Time and Dose-Dependent Effects of Viscum Album Quercus on Rabbit Spermatozoa Motility and Viability *in Vitro*

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Summary

The target of this study was to evaluate the effect of extract of the European mistletoe – *Viscum album quercus* L. on spermatozoa motility and viability *in vitro*. The CASA system was used to determine the spermatozoa motility parameters at different time intervals (0, 1, 2 and 3 h) and spermatozoa viability was determined in five different doses of *Viscum album quercus* L [10 (QA), 6.6 (QB), 3.3 (QC), 2.5 (QD) and 2 (QE) mg/ml]. Results in experimental groups detected a significant deterioration on rabbit spermatozoa after 1, 2 and 3 hours, compared to the control. The initial total spermatozoa motility showed increased value for all doses of *Viscum album quercus* in comparison to control. After *in vitro* culture a dose–dependent decrease (QA: reduction of 69.7 %, QB: reduction of 40.9 %) was found. For the progressive spermatozoa most significant decrease (86.8 % for QA vs. 48.5 % for QB) was detected compared to the control after 3 hours of culture. Spermatozoa viability (MTT test) was decreased in all experiment groups at the end of experiment, but the differences were not significant. Significant alterations of membrane integrity were found in groups with the highest *Viscum album quercus* concentration (QA, QB), but acrosome integrity showed no significant changes. Results suggest negative dose– and time–dependent effect of *Viscum album quercus* at higher doses on spermatozoa motility and viability parameters *in vitro*.

Key words

Viscum album • Spermatozoa • CASA • Viability • *in vitro*

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Introduction

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.

Extracts of the European mistletoe (*Viscum album* L.) have been widely used for decades as alternative, complementary treatment (Kovacs *et al*. 2006, Felenda *et al*. 2019, Suveren *et al*. 2017). In clinical practice mistletoe therapy is often given concomitantly to conventional chemotherapy. Mistletoe plants are generally growing on different host trees, like apple, oak, or pine. Cytotoxic glycoproteins, the mistletoe lectins, are active component of mistletoe extracts and can stimulate effector cells of the innate and adaptive immune system (Stein *et al.* 2002, Braedel-Ruoff 2010, Gren and Formicki 2013, Gren and Massanyi 2016).

Experiments also indicate a statistically significant increase in albumin fraction level and lymphocyte count. Moreover, decrease of the total protein

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content, protein fractions globulins alpha2, beta, gamma and neutrophil, monocyte count in mouse serum was observed (Gren 2009).

The reproductive ability and the semen quality of animal species can be affected by many environmental sources, as well as age, stress, hormonal status, nutrition and toxins (Mangelsdorf *et al*. 2003, Lukac *et al*. 2011, Mousa-Balabel and Mohamed 2011, Fallas-López *et al*. 2011, Tirpak *et al*. 2017, Saha *et al*. 2019). In the many years, following the increased success rate of cancer treatments, great efforts have been made to improve quality of life in survivors, including fertility preservation in young patients (Masopotova *et al.* 2018). Because of their gonadotoxic effects, chemo– and radiotherapy can temporarily or permanently compromise fertility (Di Bisceglie *et al*. 2013). Oncological treatments present severe gonadotoxic effects on both germ and Leydig cells. Of note, in a significant percentage of patients (20-50 %) spermatogenesis is impaired even before cancer treatments, probably due to the malignancy itself. The recovery of normal spermatogenesis after treatment may require several years, and mainly depends on various factors – initial spermatozoa count, type and dose of specific oncological treatments and patient age. Disturbance of homeostasis of reproductive system in some diseases can be seen from the decline in physical and chemical parameters of spermatozoa, such as pH, semen volume, concentration, motility, and the percentage of spermatozoa viability. These data justify the increasing efforts in identifying prevention and treatment strategies to preserve reproductive functions in young men with malignancies (Colpi *et al*. 2004, Maltaris *et al*. 2006, Vitku *et al.* 2015, Heráček *et al.* 2018). The mechanisms underlying the male infertility of *Viscum album* extracts have not been investigated.

The objective of this *in vitro* study was to determine the effect of various concentrations of *Viscum album quercus* during various time periods (0-3 h) on the selected parameters of rabbit spermatozoa motility and viability.

Methods

Animals, semen samples and in vitro culture

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

Semen samples (n=5) in five replicates were collected on a single day (early in the morning) with the help of artificial vagina (Krockova *et al*. 2012, Parkanyi *et al*. 2015). Immediately after collection the individual doses of semen exhibiting a white color without presence of any gel and artificial particles, were mixed together to obtain pooled sample. The spermatozoa concentration in semen was 0.40-0.63 × 10^9 per ml. The obtained semen samples were diluted according to routine methods (Chrenek *et al*. 2007, Roychoudhury and Massanyi 2008). Later the spermatozoa were incubated in thermostat (37±0.5 °C) with various concentrations of *Viscum album*

Table 1. Concentrations of *Viscum album quercus* used in the study.

Group	Semen (µl)	Iscador Qu 10 mg (µl)	Physiological solution (µl)	Concentration of Iscador Qu in samples (mg/ml)
QK – control	100	0	300	0
QA	100	300	0	10
QB	100	200	100	6.6
QC	100	100	200	3.3
QD	100	100	300	2.5
QE	100	100	400	2

quercus (Iscador Qu 10 mg, Weleda, Verein für Krebsforschung Institute Hiscia - Arlesheim, Switzer-land) dissolved in physiological solution. Concentrations of the Iscador Qu were based on manufacturer´s values and later diluted with physiological solution to reach decreased experimental concentration (6.6-2.0 mg). The control (QK) group was cultured only with physiological solution. The scheme of experiments is presented in Table 1.

Computer-assisted semen analysis

Spermatozoa motility was used as an indicator of cell quality. The motility analysis was carried out using a CASA (Computer Assisted Semen Analysis) system – Sperm Vision TM 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 (Massanyi *et al*. 2008). This study was performed in five replicates at each concentration. At least 1000 spermatozoa were analyzed in each sample (Lukac *et al*. 2013, Halo *et al*. 2018). Using the rabbit specific set up the following parameters have been evaluated – total motility (MOT, %), 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), velocity straight line (VSL, 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 Adamkovicova *et al*. 2016).

