**ABSTRACT**

Mistletoe (*Viscum album L.*) was previously described as an effective additive in the therapy of patients with cancer after medical therapy of the primary neoplasia. The defence activity and also direct cytotoxic stimulation was found to be upstanding for the anti-carcinogenic action. The main target of this study was to detect the effect of *Viscum album pini* at various concentration 1.0 (PA); 0.66 (PB); 0.33 (PC); 0.1 (PD); 0.066 (PE) and 0.033 (PF) mg/mL on the fine rabbit spermatozoa motility parameters at various time periods (0; 1; 2 and 3 h) during the cultivation in vitro. The CASA methodology was applied to estimate the spermatozoa motility parameters. The spermatozoa motility at the beginning of the experiment (Time 0) found decreased rate for examined doses of *Viscum album pini* compared to the control, but the statistical significant reduction was observed (p<0.01) only in the sample PD compared to groups PK (control). After 3 hours of in vitro cultivation interesting but not significant tendency of spermatozoa motility increase was found. Only in the group with the highest concentration (PA) a non-significant decrease was detected. Also, other parameters confirm this finding. After 3 hours of incubation, viability of rabbit spermatozoa showed decreased values in all doses of *Viscum album pini* in comparison to the control group. A significant decrease (p<0.01) of membrane integrity was found in group PA compared to control group. Also, in groups PB, PC, PD lower values in comparison to control group were detected. Acrosomal integrity showed very similar tendency in all groups. Our results detected any dose and/or time dependent significant effect of *Viscum album pini* on rabbit spermatozoa motility and viability in vitro. Positive impact on motility parameters after cultivation of rabbit semen with *Viscum album pini* was not found, but indications of changes on membrane integrity were observed.

**Keywords:** *Viscum album pini*, spermatozoa, CASA, motility, in vitro

**INTRODUCTION**

Herbs have been used widely across cultures for centuries. At the beginning, they were collected, later – cultivated. An important part in herb cultivation was played by Benedictine monks, the Knights Hospitallers and the Capuchin order. A rapid development of pharmacology based on active chemical compound synthesis (started with laboratory preparation of phenacetin by Bayer and pyramidon by Hoechst) significantly diminished the importance of phytotherapy. However, in recent years, the number of ethnopharmacological scientific publications has increased; they analyse agents of plant origin used in traditional medicine (Modak et al., 2007; Greiß, 2009; Cravotto et al., 2010; Greiß et al., 2011). Nowadays, medical plants are treated as the source of biologically active compounds, which are often extracted on a commercial scale and used in targeted therapies of many diseases. Research outcomes confirm that antioxidants, able to neutralize free radicals, are effective in preventing the development of experimental diabetes in animal models (Kubisch et al., 1997; Naziroglu and Cay, 2001; Karunakaran and Park, 2013).

*Viscum album L.*, the European mistletoe, is an ordinary kind from the *Viscaceae* family. This usual hemiparastic bush occurs on different trees and included various biologically active substances. Its chemical compound is different depending on the period of gather, species of the host tree and the industrial procedure. Under well-studied and described active phytochemicals identified in *V. album* are lectins and viscotoxins, which perform important part in cancer therapy according to apoptotic and cytotoxic effects. Other parts of compounds occurring in mistletoe are phenolic acids, phenylpropanoids and flavonoids having antioxidant and anti-inflammatory functions. Other mistletoe parts are triterpenes with cytotoxic and apoptotic properties, and phytosterols, oligo- and polysaccharides. Plant extracts, especially aques, are being used in traditional and formal medicine, as well as in therapy of hypertension or arthritis. In theory, they can also be used as a hepatoprotective or sedative medicaments (Naziaruk and Orlikowski, 2016).

Systematic reviews of mistletoe therapy (MT) trials in cancer show promising results in improvement of patients’ quality of life during chemotherapy and reduction of fatigue. However, patients’ experiences of side effects and the acceptability, tolerability, and perceived benefits of MT have not been systematically reviewed. Some patients reported demonstrable changes to their physical, emotional, and psychosocial well-being following MT, as well as a reduction in chemotherapy side effects (Evans et al., 2016). The mistletoe (*Viscum album L.*) extract (Iscador) was previously reported as a useful supplementary medication in the therapy of cancer subjects after surgical excision of the primary tumour. This enhanced survival and recovery from injury caused by radiation or cytostatic treatment, as well as the quality of life. In animal experiments, exact anti-carcinogenic action was described (Maldacker, 2006; Gardin, 2009).

