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Potential use of dimethyl sulfoxide (DMSO) in artificial insemination of rabbits

Martin Fik[1], Radovan Kasarda[2], Ľubomír Ondruška[3], Dariusz Kokoszyński[4], Norbert Lukáč[5], Ewa Czerniawska-Piątkowska[6], Barbara Biesiada-Drzazga[7], Katarzyna Andraszek[7], Dorota Banaszewska[7#]

[1] Institute of Animal Husbandry, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Nitra, 949 76, Slovakia

[2] Institute of Nutrition and Genomics, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Nitra, 949 76, Slovakia

[3] Institute of Small Farm Animals, Institute for Animal Production Nitra, Lužianky, 951 41, Slovakia

[4] Department of Animal Breeding and Nutrition, Faculty of Animal Breeding and Biology, Bydgoszcz University of Science and Technology, Bydgoszcz, 85-084, Poland

[5] Institute of Applied Biology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Nitra, 949 76, Slovakia

[6] West Pomeranian University of Technology, Laboratory of Molecular Cytogenetics, Szczecin, 71-270, Poland

[7] Faculty of Agricultural Sciences, University of Siedlce, Siedlce, 08-110, Poland

SUMMARY

The aim of this work was to analyse the effect of incubation of a rabbit insemination dose (ID) with dimethyl sulfoxide (DMSO) on reproductive parameters (kindling rate, number of live-born and stillborn kits, and birth weight of kits). The experiments were carried out using two rabbit breeds. Each ejaculate was diluted to a concentration of 40 to 50 million sperm/0.5 ml ID. DMSO was added to the ID in a concentration of 0.5 M. The control ID did not contain any DMSO. Kindling rates obtained from two different breeds (P1, P2) showed minor improvement (+4.36% P1; +4.06% P2) compared to the controls, but without statistically significant differences. The number of live-born kits per litter obtained from breeds P1 and P2 showed minor improvement (P1 +0.61; P2 +0.13) compared to the control groups ($p > 0.05$). The weight of live-born kits was higher in the experimental groups (+3.85 g P1 = $p \leq 0.001$; +1.18 g P2 = $p>0.05$) than in the control groups. Monitoring of sperm quality indicators (1, 3 and 6 hours incubation) in *in vitro* conditions (CASA – Computer Assisted Semen Analysis) showed the best results in the control group that was not treated with DMSO. The data obtain-

Corresponding author e-mail: dorota.banaszewska@uws.edu.pl
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ed in our experiments suggest positive effects of DMSO incubation on reproduction parameters, especially *in vivo*.

KEY WORDS: spermatozoa, rabbit, rabbit insemination, DMSO, insemination

INTRODUCTION

Expanding rabbit production is attributable to rabbits' high reproduction rate, rapid growth rate, early maturation, high genetic selection potential, efficient feed utilization, and high-quality nutritious meat (Okab et al., 2013). Semen extenders are substances added to the insemination dose to preserve the fertilization ability of sperm and prolong their viability. Anchordoguy et al. (1987) confirmed that semen additives affect sperm stability and protect individual sperm components. The positive effect of various semen extenders on livestock insemination is discussed by various authors. Studies have focused on insulin growth factor in rat (Vickers et al., 1999) and equine spermatozoa (Champion et al., 2002); on caffeine in buffalo and cattle (Tathan et al., 2003) and in sheep (Matejašáková et al., 2005, Riha et al., 2006); on heparin and hyaluronan in cattle (Januskauskas et al., 2000); on heparin in cattle (Lapointe et al., 1996, Parrish et al., 1993) and in rabbits (Lapointe et al., 1996, Parrish et al., 1993, Fik et al., 2008a, Fik et al., 2008b, Fik et al., 2008c, Fik and Malíková, 2013, Fik et al., 2014); and on buserelin acetate in rabbits (Vicente et al., 2011).

In general, oxidative stress (OS) is characterized as an imbalance between the production of reactive oxygen species (ROS) and the ability of biological systems to readily detoxify the reactive intermediates or easily repair the resulting damage. It occurs when the system is not able to eliminate and neutralize free radicals and active intermediates at the cellular or organismal level (Liu et al., 2018). OS in sperm is caused by ROS, which include peroxides and free radicals, deemed the most dangerous factors affecting semen quality (Bansal and Bilaspuri, 2010). Hydroxyl radicals, superoxide anion and hydrogen peroxide are the principal ROS presented in seminal plasma (Agarwal and Sekhon, 2010). However, it is important to note that ROS production is a normal physiological process in spermatozoa which is essential for fertilization, hyperactivation, the acrosome reaction, capacitation and motility (Agarwal et al., 2008). Nevertheless, an imbalance between ROS and antioxidants, which are unable to scavenge excessive ROS, is noxious to spermatozoa and can cause male infertility (Bansal and Bilaspuri, 2010). Sperm are highly sensitive to OS, especially to lipid peroxidation, because of the high concentration of unsaturated fatty acids in their plasmatic membrane. These fatty acids are necessary for the male germ cell to maintain normal function (Hekimoglu et al., 2009); however, the imbalance mentioned above causes damage to the sperm cell. OS affects spermatozoa in three principal ways: through DNA damage, membrane lipid peroxidation, and induction of apoptosis (Durairajanayagam et al., 2014).

