Improving liquid semen preservation and fertilizing ability of frozen-thawed Mangalica semen with antioxidant treatments

Verbesserung der Flüssig- und Tiefgefrierkonservierung von Samen von Mangalica Ebern durch Behandlung mit Antioxidanten

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Introduction

It is essential to introduce up to date keeping, feeding and reproductive technologies in native farm animal breeding too for improving productivity of them. Selection before breeding is one of the most important for genetic progress. In pig sector boars are selected for high fertility. The best result can be realized, if the breeds are characterized in details, for example anatomical parameters of the genital tracts are described, semen production, and quantitative and qualitative parameters of the ejaculate are controlled regularly, furthermore liquid preservation of the semen are tested.

Aim of the study was to adapt modern zootechnological-biotechnological applications for the breeding of native Mangalica pig. These methods could be beneficial for production and commercial success in breeding. It is necessary to obtain breed-specific reproductive biological characterization of Mangalica in both sexes.

In contrast to female Mangalica pigs we had no previous information about the reproductive parameters of Mangalica boars, however the male animals have almost the same influence on the success of reproduction as the females. Furthermore with this new knowledge it becomes feasible to elucidate the causes of possible differences between boars of Mangalica and modern breeds.

We planned to elaborate a special treatment of semen which can help to protect membrane structures of the spermatozoa during liquid preservation and before cryo-conservation. Recently there is no routinely used method for cryo-conservation of boar semen which yields comparable results to the insemination with fresh semen. During semen freezing occur several detrimental processes, one of the most important is the peroxidation of the sperm plasma membranes' phospholipids. In the project we planned to develop/adapt a pre-freezing treatment of the semen using previous experiences in this field to improve the freezability of boar semen.

A further goal was to detect pregnancy as soon as possible after insemination in Mangalica sows. Ultrasound investigation was planned between days 23-28 after AI and repeated checking was outlined if it was necessary.

Material and methods

Semen conservation experiments were performed with semen from 4 mature boars in 4 replicates. Mixed samples of fresh ejaculates from 4 boars were liquid preserved. Concentration was determined in Buerker-chamber and adjusted to $0.1x10^9$ sperm cells/ml. After dilution different antioxidants were added to the samples. The treated semen was stored at 16 ^oC for 8 days, motility was checked daily with a computer assisted semen analyzer (CASA).

Freezing trial 1

Freezing experiments were carried out with ejaculates of selected boars using the method of Westendorf et al. (1975). Semen samples were loaded in 0.5 ml straws and frozen in a programmable freezer (Planer Cryo 360-1.7). After thawing samples were diluted with BTS to $0.1x10^9$ sperm cells/ml then motility parameters were evaluated in Makler-chamber with CASA. Acrosomal damages were estimated after fluorescence staining.

Insemination trial 1

Estrus of 10 Mangalica gilts was synchronized by Regumate feeding (15 days, 20 mg/animal/day), 24 hours after the last Regumate feeding 1000 IU eCG (Folligon) was injected to stimulate follicular development and 72 hours later ovulation was induced by 50µg GnRH (Fertagyl). Laparoscopic insemination (LAI) was performed 34 h after GnRH. Straws were thawed at 50°C for 12 s and diluted 1 to 1 in BTS. Motility was checked under phase-contrast microscope before insemination. Two-two straws per uterine horn were used. Pregnancy check was done by ultrasonic on 28 days after LAI.

According to the poor results of insemination trial 1 the protocol was adapted for

Freezing trial 2

During this trial period 153 ejaculates from 16 Mangalica boars were frozen. Six boars were discarded due to above mentioned reasons or not appropriate semen parameters. Boars were selected according to the following parameters from 3 ejaculates: >80% motility 0 h; >70% motility 24 h and <15% all sperm morphological defects.

Freezing protocol (Egerszegi et al., 2009):

The ejaculate was diluted 1:1 with Standard (Pigletplusz 2004 Ltd.) extender after microscopic evaluation. Diluted semen was incubated at 15 °C for 3 hours, then it was centrifuged with 400xg at 15 °C for 10 min. The pellet was re-diluted with lactose-egg yolk extender (LEY) and incubated another 2 hours at 5 °C. The final concentration was adjusted with LEY+glycerine+equex es paste combination for 10^9 sperm cells/ml. Straws were frozen on N_2 vapour (4 cm) for 8 min then plunged to liquid N_2.

Motility assessment was performed after thawing at 38 °C for 20 s and a second time after 10 min incubation at 38 °C. Morphology, live and dead cells were evaluated after staining as described by Kovács and Foote (1992).

<u>Insemination trial 2</u>

Altogether 64 sows were inseminated with frozen/thawed semen cervically, whereas the deep intra-uterine method could not be used because of the nervous, sometimes aggressive behavior of the Mangalica sows. More than three years of experience in artificial insemination of Mangalica showed that 10-15% of the sows were unsuitable for AI. So we decided to use the cervical method in our program.

Selection of the sows:

Estrus sows were searched twice a day with teaser boar in the morning and late afternoon. Selected sows were checked with ultrasound and were inseminated with 2.5x 10^9 motile cells AI dose 24 and 36 h after selection.

