THE IN VITRO EFFECT OF ELDERBERRY (SAMBUCUS NIGRA) EXTRACT ON THE ACTIVITY AND OXIDATIVE PROFILE OF BOVINE SPERMATOZOA

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ABSTRACT

The paper presents the overall results and experimental details of the in vitro assessment of the elderberry (Sambucus nigra) extract on the motility, viability and reactive oxygen species (ROS) production of bovine spermatozoa in different time periods (0, 2, 6 and 24 hours). Sambucus nigra is often used for medicinal purposes throughout the world. Modern research reveals that Sambucus nigra extracts may have anti-inflammatory, antiviral, anticancer and antioxidant properties, because of a high content of biologically active components. Spermatozoa motility was assessed using the Computer-assisted sperm analysis (CASA) system. Cell viability was examined using the metabolic activity MTT assay and ROS generation was quantified using luminometry. The CASA analysis revealed that the motility in the experimental groups supplemented with 100, 50 and 1 μg/mL elderberry extract was lower in comparison with other samples. The experiment showed that the elderberry extract had a considerable in vitro effect on the sperm motility, vitality and oxidative profile. The ROS production as well as the CASA assessment proved that the optimal concentration of both extracts was 10 μg/mL in every time with statistically significant results. The MTT test showed a statistically significant increase of mitochondrial at all time periods with 10 μg/mL elderberry extract when compared to the control group. When lower concentrations of the elderberry extract were used (5 and 1 μg/mL), the mitochondrial activity was higher than in the control group but lower than in the group supplemented with 10 μg/mL of the extract. In these groups this indicator increased maximally after 24 h. The findings of the present study indicate that Sambucus nigra extract possesses activity promoting properties on bovine spermatozoa at 10 and 5 μg/mL.

Keywords: Elderberry, Sambucus nigra, spermatozoa, bull, motility, mitochondrial activity, reactive oxygen species

INTRODUCTION

Several commonly used plants have been reported to affect male reproductive functions in wildlife and humans. The effects observed with most of the plant and plant-based products have been attributed to a wide variety of properties of one or more active compounds present in ethnopharmacologically important medicinal herbs (D’Cruz et al., 2010). Evaluation of herbs has been in progress worldwide for several decades to identify effective and safe substances for fertility regulation. This approach proved to be a good alternative to synthetic drugs as the chemicals of plant origin have limited side effects. Various medicinal plants extracts were investigated for their fertility-related activity both in male and female animal models (Sharma et al., 2013). Sambucus nigra Linn, frequently known as ‘Sweet elder’ belongs to family Caprifoliaceae. Sambucus species are being investigated for their potential health benefits. It is one of the most attractive trees being put to some useful purpose in Ayurveda, homeopathic medicine and has become a cynosure to modern treatment options. The plant is highly used traditionally in curing diverse disorders. Commonly it is used as an astringent, antiviral and diuretic. The antioxidant activity of elderberry extracts has been evaluated before, and it is often used for medicinal purposes throughout the world. Modern research reveals that Sambucus nigra extracts may have anti-inflammatory, antiviral, anticancer and antioxidant properties, because of a high content of biologically active components. Spermatozoa motility was assessed using the Computer-assisted sperm analysis (CASA) system. Cell viability was examined using the metabolic activity MTT assay and ROS generation was quantified using luminometry. The CASA analysis revealed that the motility in the experimental groups supplemented with 100, 50 and 1 μg/mL elderberry extract was lower in comparison with other samples. The experiment showed that the elderberry extract had a considerable in vitro effect on the sperm motility, vitality and oxidative profile. The ROS production as well as the CASA assessment proved that the optimal concentration of both extracts was 10 μg/mL in every time with statistically significant results. The MTT test showed a statistically significant increase of mitochondrial at all time periods with 10 μg/mL elderberry extract when compared to the control group. When lower concentrations of the elderberry extract were used (5 and 1 μg/mL), the mitochondrial activity was higher than in the control group but lower than in the group supplemented with 10 μg/mL of the extract. In these groups this indicator increased maximally after 24 h. The findings of the present study indicate that Sambucus nigra extract possesses activity promoting properties on bovine spermatozoa at 10 and 5 μg/mL.

