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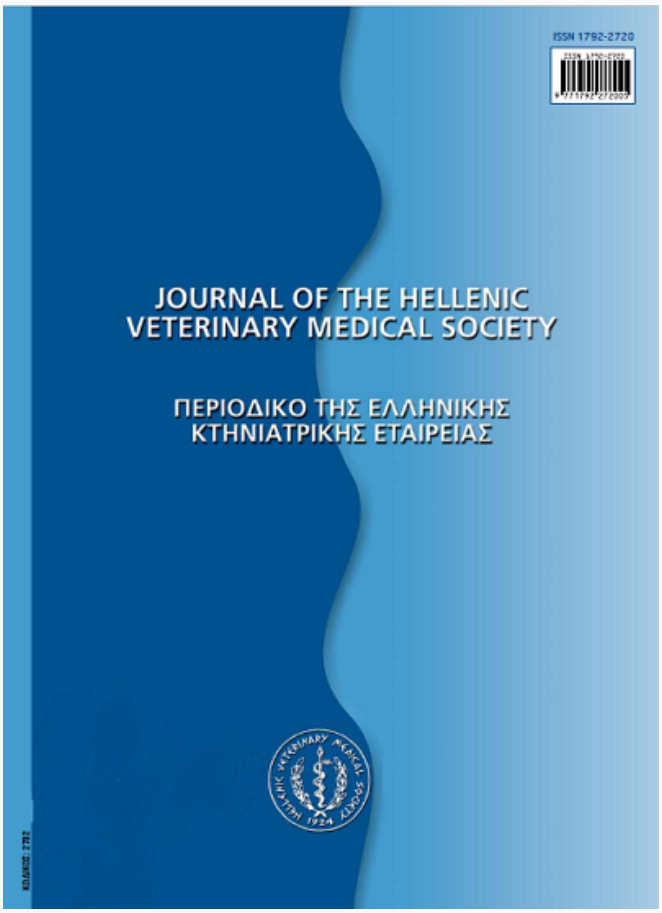

Crude glycerol negatively affects rabbit spermatozoa motility in vitro

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Research article
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Crude glycerol negatively affects rabbit spermatozoa motility in vitro

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ABSTRACT. In recent years the conventional diet for farmed animals has been increasingly substituted by alternative energy sources such as crude glycerol. There is an urgent need for investigation of the impact of crude glycerol on the male reproductive tract. Rabbit semen was cultured in a TRIS/NaCl-based medium containing 0% (control), 5%, 10%, 15% and 20% crude glycerol. Sperm kinetic characteristics were assessed immediately and 30 min, 60 min, 120 min and 180 min upon treatment by computer assisted analyzer. In general, a decrease in motility was recorded for the spermatozoa treated with 5% glycerol ($P<0.001$). A substantial reduction in spermatozoa motility was observed in the samples containing 10% and 15% crude glycerol ($P<0.05$). The lowest motility was observed for spermatozoa incubated with 20% crude glycerol. Presented data suggest toxic effects of crude glycerol on the rabbit spermatozoa kinetics in vitro.