Since decades, *Viscum album* preparations have been used in Europe in oncology. They show multi-faceted anti-tumor in vitro activities, which include inhibition of tumour cell proliferation, induction of apoptosis, inhibition of angiogenesis, modulation of immune competence and gene signature expression. Recently, it was demonstrated in vitro that *Viscum album* exerts an anti-inflammatory effect, mostly directed to chronic inflammation by selectively inhibiting cytokine-induced expression of cyclooxygenase-2 (Seibert et al., 1989; Bussing, 2006; Bussing et al., 2008; Hegde et al., 2011; Hajto et al.,
2011). It has been stated that anticaner drugs could cause harmful effects on the spermatozoa quality and spermatogenic cell arrangement of male.

Rabbits were chosen as the experimental animal in this research for their well-defined reproductive systems (Paal et al., 2014; Rafay and Parkányi, 2016). Some herbal extracts have been proven to have effects on male infertility, for example, gossypol, papaya seed and neem oil and neem seed (Mosher and Pratt, 1991; Dehghan et al., 2005; Roychoudhury et al., 2009). For the in vitro effects of Viscum album on rabbit/animal spermatozoa in order to assess possible beneficial and/or toxic effects of this compound are completely missing (Figure 1).

**Figure 1** The occurrence/number (Y-axis) of articles related to Iscador and Viscum album related to health, reproduction and spermatozoa in PubMed (09/2018).

The target of our *in vitro* experiment was to describe the effect of different doses of *Viscum album* pini during several time intervals (0 - 3 h) on the fine parameters of rabbit spermatozoa motility and viability.

**MATERIAL AND METHODS**

**Animals, semen samples and *in vitro* culture**

Male breeding rabbits (n=10; New Zealand White) kept under standard conditions at the experimental breeding and production centre (Animal Production Research Centre Nitra, Slovak Republic) were selected on the basis of age normally associated with reproduction (12–14 months). Rabbits were kept in a partially air-conditioned area under a photoperiod 16L:8D (minimum light intensity of 80 lux). Animals were housed in single cages, fed with a usual diet and were the water was available ad libitum. The air temperature was 20±2°C and relative humidity 70±5%. Conditions of their care, manipulations and use corresponded to the instruction of EC no. 178/2002 and related EC documents, and were approved by local ethics committee.

Semen samples in five replicates were collected (early in the morning) on a single day with the help of artificial vagina (Kročková et al., 2012; Parkányi et al., 2015). Promptly after collection the single semen showing a clear white colour without contain of gel and artificial particles, were processed to acquire pooled sample. The spermatozoa concentration in semen was 0.40 – 0.63 x 10⁶ per mL. The obtained semen samples were diluted according to routine methods (Chrenek et al., 2007; Roychoudhury and Massányi, 2008).

Later the spermatozoa were incubated in thermostat (37±0.5°C) with various concentrations of *Viscum album* pini (Iscador P; Weleda, Verein für Krebsforschung Institute Hiscia – Arlesheim, Switzerland) dissolved in physiological solution. The control (PK) group used in the study was diluted only with physiological solution. The organization of experiments is listed in Table 1.

**Table 1** Concentrations of *Viscum album* pini used in the study

<table>
<thead>
<tr>
<th>Group</th>
<th>Semen (µL)</th>
<th>Iscador P (µL)</th>
<th>Physiological solution (µL)</th>
<th>Concentration of Iscador P in samples (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK – control</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>300</td>
</tr>
<tr>
<td>SA</td>
<td>100</td>
<td>100</td>
<td>300</td>
<td>Iscador P</td>
</tr>
<tr>
<td>PC</td>
<td>100</td>
<td>200</td>
<td>1 mg</td>
<td>0</td>
</tr>
<tr>
<td>PE</td>
<td>100</td>
<td>300</td>
<td>Iscador P</td>
<td>0</td>
</tr>
<tr>
<td>PE</td>
<td>100</td>
<td>200</td>
<td>0.1 mg</td>
<td>0</td>
</tr>
<tr>
<td>PE</td>
<td>100</td>
<td>100</td>
<td>0.033</td>
<td></td>
</tr>
</tbody>
</table>

Legend: Total motility index (MI) content in Iscador P preparations is 35 mg/mL and viscotoxin 160 µg/mL (Podlich et al., 2012).