Viability analysis – MTT test

Viability of rabbit spermatozoa cultured with *Viscum album quercus* was evaluated by the metabolic activity (MTT) assay after 3 hours of culture. This colorimetric assay measures the conversion of 3-(4.5-dimetylthiazol-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, St. Louis, USA). For each slide 300 cells were counted under a light microscope (1000×, Leica DMIL 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, St. Louis, USA), 1 % rose bengal (Sigma-Aldrich, St. Louis, USA) and 40 % ethyl alcohol (Centralchem, Bratislava, Slovak Republic) in 0.1 M citric acid – 0.2 M disodium phosphate buffer (Sigma-Aldrich, St. Louis, USA). Twenty microliters of the sample were mixed with 20 μl of the staining solution and incubated for 70 s at room temperature. Ten microliters of the mixture were smeared on a tempered glass slide and air-dried at 37 °C. Acrosomal integrity was evaluated using bright field microscopy at 1000× 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 quercus*) was compared to the experimental groups. Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows, GraphPad Software, La Jolla California USA). Descriptive statistical characteristics (mean, standard deviation) were evaluated at first. One-way ANOVA with Dunnett's post–test was used for statistical evaluations. The level of significance was set 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

Spermatozoa motility

The initial spermatozoa motility (Time 0) showed increased value for all doses of *Viscum album quercus* in comparison to the control group. Statistically significant increase was observed ($p<0.05$) in the sample QA (77.23±8.64 %), and also ($p<0.001$) for QB (81.17±8.08 %) and QC (80.87±8.83 %). After 1 h of the culture the average spermatozoa motility in control group was 72.17±11.94 %. At the same time a statistically significant ($p<0.001$) decrease (23.8 %) was observed in group QA (54.98±15.53 %). After 2 hours of *in vitro* cultivation significantly decreased ($p<0.001$ and 0.01) total spermatozoa motility in comparison to the control group was observed in groups QA and QB (doses 10 and 6.6 mg/ml). Also, after 3 hours of *in vitro* cultivation a dose-dependent decrease (QA: 19.39±7.7 %, reduction of 69.7 %, QB: 37.84±18.29 %, reduction of 40.9 %) was found (Fig. 1).

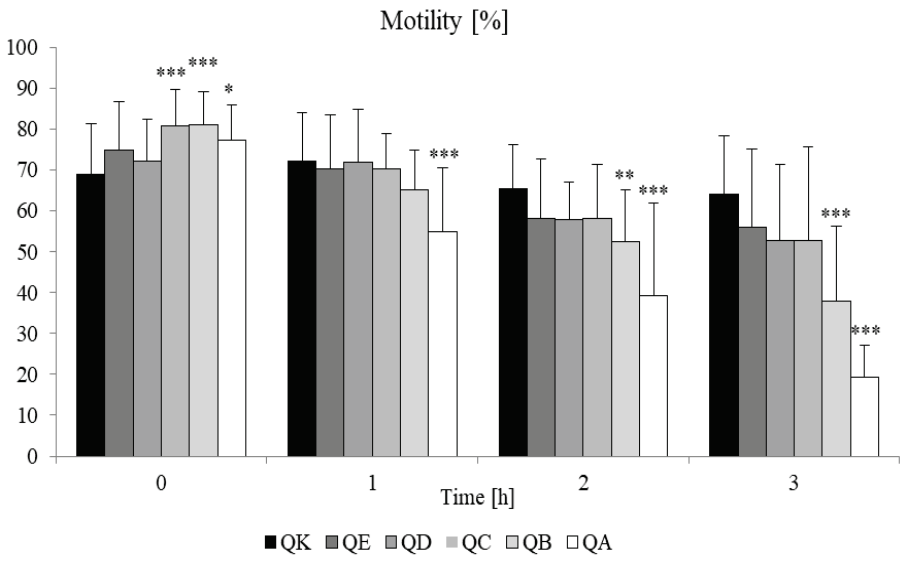

Fig. 1. The effect of *Viscum album quercus* on the total spermatozoa motility (in %).
QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu. The level of significance was set at ***($p<0.001$), **($p<0.01$) and *($p<0.05$).

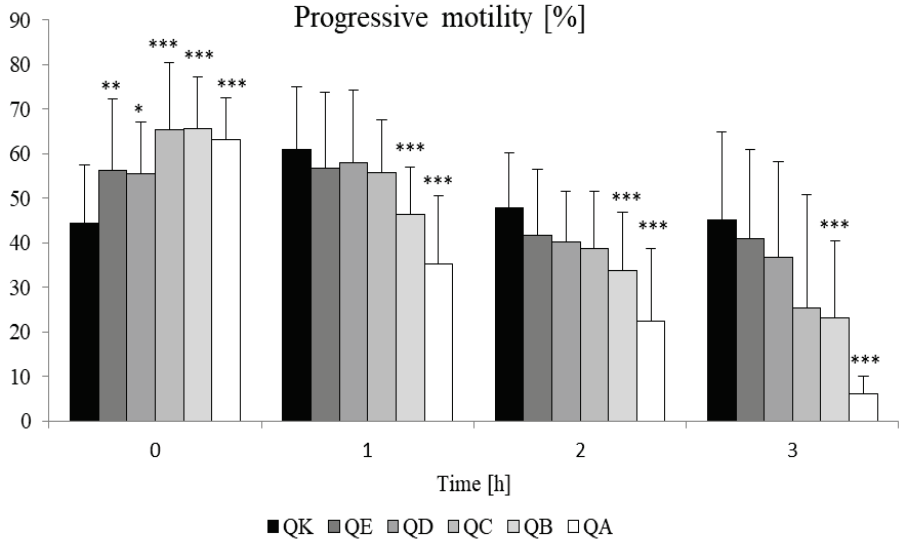

Fig. 2. The effect of *Viscum album quercus* on the progressive spermatozoa motility (in %).
QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu. The level of significance was set at ***($p<0.001$), **($p<0.01$) and *($p<0.05$).