Keywords: *Crude glycerol, Motility, Spermatozoa, Rabbit, Toxicity*

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INTRODUCTION

Glycerol (1, 2, 3-propanetriol) is naturally present in living organisms, from unicellular organisms to the mammalian body (McCabe, 1995). The total glycerol pool in the organism is sustained by intracellular turnover of glucose, proteins, pyruvate and triacylglycerol, or via digestion of dietary fats (Lin, 1977; Bortz et al., 1972). On the other hand, artificial production of glycerol relies solely on industrial synthesis from various sources. The chemical composition of produced glycerol predestines its further application. Pure glycerol contains low amount of chemical residues and has been extensively used as a permeating cryoprotectant since Polge et al. (1949) discovered its protective effects on cryopreserved spermatozoa. Crude glycerol is rich in fatty acids and contains chemical impurities such as methanol, sodium and potassium salts (Retore et al., 2012). Biodiesel industry is constantly producing crude glycerol as a by-product from biofuel fabrication through the NaOH- or KOH-catalysed transesterification of triacylglycerols (Ma and Hanna, 1999; Van Gerpen, 2005; Thompson and He, 2006). Rising energy prices and environmental pollution from the combustion of fossil fuels enforce global production of biodiesel (Ma and Hanna, 1999; Hill et al., 2006; Kurki et al., 2010) so that 2 millions of tons of crude glycerol constantly reach the market every year (Expedito, 2003; Ciriminna et al., 2014). The ready availability of crude glycerol makes it a promising alternative to the energy-rich diet used in animal nutrition, which could cover the energy needs of livestock (Kijora et al., 1995; Rosebrough et al., 1996; Simon et al., 1996; Cerrate et al., 2006). Therefore, numerous studies have focused on assessing the nutritional value of crude glycerol and optimizing its use in animal feeding (Retore et al., 2012). In non-ruminants, including rabbits, glycerol is absorbed through the gastrointestinal tract (Tao et al., 1983) and subsequently utilized as an energy source (Cryer and Bartley, 1973). The cecum in the rabbit digestive tract allows rabbits to be fed a diet enriched by crude glycerol (Mateos and Vidal, 1996). However, crude glycerol as a side product from biodiesel may contain impurities such as alcohols or salts, which are generally considered toxic (Chatzifragkou et al., 2010; Venkataramanan et al., 2012). Monovalent salts are responsible for reducing the van der Waals forces between the lipid tails within the cell membrane (Petrache et al., 2006). Alcohols have been shown to inhibit the membrane ATPase and transport mechanisms (Shimizu et al., 1988). Numerous works suggest toxic actions of alcohols suppressing the transmembrane pH gradient (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985). Although crude glycerol represents a promising alternative to the conventional high-energy diets for rabbits, there is still a lack of data on the toxicity of crude glycerol on the male reproductive tract. Therefore, this study was designed to assess the impact of crude glycerol on rabbit spermatozoa motility in vitro.

MATERIALS AND METHODS

Semen samples were collected from five sexually mature and healthy New Zealand White rabbits using an artificial vagina. The males were housed in cages, allowing free access to fresh water and fed a granular diet ad libitum. Only samples exhibiting spermatozoa total motility ≥ 80% and concentration ≥ 500.106 per mL were forwarded for further manipulation. These five ejaculates were pooled to create one heterospermic sample. In total, three pooled samples were diluted in a sodium citrate-based medium containing 0% (control), 5%, 10%, 15% and 20% crude glycerol (glycerol stock solution composition: 80% glycerol; 7.15% NaCl; 8% water; 0.001% methanol; 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; Cr = 7.5 mg/L) to a final concentration 100-200.106 cells per ml, followed by incubation at 37°C. Motility analyses were performed immediately, 30 min, 60 min, 120 min and 180 min upon treatment. Spermatozoa movement was assessed using the CASA technology consisting of a phase contrast microscope (Olympus BX 51, Japan) with the microscope stage pre-warmed at 37°C, SpermVision v. 3.5 imaging software (Minitüb, Germany) and a Makler Counting Chamber (Sefi-Medical Instruments, Germany). A minimum of 1000 spermatozoa were

