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Crude Glycerol Affects Canine Sperm Motility: Computer Assisted Semen Analysis *in vitro*

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Abstract**—**Target of this study was the analysis of the impact of crude glycerol on canine spermatozoa motility, morphology, viability, and membrane integrity. Experiments were realized *in vitro*. In the study, semen from 5 large dog breeds was used. They were typical representatives of large breeds, coming from healthy rearing, regularly vaccinated and integrated to the further breeding. Semen collections were realized at the owners of animals and in the veterinary clinic. Subsequently the experiments were realized at the Department of Animal Physiology of the SUA in Nitra. The spermatozoa motility was evaluated using CASA analyzer (SpermVision^{TM}, Minitub, Germany) at the temperature 5 and 37°C for 5 hours. In the study, 13 motility parameters were evaluated. Generally, crude glycerol has generally negative effect on spermatozoa motility. Morphological analysis was realized using Hancock staining and the preparations were evaluated at magnification 1000x using classification tables of morphologically changed spermatozoa. Data clearly detected the highest number of morphologically changed spermatozoa in the experimental groups (know twisted tails, tail torso and tail coiling). For acrosome alterations swelled acrosomes, removed acrosomes and acrosomes with undulated membrane were detected. In this study also the effect of crude glycerol on spermatozoa membrane integrity were analyzed. The highest crude glycerol concentration significantly affects spermatozoa integrity. Results of this study show that crude glycerol has effect of spermatozoa motility, viability, and membrane integrity. Detected changes are related to crude glycerol concentration, temperature, as well as time of incubation.

Keywords**—**Dog, semen, spermatozoa, acrosome, glycerol, CASA, viability.

I. Introduction

GLYCEROL ($CH_3H_8O_3$), a highly permeable polyhydric alcohol is the cryo-protector most frequently used in semen freezing in different species [1]. However, this cryo-protector exhibits toxic effects on spermatozoa, such as physicochemical alterations that can lead to rupture of the plasma membrane or removal of important membrane proteins, as well as cause acrosome damage, which will be reflected in reduced fertility [2], [3]. Since glycerol has a relative high molecular weight (92 kDa) and does not readily penetrate into the spermatozoa membrane, it causes tremendous osmotic stress during the thawing procedure, because it is easily removed from the cell membranes [4]. The ideal concentration of glycerol in the extender represents a balance between its toxicant and protecting effects; high concentrations can also affect the fertilizing capacity of the spermatozoa [5]. However, the sensitivity of spermatozoa to glycerol is highly species dependent [6]. The aim of this study was to analyze the influence of different crude glycerol concentrations on canine spermatozoa motility parameters during short *in vitro* cultivation.

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II. Material and Methods

In this study, semen was obtained from sexually mature male of five large dog breeds: Boxer, Border collie, Rottweiler, Greyhound and Husky. Semen was diluted in a ratio of 1 part of semen and 10 parts of physiological solution (Sodium chloride 0.9% Braun, B. Braun Melsungen AG, Melsungen, Germany) – control sample (GK), or commercial diluent (GR). In the same ratio, the semen was diluted with different concentration of crude glycerol solution: GA: 0.11 ml/ml, GB: 0.05 ml/ml, GC: 0.027 ml/ml, GD: 0.013 ml/ml, GE: 0.0069 ml/ml, GF: 0.0034 ml/ml, GG: 0.0017 ml/ml diluted in the physiological solution. Samples were cultured at 5 and 37°C (T) and recorded at five time periods: 0, 1, 2, 3, and 4 hours.

Characteristic of used glycerol – crude glycerol: content at least 80.06%; NaCl content 7.15%; water 8.0%; methanol 0.001%; Cd<0.01 mg/l, Pb<0.1 mg/l, Cu<0.04 mg.l, Mn<0.03 mg/l, Zn 2.5 mg/l, Fe 15 mg/l, Ni 2.5 mg/l, Co 12.5 mg/l and Cr 7.5 mg/l.

