained from Institutional Animal Ethics Committee of University of Mysore, India (reference number: UOM/IAEC/04/2018) and the study was carried out as per the guidelines of the committee.

Experimental Design

An in vitro experiment was conducted to assess the effects of two different concentrations of $NaNO_3$ on sperm motility and abnormality at different time intervals viz.,0, 5, 10, 15, 20, 25 and 30 min. The epididymal sperm suspension (60 million/ml) was incubated with different concentrations of $NaNO_3$ (10mg/ml and 100mg/ml) at different time intervals. The group devoid of $NaNO_3$ was treated as control. After incubation, sperm motility and sperm abnormalities were analysed at regular time intervals.

Isolation of spermatozoa

Epididymal spermatozoa were isolated from cauda epididymis of adult male rats. The epididymis was minced in 1 ml of phosphate buffered saline and the suspension was filtered through muslin cloth. The filtered sperm suspension was used for the *in vitro* experiment.

Estimation of sperm motility

Progressive sperm motility was considered for estimating sperm motility. Motility was estimated by placing the sperm suspension on a slide and number of motile spermatozoa was counted from three different microscopic fields under light microscope. The mean of the three estimations was calculated in percentage [23].

Estimation of sperm abnormality

Sperm abnormality was analysed by staining the sperm suspension with eosin and a uniform smear was made on a glass slide. One thousand spermatozoa were observed under higher magnification (40X) and the number of spermatozoa showing head and tail abnormalities were counted. The aggregate of different types of spermatozoa showing abnormality were considered to compute percentage of spermatozoa with abnormal morphology [24,25].

Statistical Analysis

The mean ± standard error of each parameter was computed by considering the data and the mean values of each parameter of different groups were compared using one way analysis of variance followed by Duncan's multiple range test and judged significant if $p< 0.05$.

Results

Effect of different concentration of $NaNO_3$ on sperm motility at 0, 5, 10, 15, 20, 25 and30min intervals

There was a significant decrease in sperm motility in

sperm suspension treated with 100 mg/ml of $NaNO_3$ at 5, 10, 15, 20, 25 and 30 min of time interval compared to control. However, 10mg/ml of $NaNO_3$ significantly decreased the sperm motility only at 25 and 30 min time interval compared to control. No significant changes in sperm motility was observed at 5, 10, 15 and 20 min time interval of 10mg/ml of $NaNO_3$ treated groups compared to controls. Treatment of 100mg/ml of $NaNO_3$ caused complete loss of sperm motility at 15, 20, 25 and 30 min of incubation (Table 1).

Groups	Mean percentage of motility ± SE						
	0 min	5 min	10 min	15 min	20 min	25 min	30 min
Control	91.00±3.21[a]	91.33±2.72[a]	94.33±2.33[a]	89.00±3.05[a]	76.66±2.40[a]	67.33±1.76[a]	45.33±3.711[a]
10mg/ml of NaNO3	92.33±1.76[a]	95.33±0.88[a]	91.66±0.88[a]	82.00±3.05[a]	69.33±2.96[a]	45.66±2.33[b]	27.66±4.33[b]
100mg/ml of NaNO3	91.00±2.64[a]	37.66±6.48[b]	15.00±5.13[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[c]	0.00±0.00[c]
ANOVA F value(df=2,6)	0.87 *p>0.918	84.31 ***p<0.001	187.04 ***p<0.001	393.58 ***p<0.001	368.88 ***p<0.001	414.27 ***p<0.001	48.11 ***p<0.001

Table 1: Effect of different concentration of $NaNO_3$ on sperm motility.
Note: Mean values were compared by one-way ANOVA followed by Duncan's multiple range tests. Values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (*p< 0.05) different from each other. df - degree of freedom, $NaNO_3$ - sodium nitrate.

Effect of different concentration of $NaNO_3$ on sperm abnormality at 0, 5, 10, 15, 20, 25 and 30 min interval

A significant increase in sperm abnormality was observed in 100mg/ml of $NaNO_3$ treated groups compared to that of controls and 10mg/ml of $NaNO_3$. However, no significant difference in sperm abnormality was observed between control groups and 10mg/ml of $NaNO_3$ with increase in duration of incubation (Table 2).

Groups	Mean percentage of abnormality ± SE						
	0 min	5 min	10 min	15 min	20 min	25 min	30 min
Control	0.00±0.00[a]	0.66±0.33[a]	0.66±0.33[a]	0.66±0.33[a]	0.66±0.33[a]	0.66±0.33[a]	0.66±0.33[a]
10mg/ml of $NaNO_3$	0.00±0.00[a]	1.33±0.33[a]	1.66±0.66[a]	2.00±0.57[a]	2.66±1.20[a]	3.00±0.57[a]	3.33±0.33[a]
100mg/ml of $NaNO_3$	1.00±0.57[a]	11.00±1.15[b]	14.66±1.76[b]	23.66±3.48[b]	23.66±3.48[b]	23.66±3.48[b]	23.66±3.48[b]
ANOVA F value (df=2,6)	3 *p<0.125	64.5 ***p<0.001	49.9 ***p<0.001	39.83 ***p<0.001	35.63 ***p<0.001	38.29 ***p<0.001	38.49 ***p<0.001

Table 2: Effect of different concentration of $NaNO_3$ on sperm abnormality.
Note: Mean values were compared by one-way ANOVA followed by Duncan's multiple range tests. Values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (*p< 0.05) different from each other. df - degree of freedom, $NaNO_3$ - sodium nitrate.