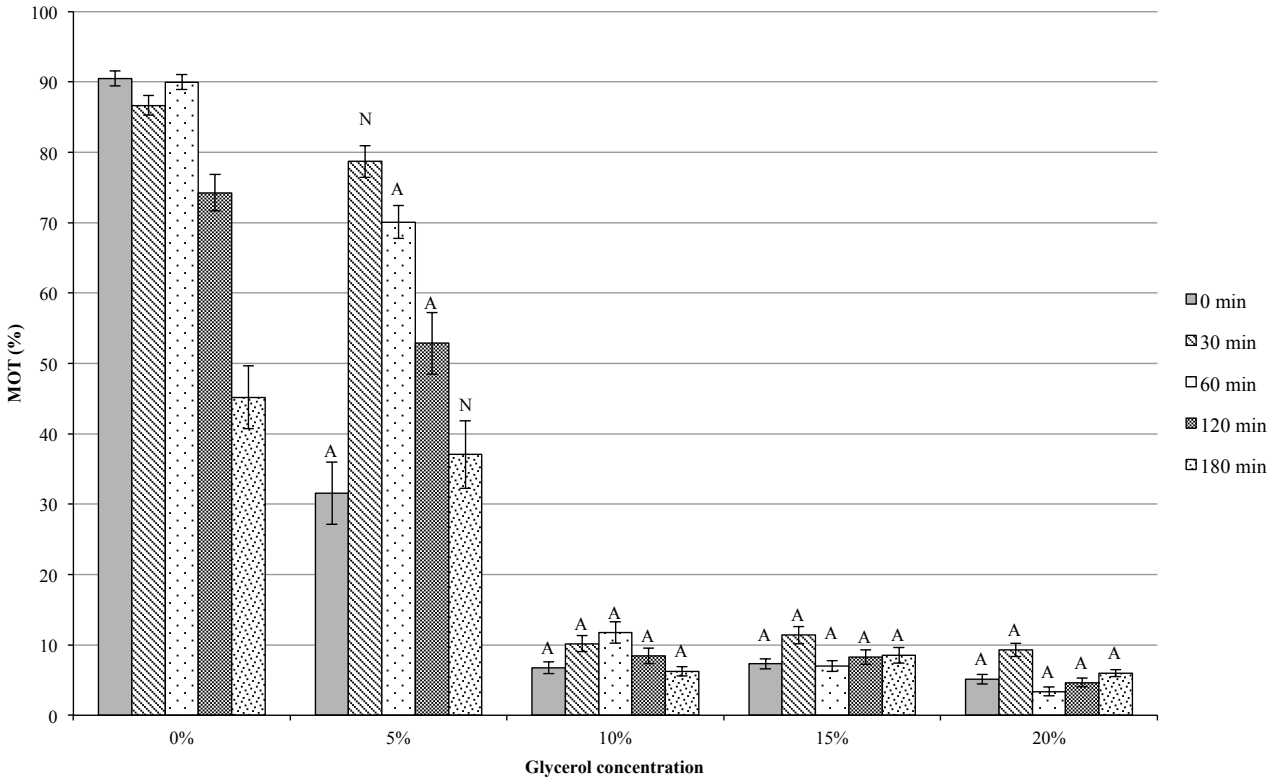

Figure 1. Total spermatozoa motility (MOT) recorded in three pooled samples at incubation times 0, 30, 60, 120 and 180 min after the treatment with crude glycerol at 0%, 5%, 10%, 15% and 20 %. A: P<0.001; N: non-significant. The significance relates to the control group.

recorded at 30 frames per second and analyzed for 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 (VSL/VAPx100), linearity-LIN (VSL/VCLx100), wobble-WOB (VAP/VCLx100), amplitude of lateral head displacement (ALH; μm) and beat-cross frequency (BCF; Hz) as described previously (Massanyi et al., 2008; Roychourdhury and Massanyi, 2008; Paal et al., 2014; Slanina et al., 2015). Total motility (MOT) mirrors the percentage of highly motile spermatozoa (> 5 μm/s) in a sample. Progressive motility (PRO) refers to the forward movement of spermatozoa (> 20 μm/s). The beat-cross frequency (BCF) is the number of times the spermatozoa head crosses the direction of movement, and is related to the development of another flagellum wave. The curvilinear velocity (VCL) refers to the total distance that the spermatozoa head covers in the observation period. The amplitude of lateral head displacement (ALH) is the width of the lateral movement of the spermatozoa head. It is calculated as the total width of the head trajectory and is expressed in micrometers. The straight-line velocity (VSL) is determined from the straight-line distance between the first and last points of the trajectory and gives the net space gain in the observation period. The average path velocity (VAP) is the distance the spermatozoon has travelled in the average direction of movement in the observation period. Other parameters such as linearity (LIN), straightness (STR), and wobble (WOB) describe straightness of spermatozoa movement (Mortimer, 2000). Each measurement was performed threefold. Animals were carefully handled according to the ethical rules of Animal Production Research Centre Nitra.

