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In vitro Effects of *Viscum album* on the Functionality of Rabbit Spermatozoa

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***Abstract*—**This study aimed to assess the *in vitro* effects of different concentrations of the *Viscum album* extract on the motility, viability, and reactive oxygen species (ROS) production by rabbit spermatozoa during different time periods (0, 2, and 8h). Spermatozoa motility was assessed by using the CASA (Computer aided sperm analysis) system. Cell viability was evaluated by using the metabolic activity MTT assay, and the luminol-based luminometry was applied to quantify the ROS formation. The CASA analysis revealed that low *Viscum* concentrations were able to prevent a rapid decline of spermatozoa motility, especially in the case of concentrations ranging between 1 and 5 μg/mL ($P<0.05$ with respect to time 8h). At the same time, concentrations ranging between 1 and 100 μg/mL of the extract led to a significant preservation of the cell viability ($P<0.05$ in case of 5, 50 and 100 μg/mL; $P<0.01$ with respect to 1 and 10 μg/mL, time 8h). 1 and 5 μg/mL of the extract exhibited antioxidant characteristics, translated into a significant reduction of the ROS production, particularly notable at time 8h ($P<0.01$). The results indicate that the *Viscum* extract is capable of delaying the damage inflicted to the spermatozoon by the *in vitro* environment.

***Keywords*—**CASA, mistletoe, mitochondrial activity, motility, reactive oxygen species, rabbits, spermatozoa, *Viscum album*.

I. Introduction

OVER the last decade, diverse studies have reported about the beneficial effects of oral supplementation of natural substances isolated from plants on semen quality and male fertility in animals and humans [1], [2]. Nevertheless, knowledge concerned with the *in vitro* effects of potentially stimulating or protective molecules on the sperm cell is still substantially limited or controversial. Meanwhile, *in vitro* data are crucial for further progress in practical andrology, as it has been repeatedly shown that numerous biologically active compounds may protect male gametes against the loss of functionality. Subsequently, this information may be viable for semen handling protocols for long-term semen preservation or artificial insemination.

The *in vitro* environment represents a hazard to the sperm survival, as it provides suitable conditions for ROS overgeneration and a subsequent structural or functional damage to the cell [3]. Administration of synthetic antioxidants to extenders represents a traditional way to prevent structural or functional alterations to spermatozoa. Nevertheless, the safety of synthetic additives has been questioned leading to the renaissance of naturally occurring substances with numerous beneficial properties. The chemical diversity, structural complexity, availability, or lack of substantial toxic effects of natural products convert them into ideal candidates for new research strategies [4].

Viscum album, commonly known as mistletoe, belongs to the family Santalaceae, and is originally native to Europe, North Western, and Southern Africa [5]. It is a semi-parasitic evergreen growing on host trees, depending on the host plant for minerals and water. Its green leathery, oblong leaves [6] are rich in a diversity of chemical constituents including caffeic acid, alkaloids, amines, phenols, flavonoids, terpenoids and viscotoxins, lecitins, triterpenes, saponins, vitamin C, histamine, resins, thionins, and cardenolides [7]. Mistletoe has been commonly used in traditional medicine as a treatment for menopausial symptoms, infertility, cancer, nervous tension, asthma, hypertension, headache, diabetes, and dermatitis [5], [7], [8].

Based on this body of evidence, this *in vitro* study was aimed to assess the efficacy of the *Viscum album* extract on rabbit spermatozoa motility, viability and ROS production during an 8-hour *in vitro* cultivation, in order to provide information on its behavior in the male reproductive cell, as well as to define optimal concentrations of this extract for further experiments in veterinary andrology.

II. Material and Methods

A. Plant Material

Leaves from *Viscum album* 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) of 100.4 mg/mL as a stock solution [9].

B. Sample Collection and Processing

Ten male rabbits (New Zealand white broiler line; approx. four months of age; 4.0±0.2 kg of weight) used in the

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experiment were obtained from the experimental farm of the Animal Production Research Centre Nitra (APRS Nitra), Slovak Republic. The animals were housed in individual cages in a partially air-conditioned rabbit house under a photoperiod of 16L:8D (minimum light intensity of 80 lux), air temperature of 20-24 °C and relative humidity of 65%. The rabbits were fed with a commercial diet, and water was provided ad libidum.