Progressive spermatozoa motility

At Time 0 the average progressive spermatozoa motility was higher in all experimental groups with *Viscum album quercus* in comparison with the control (44.67±13.03 %). These changes were statistically significant. After 1 hour of culture the progressive spermatozoa motility was the highest in the control group QK (60.97±13.98 %) and a statistically significant decrease was detected in groups QA and QB. This decrease was compared to control 42.4 % for QA and

24.2 % for group QB. After 2 hours of *in vitro* culture, the progressive spermatozoa motility was significantly reduced in groups QA and QB with a decrease up to 53 % in QA (22.43±16.34 %) and 29.5 % in group QB (33.63±13.31 %). Even more dramatic reduction (86.8 % for QA vs. 48.5 % for QB) was detected compared to the control (45.12±19.61 %) after 3 hours of culture (Fig. 2).

Distance parameters

Spermatozoa distance path parameters confirm the negative dose and time-dependent effect of *Viscum album quercus*. Spermatozoa distance average path significantly decreased at Time 1 in experimental groups QA, QB and QC. Later (Time 2) a significant decrease was detected in almost all experimental groups (except the lowest concentration) and at Time 3 only in groups with the highest *Viscum album quercus* concentrations (Table 2).

Spermatozoa distance curvilinear line showed similar trends as spermatozoa distance path with various significant decrease at all time periods in experimental groups compared to control (Table 3).

Table 2. Spermatozoa distance average path (DAP, μm) in experimental groups and time periods.

Group	Mean	S.D.	C.V.	Minimum	Maximum
Time 0					
QK	22.90	4.43	19.36	16.02	32.75
QA	24.56	3.79	15.43	16.82	31.60
QB	25.69	3.92	15.25	18.35	32.61
QC	25.41	5.21	20.52	11.97	33.70
QD	26.14	5.76	22.02	14.05	38.53
QE	22.97	3.99	17.37	16.40	31.49
Time 1					
QK	28.91	3.69	12.75	21.19	36.69
QA	18.05***	2.22	12.30	14.14	22.83
QB	20.24***	2.25	11.14	16.41	26.01
QC	22.92***	2.38	10.38	18.70	27.44
QD	26.23	2.42	9.21	21.15	30.61
QE	26.49	3.44	12.99	21.54	32.76
Time 2					
QK	22.51	3.34	14.84	15.29	31.10
QA	13.27***	3.36	25.34	0.00	16.81
QB	17.26***	2.41	13.97	12.68	21.90
QC	17.45***	1.88	10.80	13.81	21.56
QD	19.02**	2.72	14.30	14.45	27.30
QE	20.55	2.80	13.60	13.64	26.45
Time 3					
QK	19.54	4.31	22.06	11.80	27.42
QA	9.40***	3.99	42.45	0.00	16.90
QB	14.17***	4.06	28.65	0.00	22.14
QC	16.70	4.42	26.48	8.95	22.17
QD	18.56	4.40	23.72	11.09	27.61
QE	18.44	3.18	17.25	12.06	22.85

Legend: S.D. – standard deviation, C.V. – coefficient of variation, *** ($p<0.001$), ** ($p<0.01$) (experimental group vs. control). QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu.

Spermatozoa distance straight line significantly decreased at all time periods in groups QA - QC. Only at Time 2 group QD showed significant decrease (Table 4).

Velocity parameters

Also the spermatozoa velocity parameters were affected by the addition of *Viscum album quercus* (Iscador Qu) to the culture medium. The spermatozoa velocity curved line at all time periods in control group was 97.58-131.47 μm/s. After a significant increase at Time 0 (groups QB, QD) a signifacnt decrease was found in all experient groups. Later the most significant decrese was detected in group QA compared to control (131.47±16.38 vs. 93.25±10.19 at Time 1, 108.93±17.72 vs. 70.77±19.69 at Time 2 and 97.58±21.79 vs. 47.86±21.66 at Time 3). Also in group QB a significant decrease was detected after 1, 2 and 3 hours of culture (Fig. 3).

Table 3. Spermatozoa distance curvilinear line (DCL, μm) in experimental groups and time periods.

Group	Mean	S.D.	C.V.	Minimum	Maximum
Time 0					
QK	47.84	7.80	16.30	35.09	67.59
QA	52.25	6.81	13.03	36.55	65.73
QB	52.76	6.52	12.35	38.31	65.55
QC	50.01	7.19	14.37	34.42	63.38
QD	56.03**	14.35	25.61	35.85	92.81
QE	47.66	6.48	13.59	33.89	62.09
Time 1					
QK	56.01	7.39	13.19	44.27	77.16
QA	41.19***	4.74	11.52	33.71	53.96
QB	43.79***	4.70	10.74	36.12	53.41
QC	45.98***	4.07	8.86	40.28	54.68
QD	52.54	5.33	10.14	40.52	61.28
QE	53.61	7.27	13.56	41.29	66.05
Time 2					
QK	46.87	7.61	16.24	33.81	68.31
QA	31.38***	9.01	28.71	0.00	44.65
QB	40.40***	4.93	12.21	31.29	50.00
QC	38.71***	5.07	13.09	29.61	50.94
QD	41.62*	5.56	13.35	28.80	59.57
QE	45.42	5.46	12.03	31.92	58.97
Time 3					
QK	42.31	9.20	21.76	27.39	60.82
QA	20.69***	9.53	46.07	0.00	37.80
QB	34.22**	10.26	29.97	0.00	52.86
QC	37.83	9.40	24.85	21.49	49.89
QD	43.07	8.81	20.45	25.50	58.38
QE	41.58	7.61	18.31	25.81	54.31

Legend: S.D. – standard deviation, C.V. – coefficient of variation, *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$) (experimental group vs. control). QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu.

Table 4. Spermatozoa distance straight line (DSL, μm) in experimental groups and time periods.