Statistics

One Way ANOVA followed by Scheffe's test

(P<0.05, P<0.01 and P<0.001) was computed to determine the differences among the controls and treatments. Obtained data are presented as mean ± standard error (SE).

RESULTS

A significant decrease in MOT was observed for the spermatozoa cultured in 5% glycerol compared to control (P<0.001) immediately, 60 min and 120 min upon treatment. The most detrimental impact of crude on MOT was recorded in the samples containing 10%, 15% and 20% glycerol (P<0.001) (Figure 1).

A rapid drop in PRO was recorded in almost all samples treated with 5-20% glycerol over the whole incubation period (P<0.001), except the treatment with 5% glycerol at 180 min. Higher glycerol concentrations (10-20 %) caused that most of the spermatozoa were unable to move progressively (Figure 2).

Regarding the BCF crude glycerol did not induce any changes in the samples containing 5% glycerol compared to control (P>0.001). Nevertheless, higher concentrations (10-20%) considerably decreased BCF (P<0.001) during cultivation (Figure 3).

With respect to VCL, our results revealed a moderate, although not significant decline (P>0.05) in most of spermatozoa cultured with 5% glycerol. However, the treatments with higher glycerol concentrations (10-20%) exhibited a dramatic reduction in VCL (P<0.001). Regardless of concentration used, crude glycerol caused a sharp drop in VCL (P<0.001) immediate upon treatment (Figure 4).

Although 5% glycerol decreased the ALH of treated spermatozoa, recorded differences were not significant compared with the control (P>0.05). However, increasing of glycerol concentration (10-20%) caused a substantial decline in ALH (P<0.001; P<0.01). In general, the most dramatic reduction of ALH was found in the samples cultured with 20% glycerol (Figure 5).

While VSL and VAP were lowered in the samples containing 5% glycerol (P<0.001; P<0.01), higher concentrations decreased the both VSL and VAP considerably (P<0.001). The LIN, STR and WOB were in most cases not significantly affected by any glycerol concentration (P>0.05) (data not present).