Semen samples were collected on a single day (early in the morning) using an artificial vagina. Immediately after collection, each sample was diluted in physiological saline solution (PS) (sodium chloride 0.9% w/v, Bieffe Medical, Italia) supplemented with 0 (Control group), 1, 5, 10, 50, 100, and 500 µg/mL Viscum extract. The samples were cultured at 37 °C. At cultivation times of 0, 2 and 8 h, further tests were performed.

C. Spermatozoa Motility Analysis

Spermatozoa motility (%; MOT) was assessed by using the computer-aided sperm analysis (CASA, Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). Ten µL of each sample were placed into the Makler counting chamber (depth 10µm, 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.

D. Mitochondrial Activity (MTT Test)

Viability of the cells exposed to Viscum 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) was dissolved in phosphate-buffered saline (Dulbecco's PBS; Sigma-Aldrich) at 5 mg/mL. 10 µL of the solution was added to the cells (in 100 µL medium per well). After 2h of incubation (shaker, 37 °C, 95 % air atmosphere, 5% CO_2), the cells and the formazan crystals were dissolved in 150 µL of acidified (0.08 M HCl; Centralchem) 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% [10].

E. ROS Generation

ROS levels in samples were assessed by the chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma-Aldrich) as the probe [11]. 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 Dulbecco's PBS (Sigma-Aldrich). Positive control included 400 µL Dulbecco's PBS and 50 µL of hydrogen peroxide (30%; 8.8 M; Sigma-Aldrich) in triplicates. Chemiluminescence was measured on a 48-well plate for 15 min by using the Glomax Multi$^+$ Combined Spectro-Fluoro-Luminometer (Promega, Madison, WI, USA). The results were expressed as relative light units (RLU)/sec/10^6 sperm [11], [12].

F. 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 Viscum 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 *** ($P<0.001$); ** ($P<0.01$); * ($P<0.05$).

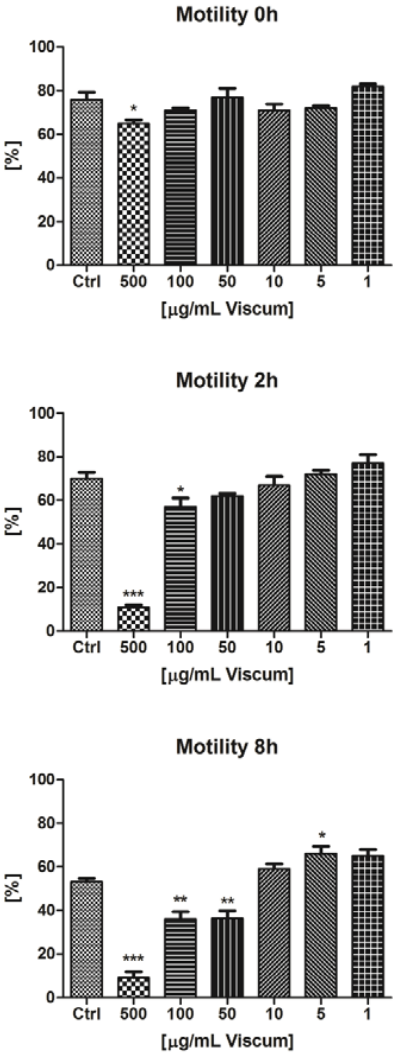
III. Results and Discussion

Recently, natural plant extracts have attracted the scientific attention due to their vast range of antimicrobial, anti-cancer, anti-inflammatory or antioxidant properties [4], [7], [9], [12], [13].

Different in *vitro* studies have reported that *Viscum* extracts are well absorbed and tolerated, and no distinct toxicity was reported [14]–[16]. On the other hand, *in vivo* studies on the impact of mistletoe extracts on male reproduction suggest potentially toxic effects of *Viscum* on male reproductive function [17]. Due to the existing controversy on the exact *in vitro* behavior of *Viscum* on male gametes, we focused on the *in vitro* impact of *Viscum* extracts on the structural and functional competence of rabbit spermatozoa.

The CASA assessment showed a continuous decrease of spermatozoa motility in all groups over the course of the *in vitro* culture (Fig. 1). The initial (time 0h) as well as short-term (time 2h) MOT was higher in the experimental groups supplemented particularly with low *Viscum* extract concentrations (1 and 5 µg/mL) when compared to the control group, although without any statistical significance ($P>0.05$). Moreover, 500 µg/mL *Viscum* extract caused an instant significant decrease of the spermatozoa motility ($P<0.05$). After 2h, the decline of spermatozoa MOT became more significant when administering 100 and 500 µg/mL *Viscum*. At the end of the experiment (time 8h), the MOT was significantly decreased in relation to a concentration range of 50-500 µg/mL *Viscum* extract.