Group	Mean	S.D.	C.V.	Minimum	Maximum
Time 0					
QK	17.63	3.87	21.97	10.02	26.30
QA	16.62	3.16	19.00	12.07	23.31
QB	18.15	3.57	15.25	11.38	24.51
QC	19.18	4.75	20.52	8.00	26.47
QD	17.84	3.59	20.11	9.68	24.21
QE	16.91	3.24	19.17	11.06	23.93
Time 1					
QK	22.28	3.37	15.13	16.09	29.73
QA	12.84***	1.52	11.88	9.67	16.74
QB	15.33***	1.81	11.78	12.13	19.33
QC	17.72**	1.94	10.92	13.87	22.38
QD	19.85	1.74	8.75	15.93	23.06
QE	19.88	2.18	10.95	16.33	23.88
Time 2					
QK	17.38	2.66	15.29	12.61	24.09
QA	9.60***	2.48	25.82	0.00	13.09
QB	12.73***	2.04	16.05	8.87	17.83
QC	13.53***	1.57	11.62	10.57	17.16
QD	14.82**	2.58	17.40	11.06	22.86
QE	15.58	1.95	12.52	10.86	19.14
Time 3					
QK	14.86	3.12	20.97	8.69	20.65
QA	6.72***	2.81	41.89	0.00	10.76
QB	10.08***	2.75	27.26	0.00	15.48
QC	12.12**	3.16	26.10	5.58	16.52
QD	13.31	3.37	25.33	7.81	22.37
QE	13.60	2.12	15.59	9.66	17.87

Legend: S.D. – standard deviation, C.V. – coefficient of variation, *** ($p<0.001$), ** ($p<0.01$) (experimental group vs. control). QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu.

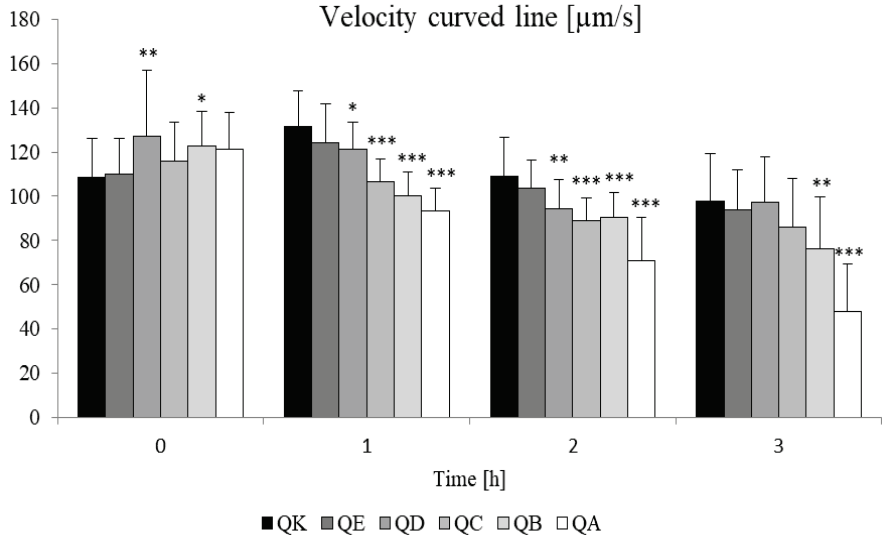

Fig. 3. The effect of Viscum album quercus on the spermatozoa velocity curved line (μm/s).
QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu. The level of significance was set at ***($p<0.001$), **($p<0.01$) and *($p<0.05$).

Table 5. Spermatozoa velocity average path (VAP, μm/s) in experimental groups and time periods.

Group	Mean	S.D.	C.V.	Minimum	Maximum
Time 0					
QK	52.47	10.24	19.51	33.87	73.56
QA	57.45	9.21	16.03	39.74	73.75
QB	60.27	9.37	15.55	43.24	79.03
QC	59.22	12.53	21.16	27.74	76.57
QD	60.01	12.74	21.22	30.63	82.13
QE	53.54	9.79	18.28	37.27	73.56
Time 1					
QK	68.12	8.16	11.99	50.96	86.26
QA	41.16***	5.22	12.68	31.48	51.78
QB	46.62***	5.25	11.26	36.14	59.89
QC	53.53***	6.09	11.37	43.38	64.99
QD	60.81***	5.87	9.65	48.00	73.53
QE	61.79**	8.34	13.50	50.35	78.56
Time 2					
QK	52.56	7.84	14.91	35.50	72.26
QA	30.32***	7.56	24.93	0.00	38.99
QB	38.92***	5.39	13.84	29.51	49.88
QC	40.41***	4.07	10.07	32.51	48.94
QD	43.58***	6.71	15.41	31.77	60.93
QE	47.11*	6.80	14.43	33.09	64.36
Time 3					
QK	45.30	10.31	22.75	27.50	64.75
QA	22.61***	10.05	44.43	0.00	40.14
QB	31.86***	9.39	29.48	0.00	49.84
QC	38.17	10.45	27.38	19.32	52.12
QD	42.18	10.34	24.51	25.95	61.73
QE	41.81	7.56	18.08	28.83	52.11

Legend: S.D. – standard deviation, C.V. – coefficient of variation, *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$) (experimental group vs. control). QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu.

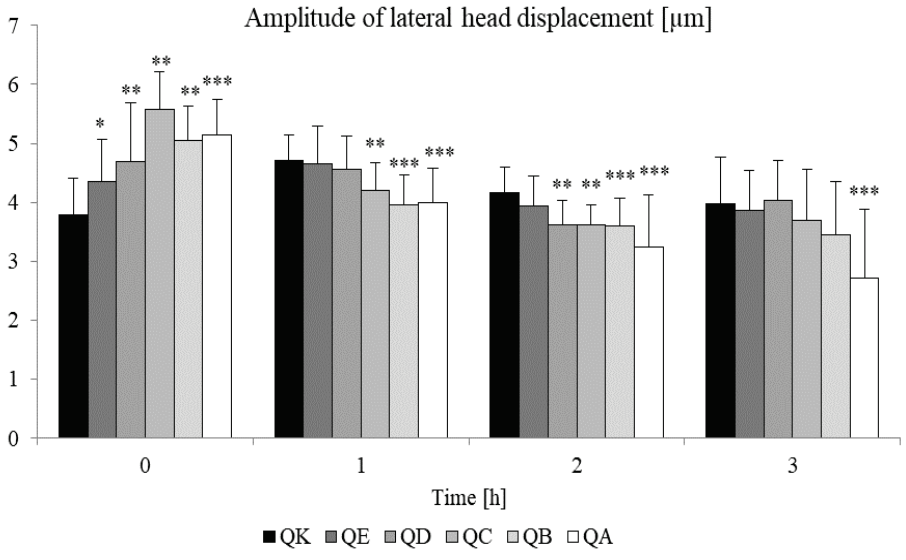

Fig. 4. The effect of *Viscum album quercus* on the amplitude of lateral head displacement (μm).
QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu. The level of significance was set at ***($p<0.001$), **($p<0.01$) and *($p<0.05$).