To evaluate spermatozoa motility parameters Computer Assisted Semen Analyzer (CASA) system – SpermVision program (Minitube, Germany) equipped with a microscope Olympus BX 51 (Olympus, Japan) was used. Each sample was placed into Makler Counting Chamber (depth of 10 μm, Sefi–Medical Instruments, Germany) and then placed in a microscopic field. Using the canine specific set up motility parameters were evaluated (mainly total motile spermatozoa and progressively motile spermatozoa). For morphological analysis, the staining according to Hancock was used. The preparations were evaluated at magnification 1000x. The changes were classified according to the classification tables of morphologically changed forms of spermatozoa. Obtained data were statistically analyzed with the help of the PC program Excel and a statistics package SAS 9.1 (SAS Institute Inc., USA) using Student's t–test and Scheffe test. Statistical significance was indicated by P values of less than 0.05; 0.01 and 0.001.

III. Results and Discussion

The percentage of total spermatozoa motility (MOT) cultured at 5°C in the control sample (C) was in range from 71.75 to 84.10% (Fig. 1). In the experimental groups motility ranged from 0 to 75.95%. A significantly lower ($p<0.001$) values were detected at the beginning of cultivation only in the samples GA with concentration of glycerol 0.11 ml/ml. In times 1, 2, 3, 4 significantly decrease ($p<0.001$) of spermatozoa motility was observed in all experimental groups compared to the control group. Spermatozoa progressive motility (PRO) followed the tendency of spermatozoa motility. Progressive motility in the control sample ranged between 68.80 and 81.46%. In the experimental groups, motility ranged from 0 to 72.39%. Statistically significant difference ($p<0,001$) was detected at the time 1 hour only between sample C and GA, where the highest progressive motility was recorded in sample C. When compared the experimental groups to the control sample, significantly lower values ($p<0,001$) were observed after 1, 2, 3, 4 hours of culture in all samples. Analysis of velocity-curved line (VCL) showed no significant differences between samples GB, GC, GD and the control at the beginning of *in vitro* cultivation. Only in the sample GA significantly lower ($P<0.001$) value was observed. However, other cultivation times showed a significant decrease ($P<0.001$) of this parameter in all experimental samples when compared to the control.

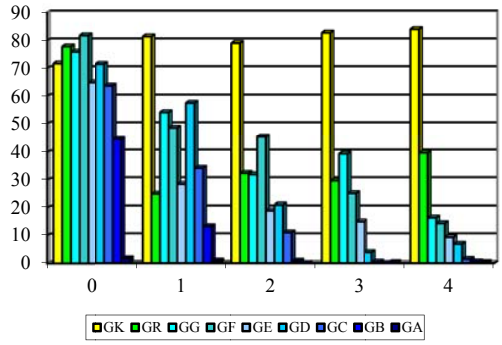

Fig. 1 Spermatozoa motility (%) after glycerol addition at 5^0C in different time periods

Total spermatozoa motility cultured at 37°C in the control group ranged in 5 time periods from 68.75 to 83.74% (Fig. 2). In the GA the group (the group of glycerol concentration 0.11 ml/ml) reached 1.64–0.27%, GB 36.50–0.13%, GC 73.71–1.58%, GD 59.35–4.69%, GE 65.68–34.89%, GF 65.15–39.56% and GG 67.26–29.52%. For GB and GA significant decrease ($p <0.001$) at time 0 was recorded. In the group of GCT, increase of the motility at the time 0, but at other times and decrease was significantly. The progressive spermatozoa motility incubated at 37°C in the control GKT ranged from 65.79 to 80.86%. In the group with a commercial diluent GRT ranged from 63.89 to 74.19%, in the group GAT was from 0.54 to 0%. Velocity straight line (VSL) in the GKT group reached value from 81.34 μm/s (time 0) to 74.42 μm/s (time 4). In the monitored parameters a conclusive decrease ($p<0.001$) in groups GBT GAT was detected from time 0.

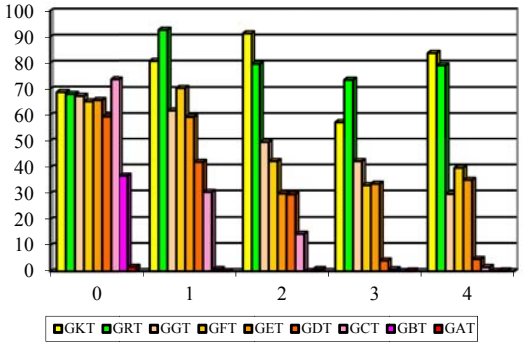

Fig. 2 Spermatozoa motility (%) after glycerol addition at 37^0C in different time periods

Analysis detected that the highest number of morphologically changed spermatozoa occurred in the experimental groups (Figs. 3 and 4). Main changes were twisted tails, tail torso, and tail coiling.