According to the MTT assay, instant *Viscum* supplementation (time 0h and 2h) had no significant effects on the sperm cell viability in most of the experimental groups, with the exception of the highest concentration of the plant extract used (500 µg/mL; $P>0.05$; Fig. 2).

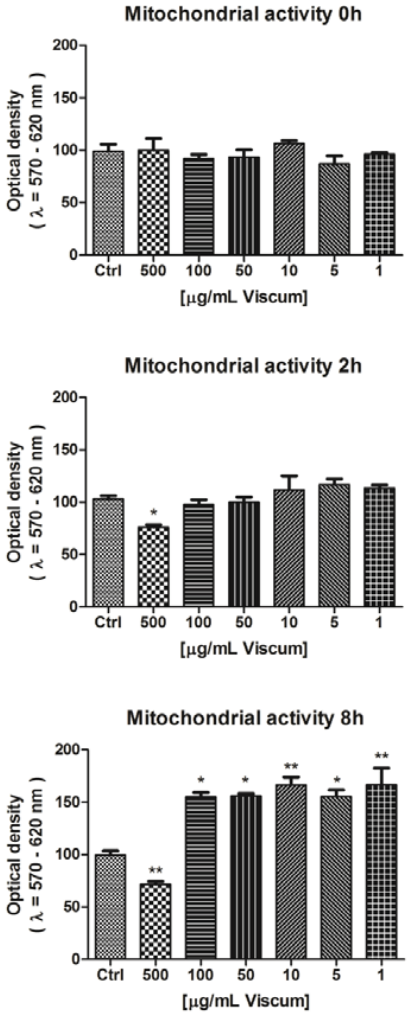


Mean±SEM; *** (P<0.001); ** (P<0.01); * (P<0.05)

Fig. 1 Spermatozoa motility (%) in the absence (Ctrl) or presence of the *Viscum album* extract in different time periods

At 8h, it was revealed that 1-100 µg/mL extract had a viability promoting effect on rabbit spermatozoa, alongside with statistically significant results when compared to the control group (P<0.05 in case of 5, 50 and 100 µg/mL; P<0.01 with respect to 1 and 10 µg/mL). Similar to the CASA analysis, the MTT test revealed an inhibition of the cell viability followed by the administration of 500 µg/mL *Viscum* extract, particularly at times 2h and 8h (P<0.05 with respect to time 2h; P<0.01 with regards to time 8h).

It has been previously stated that *Viscum album* contains a variety of flavonoids, such as isoflavones, flavanones, flavonols and dihydrochalcon [7], all of which have been extensively studied for their potential roles on spermatogenesis or *in vitro* sperm survival. Improved spermatozoa motility and mitochondrial activity after flavonoid administration were recorded in different studies on fresh as well as frozen goat, mouse and human semen [18]–[20].



Mean±SEM; *** (P<0.001); ** (P<0.01); * (P<0.05)

Fig. 2 Spermatozoa mitochondrial activity (%) in the absence (Ctrl) or presence of the *Viscum album* extract in different time periods. Each bar represents mean (±SEM) optical density as the percentage of controls, which symbolize 100%. The data were obtained from five independent experiments

Ofem et al. [21] revealed that modest and restricted doses of *Viscum album* extract at 150 mg/kg, 300 mg/kg, and 450 mg/kg, respectively, increased the serum levels of testosterone, LH and FSH but decreased prolactin concentrations in rats. On the other hand, Ojezele et al. [17] investigated the effects of aqueous extracts of *Viscum album* obtained from three host plants (cocoa, kola, and coffee) on the semen parameters of Wistar rats. The experimental groups received 400, 800, 1600, or 3200 mg/kg doses of the extract daily for 24 days. The study revealed that the administration of all selected doses of the *Viscum album* extract caused a significant dose-dependent decrease in the sperm count, motility, morphology, concentration and viability, although the semen volume was not significantly altered. This report

