

IN VITRO EFFECT OF RESORCINOL ON BOVINE SPERMATOZOA IN PROCESS OF CRYOPRESERVATION

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ABSTRACT

The purpose of present study was to observe the effect of resorcinol on bovine spermatozoa before and after cryopreservation. Fresh semen was obtained from six randomly chosen breeding bulls. Experimental samples were prepared by diluting the semen with six different concentrations of resorcinol (REZ1 – 4; REZ2 – 2; REZ3 – 1; REZ4 – 0.5; REZ5 – 0.25 and REZ6 – 0.152 mg.ml⁻¹). Experimental groups were compared against control group (REZK). Using CASA method spermatozoa motility was evaluated before cryopreservation and also after thawing, during incubation at 37°C in time periods 0, 5, 10, 15 and 60 minutes. Negative effect of *in vitro* resorcinol addition was observed in all analysed parameters (MOT, PRO, VCL, ALH and BCF) in groups REZ1, REZ2 and REZ3. In contrast, positive impact mainly on motility and progressive motility was found in experimental groups with the lowest resorcinol concentration addition (REZ4, REZ5 and REZ6) in comparison to the control group. After thawing significant effect of resorcinol on motility parameters were observed within each experimental group. Group with the highest concentration of resorcinol (REZ1) had markedly negative impact on evaluated motility parameters. Results of our study clearly confirm toxic effect of resorcinol on motility parameters of spermatozoa, which depend on concentration and time period.

Keywords: resorcinol, bull, spermatozoa, cryopreservation, CASA

INTRODUCTION

Breeding of animals underwent significant changes in the past years. Insemination is a process which helped in genetic development of direct and indirect utility attributes and can be marked as one of classic biotechnologies. Nowadays, breeders cannot imagine reproduction without artificial insemination. Insemination is executed using fresh diluted semen or conserved semen. Conservation of semen can be done by chilling or freezing process (Diskin, 2018). Aim of cryopreservation is to maintain spermatozoa viability and functionality for decades at sub-zero temperature, despite harmful effects on cell structure (Grötter *et al.*, 2019). During cryopreservation, cells are exposed to osmotic and thermic shocks that underway during dilution, cooling-freezing and thawing process. Mainly cellular membranes are affected during the freezing-thawing process (Andrabi, 2009). Some individuals can have problem with cryotolerance and it only depends on a suitable freezing buffer (Sichtař *et al.*, 2017). Several substances were examined, which can be a potential part of freezing extender (Akhter *et al.*, 2012; Mousavi *et al.*, 2019; Contreras *et al.*, 2019).

Resorcinol (CAS no. 108-46-31; 1,3-benzenediol; *m*-hydroxybenzene; *m*-dihydroxyphenol) is widely used in the manufacture of tires and rubber goods. In a smaller quantities is added to pharmaceutical creams, used for the treatment of acne and to cosmetic products. Its occurrence has been recorded in canned molasses, roasted barley, cigarette smoke and coffee (Lych *et al.*, 2002). Into medical use was resorcinol introduced in 19th century (Welsh *et al.*, 2008). Resorcinol was placed on a list of chemicals with potential of endocrine disruption by the European Union's Committee of Toxicity, Eco-toxicity and the Environment (CSTEE, 2003). Administration of orally taken resorcinol can be very hazardous on human health (Duran *et al.*, 2004). Also it has been reported, that resorcinol can induce various toxic effect on central nervous system followed

by myocardial depression, central necrosis of liver, tubular and glomerular degeneration of kidney (Thienes and Haley, 1972). EFSA established the lowest health-based limit for resorcinol on 0.12 mg/kg bodyweight (EFSA, 2010).

The objective of present *in vitro* study was to evaluate the effect of resorcinol on bull spermatozoa motility parameters before cryopreservation and following thawing.

MATERIAL AND METHODS

Semen collection and processing

Fresh semen was obtained from six randomly chosen breeding bulls using artificial vagina (Slovak Biological Services, a.s., Lužianky, Slovak Republic). Aliquots of ejaculates used as control groups (REZK) were diluted with physiological solution (NaCl 0.9% Braun, B. Braun Melsungen AG, Germany) in ratio 1:40. Experimental samples were prepared according to the same dilution rate, using 6 different concentrations of resorcinol (Table 1), dissolved in the physiological solution. In advance were prepared resorcinol solutions to ensure the proper dissolution of resorcinol.