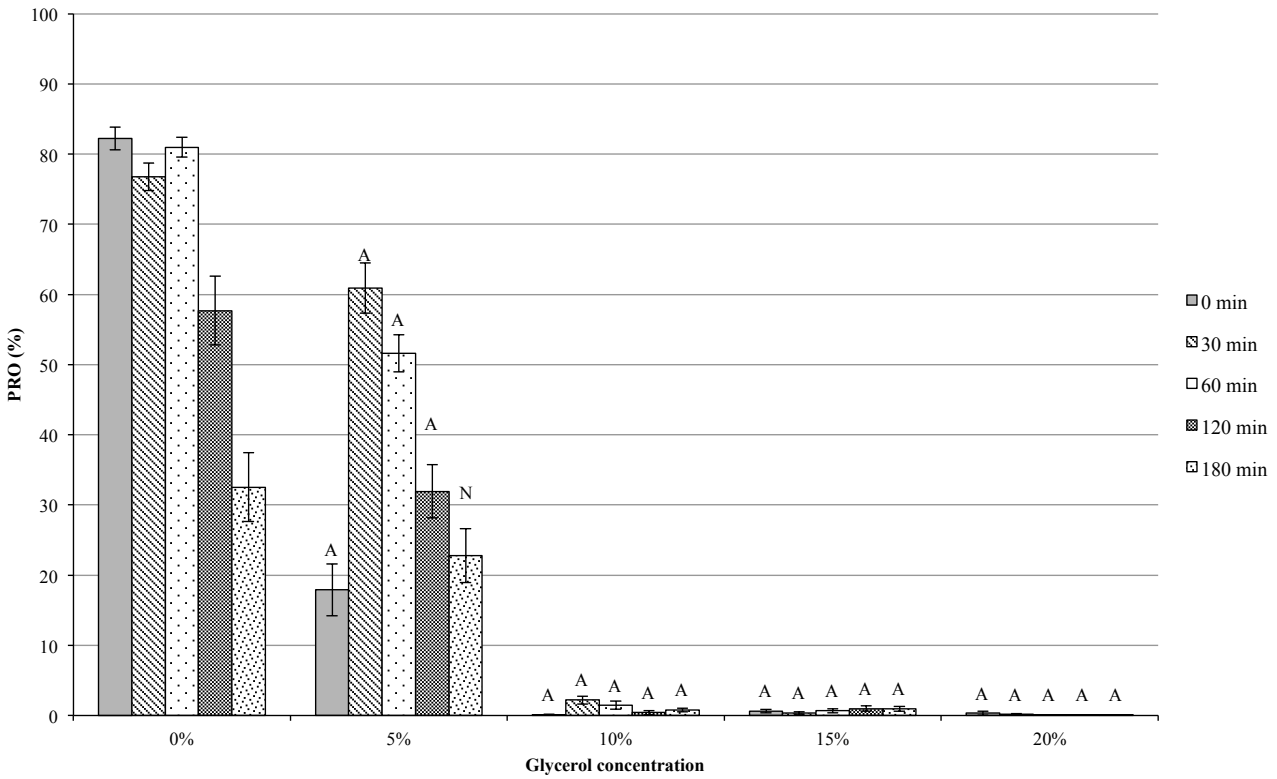

Figure 2. Progressive spermatozoa motility (PRO) recorded in three pooled samples at incubation times 0, 30, 60, 120 and 180 min after the treatment with glycerol at 0%, 5%, 10%, 15% and 20%. A: P<0.001; N: non-significant. The significance relates to the control.

DISCUSSION

The effects of pure glycerol on animal and human spermatozoa have been previously well documented (Kashizawaki et al., 2006; Rosato and Iaffaldano, 2013). This study was designed to investigate the effects of crude glycerol on rabbit spermatozoa motility in vitro. Our results reveal a negative impact of crude glycerol on the spermatozoa kinetics. Rosato and Iaffaldano (2013) observed a beneficial effect of 5% and 10% glycerol on rabbit spermatozoa, where glycerol preserved 60-65% of spermatozoa motility for the first 45 minutes upon treatment. Then, however, the motility dropped to ~40% after 15 min incubation. In our study, similar trends were observed for total motility (~79%) at 5% crude glycerol after 30 min incubation. At the same time, 10% glycerol reduced MOT to ~10%. A work of Kashizawaki et al. (2006) describes detrimental actions of glycerol at 1.0 M on cryopreserved rabbit semen, accompanied with motility decline and disruption of the plasma membrane. According to our findings ≥ 10% crude glycerol led to a rapid drop in the kinetics of treated spermatozoa. These deleterious effects of glycerol were further augmented with time exposition. García et al. (2012) observed a pronounced toxicity of ≥ 3.5% glycerol on stallion spermatozoa in vitro, with the maximal toxicity at 5% glycerol. Compared to our results it can be speculated that the toxicity of a glycerol concentration is species-dependent, supported by findings of Silva et al. (2012), where 5% glycerol still protected frozen-thawed ram spermatozoa against loss of progressive motility. It is generally accepted that the toxicity of cryopermeating agents such as glycerol strongly correlates with increasing concentration and exposure time, as there is time latency for glycerol to permeate spermatozoa and reach equilibrium between extracellular and intracellular environment. The chemical composition of particular permeating substance and species specificity might contribute to the overall crude glycerol toxicity on spermatozoa. Glycerol may induce cellular damage through two distinct mechanisms, either the physical-osmotic effect or the biochemical one (Fahy, 1986, 1987). The permeability of the spermatozoa plasma membrane to glycerol influences subsequent osmotic damage

