EFFECT OF NATURAL SUBSTANCES AS A POTENTIAL SUBSTITUTE FOR ANTIBIOTICS IN BOAR SEMEN EXTENDER ON SEMEN SURVIVAL TIME

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Abstract

The objective of the experiment was to determine the effect of natural substances as a potential substitute for antibiotics in the boar extender on the semen survival time. Thirteen natural substances were tested. Natural substances were dissolved in 4% DMSO with a minimum bactericidal effective concentration of between 300 and 4,800 μg/ml. The control sample was diluted with a semen dilution ratio of 1:1 in a BTS extender without antibiotics. Sperm motility was evaluated at 0h, 1h and 24h after semen dilution. The best sperm motility was only recorded at thymol 25 % and carvacrol 40 % after 24h storage time. Other tested natural substances had sperm motility between 0 and 5 %. The sperm motility was significantly decreased over the storage time for all the tested natural substances ($P < 0.01$). Results of the present study indicate a negative effect of the concentrations of tested natural substances on boar semen survival time. At present, practical utilization of the natural substances we tested as a potential substitute for antibiotics in boar semen extenders is not possible owing to its reduced sperm motility.

Key Words: Boar, semen, natural substance, survival time

Microorganisms contaminating boar semen are one of the most important factors that negatively affect the biological quality of spermatozoa. Their strong biochemical activity leads to the decrease in energetic sources of seminal plasma and produces toxic metabolites to reproductive cells (Mazurová *et al.,* 2007). Bacterial contamination mainly leads to a series of alterations including diminished sperm motility, sperm agglutination, or «clumping», an increased proportion of altered acrosomes and pH lowering to acidic levels (5.7 – 6.4) (Althouse *et al.,* 2000).

Curative effects of natural substances (NS) have been known for many centuries. Their biological activity includes antimicrobial, antimycotic, antivirus and antiparasitic effects. Therefore NS have great potential for utilization in medicine, food industry, cosmetic and pharmacology (Kalemba and Kunicka, 2003). The systematic screening of antibacterial plant extracts represents a continuous effort on the part of many laboratories to find new compounds with the potential to replace antibiotics (Mazurová *et al.,* 2006).

This study is connected to the work of Mazurová *et al.* (2006; 2007) focused on the bactericidal activity of various NS against microorganisms contaminating raw boar ejaculates. Tested microorganisms isolated from boar ejaculates were *Staphylococcus aureus* CCM 3953, *Escherichia coli* CCM 3954, *Enterococcus faecalis* CCM 4224 and *Pseudomonas aeruginosa* CCM 3955 reference strains, and *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus hyicus* strains. On the basis of assessing the minimum bactericidal activity of NS by Mazurová *et al.* (unpublished), we could study the potential of NS as a substitute for antibiotics in boar extenders. These NS were chosen for our experiment with the minimum bactericidal effective concentration: essential oils (*Carvi aetheroleum, Caryophylli aetheroleum, Terebinthinae aetheroleum, Lavandulae aetheroleum, Foeniculi aetheroleum, Rosmarini aetheroleum, Eucalypti aetheroleum),* organic acid (ethylgallate), natural monoterpene phenol (thymol), organic compounds (hydroquinone, hydroquinone monomethylether), monoterpens (carvacrol) and diterpene (cnicin).

The objective of this study was to investigate the effect of NS as a potential substitute for antibiotics in boar semen extender on semen survival time.

Material and Methods

The NS used in this study were *Carvi aetheroleum, Caryophylli aetheroleum, Terebinthinae aetheroleum, Lavandulae aetheroleum, Foeniculi aetheroleum, Rosmarini aetheroleum, Eucalypti aetheroleum* (prepared in the laboratory of the Department of Pharmaceutical Botany and Ecology, Pharmaceutical Faculty in Hradec Králové, Charles University in Prague) ethylgallate, thymol, hydroquinone, hydroquinone monomethylether, carvacrol, cnicin (Sigma-Aldrich Co., Praha, Czech Republic). Tested effective concentrations of NS are summarized in Table 1. For solution NS were used 4% DMSO (Sigma-Aldrich Co., Praha, Czech Republic) that was prepared by dilution in extender Beltsville Thawing Solution (BTS) without antibiotics (Minitüb, Germany). In Table 2 is presented solubility of NS in 4% DMSO.