In fact, OS targets all cellular components, including lipids, proteins, sugars, and nucleic acids. OS damage depends on the nature and amount of ROS produced, the duration of ROS exposure, and extracellular factors such as oxygen tension, temperature and the composition of the surrounding environment (e.g. ions, proteins, and ROS scavengers) (Agarwal et al., 2008). The use of synthetic antioxidants can be one solution; however, naturally occurring substances are preferable due to their structural complexity, chemical diversity, intrinsic biological activity, availability, and lack of substantial toxic effects (Tvrdá et al., 2015a). Zibrín et al. (2005) demonstrated that the sperm cell membrane, which protects sperm against oxidative damage induced by ROS, is weakened by the

dilution of semen during preparation of insemination doses. Tvrdá et al. (2015b) confirmed the antioxidant efficiency of resveratrol on oxidative stress-induced damage in bovine spermatozoa. Hashim et al. (2017) showed that natural antioxidants such as vitamins C and E protect and improve the post-thaw quality of bovine insemination doses. The value of adding antioxidants to the semen of rams was also confirmed by Sarlós et al. (2002), who found that they demonstrably improved sperm motility and decreased the frequency of acrosome defects. Hypotaurine and dimethyl sulfoxide (DMSO) are antioxidants that scavenge hydroxyl radicals involved in the initiation of peroxidative damage (Gordon et al., 1996). Fik and Malíková (2013) showed that dimethyl sulfoxide affects selected quality indicators of rabbit semen. Fik et al. (2011), following the addition of GnRH to rabbit insemination doses, observed non-physiological circular movements of the sperm, as well as significant sperm agglutination (at both tested concentrations of GnRH). However, these abnormalities could not be evaluated in the CASA (Computer Assisted Semen Analysis) system. Several authors have demonstrated that sperm with higher levels of DMSO (0.8 to 1.2 M) are better protected (Castellini et al., 1992, Martin, 1993). From 1 to 1.5 M of DMSO has been used in the extender to obtain 40–50% motility and 30–70% normal acrosomes (Castellini et al., 1992, Martin, 1993). DMSO is commonly used in cryopreservation of sperm. Neither the combination of vitamin C and E nor DMSO had a significant protective effect against damage inflicted on human spermatozoa by activated polymorphonuclear leukocytes. Catalase and catalase plus DMSO had a significant effect on total percentage motility, and the latter combination also improved space-gain motility (Gordon et al., 1996). Tsuzuki et al. (2000) monitored the effect of cryoprotectants (dimethyl sulfoxide – DMSO, propylene glycol – PG, and ethylene glycol – EG) on the survivability of bovine oocytes maturing *in vitro* with or without freezing. The authors reported that the cleavage rate in the PG + DMSO group without freezing was significantly lower than in the control group. However, in oocytes frozen by either method, all the cryoprotectants resulted in a poor cleavage rate as compared to the control group oocytes. This suggests that cytoplasmic changes and plasma membrane collapse are not the only factors influencing oocyte development after freeze-thaw. The cleavage rates from the 2 to 16 cells stage in the DMSO and EG + PG groups were slightly lower than in the control group ($p > 0.05$). However, the development rates up to the blastocyst stage in each cryoprotectant-treated group were significantly lower ($p \leq 0.05$) than in the control group. When bovine oocytes maturing *in vitro* were exposed to 1.5 M DMSO + 0.25 M sucrose for 15–20 min, some of the cell organelles were changed (unpublished data). Failure of cytoplasmic competence might be revealed more clearly beyond the blastocyst stage (Sirard et al., 1996). Parkányi et al. (2005) report that intravaginal administration of DMSO (0.50 M and 1.75 M in ID) in rabbits statistically significantly improved conception rates versus the control group. The aim of this work was to analyse the impact of DMSO extender in rabbit insemination doses on selected reproductive parameters *in vivo* (conception rate, number of live-born and stillborn kits, and birth weight of kits) and *in vitro* (Computer Assisted Semen Analysis).