Keywords: Elderberry, Sambucus nigra, spermatozoa, bull, motility, mitochondrial activity, reactive oxygen species

MATERIAL AND METHODS

Plant Material

Sambucus nigra berries were obtained from the Botanical Garden at the Slovak University of Agriculture in Nitra. After drying, the plant tissues were crushed, weighed and soaked in ethanol p.a. (96%, Centralchem, Bratislava, Slovak Republic) during two weeks at room temperature in the dark. Exposure to sunlight was avoided to prevent the degradation of active components. The ethanolic plant extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove any residual ethanol (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany). Crude plant extracts were dissolved in DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA) to equal 100.4 mg/mL as a stock solution.

Sample Collection and Processing

Bovine semen samples were obtained from 10 adult Holstein breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The animals were of similar age and were kept under uniform feeding and housing conditions. Two
samples were obtained from each bull on a regular collection schedule with the help of an artificial vagina. Subsequently, sperm concentration and motility was evaluated using phase contrast microscopy (200 x). Only semen samples with a minimum 70% progressive motility were used for the experiments. Each sample was diluted in physiological saline solution (PS; sodium chloride 0.9% w/v; Bieffe Medital, Italia) containing different concentrations of the elderberry extract (1, 5, 10, 50 and 100 μg/mL) using a dilution ratio of 1:40. The samples were cultured at laboratory temperature (22-25°C). The control (Ctrl) group (medium without Sambucus nigra extract supplementation, containing 0.5% DMSO) was compared with the experimental groups.

Spermatozoa Motility Analysis

Spermatozoa motility (%; MOT) was assessed by using the computer-aided sperm analysis (CASa, Version 14.0 TOX IVOS II.; Hamilton-Thomson Biosciences, Beverly, MA, USA). Ten μL of each sample were placed into the Makler counting chamber (depth 10 μm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. Ten microscopic fields were subjected to each analysis in order to include at least 300 cells.

Mitochondrial Activity (MTT Test)

Viability of the cells exposed to Sambucus nigra was evaluated by the metabolic activity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT test. This colorimetric assay measures the conversion of a yellow tetrazolium salt (MTT) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. Formazan can be measured spectrophotometrically.

The MTT tetrazolium salt (Sigma-Aldrich, St. Louis, USA) was dissolved in phosphate-buffered saline (Dubecco's PBS; Sigma-Aldrich) at 5 mg/mL. A 10 μL of the solution was added to the cells (in 100 μL medium per well). After 2 h of incubation (shaker, 37 °C, 95 % air atmosphere, 5% CO2), the cells and the formazan crystals were dissolved in 150 μL of acidic (0.08 M HCl; Centralchem, Bratislava, Slovak Republic) isopropanol (Centralchem). The optical density was determined at a wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Anthos MultiRead 400, Austria). The data were expressed as percentage of the control, set to 100% (Knaizkka et al., 2012).

ROS Generation

ROS levels in samples were assessed by the chemiluminescence assay using luminol (5-amino-2, 3-dihydroxy-4-phthalaldehydeinized; Sigma-Aldrich) as the probe. The test samples consisted of luminol (10 μL, 5 mM) and 400 μL of control or experimental sample. Negative controls were prepared by using 400 μL of Dubecco's PBS (Sigma-Aldrich). Positive control included 400 μL Dubecco's PBS and 50 μL of hydrogen peroxide (30%; 8.8 M; Sigma-Aldrich) in triplicates.