**Computer-assisted semen analysis**

Spermatozoa motility was used as an indicator of semen quality. The motility analysis was carried out using a CASA (Computer Assisted Sperm Analyzer) system—Sperm Vision™ program (MiniTub, Tiefenbach, Germany) with the Olympus BX 51 microscope (Olympus, Tokyo, Japan) at cultivation times 0, 1, 2 and 3 hours (Time 0 – 3). Each sample was placed into the Makler Counting Chamber (depth 10 µm, Sefi-Medical Instruments, Haifa, Israel (Massányi et al., 2008). This study was performed in five replicates at each concentration. At least 1000 spermatozoa were analyzed in each sample (Lukáč et al., 2013). For the analysis a specific set up for rabbit semen was used and the selected parameters were analysed – total motility (MT; %), progressive motility (PRO; %), distance average path (DAP; µm), distance curved line (DCL; µm), distance straight line (DSL; µm), average path velocity (VAP; µm/s), velocity curved line (VCL; µm/s), straightness (STR), linearity (LIN), wobble (WOB), amplitude of lateral head displacement (ALH; µm) and beat-cross frequency (BCF; Hz) as described previously (Roychoudhury et al., 2010; Tvrda et al., 2015; Adamkovičová et al., 2016; Paal et al., 2016).

**Viability analysis – MITT test**

Viability of rabbit spermatozoa cultured with *Viscum album pini* was evaluated by the metabolic activity (MTT) assay after 3 hours of culture. This colorimetric assay measures the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Formazan was measured spectrophotometrically by a microplate ELISA reader (Multiskan FC, Thermofisher Scientific, Finland). The data are expressed in percentage of control. Results from the analysis were collected during four repeated experiments for each concentration (Slanina et al., 2016).

**Viability – membrane integrity – Eosin-nigrosin**

The spermatozoa viability was evaluated using eosin-nigrosin staining methods (Slanina et al., 2018). From all the samples smears were prepared after 3 hours of culture. Experimental samples and the control sample were diluted in the ratio 1 : 2 : 2 with 5% eosin (Eosin Y) and 10% nigrosin (Nigrosin) solution (both Sigma-Aldrich). For each slide 300 cells were counted under a light microscope (1000x, Leica DML LED; Leica Microsystems CMS GmbH, Germany) and classified as viable (intact membrane) and dead (damaged membrane). The experiment was realized in six replicates. The results of viability evaluation were expressed as the percentage of viable and dead spermatozoa (in %).

**Acrosomal integrity**

The acrosomal status was assessed after 3 hours of culture following the fast green-rose Bengal staining protocol designed by Pope et al. (1991). This single-step staining method applies a mixture consisting of 1% fast green (Sigma-Aldrich), 1% rose bengal (Sigma-Aldrich) and 40% ethyl alcohol (Centralchem, Bratislava, Slovak Republic) in 0.1 M citric acid – 0.2 M disodium phosphate buffer (Sigma-Aldrich). Twenty microliters of the thawed sample were mixed with 20 µl of the staining solution and incubated for 70 s at room temperature.

Ten microliters of the mixtures were smeared on a tempered glass slide and air-dried at 37°C. Acrosomal integrity was evaluated using bright field microscopy at 1000x using oil immersion. At least 200 cells per slide were evaluated for the presence or absence of acrosome, and expressed as a percentage rate (Tvrda et al., 2017).

**Statistical analysis**

The control group (medium without *Viscum album* pini) was compared to the experimental groups. Statistical analysis was done by the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA). For the analysis the descriptive statistical parameters (mean, standard error) were primary evaluated. One-way ANOVA with Dunnett’s post-test was used for statistical evaluations. The level of significance was estimated at \( *p<0.001\), \( **p<0.01\) and \( *p<0.05\). For individual measurements average value (x), minimum (min) and maximum (max) value, standard deviation (SD) and coefficient of variation (CV) were recorded.