Table 6. Spermatozoa velocity straight line (VSL, μm/s) in experimental groups and time periods.

Group	Mean	S.D.	C.V.	Minimum	Maximum
Time 0					
QK	40.53	8.81	21.73	21.06	60.28
QA	39.07	7.71	19.72	27.11	54.48
QB	42.93	8.68	20.22	26.91	59.89
QC	44.94	11.41	25.39	18.85	60.35
QD	41.47	9.28	22.36	21.05	58.10
QE	39.72	7.99	20.12	25.20	56.26
Time 1					
QK	52.75	7.60	14.41	38.78	70.71
QA	29.35***	3.70	12.60	21.52	37.00
QB	35.44***	4.17	11.78	27.66	44.88
QC	41.50***	5.04	12.14	31.08	52.56
QD	46.26***	4.27	9.24	36.38	55.44
QE	46.64***	5.25	11.25	39.21	57.29
Time 2					
QK	40.72	6.28	15.41	29.37	56.76
QA	21.99***	5.56	25.60	0.00	30.47
QB	28.76***	4.53	15.76	20.79	40.55
QC	31.40***	3.55	11.31	24.63	39.57
QD	34.09***	6.39	18.75	23.67	51.32
QE	35.68**	4.51	12.63	26.71	44.68
Time 3					
QK	34.52	7.51	21.77	20.31	49.01
QA	16.49***	7.56	45.86	0.00	31.82
QB	22.65***	6.40	28.23	0.00	34.89
QC	27.81**	7.45	26.80	12.09	38.72
QD	30.22	7.86	23.01	18.52	50.15
QE	30.80	4.97	16.14	21.21	40.57

Legend: S.D. – standard deviation, C.V. – coefficient of variation, *** ($p<0.001$), ** ($p<0.01$) (experimental group vs. control). QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu.

The spermatozoa velocity average path clearly confirm the data for spermatozoa velocity curved line with similar significant decrease (Table 5).

Also the spermatozoa velocity straight line was negative affected by the *Viscum album quercus* addition. In group with the highest concentration this parameter decreased at Time 1 to 55.64 %, at Time 2 to 54.00 % and at Time 3 to 47.77 % compared to control (Table 6).

Other fine motility parameters

The initial amplitude of lateral head displacement (ALH) was 3.78±0.63 μm. At Time 0 a statistically significant increase was detected in all experiment groups. After 1 hour of cultivation the ALH was in control group 4.70±0.44 μm and the values decreased by 15.1 % for group QA and 15.9 % for QB. After 2 hours the ALH was the highest in control groups (4.16±0.44 μm) and the most significant decrease was noted in group QA (3.24±0.88 μm, $p<0.001$) with a decrease of 22.1 %. After 3 h of *in vitro* cultivation, the lowest value was found in the sample QA (2.72± 1.16 μm) compared to control (3.98±0.79 μm) and was statistically significant (Fig. 4).

The average value of the initial beat cross frequency (BCF) was 29.79±2.98 Hz. After 1 h of *in vitro* cultivation the BCF in QK group increased to 31.91±2.60 Hz. In all experimental samples a statistically significant decreased was found (time-

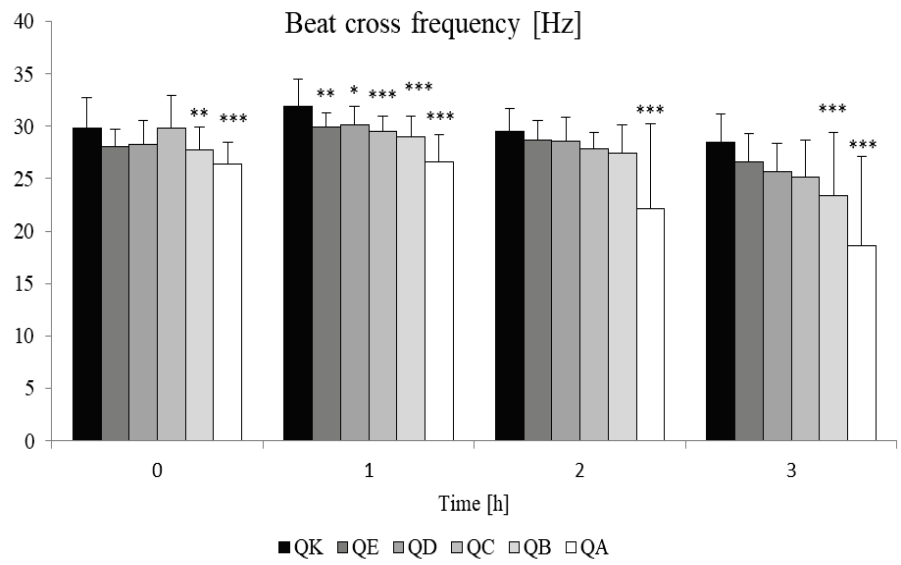

Fig. 5. The effect of *Viscum album quercus* on the beat cross frequency (Hz). QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/mL of Iscador Qu. The level of significance was set at ***(p<0.001), **(p<0.01) and *(p<0.05).