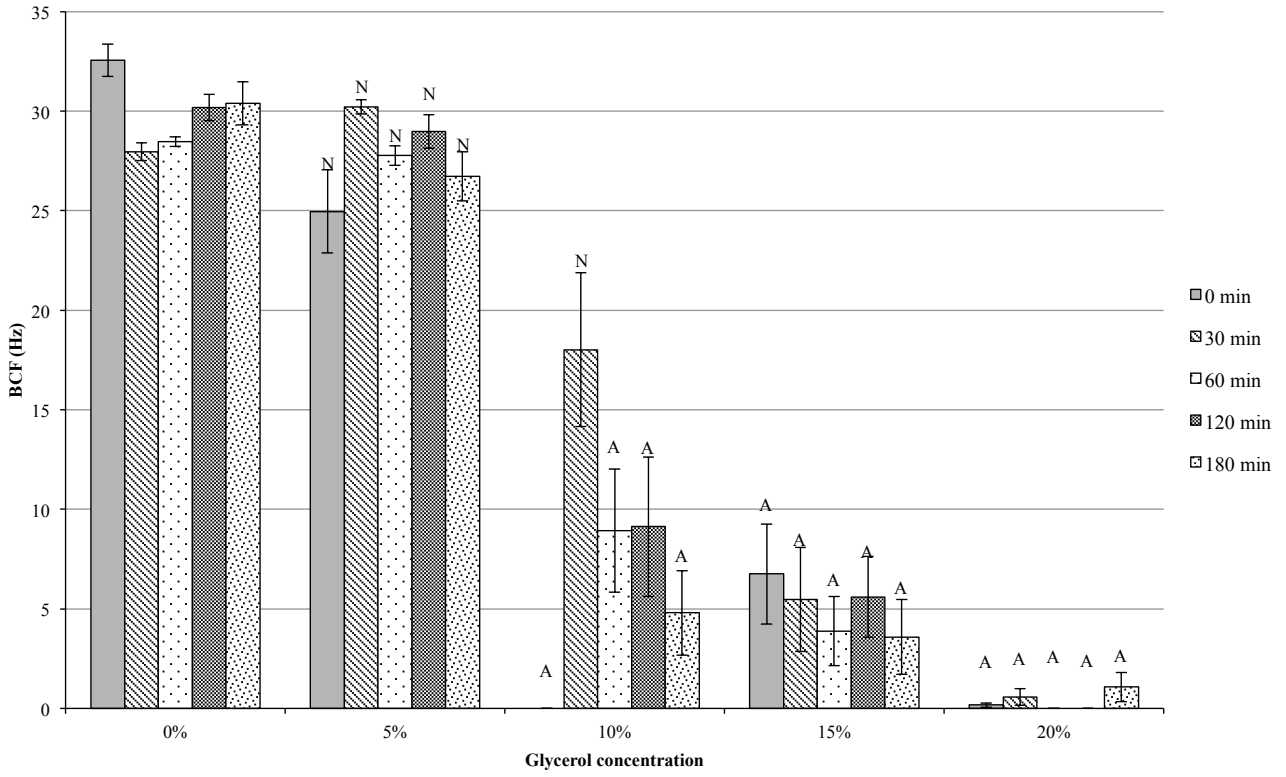

Figure 3. Beat cross frequency (BCF) recorded in three pooled samples at incubation times 0, 30, 60, 120 and 180 min after the treatment with glycerol at 0%, 5%, 10%, 15% and 20%. A: P<0.001; N: non-significant. The significance relates to the control.