MATERIAL AND METHODS

The study was carried out in strict compliance with the recommendations in Directive 63/2010/EU and the Journal of Laws of the Republic of Poland of 2015 on the protection of animals used for scientific or educational purposes. The study was approved by the Local Ethics Committee, Warsaw, Poland, and by the Polish Laboratory Animal Science Association.

The experiments were carried out using two rabbit breeds (production breed 1 and production breed 2). Semen was collected using an artificial vagina (50°C). In production breed 1 (P1), breeding was focused on the production of broilers (Močcenok – Šaľa District, Nitra Region). Does (1800) of the Hycole broiler strain (commercial hybrid) were inseminated on the 19th day post-partum. A total of 646 females (experimental group 220, control group 466) were used in the study. Fresh IDs (Hycole strain, 22 males) were used. In production breed 2 (P2), breeding was focused on the production of dwarf rabbits for sale in pet shops (Žitavany – Zlaté Moravce District, Nitra Region). Does (170) of the dwarf rabbit breed were inseminated on the 25th day post-partum. A total of 74 females (experimental group 41, control group 33) were used in the study. Fresh IDs (from 10 male dwarf rabbits) were used. On each farm (in each breed) females (primiparous and multiparous up to 7 litters) were exposed to hormonal treatment before AI (48 hours; Sergon – PMSG). On each farm (P1, P2) the does were divided into a control group and an experimental group. Rabbit semen samples for *in vitro* analysis were obtained from 10 adult breeding males (meat line – P 91). Males were bred at the Research Institute for Animal Production Nitra, National Agricultural and Food Centre.

The control groups of does (P1, P2) were inseminated with ID without DMSO. The experimental groups of does (P1, P2) were inseminated with ID exp. (experimental insemination dose), with DMSO. Fresh insemination doses (fresh sperm diluted with a commercial diluent from the company Minitübe), at sperm concentrations of 40–50 million/0.5 ml ID, were divided into a control ID (ID control, without DMSO) and experimental ID (ID exp.). DMSO was added to ID exp. at a concentration of 0.5 M DMSO/0.5 ml ID (min. 30–60 min. incubation before AI). Immediately after AI, GnRH was administered intramuscularly at 0.1 ml = 2.5 µg /doe (control and experimental does).

Monitored parameters:

- kindling rate (%)
- number of live-born kits
- number of stillborn kits
- weight of kits (in g, immediately after birth)

Rabbit semen samples were obtained from 10 adult breeding males (meat line – P 91) on a regular collection schedule using an artificial vagina. Fresh insemination doses (fresh sperm diluted with commercial diluent from Minitübe), 50–60 million sperm/0.50 ml ID, were divided into a control ID (control without DMSO) and two experimental IDs (E 1.75; E 0.50). DMSO was added in two concentrations. The sample labelled E 1.75 was adjusted to a concentration of 1.75 M DMSO in ID. The sample labelled E 0.50 was adjusted to a concentration of 0.50 M DMSO in ID. Qualitative indicators (sperm motility, progressive sperm motility, average sperm velocity, sperm motion linearity, and curvilinear velocity of sperm) were examined by the Computer Assisted Semen Analysis (CASA) system using SpermVisionTM software (Minitübe, Tiefenbach, Germany) and an Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan). The samples were placed in the Makler Counting Chamber (depth 10 µm, 37°C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. At least 1000 cells were evaluated in each sample (Massanyi et al., 2008). Qualitative sperm parameters were analysed 1, 3 and 6 hours after incubation with DMSO. We compared the control sample with the experimental samples (E 1.75; E 0.5).

Kindling rates (relative values) were compared using the chi square test (χ^2). The significance of differences between groups was determined by the t-test. Statistical analysis was performed in Microsoft Excel software.

RESULTS

The number of live-born kits in the experimental groups was higher in both production breeds included in the study. The difference was more pronounced in the broiler rabbits (P1), in which the average number of live-born kits per litter was 10.07 ± 2.91 in the experimental group and 9.46 ± 3.54 in the control group (Table 1). However, neither of these differences was statistically significant, nor were the differences in the number of stillborn kits. We recorded a lower average number of stillborn kits in the experimental group compared with the control group (0.23 vs. 0.25). We found the same difference in P2, but the difference was slightly greater (0.03). We recorded statistically highly significant differences ($p \leq 0.001$) in the weight of live-born kits in P1: 57.57 ± 7.54 g in the control group and 61.42 ± 8.42 g in the experimental group. The use of DMSO in the ID also clearly improved the weight of live-born kits of the dwarf lop breed, which was 41.14 ± 10.11 g in the experimental group and only 39.96 ± 8.69 g in the control group it was. However, this finding was not statistically significant ($p > 0.05$). Kindling rates obtained from the two breeds (P1, P2) showed minor improvement (+4.36% P1; +4.06% P1) in the experimental groups, but without statistically significant differences (P1 χ^2 = 0.25(-); P2 χ^2 = 0.25 (-)).