**RESULTS AND DISCUSSION**

**Spermatozoa motility**

The initial spermatozoa motility (Time 0) showed decreased value for all doses of *Viscum album pini* in comparison to the control group, but only significant decrease was observed \( (p<0.01) \) in the sample PD \( (73.04\%\pm8.70\%) \) compared to control \( (87.78\%\pm5.69\%) \). After 1 h of the culture the average spermatozoa motility in control group \( (78.47\%\pm12.51\%) \) was very similar as in all experimental groups \( (73.28 – 83.85\%) \) and no significant differences were found. After 2 hours of *in vitro* cultivation no significant differences were found, but all values in experimental groups were higher compared to control. After 3 hours of *in vitro* cultivation interesting but not significant tendency of spermatozoa motility...
increase was found. Only in the groups with the highest Iscador P concentration (PA) a non-significant decrease was detected (Figure 2).

![Figure 2](image2.png)

**Figure 2** The effect of *Viscum album pini* on the total spermatozoa motility (in %), B - p<0.01.

**Progressive spermatozoa motility**

At the beginning of the experiment (Time 0) the average progressive spermatozoa motility was decreased in all experimental groups compared to the control (76.78±8.15%), but a significant values were detected only in group PD. After 1 hour of culture the progressive spermatozoa motility was in the control group (PK) 67.38±15.53% and no significant differences were observed in experimental groups. After 2 hours of *in vitro* culture the progressive spermatozoa motility was higher in all groups with addition of Iscador P, but the differences were not significant. After 3 hours of culture for groups with lower Iscador P concentration (PD and PE) similar values were found, and in groups with higher Iscador P (PA, PB, PD) non-significant decrease was evident (Figure 3).

![Figure 3](image3.png)

**Figure 3** The effect of *Viscum album pini* on the progressive spermatozoa motility (in %), C – p<0.001.

**Distance parameters**

Spermatozoa distance average path was not significantly affected at Time 0 and the values were in range 23.18 – 29.64 µm (Figure 4). Very slightly lower values were detected after 1 hour of culture as well as after 2 hours of culture. At Time 3 not significant decrease was found in groups with higher *Viscum album pini* concentration (PA: 16.18±6.48 µm; PB: 18.53±4.96 µm; PC: 18.87±5.28 µm; 19.83±6.98 µm) compared to control (21.43±9.43 µm). Similar tendency was found also for distance curved line and distance straight line (Figure 5, 6).

![Figure 4](image4.png)

**Figure 4** The effect of *Viscum album pini* on the spermatozoa distance average path (µm).

![Figure 5](image5.png)

**Figure 5** The effect of *Viscum album pini* on the spermatozoa distance curvilinear line (µm).

![Figure 6](image6.png)

**Figure 6** The effect of *Viscum album pini* on the spermatozoa distance straight line (µm), A – p<0.05.

**Velocity parameters**

At Time 0 the spermatozoa velocity average path was the highest in control group (69.58±3.88 µm/s) and lower values were detected in experimental groups (58.72 – 67.97 µm/s). Significant differences were found in experimental group PB in velocity average path (VAP) as well as in velocity curvilinear line (VCL) at Time 0 (Figure 7, 8). After 1 hour of culture the values were very similar in all groups (59.52 – 68.27 µm/s). At Time 2, interestingly, all values were higher (42.40 – 50.83 µm/s) in *Viscum album pini* groups compared to control (41.13±6.32 µm/s), but the differences were not significant. At the end of the experiment the lowest velocity average path was found in group with the highest *Viscum album pini* concentration (37.77±14.96 µm/s) and difference compared to control was 11.91µm/s (Figure 7). The values of spermatozoa velocity curvilinear and straight line showed very similar tendency (Figure 8, 9).

![Figure 7](image7.png)

**Figure 7** The effect of *Viscum album pini* on the spermatozoa velocity average path (µm/s), A – p<0.05.

![Figure 8](image8.png)

**Figure 8** The effect of *Viscum album pini* on the spermatozoa velocity curvilinear line (µm/s), A – p<0.05.

![Figure 9](image9.png)

**Figure 9** The effect of *Viscum album pini* on the spermatozoa velocity straight line (µm/s).
Figure 9 The effect of *Viscum album pini* on the spermatozoa velocity straight line (µm/s).

**Other fine motility parameters**

For selected motility ratios – spermatozoa straightness and wobble any dose and/or time dependent significant positive or negative effects were found (Figure 10, 12). After one hour of incubation linearity (VSL/VCL) showed significant difference in experimental group PC.