Discussion

Present study was undertaken to investigate the toxic effect of two different concentration of $NaNO_3$ on sperm motility and sperm abnormality at different time intervals. The study revealed that treatment of $NaNO_3$ decreased the sperm motility and increased the number of abnormal spermatozoa compared to controls.

Sperm motility is an important aspect of spermatozoa to reach the fallopian tube during fertilization. The quality of spermatozoa depends on its motility and number of abnormal spermatozoa [26]. Earlier *in vivo* studies in rat and mice showed a decline in sperm motility under NO_3 treatment [9-12]. Similarly, present study showed NO_3 induced reduced sperm motility in *in vitro* condition. Between the two doses of $NaNO_3$, a higher concentration that is 100mg/ml showed severe toxicity as it resulted in complete cessation of sperm motility at 15 min of incubation with epididymal spermatozoa. However, the lower dose of $NaNO_3$, that is 10mg/ml showed a time dependent decrease in sperm motility from 5 min to 30 min compared to controls.

A significant dose dependent effect was observed between two doses of $NaNO_3$ at different durations of incubations studied. Thus, the present study clearly demonstrates that treatment of $NaNO_3$ affected sperm motility in dose and time dependent manner. Similarly, earlier study have reported the dose dependent inhibition of sperm motility by NO donors viz, nitroprusside and pure NO gas [27]. In contrast, few investigations reported the beneficial aspects of NO_3 on spermatozoa wherein treatment with NO_3 caused enhanced capacitation [28], motility, viability [29] and vigour [30] of spermatozoa. In addition, it has been reported that NO and nitric oxide synthase (NOS) is essential for capacitation and fertilization [31].

The NO_3 induced decrease in sperm motility is due to increased level of NO, a product of NO_3 and NO_2 [2]. Higher concentration of NO_3 enhances the synthesis of NO which in turn affects sperm motility. The peroxy nitrate ($ONOO^-$) formed due to the interaction between NO and reactive oxygen species (ROS) can cause damage to lipids and thiol proteins of sperm membrane [32] thereby affects sperm motility. In addition, NO inhibit sperm motility by affecting mitochondrial electron transport protein and thereby obstruct cellular respiration and ATP production in spermatozoa [27,33]. Further, NO_3 inhibit sperm motility by inducing oxidative stress [34]. Spermatozoa are very sensitive to ROS and $ONOO^-$ which can readily damage the cell membrane [32,34].These are the possible mechanisms through which NO_3 can affect sperm motility.

The morphology of spermatozoa is another important aspect which plays a vital role in fertilization. Earlier *in vivo* studies have proved enhanced number of abnormal spermatozoa under NO_3 treatment [9-12]. In the present *in vitro* study, treatment of 100mg/ml of $NaNO_3$ caused a significant increase in the number of abnormal spermatozoa compared to 10mg/ml of $NaNO_3$ and control groups. The $NaNO_3$ induced increase in abnormal spermatozoa increased with increasing duration of exposure. These results clearly indicate that $NaNO_3$ affect the sperm morphology by increasing head and tail abnormalities, thereby affect the fertilization capacity of spermatozoa. The NO_3 induced sperm abnormality may be due to obstruction in cellular respiration and production of ATP and formation of ROS which can cause damage to the lipid and thiol proteins present in the sperm membrane [27,32-34]. Therefore, NO_3 exposure increases the number of abnormal spermatozoa by affecting cell membrane.

The present *in vitro* study clearly demonstrated that $NaNO_3$ has adverse effect on spermatozoa and the effect is time and dose dependent. The study reports the toxic effects of $NaNO_3$ on spermatozoa in *in vitro* condition for the first time as earlier *in vitro* studies were focused on effect NO on human spermatozoa [19,21,27]. The study also provides evidence that higher concentration of $NaNO_3$ that is 100mg/ml has severe effect on sperm motility and abnormality. These results gain importance in male infertility of human beings residing in the areas where there is high level of NO_3 in drinking ground water. Therefore, present investigation appears to be an evidence for the effect of NO_3 on male reproduction. However, future studies are needed to reflect mechanistic action of NO_3 in affecting sperm motility.

Conclusion

The present study demonstrated dose and time dependent effect of NO_3 on spermatozoa. The higher dose of $NaNO_3$ that is 100mg/ml adversely affected sperm motility and abnormality compared to lower dose. The results of the present study for the first time showed reproductive toxicity of $NaNO_3$ in *in vitro* condition.

Conflicts of Interest

The authors declare no conflicts of interest.

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