Table 1
Reproductive parameters

Parameter	Group	Number of AI does	Number of live-born kits per litter	Number of stillborn kits per litter	Weight of live-born kits	Kindling rate (%)
		n	Mean±SD	Mean	Mean ±SD	
Production breed P1 (broiler rabbits)	Experimental	210	10.07 ± 2.91	0.23	1.42 ± 8.42	81.82
	Control	466	9.46 ± 3.54	0.25	7.57 ± 7.54	77.46
	Statistical differences		p>0.05	p>0.05	≤0.001	χ^2= 0.25(-)
Production breed P2 (dwarf lop)	Experimental	41	3.25 ± 1.46	0.28	1.14 ± 10.11	70.73
	Control	33	3.12± 1.52	0.31	9.96 ± 8.69	66.67
	Statistical differences		p>0.05	p>0.05	>0.05	χ^2 =0.25 (-)

(-) statistically non-significant, (+) statistically significant

Comparison of the qualitative parameters of insemination doses of rabbits treated with DMSO using the CASA system is shown in Table 2.

Table 2
Comparison of qualitative parameters of rabbit insemination doses treated with DMSO using the CASA system

Parameter	Incubation time (hours)	Sample label		
		Control Mean±SD	E 1.75 Mean±SD	E 0.5 Mean±SD
Sperm motility (%)	1	47.30 ± 7.99	60.21 ± 6.52	36.84 ± 4.52
	3	57.09 ± 23.36	43.4 ± 3.21	35.65 ± 11.64
	6	64.65 ± 8.60	3.91 ± 1.25	39.33 ± 8.47
Progressive sperm motility (%)	1	30.50 ± 7.35	33.32 ± 5.83	18.06 ± 4.01
	3	42.06 ± 22.69	28.92 ± 4.62	19.68 ± 8.04
	6	56.34 ± 8.88	0.00 ± 0.00	31.61 ± 10.05
Average sperm velocity (µm/s)	1	71.86 ± 8.19	48.11 ± 6.42	56.72 ± 8.38
	3	62.35 ± 7.89	52.43 ± 4.22	58.48 ± 10.14
	6	73.93 ± 8.18	0.00 ± 0.00	69.51 ± 7.11
Sperm motion linearity (VSL/VCL)	1	0.43 ± 0.03	0.34 ± 0.02	0.36 ± 0.02
	3	0.42 ± 0.001	0.34 ± 0.01	0.37 ± 0.02
	6	0.32 ± 0.001	0.00 ± 0.00	0.35 ± 0.02
Curvilinear velocity of sperm movement (µm/s)	1	136.83 ± 14.28	113.80 ± 9.82	119.97 ± 12.01
	3	120.69 ± 16.36	121.00 ± 10.27	119.37 ± 28.26
	6	172.72 ± 20.04	0.00 ± 0.00	154.33 ± 14.79
Beat cross frequency (Hz)	1	33.95 ± 3.24	32.36 ± 0.86	29.88 ± 1.23
	3	31.94 ± 2.46	29.64 ± 0.91	28.90 ± 2.25
	6	29.28 ± 1.39	0.00 ± 0.00	30.15 ± 2.24

E 1.75 – concentration of 1.75 M DMSO in ID
E 0.5 - concentration of 0.5 M DMSO in ID

DISCUSSION

Parkányi et al. (2005) monitored the influence of DMSO in ID (two concentrations, 0.5 M and 1.75 M) on the conception rate and the number of live births and stillborn kits. They reported an improvement in the conception rate ($p \leq 0.01$) and number of live births (+0.63 and 0.59, $p > 0.05$) at both DMSO concentrations. Fik et al. (2014) found improvement in the conception rate after adding heparin to the insemination dose (74.50 vs 81.80%), but without statistically significant differences. In the present study, the number of live-born kits per litter obtained from breed P1 showed minor improvement (+ 0.61) compared to the control group ($p > 0.05$). Our results are in agreement with the findings of Parkányi et al. (2005), who showed that incubation of ID with DMSO positively influences the number of live-born kits per litter (+ 0.61) compared to the control group ($p > 0.05$). Fik (2018) reported that intravaginal administration of GnRH to rabbits in the form of Supergestran at concentration of 0.4 in the ID fully replaces intramuscular administration. Experiments on nulliparous females showed slight improvement in the conception rate in favour of the group inseminated with the treated ID (77.66%) versus the group of females inseminated with the control ID (74.55%) (no statistically significant differences). Gogol (2016) reported a negative