Cryopreservation and motility analyses

Amount of ejaculate, which was set by spectrophotometer was poured into a beaker in which was prepared 5 ml of thinner. With the use of suction tubes, the straws were filled with ejaculate and subsequently inserted into freezing ramps. Ramps were afterwards inserted in refrigerator, where were kept for 4 – 6 hours to undergo the equilibration process. After equilibration process straws were frozen in freezer by vapours of liquid nitrogen for the duration of 15 minutes. For 15 minutes glycerine based diluent was added and after diluting of the ejaculate,

the activity of spermatozoa was checked. Ejaculate used to be frozen has to have activity of spermatozoa at the level of at least 60%. Semen was inserted in liquid nitrogen in so called “baskets” for 7 minutes. After this time passed the “basket” with the straws inside liquid nitrogen where were stored for 5 minutes. After freezing straws were quickly moved into storage containers filled with liquid nitrogen which were transported to a laboratory after 24 hours. After pulling the samples out of a container, samples were defrosted for 30 seconds at the temperature of 37°C and the motility parameters were measured in time periods of 0, 5, 10, 15 and 60 minutes using CASA system (Gamčík et al., 1992).

Table 1 Concentration of resorcinol

Group	Volume of ejaculate (µl)	Resorcinol (mg)	Physiological saline (µl)
REZK	20	0	800
REZ1	20	4	800
REZ2	20	2	800
REZ3	20	1	800
REZ4	20	0.5	800
REZ5	20	0.25	800
REZ6	20	0.152	800

Semen was evaluated using the Computer assisted semen analysis (CASA) method with SpermVision software (Minitube, Tiefenbach, Germany) and the microscope Olympus BX 51 (Olympus, Japan). Each sample was placed into Makler counting chamber (10 µm, Sefi-Medical Instruments, Germany). Following parameters in each sample were observed: MOT – motility of spermatozoa (%); PRO – progressive motility percentage of spermatozoa (%); VCL – velocity curved line (µm.s⁻¹); ALH – amplitude of lateral head displacement (µm) and BCF – beat cross frequency (Hz) (Tirpák et al., 2019; Halo Jr. et al., 2019).

Statistical analysis

All data were analysed using the Statistical Analyses System (SAS 9.2. using of application Enterprise guide 5.1). Pearson’s correlations between motility parameters of bovine spermatozoa before cryopreservation and after thawing in different time period were used. The level of significance was set at *** (p<0.001), ** (p<0.01) and * (p<0.05) and results were interpreted as means and expressed with SD.

RESULTS AND DISCUSSION

CASA analyses before cryopreservation

Evaluation of motility parameters was realised before cryopreservation. Negative effect of *in vitro* resorcinol addition was observed in all analysed parameters (MOT, PRO, VCL, ALH and BCF) in groups REZ1, REZ2 and REZ3. But positive impact mainly on motility and progressive motility was recorded in experimental groups with the lowest concentrations of resorcinol addition (REZ4, REZ5 and REZ6) in comparison to the control group. (Table 2).

Table 2 Motility parameters of bovine spermatozoa before cryopreservation

Group	Parameter	\bar{X}	SD
REZK	MOT (%)	87.20	4.13
	PRO (%)	83.79	4.27
	VCL (µm.s ⁻¹)	150.64	12.02
	ALH (µm)	4.25	0.29
	BCF (Hz)	36.38	0.87
REZ1	MOT (%)	23.71*	3.19
	PRO (%)	9.37*	5.70
	VCL (µm.s ⁻¹)	82.29*	21.11
	ALH (µm)	3.82	0.53
	BCF (Hz)	21.35*	2.14
REZ2	MOT (%)	65.94*	14.56
	PRO (%)	53.25*	13.88
	VCL (µm.s ⁻¹)	137.29	19.59
	ALH (µm)	4.14	0.50
	BCF (Hz)	27.05*	1.32
REZ3	MOT (%)	63.74*	10.85
	PRO (%)	50.58*	17.98
	VCL (µm.s ⁻¹)	140.32	27.97
	ALH (µm)	3.99	0.71
	BCF (Hz)	28.17*	4.24
REZ4	MOT (%)	94.57	1.94
	PRO (%)	91.23	2.82
	VCL (µm.s ⁻¹)	126.56	2.69
	ALH (µm)	4.66	0.18
	BCF (Hz)	29.81*	1.06