Semen was collected using the gloved-hand technique from fertile boars (n=3) with the average values of sperm

motility 87 % and 16.3 % morphologically abnormal spermatozoa. Control sample was prepared from native boar semen and extender BTS without antibiotics in dilution ratio 1:1 and was stored at a temperature 17°C up to 24h. The pH of fresh semen, BTS extender and semen dilution samples were measured at 17°C by means of Microprocessor pH Meter 211 (Martes, Praha, Czech Republic) calibrated with pH 4.01 and 9.21 standard solutions.

Boar sperm survival in the NS was evaluated according to sperm motility. The sperm motility was evaluated subjectively by microscopic estimation of the number of sperm moving in a visual filed of phase-contrast microscopy with a heating stage (38°C) at 100× magnification. Each sample was examined at three different microscopic fields and sperm motility was expressed as percentage of sperm showing normal forward progressive movements. Sperm motility was evaluated at 0h, 1h and 24h after semen dilution.

Basic statistical characteristics of the results were calculated by the QC Expert program (TriloByte Statistical Software, s.r.o., Pardubice, Czech Republic). Statistical significance analysis was checked using the t-test and variance ANOVA - Tukey test at significance levels of $P < 0.05$ and $P < 0.01$.

Results and Discussion

Boar sperm viability in NS was evaluated according to sperm motility, because sperm motility is one of the main parameters of boar sperm quality. Sperm motility is a good indicator of an active metabolism and the integrity of membranes (Johnson *et al*., 2000), and is considered to be of great importance to fertilization. The sperm motility was evaluated only during 24h because the reached motility below 30% of normal motile sperm signifies the end of survival period.

Changes in semen survival time after the adding of the NS into boar semen are shown in Table 2. The same sperm motility as in control sample (70 %) was assessment at *Terebinthinae aetheroleum,* thymol and carvacrol after dilution (0h). A statistically significant decrease of sperm motility were observed in *Lavandulae aetheroleum, Eucalypti aetheroleum*, *Rosmarini aetheroleum,* hydroquinone monomethylether ($P < 0.05$) and in *Caryophylli aetheroleum,* ethylgallate and hydroquinone ($P < 0.01$) in comparison with control sample after dilution (0h). Reduced sperm motility was also noted during the first hour after semen dilution with NS where only carvarol had the same sperm motility as control sample. Sperm motility was significantly decreased in thymol about 33 % and hydroquinone monomethylether about 50 % in comparison with control sample after 1h storage time ($P < 0.01$). In the other NS were detected significant differences in sperm motility at 1h and 24h storage time in comparison with control sample ($P < 0.01$). Sperm motility was only recorded in thymol 25 % and carvarol 40 % after 24h storage time. Sperm motility was significantly decreased over the storage period for all the tested NS ($P < 0.01$).

The presented results document that the addition of the NS we tested has a negative effect on boar sperm viability during short storage time, because sperm motility was significantly reduced during storage time. Averaged sperm motility was before dilution 87 % which is typical for fresh boar ejaculates (Strzeżek *et al.,* 1998). Britt *et al.* (1999) stated that sperm motility scores of ≤ 60 % lead to fewer fertilized eggs and lower farrowing rates. Smital (2003) noted average values of sperm motility between 63 and 69 % after a 24h storage time for different breeds of boars. In this study, thymol and carvarol only showed the sperm motility above 25 % after 24h storage period. Other tested NS had sperm motility between 0 and 5 %. Malo *et al.* (2011) studied the antioxidant effect of rosemary (*Rosmarinus officinalis*) on protecting of epididymal spermatozoa during cryopreservation. Their results showed that the rosemary (10g/100ml) as added supplement to the freezing extender has a significant beneficial effect on improving sperm motility and preventing spermatozoa peroxidation after thawing.

Sperm motility could be also influenced by the incomplete solubility of the NS, because thymol formed the suspension, essential oils formed the emulsion and cnicin was insoluble in 4% DMSO. The similar results in insolubility of cnicin, thymol were noted by Mazurová *et al.* (2007) and Sobková (2009) in solvent DMSO. However, the minimum bactericidal concentrations of the NS used against microorganisms contaminating boar ejaculates probably also reduced the viability of boar spermatozoa.