effect of goserelin and leuprolide added to rabbit semen. The kindling rate was 80.5% in group G10 (10 µg of goserelin) and 75.0% in Group L10 (10 µg of leuprolide), while the rate in the control group was 85.9%. The kindling rates in groups G5 (5 µg of goserelin) and L5 (5 µg of leuprolide) were significantly lower than in the control group (60.0%, 54.2% and 85.9%, respectively). Ondruška et al. (2008) showed that intravaginal administration of GnRH (at 7.5 µg/doe) in the ID positively influences ovulation (+9.35% conception rate). Fik and Malíková (2013) report that incubation of rabbit sperm with heparin yields better reproduction results. The authors report an improvement in the conception rate (70.06 vs 55.94 %), the number of live-born kits/litter (8.69 vs 8.41), and the number of live-born kits/inseminated female (6.28 vs 4.89), but without statistically significant differences. Greifová et al. (2017) tested the antioxidant effects of different epicatechin (EPI) concentrations during *in vitro* cultivation on bovine sperm quality parameters. Cultivation for 24 h confirmed the protective effect of EPI in the experimental groups ($p < 0.001$) on motility as well as on viability and ROS production at concentrations ranging between 1 and 50 µM/L. Ďuračka et al. (2017) monitored the *in vitro* effects of selected biologically active compounds on rabbit spermatozoa motility. After 8 h of *in vitro* culture, the highest sperm motility was detected in the experimental groups treated with 10 µmol/L resveratrol (RES) ($p < 0.05$); 1–10 µmol/L quercetin (QUE) ($p < 0.01$ for 1 µmol/L QUE; $p < 0.001$ for 5 and 10 µmol/L QUE); 1 µmol/L curcumin (CUR) ($p<0.01$); 1–100 µmol/L EPI ($p<0.01$ for 50, 10 and 5 µmol/L EPI; $p<0.001$ for 100 and 1 µmol/L EPI); and 10 µmol/L isoquercitrin (ISO) ($p < 0.05$) when compared to the untreated control. Tirpák et al. (2017) investigated the effect of various taurine concentrations on rabbit spermatozoa in *in vitro* conditions for potential use in routine artificial insemination. The study showed improved viability and motility in *in vitro* conditions. Nevertheless, the study alone does not confirm an improved fertilization rate. Since the highest concentration of taurine was not the most effective, we may suggest that the optimal taurine concentration in rabbit semen doses is 6.250 mM. The aim of a study by Vicente and Viudes-de-Castro (1996) was to formulate a simple extender for freezing rabbit semen. Three factors and their interactions were studied: final concentration of DMSO (1, 1.25, 1.5 and 1.75 M), egg yolk (0% of 10 v/v) and sugar (none, or 0.5 M of glucose, lactose, sucrose, or maltose). Sucrose and 1.75 DMSO significantly improved the post-thawing motility rate (sucrose 1.75 M DMSO extender: 42 ± 3%). Fik and Malíková (2013) reported that following treatment of rabbit sperm with DMSO (0.5 M and 1.75 M concentration of DMSO in ID), sperm movement patterns were not compromised.

CONCLUSION

This study casts a new light on the complexity of interactions between sperm and ovule after ID incubation with DMSO. Kindling rates obtained from two different breeds (P1, P2) showed minor improvement (+4.36 % P1; +4.06 % P2) in the experimental groups, but without statistically significant differences. The number of live-born kits per litter obtained from breeds P1 and P2 showed minor improvement (+0.61 P1; + 0.13 P2) compared to the control groups ($p > 0.05$). The weight of live-born kits was higher in the experimental groups (+ 3.85 g P1 = $p \leq 0.001$; + 1.18 g P2 = $p > 0.05$) than in the control groups. Monitoring of sperm quality indicators in *in vitro* conditions showed the best results in the control group not treated with DMSO. This confirms the differences between *in vivo* and *in vitro* conditions. The improvement in *in vivo* results is apparently due to the fact that DMSO improves the transport of all extender molecules through the sperm cell membranes

and acts as a scavenger of the hydroxyl free radical, thereby improving sperm fertilization capacity. A positive effect of DMSO on sperm penetration is also possible.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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