REZ5	MOT (%)	90.25	2.92
	PRO (%)	84.36	3.88
	VCL (µm.s ⁻¹)	135.29	5.41
	ALH (µm)	4.50	0.31
	BCF (Hz)	32.34	1.71
REZ6	MOT (%)	95.15*	1.54
	PRO (%)	91.14	2.43
	VCL (µm.s ⁻¹)	121.75	3.70
	ALH (µm)	5.17	0.30
	BCF (Hz)	28.39*	2.33

Spermatozoa motility

Motility of spermatozoa before cryopreservation was 87.20±4.13% in control group (REZK), after thawing motility decreased to 47.18±11.27%. After thawing (Time 0) spermatozoa motility showed decreased value for all doses of resorcinol in comparison to the control group. After 5 and 10 minutes of the culture significant differences were found (groups REZ1, REZ2, REZ3), and all values in experimental groups were lower compared to control. Only in the group REZ4 value of motility was slightly higher compared to control group after 15 minutes of *in vitro* cultivation with lower values in other experimental groups. Similar tendency as at Times 0, 5, 10 was found at Time 60 (Figure 1).

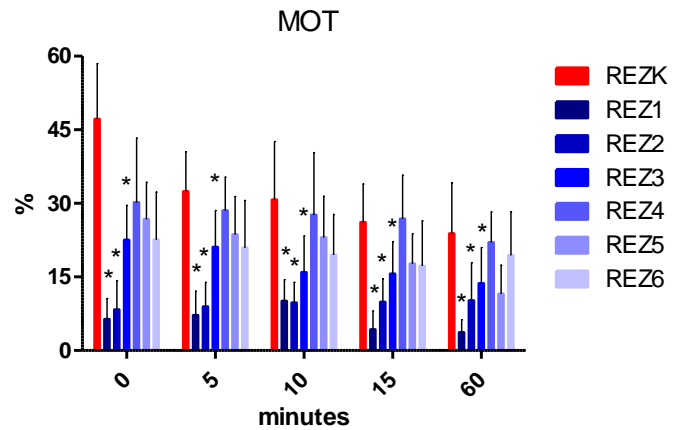


Figure 1 The effect of resorcinol on the total motility (%). (REZK – 0; REZ1– 4; REZ2 – 2; REZ3 – 1; REZ4 – 0.5; REZ5 – 0.25; REZ6 –0.152 mg.ml⁻¹ of resorcinol). Each bar represents the mean (±SD). The level of significance was set at *** (p<0.001), ** (p<0.01) and * (p<0.05).

Progressive spermatozoa motility

Markedly negative effect on spermatozoa progressive motility was found in experimental group with the highest dose of resorcinol (REZ1) during whole cultivation. At Times 0 and 60 the average progressive spermatozoa motility was decreased in all experimental groups compared to the controls (34.73±14.64%; 23.37±7.69%). After 10 and 15 minutes of cultivation slightly increased values in group REZ4 were detected compared to control. After 60 minutes of culture the progressive spermatozoa motility was in the control group (REZK) 12.63 ± 8.64% and no significant differences were found in experimental groups with lower concentration (Figure 2). On the other hand, significant decrease (p<0.05) was detected in groups with higher resorcinol concentration (REZ1, REZ2, REZ3).

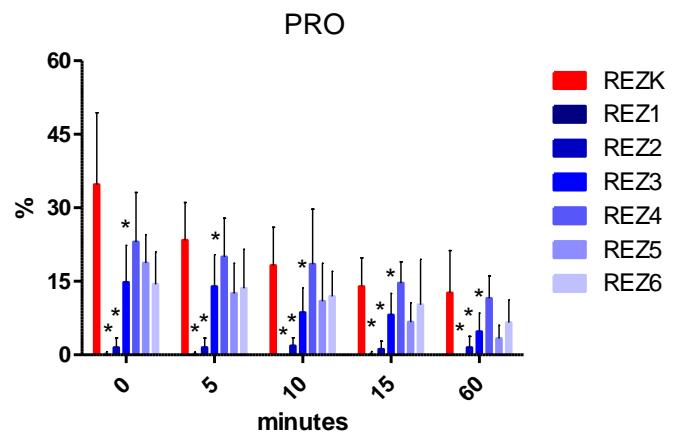


Figure 2 The effect of resorcinol on the progressive motility (%). (REZK – 0; REZ1– 4; REZ2 – 2; REZ3 – 1; REZ4 – 0.5; REZ5 – 0.25; REZ6 –0.152 mg.ml⁻¹

of resorcinol). Each bar represents the mean (\pm SD). The level of significance was set at *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$).