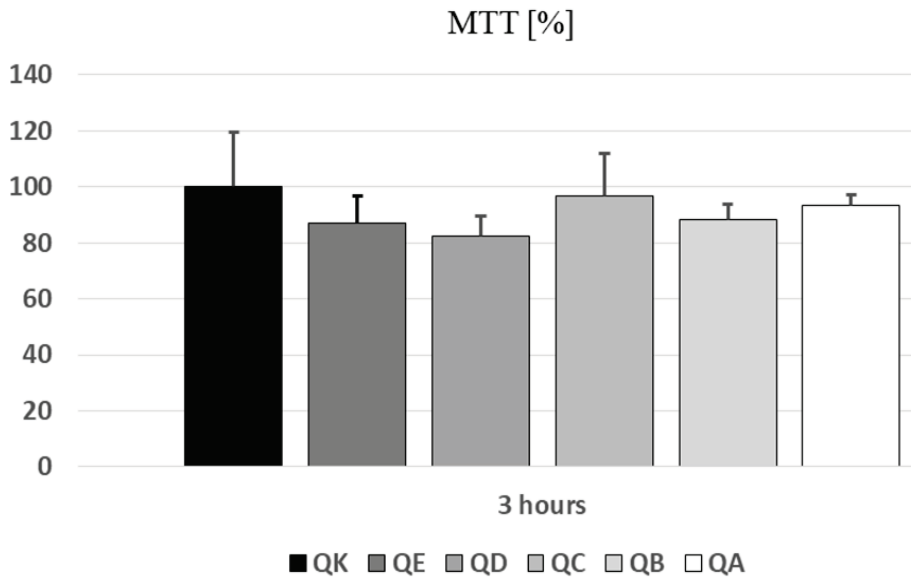

Fig. 6. The effect of *Viscum album quercus* on the viability (%) of rabbit spermatozoa after 3 hours of incubation.
QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu.

dependent). However, after 2 hours the BCF in control groups (QK) reached 29.55±2.18 Hz. The most significant decrease (25.2 %) was found for group QA (22.11±8.10 Hz). After 3 hours the BCF in the control sample was 28.46±2.75 Hz. In the group QA a statistically significant decrease (34.6 %, 18.62± 8.49 Hz), and also in the group QB (17.9 %, 23.36±6.03 Hz), compared to the control group was detected (Fig. 5).

The straightness of spermatozoa movement was in all time periods significantly decreased only in group QA (Table 7).

Very similar trends were found for spermatozoa linearity (Table 8).

The spermatozoa wobble was significantly affected only in groups QA and QB (Time 1 and 2) with the highest difference detected at Time 1 (0.51±0.03 in group QK vs. 0.44±0.03 in group QA (Table 9).

Viability, membrane integrity, acrosomal integrity

After 3 hours of incubation, viability of rabbit spermatozoa showed decreased values in all doses of *Viscum album quercus* in comparison to the control group, but the difference were not significant (Fig. 6).

Significant decrease of membrane integrity (intact) was found in groups with the highest *Viscum album quercus* concentrations QB (64.00±2.65 %, $p<0.05$) and QA (60.33±5.51 %, $p<0.01$) compared to control group (QK) (79.33±1.53 %). Also, in groups QE, QD, QC lower values in comparison to control group were detected ($p<0.05$, Fig. 7).

Table 7. Spermatozoa straightness (STR) in experimental groups and time periods.

Group	Mean	S.D.	C.V.	Minimum	Maximum
Time 0					
QK	0.77	0.06	7.88	0.61	0.89
QA	0.67***	0.06	9.28	0.57	0.77
QB	0.70**	0.06	9.17	0.59	0.80
QC	0.75	0.06	8.50	0.63	0.87
QD	0.69***	0.11	15.98	0.45	0.84
QE	0.73	0.04	5.16	0.67	0.82
Time 1					
QK	0.77	0.04	5.57	0.62	0.86
QA	0.71***	0.05	6.91	0.58	0.79
QB	0.76	0.04	5.45	0.69	0.86
QC	0.77	0.03	3.59	0.71	0.81
QD	0.76	0.05	6.26	0.64	0.85
QE	0.75	0.03	4.51	0.68	0.82
Time 2					
QK	0.77	0.04	5.42	0.62	0.85
QA	0.70***	0.14	19.46	0.00	0.89
QB	0.73	0.05	6.56	0.65	0.84
QC	0.77	0.02	3.10	0.72	0.82
QD	0.77	0.05	5.89	0.69	0.85
QE	0.75	0.04	5.50	0.66	0.84
Time 3					
QK	0.76	0.05	6.45	0.66	0.91
QA	0.64C	0.26	40.91	0.00	31.82
QB	0.69	0.14	20.23	0.00	0.92
QC	0.73	0.05	6.66	0.61	0.80
QD	0.71	0.06	8.58	0.61	0.81
QE	0.74	0.04	5.89	0.66	0.81

Legend: S.D. – standard deviation, C.V. – coefficient of variation, *** ($p<0.001$), ** ($p<0.01$) (experimental group vs. control). QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/mL of Iscador Qu.

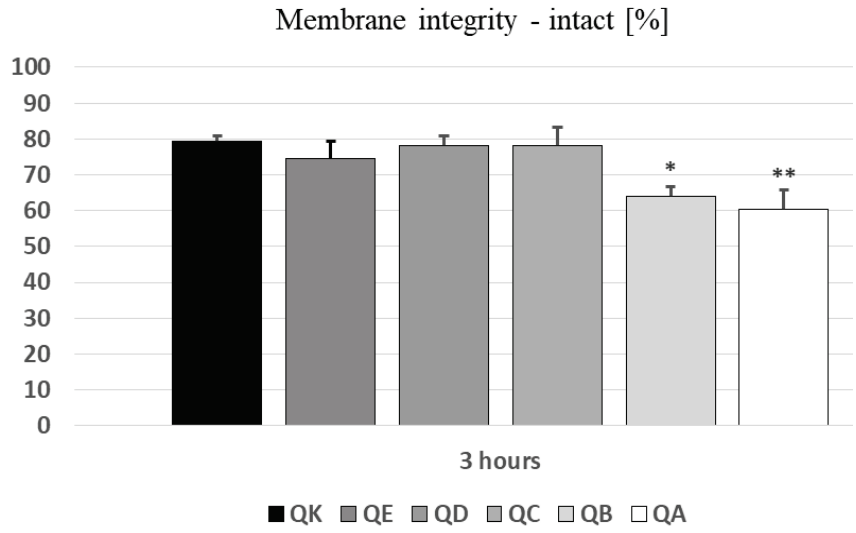

Fig. 7. The effect of *Viscum album quercus* on the membrane integrity (%) of rabbit spermatozoa assessed after 3 hours of incubation.
QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu. The level of significance was set at ** ($p<0.01$) and * ($p<0.05$).

