Effect of Sodium Nitrate (NaNO₃) on Sperm Motility and Abnormality: An In vitro Approach

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Abstract

Nitrates (NO₃⁻) are the naturally occurring inorganic ions with beneficial effects in both plants and animals. However, NO₃ 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 (NaNO₃) 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 NaNO₃ significantly reduced the sperm motility only at 25 and 30 min time intervals compared to controls. However, 100mg/ml of NaNO₃ 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 NaNO₃ treated groups compared to controls and 10 mg/ml of NaNO₃. These results clearly reflected the toxic effect of NO₃ on spermatozoa and its capability in fertilization.

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

Introduction

Nitrate (NO₃⁻) and nitrite (NO₂⁻) 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 NO₃ by commensal bacteria in mouth and gastrointestinal tract [1]. In biological system, NO₃ gets converted to NO₂ and NO and are interchangeable [2].

Besides having beneficial effect, NO₃ has adverse effects on the body. The connection between human health and harmful effect of NO₃ was first reported by Comly in 1945 after observing cyanosis in residents of Iowa (USA). The primary effect of NO₃ 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, NO₃ causes improper thyroid functioning via inhibition of sodium iodide symporter [5]. Increase in NO₃ 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 NO₃ on reproductive system [8-12]. It is evident that NO₃ is a potential endocrine disruptor of reproductive endocrinology [13]. The adverse effects of NO₃ on reproductive system viz, mummified foetuses, lesions on the cervix, uterus and placenta and maternal death were observed in NO₃.
Exposure to sodium nitrate (NaNO\textsubscript{3}) has been associated with higher incidence of ovarian and endometrial cancers in women drinking NO\textsubscript{3} contaminated water [15] and increased incidences of abortions in potassium nitrate (KNO\textsubscript{3}) administered pregnant cows [16,17]. In male rat and mice models, treatment of different doses of sodium nitrate (NaNO\textsubscript{3}), sodium nitrite (NaNO\textsubscript{2}) and KNO\textsubscript{3} caused a significant reduction in testis and epididymis, sperm count and sperm motility and increased sperm abnormality [9-12]. In addition NO\textsubscript{3} causes lesions in spermatocytes and spermatids of germ layers, degenerations of Leydig cells and arrest of spermatogenesis [9,10]. Further, NO\textsubscript{3} affects steroidogenesis of male reproductive system by decreasing the activities of 3\textbeta HSD (3-beta-hydroxysteroid dehydrogenase) and 17\beta HSD (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 [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\textsubscript{3} on spermatozoa in in vitro is lacking. Hence, there is a need for investigation to assess the toxic effect of NaNO\textsubscript{3} (source of NO\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{3} (10mg/ml and 100mg/ml) at different time intervals. The group devoid of NaNO\textsubscript{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

Progressivesperm 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\textsubscript{3} on sperm motility at 0, 5, 10, 15, 20, 25 and 30 min intervals**

There was a significant decrease in sperm motility in
sperm suspension treated with 100 mg/ml of NaNO₃ at 5, 10, 15, 20, 25 and 30 min of time interval compared to control. However, 10mg/ml of NaNO₃ 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 NaNO₃ treated groups compared to controls. Treatment of 100mg/ml of NaNO₃, caused complete loss of sperm motility at 15, 20, 25 and 30 min of incubation (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 min Mean percentage of motility ± SE</th>
<th>5 min Mean percentage of motility ± SE</th>
<th>10 min Mean percentage of motility ± SE</th>
<th>15 min Mean percentage of motility ± SE</th>
<th>20 min Mean percentage of motility ± SE</th>
<th>25 min Mean percentage of motility ± SE</th>
<th>30 min Mean percentage of motility ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>91.00±3.21</td>
<td>91.33±2.72</td>
<td>94.33±2.33</td>
<td>89.00±3.05</td>
<td>76.66±2.40</td>
<td>67.33±1.76</td>
<td>45.33±3.71</td>
</tr>
<tr>
<td>10mg/ml of NaNO₃</td>
<td>92.33±1.76</td>
<td>95.33±0.88</td>
<td>91.66±0.88</td>
<td>82.00±3.05</td>
<td>69.33±2.96</td>
<td>45.66±2.33</td>
<td>27.66±4.33</td>
</tr>
<tr>
<td>100mg/ml of NaNO₃</td>
<td>91.00±2.64</td>
<td>37.66±6.48</td>
<td>15.00±5.13</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>ANOVA F value(df=2,6)</td>
<td>0.87 *p&gt;0.918</td>
<td>84.31</td>
<td>187.04</td>
<td>393.58</td>
<td>368.88</td>
<td>414.27</td>
<td>48.11</td>
</tr>
</tbody>
</table>

Table 1: Effect of different concentration of NaNO₃ on sperm motility.