In our study, we were also able to rule out the influence of 4% DMSO as an NS solution on sperm motility. The quantity of 4% DMSO that was used had no influence on sperm motility ($P < 0.01$). Short-term BTS without antibiotics was chosen as extender. Classical extender BTS with antibiotics is commonly used for liquid storage of porcine semen and can keep fresh semen in good conditions for at least 72h.

The values measured of pH was 7.22, 7.20 pH in the fresh boar ejaculate and in control sample, respectively. The initial pH of semen dilution samples in NS are shown in Table 2. The pH values of semen dilution samples in NS were detected between 8.46 and 8.81 pH. The fresh boar ejaculate had 7.22 pH. Johnson *et al.* (2000) mentioned that the pH of freshly ejaculated boar semen varies between 7.20 and 7.50 and also found a reduction of sperm motility and metabolism for pH values under 7.20. In the semen dilution samples in the NS was assessment pH between 8.46 and 8.81 pH in comparison with control sample. Gatti *et al.* (1993) found that internal pH of porcine sperm cells changed rapidly towards the pH of the dilution medium. So spermatozoa kept in a medium with higher pH will have a higher basal motility at storage temperature. In our experiment we observed the higher initial values pH of semen samples after adding of the NS in comparison with control sample. Thence sperm motility could be affect pH of the NS.

Table 1. Tested minimum bactericidal concentration of natural substances on boar sperm motility

Natural substance	Concentration (µg/ml)
Carvi aetheroleum	4,800
Caryophylli aetheroleum	4,800
Terebinthinae aetheroleum	4,800
Lavandulae aetheroleum	4,800
Foeniculi aetheroleum	4,800
Rosmarini aetheroleum	4,800
Eucalypti aetheroleum	4,800
Ethylgallate	4,800
Thymol	1,200
Hydroquinone monomethylether	2,400
Hydroquinone	4,800
Carvacrol	300
Cnicin	3,000

Table 2. Effect of minimum bactericidal concentration of natural substances on pH and sperm motility (mean values)

		Sperm motility (%)		
Natural substance	**pH**	**0h**	**1h**	**24h**
Carvi aetheroleum [1]	8.55	50.00	1.68 ^{c}	0^{c}
Caryophylli aetheroleum [1]	-	0^{c}	0 ^{c}	0^{c}
Terebinthinae aetheroleum [1]	8.76	70.00	3.33 ^{c}	1.67 ^{c}
Lavandulae aetheroleum [1]	8.53	40.00 ^{c}	3.33 ^{c}	0 ^{c}
Foeniculi aetheroleum [1]	8.65	45.00	1.67 ^{c}	1.67 ^{c}
Rosmarini aetheroleum [1]	8.61	40.00 ^{c}	0^{c}	0^{c}
Eucalypti aetheroleum [1]	8.74	30.00 ^{c}	0^{c}	0^{c}
Ethylgallate[4]	-	0 ^{c}	0^{c}	0^{c}
Thymol [2]	8.79	70.00	40.00 ^{c}	25.00^{c}
Hydroquinone monomethylether[4]	8.81	40.00 ^{b}	30.00 ^{c}	5.00 ^{c}
Hydroquinone[4]	-	0 ^{c}	0 ^{c}	0 ^{c}
Carvacrol[4]	-	70.00	60.00	40.00 ^{c}
Cnicin [3]	8.46	56.60	0 ^{c}	0 ^{c}
Control sample	7.20	70.00 ^{a}	60.00 ^{a}	60.00 ^{a}

^{a,b,c} Means with different superscript letters within a column differ among sperm motility of control sample and sperm motility in natural substances: ^{a,b} $P < 0.05$, ^{a,c} $P < 0.01$.
[1,2,3,4] Solubility of the natural substances in 4% DMSO: [1] emulsion, [2]suspension, [3]insoluble, [4]soluble.

Conclusion

The present study showed the negative effect of the NS we tested on boar sperm motility when being stored. Higher sperm motility would need to be achieved for practical AI utilization. At present, practical utilization of the NS as a potential substitute for antibiotics in boar semen extenders is not possible owing to its reduced sperm motility. Therefore, it is necessary to research other NS and effective concentrations thereof for a possible replacement for antibiotics in boar extenders.

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This study was supported by Ministry of Agriculture of the Czech Republic QI111A166.