Velocity parameter

After thawing (Time 0) spermatozoa velocity curved line was the highest in the group REZ6 ($121.72\pm 28.91 \mu\text{m}\cdot\text{s}^{-1}$) compared to control group REZK ($111.95\pm 18.67 \mu\text{m}\cdot\text{s}^{-1}$), other experimental groups had lower values of velocity curved line compared to control. Similar trend was observed after 5 minutes of cultivation. At Time 10, 15 and 60 minutes spermatozoa velocity curved line in all doses of resorcinol was decreased in comparison to control group with significant difference in groups with higher resorcinol (REZ1-3) concentration (Figure 3).

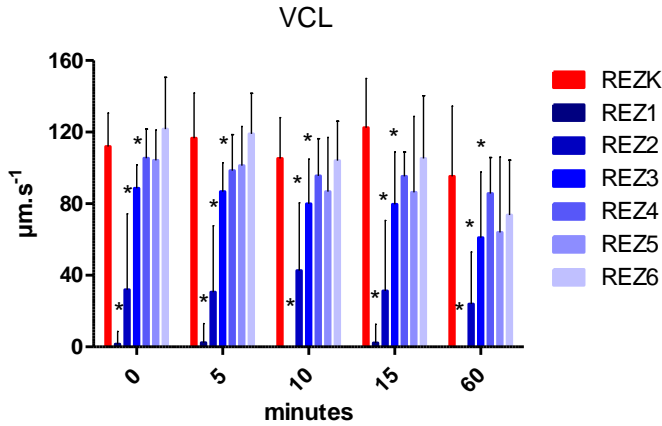


Figure 3 The effect of resorcinol on the velocity curved line ($\mu\text{m}\cdot\text{s}^{-1}$). (REZK – 0; REZ1– 4; REZ2 – 2; REZ3 – 1; REZ4 – 0.5; REZ5 – 0.25; REZ6 – 0.152 mg.ml⁻¹ of resorcinol). Each bar represents the mean (\pm SD). The level of significance was set at *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$).

Other fine motility parameters

At Time 0 amplitude of lateral head displacement in groups REZ5 and REZ6 had increased values compared to control group. This trend was also recorded after 5 minutes of cultivation, but only in the experimental group with the lowest dose of resorcinol (REZ6). After 10, 15 and 60 minutes of cultivation a negative effect of resorcinol on spermatozoa was found. Values of amplitude of lateral head displacement showed no significant differences in groups REZ6, REZ5 and REZ4 (Figure 4).

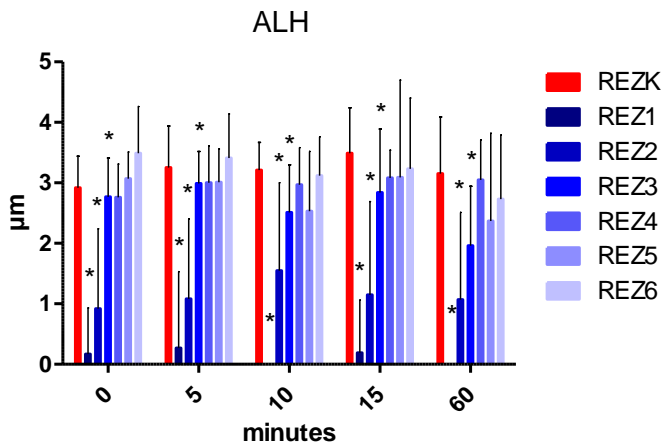


Figure 4 The effect of resorcinol on the amplitude of lateral head displacement (μm). (REZK – 0; REZ1– 4; REZ2 – 2; REZ3 – 1; REZ4 – 0.5; REZ5 – 0.25; REZ6 – 0.152 mg.ml⁻¹ of resorcinol). Each bar represents the mean (\pm SD). The level of significance was set at *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$).