Effect of different concentration of NaNO₃ 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 NaNO₃ treated groups compared to that of controls and 10mg/ml of NaNO₃. However, no significant difference in sperm abnormality was observed between control groups and 10mg/ml of NaNO₃ with increase in duration of incubation (Table 2).

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 min Mean percentage of abnormality ± SE</th>
<th>5 min Mean percentage of abnormality ± SE</th>
<th>10 min Mean percentage of abnormality ± SE</th>
<th>15 min Mean percentage of abnormality ± SE</th>
<th>20 min Mean percentage of abnormality ± SE</th>
<th>25 min Mean percentage of abnormality ± SE</th>
<th>30 min Mean percentage of abnormality ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00±0.00</td>
<td>0.66±0.33</td>
<td>0.66±0.33</td>
<td>0.66±0.33</td>
<td>0.66±0.33</td>
<td>0.66±0.33</td>
<td>0.66±0.33</td>
</tr>
<tr>
<td>10mg/ml of NaNO₃</td>
<td>0.00±0.00</td>
<td>1.33±0.33</td>
<td>1.66±0.33</td>
<td>2.00±0.57</td>
<td>2.66±1.20</td>
<td>3.00±0.57</td>
<td>3.33±0.33</td>
</tr>
<tr>
<td>100mg/ml of NaNO₃</td>
<td>1.00±0.57</td>
<td>11.00±1.15</td>
<td>14.66±1.76</td>
<td>23.66±3.48</td>
<td>23.66±3.48</td>
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</tr>
<tr>
<td>ANOVA F value(df=2,6)</td>
<td>3 *p&lt;0.125</td>
<td>64.5</td>
<td>49.9</td>
<td>39.83</td>
<td>35.63</td>
<td>38.29</td>
<td>38.49</td>
</tr>
</tbody>
</table>

Table 2: Effect of different concentration of NaNO₃ on sperm abnormality.

Discussion

Present study was undertaken to investigate the toxic effect of two different concentration of NaNO₃ on sperm motility and sperm abnormality at different time intervals. The study revealed that treatment of NaNO₃ 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 NO₃ treatment [9-12]. Similarly, present study showed NO₃ induced reduced sperm motility in in vitro condition. Between the two doses of NaNO₃, 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 NaNO₃, 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 NaNO₃ at different durations of incubations studied. Thus, the present study clearly demonstrates that treatment of NaNO₃ 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 NO₃ on spermatozoa wherein treatment with NO₃ 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 NO₃ induced decrease in sperm motility is due to increased level of NO, a product of NO₃ and NO₂ [2]. Higher concentration of NO₃ 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, NO₃ 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 NO₃ 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 NO₃ treatment [9-12]. In the present in vitro study, treatment of 100mg/ml of NaNO₃ caused a significant increase in the number of abnormal spermatozoa compared to 10mg/ml of NaNO₃ and control groups. The NaNO₃ induced increase in abnormal spermatozoa increased with increasing duration of exposure. These results clearly indicate that NaNO₃ affect the sperm morphology by increasing head and tail abnormalities, thereby affect the fertilization capacity of spermatozoa. The NO₃ 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, NO₃ exposure increases the number of abnormal spermatozoa by affecting cell membrane.

The present in vitro study clearly demonstrated that NaNO₃ has adverse effect on spermatozoa and the effect is time and dose dependent. The study reports the toxic effects of NaNO₃ 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 NaNO₃ 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 NO₃ in drinking ground water. Therefore, present investigation appears to be an evidence for the effect of NO₃ on male reproduction. However, future studies are needed to reflect mechanistic action of NO₃ in affecting sperm motility.

**Conclusion**

The present study demonstrated dose and time dependent effect of NO₃ on spermatozoa. The higher dose of NaNO₃ 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 NaNO₃ in in vitro condition.

**Conflicts of Interest**

The authors declare no conflicts of interest.

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**References**