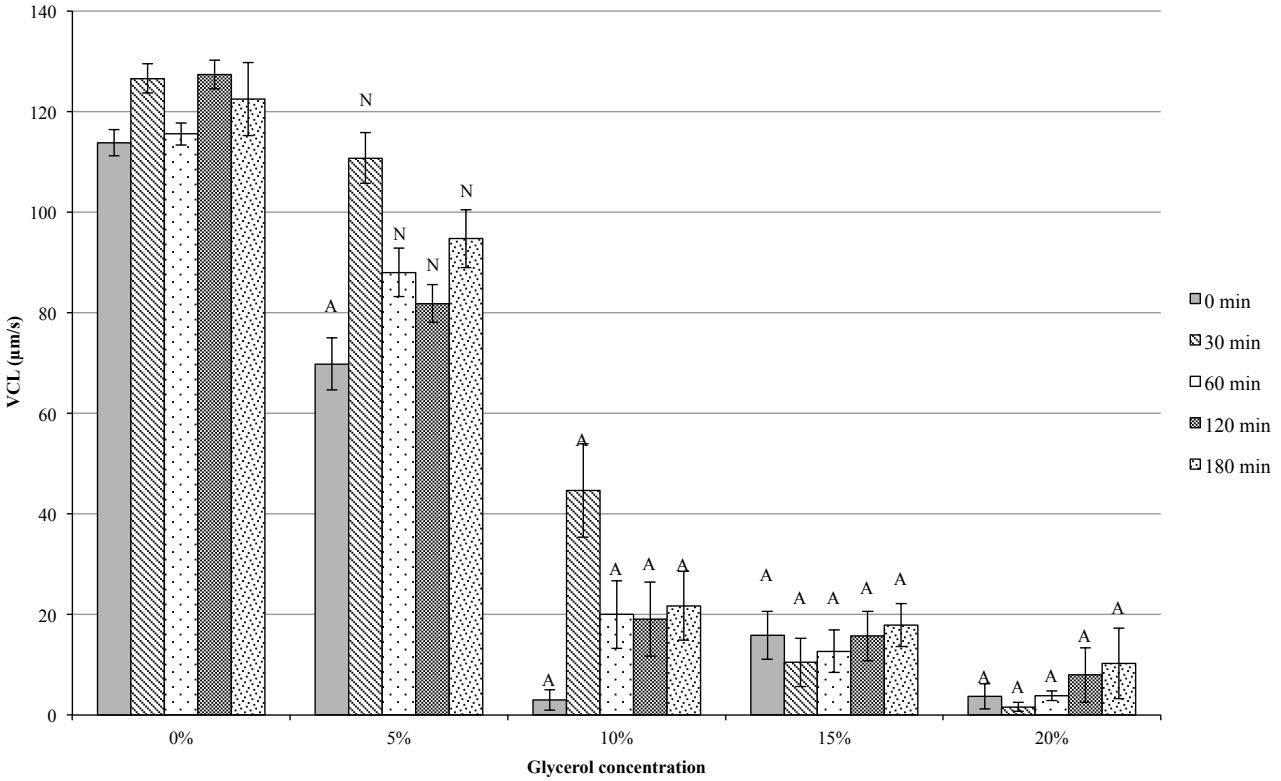

Figure 4. Curvilinear velocity (VCL) recorded in three pooled samples at incubation times 0, 30, 60, 120 and 180 min after the treatment with glycerol at 0%, 5%, 10%, 15% and 20%. A: P<0.001; N: non-significant. The significance relates to the control.