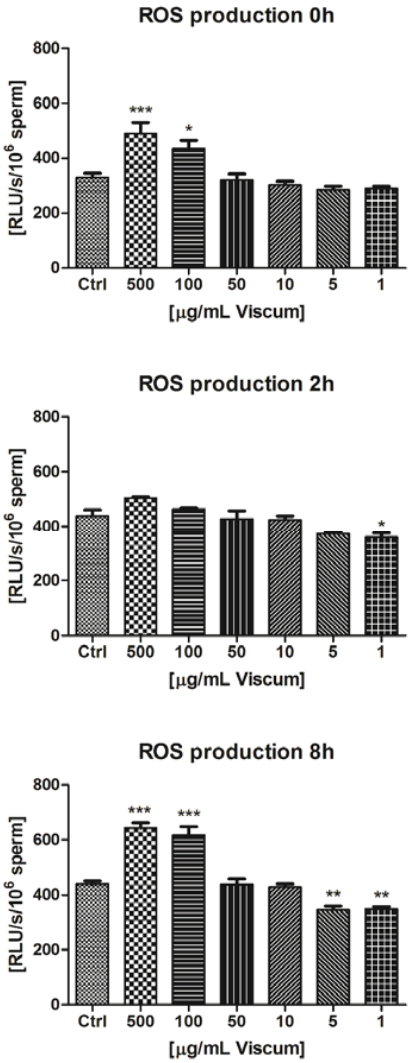
suggested that *Viscum album* could have the ability to negatively affect all essential semen parameters.

The luminometric assay revealed that high concentrations (100 and 500 µg/mL) of the *Viscum* extract exhibited pro-oxidant properties reflected in a significant ROS overgeneration, staring at time 0 ($P<0.001$ in case of 500 µg/mL; $P<0.05$ with respect to 500 µg/mL) and deepening the detrimental effects in a time-dependent manner ($P<0.001$ with respect to time 8h; Fig. 3). Inversely, experiments following 8h of cultivation revealed that the administration of 1 and 5 µg/mL extract led to a significant decline of the ROS formation in comparison to the control ($P<0.01$). *Viscum* extract concentrations ranging from 1 to 5 µg/mL exhibited a statistically significant antioxidant protection of spermatozoa and a subsequent prevention of the escalating ROS production, considered to be the essential pillar of oxidative stress.

Numerous reports have emphasized on the fact that plant extracts possess significant antioxidant activities [22], [23]. The antioxidant ability could be attributed to the exceptionally high content of phenolic compounds, particularly flavonoids with potent ROS-scavenging activities [23]. Thus, *Viscum* extracts could be a promising natural source of antioxidants, possibly used in nutritional or pharmaceutical industry for the prevention of ROS-mediated diseases.

The mistletoe cytotoxicity has been reported to be based mainly on the toxic polypeptides with low molecular mass known as viscotoxins, which have been shown to be very efficiently in inducing apoptosis in cancer cells [24]. Oxidative stress plays an essential role in the loss of mitochondrial membrane potential induced by *Viscum album* viscotoxins. A possible mechanism for ROS generation by *Viscum* is the activation of c-Jun NH2-terminal kinase - a downstream ROS target [25]. Inversely, *Viscum album* extracts have been shown to act differently on normal cells. In non-malignant cell line LLC-PK1, the mistletoe lectins down regulated the synthesis of numerous ROS, prevented lipid peroxidation, and improved the cell viability [26].

Previous studies on male reproduction performance have shown that biologically active compounds frequently found in *Viscum album* were able to significantly decrease lipid peroxidation, restore glutathione synthesis and catalase activity, associated with normal spermatogenesis and sperm viability [27]. In a different study [28], polyphenol administration led to significantly increased total antioxidant capacity, superoxide dismutase levels, as well as sperm percentage, viability, motility, accompanied by a decrease of malondialdehyde in rats, hence suggesting that flavonoids could be effective in enhancing healthy semen parameters.



Mean±SEM; *** ($P<0.001$); ** ($P<0.01$); * ($P<0.05$).

Fig. 3 ROS formation by rabbit spermatozoa (RLU/s/10^6 sperm) in the absence (Ctrl) or presence of the *Viscum album* extract in different time periods

IV. Conclusions

Our results, although preliminary, provide evidence for the dose-dependent *in vitro* biological activity and scavenger activity of the *Viscum album* extract against oxidative tension induced in rabbit spermatozoa. The design of semen extenders offering a better protection to male gametes from oxidative damage and improve their energy requirements is more than crucial. Mistletoe extracts, in small amounts, could be used as a ROS scavenging and a metabolic promoting supplement, especially in common andrology protocols including *in vitro* fertilization, artificial insemination or spermatozoa cryopreservation.

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