RESULTS AND DISCUSSION

Over the past years, natural compounds isolated from plants have emerged exhibiting a complex biological activity. Due to their broad range of effects, particularly with respect to antibacterial, anti-inflammatory protection and antioxidant mechanisms, plant extracts have attracted a widespread scientific and consumer interest (Putheti and Okigbo, 2011; Tohamy et al., 2012; Hamidpour et al., 2014). The CASA assessment showed a continuous decrease of spermatozoa motility in all groups over the course of a 24h in vitro culture (Table 1). The initial (Time 0h) MOT was higher in the experimental groups supplemented with 100 and 1 μg/mL elderberry extract and lower in other groups with extract when compared to the control group, although without any statistical significance (P>0.05). No significant differences among the control and experimental groups were recorded at Time 2h: the groups supplemented with the extract at 100 and 50 μg/mL the MOT was lower, as at the 10, 5 and 1 μg/mL – higher. A statistically significant motion-activating effect became visible after 6h in the group supplemented with 10 μg/mL of the Sambucus nigra extract whereas in other groups no significant effects of the extract were observed. At the end of the experiment (Time 24h), the motility observed in the experimental groups supplemented with 1-100 μg/mL elderberry extract was higher in comparison with the control, but a significantly highest MOT was established at the 10 μg/mL concentration. Sambucus nigra concentrations ranging between 1-100 μg/mL had an impact of activation on the sperm MOT when compared to the control. Nevertheless a significant influence was shown with respect to the extract at a concentration of 10 μg/mL at all assessment periods (Table 1).

Table 1 Bovine spermatozoa motility (MOT, %) in the absence (Ctrl) or presence of elderberry extract during different time periods (Mean±SEM; n=10)

<table>
<thead>
<tr>
<th>Time</th>
<th>Ctrl</th>
<th>100</th>
<th>50</th>
<th>5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>74.75±7.21</td>
<td>78.25±5.80</td>
<td>69.00±1.91</td>
<td>74.00±13.2</td>
<td>71.00±8.18</td>
</tr>
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<td>2h</td>
<td>63.75±1.88</td>
<td>58.25±1.73</td>
<td>58.00±5.97</td>
<td>68.75±4.87</td>
<td>67.75±1.49</td>
</tr>
<tr>
<td>6h</td>
<td>51.25±2.25</td>
<td>48.75±1.49</td>
<td>50.75±3.80</td>
<td>60.25±2.31*</td>
<td>50.00±5.34</td>
</tr>
<tr>
<td>24h</td>
<td>20.25±6.80</td>
<td>20.50±4.87</td>
<td>24.75±1.23</td>
<td>31.00±1.62*</td>
<td>27.00±2.31</td>
</tr>
</tbody>
</table>

P<0.05; P<0.01; *P<0.001

Progressive motility of control and experimental groups at 0 hours of experimental period, with different concentrations of the elderberry extract, did not exhibit significant differences. At 2h of experimental period the higher concentration of Sambucus nigra extract with 100 and 50 μg/mL decreased spermatozoa progressive motility (PRG, %). After 6 and 24 hours of experimental periods showed, that the progressive motility of the control and experimental samples with the 100, 50 and 1 μg/mL of the elderberry extract was lower, compared to 10 and 5 μg/mL (Table 2).

Table 2 Bovine spermatozoa progressive motility (PRG, %) in the absence (Ctrl) or presence of Sambucus nigra extract during different time periods (Mean±SEM; n=10)

<table>
<thead>
<tr>
<th>Time</th>
<th>Ctrl</th>
<th>100</th>
<th>50</th>
<th>5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>46.75±4.79</td>
<td>44.00±1.87</td>
<td>48.50±3.86</td>
<td>48.00±5.80</td>
<td>48.00±3.10</td>
</tr>
<tr>
<td>2h</td>
<td>45.75±1.76</td>
<td>29.00±5.76*</td>
<td>34.25±3.87</td>
<td>48.00±6.80</td>
<td>46.00±3.43</td>
</tr>
<tr>
<td>6h</td>
<td>23.00±1.46</td>
<td>17.25±1.39*</td>
<td>23.00±1.37</td>
<td>31.50±1.74*</td>
<td>31.50±1.74*</td>
</tr>
<tr>
<td>24h</td>
<td>3.50±0.46</td>
<td>1.75±0.11*</td>
<td>1.25±0.34</td>
<td>6.00±0.33*</td>
<td>6.20±0.87*</td>
</tr>
</tbody>
</table>

P<0.05; P<0.01; *P<0.001

According to the MTT assay, an instant Sambucus nigra supplementation (Time 0 h) had different effects on the sperm mitochondrial activity in any of the experimental groups (Fig. 1). It was established that 100 μg/mL extract had no specific impact on the mitochondrial activity: at 0 and 2 h it was lower and through 6 and 24 h it was almost equal to the control group. Impact of the 50 μg/mL concentration to the sperm was analogical with a higher activation at 2 and 6 h (Fig. 1). A statistically significant increase of mitochondrial activity was observed at all time periods with 10 μg/mL elderberry extract when compared to the control group. When lower concentrations of the elderberry extract were used (5 and 1 μg/mL) the mitochondrial activity was higher than in the control group but lower than in the group supplemented with 10 μg/mL of the plant extract (Fig. 1). In these groups this indicator increased maximally after 24 h (Fig. 1).