The initial amplitude of lateral head displacement (ALH) was 4.25 - 4.82 µm with the highest value detected in control group (PK). At Time 1 no significant differences were found. After 2 hours of culture the amplitude of lateral head displacement decreased in all groups but was very similar (3.31 – 4.08 µm). At the final stage of the experiment (Time 3) the decrease of ALH in comparison with control (3.95±0.67 µm) was most evident in the group with the highest Icador P concentration (group PA: 3.19±0.80 µm) but the difference was not significant (Figure 13).

Values of beat cross frequency (BCF) completely confirm previous data. At Time 0, 1 and 2 the values showed any significant difference. Also at Time 3 there were no significant differences and the difference between the control group (PK) and the group with the highest *Viscum album pini* was in average only 3.37 Hz (Figure 14).

Figure 10 The effect of *Viscum album pini* on the spermatozoa straightness.

Figure 11 The effect of *Viscum album pini* on the spermatozoa linearity, A – p<0.05.

Figure 12 The effect of *Viscum album pini* on the spermatozoa wobble.

Viability, Membrane integrity, Acrosomal integrity

After 3 hours of incubation, viability of rabbit spermatozoa showed decreased values in all doses of *Viscum album pini* in comparison to the control group. The lowest value (75.69±3.90%) was detected in group PA (Figure 15).

Significant decrease (p<0.01) of membrane integrity (intact) was found in group PA (58.67±9.60%) compared to control group (PK) (81.33±4.96). Also, in groups PB, PC, PD lower values in comparison to control group were detected. Only in groups PE and PF higher membrane integrity (intact, %) than in the control group were found, but without significant evident (Figure 16).

Figure 13 The effect of *Viscum album pini* on the amplitude of spermatozoa lateral head displacement (µm).

Figure 14 The effect of *Viscum album pini* on the spermatozoa beat cross frequency (Hz).

Figure 15 The effect of *Viscum album pini* on the viability (%) of rabbit spermatozoa after 3 hours of incubation.

Figure 16 The effect of *Viscum album pini* on the membrane integrity (%) of rabbit spermatozoa assessed after 3 hours of incubation. The level of significance was set at **P<0.01.
The values of acrosomal integrity showed very similar tendency in all experimental groups. Acrosomal integrity in control group (94.0±1.73%) detected lower value in comparison to experimental groups (PA: 96.7±6.3%; PB: 97.3±2.08%; PC: 96.3±1.15; PD: 96.3±1.33; PE: 96.3±0.58; PF: 97.3±3.53%).

Anticancer preparations made from plants have been an object of scientific interest for many years. It is worth noting that as many as 25% of cytostatics used in the anticancer chemotherapy are obtained from plants (Grèn and Massánü, 2016).

Extracts of the European mistletoe (Viscum album L.) have been widely used for decades as alternative, complementary treatment and adjuvant cancer therapy (Kovacs et al., 2006). In clinical practice mistletoe therapy is often given concomitantly to conventional chemotherapy. Iscador is extracted from mistletoe plants growing on different host trees, like apple, oak, or pine. Cytotoxic gliotoxin is an active component of mistletoe extracts and can stimulate effector cells of the innate and adaptive immune system (Stein et al., 2002; Braedel-Ruoff, S. 2010; Grèn and Fornicki, 2013). Experiments also indicate a statistically significant increase in albunin fraction level and lymphocyte count. Moreover, decrease of the total protein content, protein fractions globulins alpha2, beta, gamma and neutrophil, monocyte count in mouse serum was observed (Grèn, 2009).

Various studies clearly report that the reproductive ability and the semen quality in various animal species is influenced by various environmental causes, age, stress, hormonal status, nutrition as well as toxins (Mangelsdorf et al., 2003; Lukáč et al., 2011; Moussa-Balabel and Mohamed, 2011; Fallas-López et al., 2008). Impaired semen parameters of cancer patients, and mainly depends on various factors – biochemical pathways, enzyme dysfunction, disturbed axonemal protein function, and finally a fall in intracellular ATP levels – can exhibit cytotoxic effects. A relationship between diminished spermatozoa quality and semen motility after treatment may require several years (Kovacs, 2002; Turner, 2006; Storey, 2006).

In vitro data indicates that spermatozoa are a useful part of the biological evaluation of chemicals providing quantitative as well as qualitative data. In conclusion, the present study shows the Viscum album pini, exactly in these concentrations and in vitro conditions have no negative effect on spermatozoa motility and viability characteristics.

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