Table 8. Spermatozoa linearity (LIN) in experimental groups and time periods.

Group	Mean	S.D.	C.V.	Minimum	Maximum
Time 0					
QK	0.37	0.05	13.31	0.23	0.45
QA	0.32**	0.03	10.81	0.26	0.37
QB	0.34	0.05	14.89	0.27	0.44
QC	0.38	0.07	17.39	0.24	0.48
QD	0.33	0.09	25.70	0.18	0.47
QE	0.35	0.04	12.00	0.27	0.42
Time 1					
QK	0.40	0.04	10.28	0.29	0.50
QA	0.31***	0.03	9.77	0.24	0.36
QB	0.35***	0.03	8.88	0.29	0.46
QC	0.38	0.03	7.33	0.30	0.44
QD	0.38	0.04	9.56	0.30	0.47
QE	0.37*	0.02	6.42	0.32	0.41
Time 2					
QK	0.37	0.03	8.10	0.27	0.42
QA	0.31***	0.12	38.07	0.00	0.86
QB	0.31***	0.03	8.75	0.26	0.38
QC	0.35	0.03	7.42	0.30	0.40
QD	0.35	0.04	10.76	0.28	0.42
QE	0.34	0.02	6.99	0.29	0.39
Time 3					
QK	0.35	0.03	9.49	0.30	0.44
QA	0.32	0.18	56.85	0.00	0.74
QB	0.29	0.11	38.09	0.00	0.81
QC	0.32	0.04	12.17	0.24	0.40
QD	0.30	0.04	12.96	0.25	0.39
QE	0.33	0.03	10.36	0.27	0.40

Legend: S.D. – standard deviation, C.V – coefficient of variation, *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$) (experimental group vs. control). QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/mL of Iscador Qu.

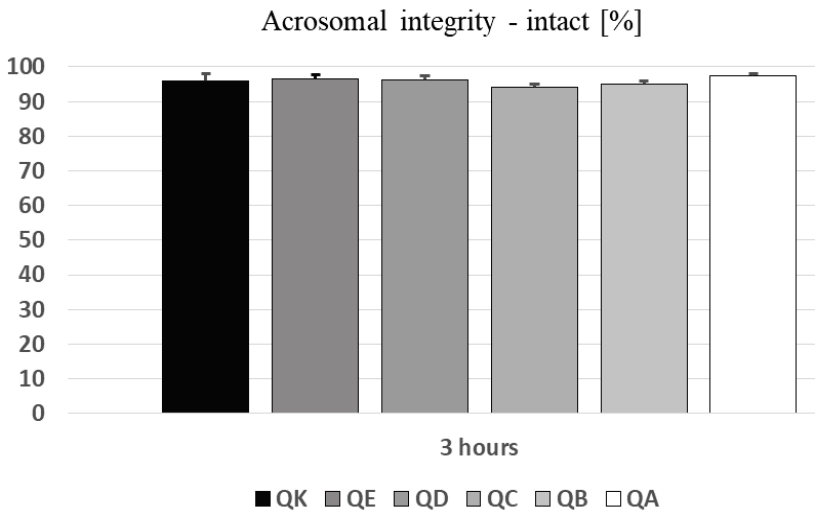

Fig. 8. The effect of Viscum album quercus on the acrosomal integrity (%) of rabbit spermatozoa assessed after 3 hours of incubation. QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu.

Table 9. Spermatozoa wobble (WOB) in experimental groups and time periods

Group	Mean	S.D.	C.V.	Minimum	Maximum
Time 0					
QK	0.48	0.04	8.73	0.38	0.55
QA	0.47	0.02	3.71	0.43	0.50
QB	0.48	0.03	7.21	0.44	0.55
QC	0.50	0.05	9.99	0.36	0.57
QD	0.47	0.06	11.90	0.39	0.56
QE	0.48	0.04	7.73	0.40	0.53
Time 1					
QK	0.51	0.03	6.17	0.44	0.60
QA	0.44***	0.03	6.29	0.38	0.49
QB	0.46***	0.02	4.89	0.42	0.53
QC	0.50	0.03	5.35	0.43	0.56
QD	0.50	0.02	4.84	0.46	0.57
QE	0.49*	0.02	4.81	0.43	0.53
Time 2					
QK	0.48	0.02	4.70	0.43	0.52
QA	0.42**	0.13	30.50	0.00	0.86
QB	0.42**	0.02	5.45	0.39	0.47
QC	0.45	0.03	6.08	0.30	0.40
QD	0.46	0.03	6.83	0.39	0.51
QE	0.45	0.02	4.99	0.40	0.50
Time 3					
QK	0.46	0.03	6.50	0.39	0.57
QA	0.42	0.20	46.81	0.00	0.80
QB	0.41	0.12	28.25	0.00	0.87
QC	0.44	0.03	7.70	0.34	0.50
QD	0.43	0.03	6.84	0.38	0.49
QE	0.44	0.03	6.16	0.38	0.52

Legend: S.D. – standard deviation, C.V. – coefficient of variation, *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$) (experimental group vs. control). QK – 0, QE – 2, QD – 2.5, QC – 3.3, QB – 6.6, QA – 10 mg/ml of Iscador Qu.

Significant decrease of membrane integrity (intact) was found in groups with the highest *Viscum album quercus* concentrations QB (64.00±2.65 %, $p<0.05$) and QA (60.33±5.51 %, $p<0.01$) compared to control group (QK) (79.33±1.53 %). Also, in groups QE, QD, QC lower values in comparison to control group were detected ($p<0.05$, Fig. 7).

The values of acrosomal integrity showed very similar tendency (94.00-97.33 %) in all experimental groups with no significant difference (Fig. 8).