Cheminoluminescence was measured on a 48-well plate for 15 min by using the Glomax MultiPlus Combined Spectro-Fluoro-Luminometer (Promega, Madison, WI, USA). The results were expressed as relative light units (RLU)/sec/10^5 sperm (Kashou et al., 2013).

Statistical Analysis

Statistical analysis was carried out by using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. As we focused to study the impact of different elderberry concentrations on the spermatozoa activity (experimental groups) in comparison to the control at a specific time frame, thus taking one factor into consideration, one-way ANOVA was used for specific statistical evaluations. Dunnett test was used as a follow-up test to ANOVA, based on a comparison of every mean to a control mean, and computing a confidence interval for the difference between the two means. The level of significance was set at *** (P<0.001); ** (P<0.01); * (P<0.05).
From these results we may hypothesize that the 10 μg/mL of the elderberry extract have a direct activating effect on the mitochondrial energy metabolism, a crucial factor supporting key spermatozoa motion. Activation of the mitochondrial function can increase sperm motility, and subsequently male fertilizing capacity.

Our investigation revealed that the lowest ROS production was observed in the group with 10 μg/mL extract, where after 2 hours cultivation with extract the ROS production was almost half of the control group (P<0.01). After 6 and 24 hours we could definitely confirm a significant positive antioxidant effect in this group compared with control and other experimental groups (P<0.05 in case of 50, 10 and 5 μg/mL).

**Table 3** Reactive oxygen species production (ROS) production by bovine spermatozoa (RLU/sec/10⁶ sperm) in the absence (Ctrl) or presence of the Sambucus nigra extract in different time periods.

<table>
<thead>
<tr>
<th>Time</th>
<th>Ctrl</th>
<th>100</th>
<th>50</th>
<th>10</th>
<th>5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>0.92±0.03</td>
<td>1.86±0.05</td>
<td>1.49±0.06</td>
<td>0.70±0.03</td>
<td>0.61±0.02</td>
<td>0.81±0.04</td>
</tr>
<tr>
<td>2h</td>
<td>1.49±0.13</td>
<td>2.95±0.44*</td>
<td>2.61±0.38</td>
<td>0.89±0.08*</td>
<td>1.11±0.09</td>
<td>1.26±0.07</td>
</tr>
<tr>
<td>6h</td>
<td>1.77±0.03</td>
<td>3.21±0.11*</td>
<td>3.01±0.07*</td>
<td>1.22±0.17*</td>
<td>1.36±0.14</td>
<td>1.58±0.09</td>
</tr>
<tr>
<td>24h</td>
<td>3.59±0.09</td>
<td>5.13±0.13*</td>
<td>4.33±0.38*</td>
<td>2.26±0.52*</td>
<td>2.33±0.08*</td>
<td>2.42±0.17</td>
</tr>
</tbody>
</table>

P<0.05; *P<0.01; **P<0.001

Our study indicates that the elderberry extract possesses activity enhancing sperm motility promoting properties on bovine spermatozoa at 10 and 5 μg/mL concentration. This study is the first laboratory based experiment. In near future more studies need to be undertaken in a similar direction to prove all the data obtained from this report.

**CONCLUSION**

The results of the present study indicate that the elderberry extracts have a considerable effect on the functional activity of bovine spermatozoa. The findings of the present study clearly indicate that Sambucus nigra extract possesses motility promoting properties on bovine spermatozoa at 10 and 5 μg/mL concentration. However, this study is the first laboratory based experiment. In near future more studies need to be undertaken in a similar direction to prove all the data obtained from this report.

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