Beat cross frequency (BCF) in control group REZK were between 35.8 Hz (Time 0) and 22.56 Hz (after 60 minutes after thawing). Negative effect of the highest dose of resorcinol (REZ1) was also found on beat cross frequency. This trend was with significant differences is the same groups as for ALH – REZ1 and REZ2 (Figure 5).

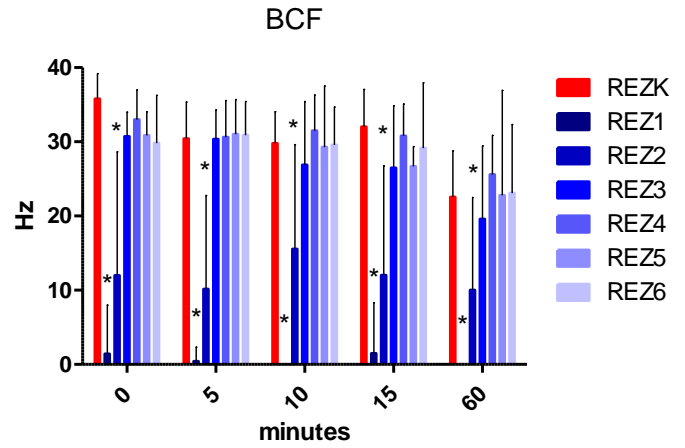


Figure 5 The effect of resorcinol on the beat cross frequency (Hz). (REZK – 0; REZ1– 4; REZ2 – 2; REZ3 – 1; REZ4 – 0.5; REZ5 – 0.25; REZ6 – 0.152 mg.ml⁻¹ of resorcinol). Each bar represents the mean (\pm SD). The level of significance was set at *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$).

Significant $p<0.05$ decrease of values was observed after thawing of samples and following cultivation (Time 0, 5, 10, 15 and 60 minutes) in monitored parameters (MOT, PRO, VCL, ALH and BCF) (Table 3).

Table 3 Differences between motility parameters of bovine spermatozoa before cryopreservation and after thawing in different time period

Group	Parameter	Time					
		0	5	10	15	60	
REZK	MOT (%)	40.02*	54.76*	56.48*	61.05*	63.36*	
	PRO (%)	49.07*	60.43*	65.55*	69.85*	71.17*	
	VCL ($\mu\text{m}\cdot\text{s}^{-1}$)	38.70	34.07	45.40*	28.16	55.43*	
	ALH (μm)	1.34*	1.01*	1.04*	0.77	1.11*	
	BCF (Hz)	0.59	5.97	6.59	4.37	13.82*	
	REZ1	MOT (%)	17.32*	16.56*	13.65*	19.45*	20.03*
		PRO (%)	9.27*	9.27*	9.37*	9.26*	9.37*
VCL ($\mu\text{m}\cdot\text{s}^{-1}$)		80.76*	79.97*	82.30*	80.03*	82.30*	
ALH (μm)		3.66*	3.55*	3.83*	3.64*	3.83*	
BCF (Hz)		19.93*	20.94*	21.36*	19.87*	21.36*	
REZ2		MOT (%)	57.62*	57.03*	56.22*	56.06*	55.77*
		PRO (%)	51.80*	51.79*	51.44*	52.12*	51.78*
	VCL ($\mu\text{m}\cdot\text{s}^{-1}$)	105.42*	106.66*	94.66*	106.01*	113.38*	
	ALH (μm)	3.22*	3.06*	2.59*	2.3*	3.07*	
	BCF (Hz)	15.08	16.91	11.50	15.05	17.05	
	REZ3	MOT (%)	41.24*	42.04*	47.83*	48.11*	50.07*
		PRO (%)	35.81*	36.67*	41.93*	42.43*	45.86*
VCL ($\mu\text{m}\cdot\text{s}^{-1}$)		51.69*	53.65*	60.34*	60.69*	79.37*	
ALH (μm)		1.22	0.99	1.48*	1.15	1.77*	
BCF (Hz)		-2.557	-2.16	1.30	1.69	8.59	
REZ4		MOT (%)	64.39*	66.4*	66.96*	67.75*	72.56*
		PRO (%)	68.19*	71.26*	72.75*	76.63*	79.71*
	VCL ($\mu\text{m}\cdot\text{s}^{-1}$)	21.11	28.09*	31.05*	31.25*	40.92*	
	ALH (μm)	1.90*	1.67*	1.69*	1.58*	1.61*	
	BCF (Hz)	-3.17	-1.81	-1.67	-0.98	4.25	
	REZ5	MOT (%)	63.55*	66.61*	67.24*	72.57*	78.72*
		PRO (%)	65.66*	71.83*	73.41*	77.70*	81.01*
VCL ($\mu\text{m}\cdot\text{s}^{-1}$)		31.10	34.02	48.59*	48.99*	71.34*	
ALH (μm)		1.43	1.49	1.97*	1.41	2.13*	
BCF (Hz)		1.49	1.34	3.07	5.68	9.57	
REZ6		MOT (%)	72.60*	74.23*	75.67*	77.92*	75.79*
		PRO (%)	76.78*	77.57*	79.21*	80.91*	84.58*
	VCL ($\mu\text{m}\cdot\text{s}^{-1}$)	0.03	2.70	17.72	16.42	48.11	
	ALH (μm)	1.68*	1.77*	1.94*	2.05*	2.45*	
	BCF (Hz)	-1.43	-2.49	-1.18	-0.74	5.33	