Discussion

Researchers are unanimous about the negative impact of various factors on male reproduction and cell/spermatozoa quality (Burton 2013, Lukac *et al*. 2013, Petrovova *et al*. 2014, Lukacova *et al*. 2015, Kolarova *et al*. 2017). The mistletoe (*Viscum album* L.) extract has been shown to be an effective complementary drug in the treatment of cancer patients after surgical removal of the primary tumor. It improved survival, recovery from damage caused by irradiation or cytostatic therapy, and quality of life. In animal tests, clear anti-carcinogenic effects of Iscador were demonstrated. Mainly immune stimulation but also direct cytotoxic activity is believed to be responsible for the anti-carcinogenic activity of Iscador. Tests examining the acute toxicity, genotoxic effects as well as effects on reproduction showed no adverse effects of Iscador preparations. Genotoxic effects and effects on reproduction, which cannot be evaluated in clinical use, have been cleared up in animal tests. Iscador was shown to be clearly non-genotoxic and free of relevant toxic effects

on reproduction *in vivo* (Maldacker 2006, Gardin 2009).

On the other hand, it has been stated that anticancer drugs could cause harmful effects on the spermatozoa quality and spermatogenic cell arrangement of male. Therefore, this study was designed to evaluate the *in vitro* effects of *Viscum album quercus* (Iscador) on rabbit spermatozoa in order to assess possible beneficial and/or toxic effects of this compound. Rabbits were chosen as the experimental animal in this research for their well-defined reproductive systems (Paal *el al*. 2014, Rafay and Parkanyi 2016). Some herbal extracts have been proven to have effects on male infertility, for example, gossypol, papaya seed, neem oil, neem seaweed and verbascoside (Mosher and Pratt 1991, Dehghan *et al*. 2005, Roychoudhury *et al*. 2009, Okab *et al*. 2013, Vizzarri *et al*. 2019).

Since decades, *Viscum album* preparations have been used in Europe in oncology. They show multi-facetted anti-tumor *in-vitro* activities, which include inhibition of tumor 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-22 (Seibert *et al*. 1989, Bussing 2006, Bussing *et al*. 2008, Hegde *et al*. 2011, Hajto *et al*. 2011). Iscador as an anticancer drug used in the treatment of a variety of neoplastic lesions. On the other hand, treatment with *Viscum album quercus* (Iscador) is accompanied by different toxic effects on different body organs.

An *in vitro* study investigated the effect of a standardized mistletoe preparation on the action of Trastuzumab, a drug used for the treatment of Her-2 positive breast cancer. A dose dependent anti-proliferative effect of *Viscum album* exctract (VAE) was observed at concentrations ≥10 µg/mL after 3 days of incubation. After 7 days a significant growth inhibition of 60 % with the clinically relevant concentration 1 µg/mL was detected and no proliferating cells were left at VAE concentrations of 10 and 100 µg/ml. With 10 µg/ml VAE after 7days the proportion of early apoptotic cells raised from 9.0 % in the control to 17.4 % and that of late apoptotic/necrotic cells from 18.7 % in control to 78.7 %, respectively (Weissenstein *et al*. 2016).

Effects of *Viscum album quercus* (VA Qu) extract in various doses were also investigated in an *in vitro* model with tumor cells: three multiple myeloma (MM) cell lines (OPM-2, RPMI-8226, U-266) and three B cell lymphoma cell lines (U-698, DOHH-2, WSU-1). *Viscum album*/Qu extract markedly downregulated the membrane expression of IL-6R and gp130 in RPMI-8226 (down to 29 % and 32 %) and the expression of gp130 in WSU-1 (down to 22 %). There was a marked reduction of viable cells of RPMI-8226 (down to 28 %) and WSU-1 (down to 8 %) at 100 µg/10^6 cells /ml. There was a clear relationship between the inhibition of proliferation and viability. VA Qu was more effective in cells having a high proliferation rate than in those with a low proliferation rate (Kovacs *et al*. 2006).

Our observations indicate that Iscador in higher doses decreases the spermatozoa motility, progressive spermatozoa motility and some other fine spermatozoa motility parameters (velocity curved line, amplitude of lateral head displacement and beat cross frequency). Thus, Iscador (in high doses *in vitro*) can have long-term effects on testicular function and can be a potent gonadal toxic drug.

Spermatozoa motility is one of the most important effective factors in male fertility. The mechanism by which Iscador affects the spermatozoa motility has not been clearly elucidated.

A decline in fructose level due to alteration in carbohydrate metabolism after Iscador Qu treatment can be suggested (Rigau *et al*. 2001, Gren *et al*. 2011). Reason for decreased spermatozoa motility, can be also a decreased level of androgen carrier proteins involved in spermatozoa motility. Mistletoe lectins have been identified as main active components and exhibit cytotoxic effects. Therefore, probably lectins have a negative effect on the spermatozoa motility found in this study. Also a relationship between diminished spermatozoa quality and anticancer treatment may a result of a series of cascade events that cause a fall in intracellular ATP levels (Luria *et al*. 2002, Turner 2006, Storey 2008), release of different apoptogenic factors (as pro-caspases, cytochrome C, and apoptosis inducing factor) (Casao *et al*. 2015) from mitochondria into the cytosol through disruption of mitochondrial membrane, inactivation of some biochemical pathways, enzyme dysfunction, disturbed axonemal protein phosphorylation, increased membrane permeability and generation of spermicidal products, which have adverse effects on the spermatozoa functions. Similarly, the spermatozoa motility is also reduced by ergonovine (Tash and Means 1982, Gallagher and Senger 1989), and the ergot alkaloids induce motility changes in bovine sperm cells

by interacting with alpha-adrenergic receptors (Wang *et al*. 2009).

Conclusion

The results of this study indicate that spermatozoa are a useful *in vitro* model for the toxicological evaluation of chemicals providing quantitative as well as qualitative data. In conclusion, the present study shows reduction of spermatozoa quality after *Viscum album quercus* addition *in vitro*.

Conflict of Interest

There is no conflict of interest.

Acknowledgments

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