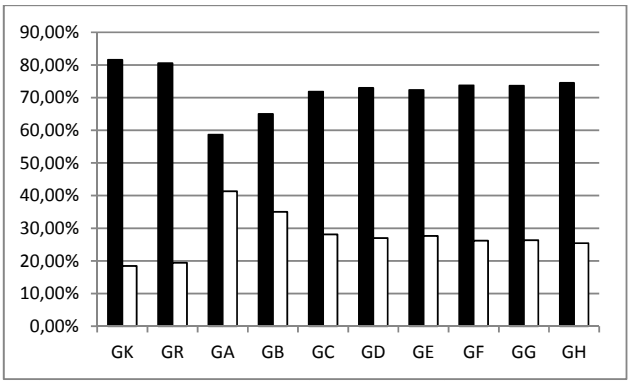

Fig. 3 Occurrence (%) of normal (black) and morphologically changed spermatozoa motility (white) after glycerol addition at 5^0C

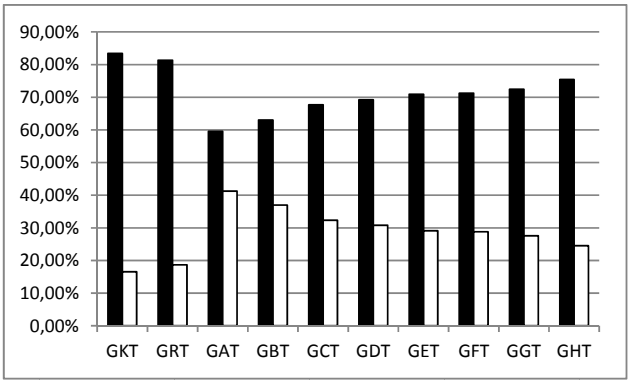

Fig. 4 Occurrence (%) of normal (black) and morphologically changed spermatozoa motility (white) after glycerol addition at 37^0C

In acrosome dominated swelled acrosomes, removed acrosomes and acrosomes with undulated membrane. The

effects of crude glycerol on membrane integrity were also analyzed. We found that the highest crude glycerol concentrations affect spermatozoa integrity. Our study proved that crude glycerol has effect of spermatozoa motility, on the analyzed parameters of spermatozoa motility, viability, and membrane integrity. Detected changes are related to additive concentration, temperature, and time of culture. A similar tendency was detected also in other species. The effect of crude and pure glycerol on rabbit spermatozoa motility parameters *in vitro* was reported [7]. The total spermatozoa motility and progressive motility in experimental groups, diluted with crude glycerol and pure glycerol were significantly lower compared to control group. Glycerol has also negative effect on the velocity curved line parameter, where samples with addition pure glycerol showed a significant decrease values compare to control [8]. The negative effects of high concentrations of crude glycerol on the rabbit spermatozoa motility were also detected. Results of their study show some interesting outcomes – the initial addition of glycerol in higher concentration decreased immediately the spermatozoa motility, but later only a negative effect of higher crude glycerol concentration was found [9]. However, results of studies showed that addition of glycerol to specific extenders had not negative effect on canine and equine spermatozoa motility [10]. The inclusion of 6% glycerol depressed ($p<0.05$) motility of canine spermatozoa, but there was no effect ($P>0.05$) of glycerol concentration on the percentage of motile equine spermatozoa. For both species, the interaction of glycerol level and extender was not significant.