Legend: (REZK – 0; REZ1– 4; REZ2 – 2; REZ3 – 1; REZ4 – 0.5; REZ5 – 0.25; REZ6 – 0.152 mg.ml⁻¹ of resorcinol). The level of significance was set at *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$).

CASA method (Computer assisted semen analyses) is often used for evaluation of motility parameters (Massányi et al., 2002; 2008; 2011). Analysing of spermatozoa motility is important factor for successful insemination but also for experimental studies (Palacín et al., 2013; Tvrdá et al., 2013).

Rafajová (2011) in her study observed effect of resorcinol on bovine spermatozoa parameters. Spermatozoa motility after *in vitro* addition of resorcinol in amount of 2 mg.ml⁻¹ was 78.95±6.80% and in amount of 1 mg.ml⁻¹ 80.47±8.99% in Time 0 whereas in our study values of motility was 65.94±14.56% and 63.74±10.85%. Welsch et al., (2008) studied two-generation toxicity of resorcinol administered via drinking water to Ctrl:CD(SD) rats. Rats were exposed of 4 different concentration of resorcinol (120, 360, 1000 and 3000 mg.ml⁻¹). After administration of 3000 mg.l⁻¹ of resorcinol had no-observed-adverse-effect, while the 1000 mg.l⁻¹ dose had the no-observed-effect level. None of the reproductive end points observed during two generations were no negatively affected by high resorcinol doses served via drinking water. Cytotoxicity of resorcinol was tested on 3T3 fibroblast for short (3 hours) and long term (72 hours or 6 weeks). Resorcinol in doses of 2500 – 4000 µg.cm⁻³ caused mitochondrial activity inhibition (Skowroń and Zapór, 2004). Study of Burnett et al., (1976) on pregnant Charles River CD rats focused on effect of topical application of hair preparation containing resorcinol did not show negative developmental effects. In following study of Burnett and Goldenthal (1988), where was evaluated effect of topical application of hair-colouring formulations containing resorcinol on Sprague-Dawley rats. There was no adverse effect on fertility observed.

As Lynch et al., (2002) states in his results, no occur of thyroid hormone synthesis after resorcinol administration to rats. In a two year bioassay these effects were not found after doses up to 520 mg/kg/day on rats.

Muñoz et al., (2008) recorded, that total motility in fresh ejaculate of Holstein bulls was 82.3±9.8% and after cryopreservation decreased at Time 0 to 75.8±12.8%, after 2 hours to 69.8±16.3% and after 4 hours to 59.4±20.9%, what confirms decreasing trend of motility after thawing in the control from 87.2±4.13% to 47.18±11.27% in our study.

CONCLUSION

Results from this *in vitro* study confirm, that resorcinol has dose- and time-dependent effect on the spermatozoa. Interestingly, low concentration of resorcinol do not negative effect spermatozoa motility spermatozoa and/or have even positive effect. But it has to be stated that higher concentrations are toxic, resulting in significant decrease of motility parameters.

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