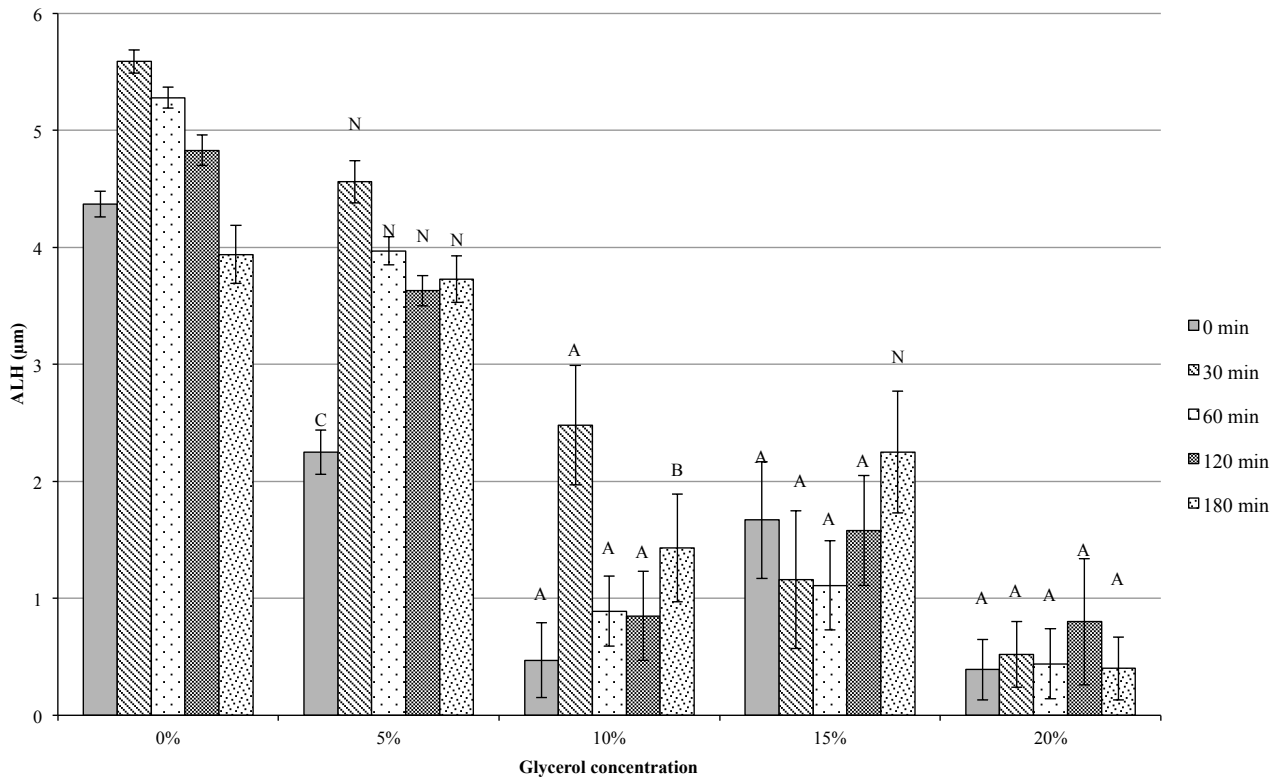

Figure 5. Amplitude lateral head displacement (ALH) recorded in three pooled samples at incubation times 0, 30, 60, 120 and 180 min after the treatment with glycerol at 0%, 5%, 10%, 15% and 20%. A: P<0.001; B: P<0.01; C: P<0.05; N: non-significant. The significance relates to the control.

(Glazar et al., 2009). Concomitant trace chemicals, such as methanol, could also contribute to crude glycerol toxicity to spermatozoa.

CONCLUSION

A little is still known of the toxicity of crude glycerol on the male reproductive tract. Recent studies have revealed a negative impact of >5% crude glycerol on spermatozoa motility. Reported toxic effects might have occurred due to an osmotic-based damage of cell membranes, impaired metabolic functions or signaling defects caused by the presence of deleterious chemical impurities in crude glycerol. Further investigations are urgently needed to elucidate the mechanism of the detrimental effects of high glycerol concentrations on spermatozoa motility.

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CONFLICT OF INTERESTS

None of the authors has any conflict of interests to declare.

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