Glycerol has been proposed as a substitute osmotic agent for glucose in peritoneal dialysis fluids. The effect of glycerol on the function of human peritoneal mesothelial cells (HPMC) *in vitro* was studied [11]. The viability of HPMC was not affected by glycerol (up to 250 mM) as assessed by the LDH release. Although the incubation of HPMC with glycerol induced a dose-dependent decrease in HPMC proliferation, the effect was significantly less inhibitory than that produced by glucose. In HPMC treated with 90 mM of glycerol or glucose the incorporation of [3H]-thymidine had reached 79.0±19.3% and 55.3±4.0% of the control ($p<0.05$ and $p<0.01$), respectively. As measured by the [methyl-14C]-choline incorporation, the intracellular amount of newly synthesized phospholipids was reduced from 147±58 in control HPMC to 59±15 in cells exposed to 90 mM of glucose ($p<0.01$), but not affected by glycerol (163±65). On the other hand, both glycerol and glucose (90 mM) decreased the synthesis of proteins (as assessed by the [3H]-proline incorporation) and interfered with potassium (86Rb) transport mechanisms in HPMC. Data suggest that there exist some possibly advantageous aspects of glycerol as far as mesothelial cell biocompatibility profile is concerned.

Clostridium pasteurianum ATCC 6013 achieves high n-butanol production when glycerol is used as the sole carbon source. The homeoviscous membrane response of *C. pasteurianum* ATCC 6013 has been examined through n-butanol challenge experiments. Lipid membrane compositions were examined for glycerol fermentations with n-butanol production, and during cell growth in the absence of n-butanol production, using gas chromatography-mass spectrometry (GC-MS) and proton nuclear magnetic resonance ((1)H-NMR). Membrane stabilization due to homeoviscous response was further examined by surface pressure-area (π-A) analysis of membrane extract monolayers. C. pasteurianum was found to exert a homeoviscous response that was comprised of an increase lipid tail length and a decrease in the percentage of unsaturated fatty acids with increasing n-butanol challenge. This led to a more rigid or stable membrane that counteracted n-butanol fluidization. This report confirms the potential to be engineered as an industrial n-butanol producer using crude glycerol [12].

The effects of dietary crude glycerin on growth performance, carcass characteristics, meat quality indices, and tissue histology in growing pigs were determined in a 138-d feeding trial. Crude glycerin utilized in the trial contained 84.51% glycerin, 11.95% water, 2.91% sodium chloride, and 0.32% methanol. Eight days post weaning, 96 pigs (48 barrows and 48 gilts, average BW of 7.9± 0.4 kg) were allotted to 24 pens (4 pigs/pen), with sex and BW balanced at the start of the experiment. Dietary treatments were 0, 5, and 10% crude glycerin inclusion in corn-soybean meal-based diets and were randomly assigned to pens. Diets were offered ad libitum in meal form and formulated to be equal in ME, sodium, chloride, and Lys, with other AA balanced on an ideal AA basis. Pigs and feeders were weighed every other week to determine ADG, ADFI, and G:F. At the end of the trial, all pigs were scanned using real-time ultrasound and subsequently slaughtered at a commercial abattoir. Blood samples were collected pre transport and at the time of slaughter for plasma metabolite analysis. In addition, kidney, liver, and eye tissues were collected for subsequent examination for lesions characteristic of methanol toxicity. After an overnight chilling of the carcass, loins were removed for meat quality, sensory evaluation, and fatty acid profile analysis. Pig growth, feed intake, and G:F were not affected by dietary treatment. Dietary treatment did not affect 10th-rib back fat, LM area, percent fat free lean, meat quality, or sensory evaluation. Loin ultimate pH was increased ($P = 0.06$) in pigs fed the 5 and 10% crude glycerin compared with pigs fed no crude glycerin (5.65 and 5.65 versus 5.57, respectively). Fatty acid profile of the LM was slightly changed by diet with the LM from pigs fed 10% crude glycerin having less linoleic acid ($P < 0.01$) and more eicosapentaenoic acid ($P = 0.02$) than pigs fed the 0 or 5% crude glycerin diets. Dietary treatment did not affect blood metabolites or frequency of lesions in the examined tissues. Experiment demonstrated that pigs can be fed up to 10% crude glycerin with no effect on pig performance, carcass composition, meat quality, or lesion scores, but the results related to reproductive parameters were not analyzed [13].

IV. Conclusion

In this study, the effects of crude glycerol on canine spermatozoa motility, morphology, viability, and membrane

integrity were analyzed. Results clearly detected that crude glycerol has negative effect on spermatozoa motility. Also highest number of morphologically changed spermatozoa occurred in the experimental groups.

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