Semen preparation for AI:

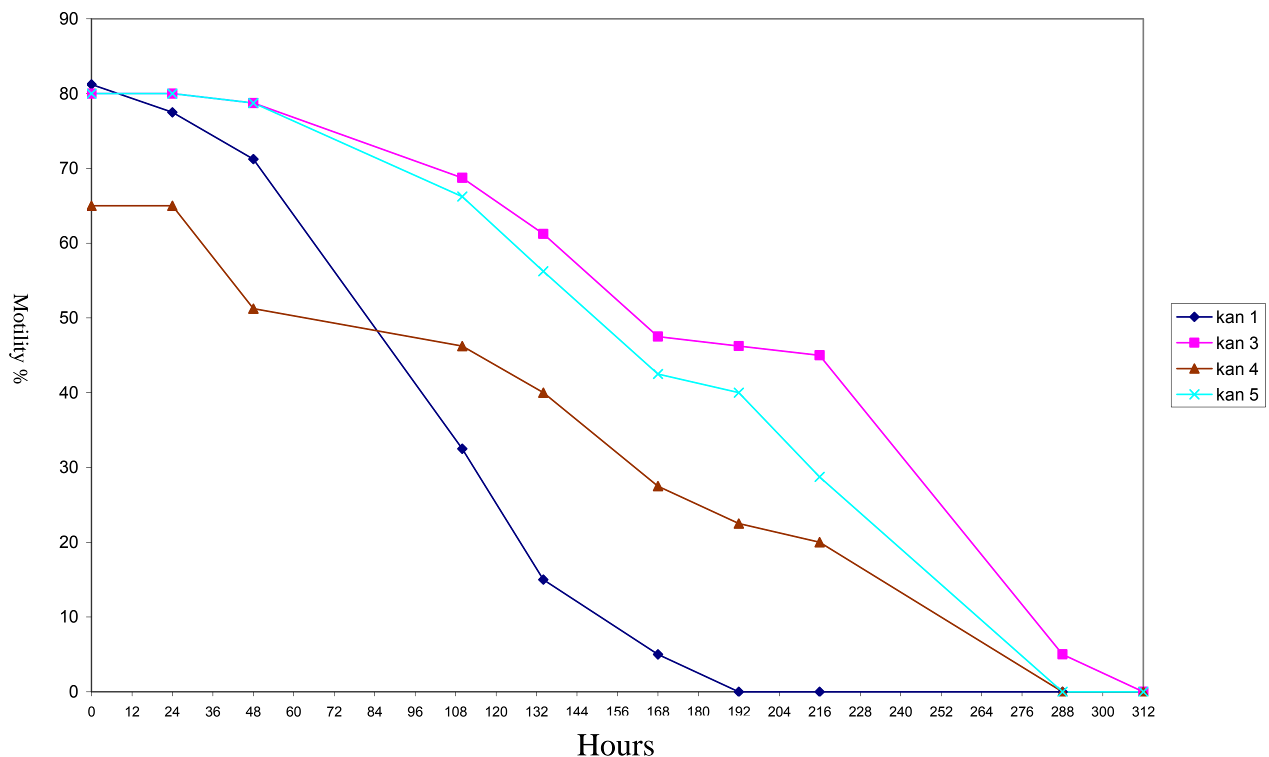
Ten straws per AI dose were thawed at 38°C for 20 s and introduced to 80 ml (38°C) Standard extender. AI was performed in 10-15 minutes after thawing.

Pregnancy diagnoses were carried out with transcutan ultrasonography (Ambisea AV-2100 portable ultrasound, 3,5 MHz sector probe) 28 days after AI.

Results

Semen motility during the preservation period was illustrated graphically (Figure 1.). The time when motility was down to 60% (trading threshold value) was: Boar 1. 79 h, Boar 3. 146 h, Boar 4. 35 h, Boar 5. 141 h.

Figure 1: Liquid preservation of Mangalica semen at 16 °C



The first freezing experiments could be considered as preliminary trials only. All steps during the freezing process have a specific temperature, which needs to be kept stable. The lack of a cooling cabinet during the filling of straws led to fluctuations in temperature, which damage the plasma membranes of the sperm cells. We performed six replicates of freezing during the trial, the highest motility after thawing being 30-35%. For this reason we modified the insemination protocol due too low motility. Insemination dose was increased to $7x10^9$ cells/ml and conventional catheter or $1x10^9$ cells/ml for endosopic intra-uterine insemination.

In the first insemination trial positive pregnancy diagnoses were found in 4 animals and 1 uncertain was recorded. Two weeks later just 3 conceptions were noticed, and after 114-116 days pregnancy these sows farrowed 5-6-5 piglets respectively.

Freezing trial 2

Motility parameters of the thawed samples are presented in table 1. Motility percent was recorded between 34.33-74.75%, whilst progressive motility was detected between 25.5-58%. Boars were classified to three groups based on motility (table 2).

Boar ID	Motility		Progressive mot		VSL		VCL		LIN		WOB	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	37	1,412	25,59	1,510	35,33	0,535	112	2,316	0,315	0,005	0,453	0,004
2	50	7,000	34,33	12,170	42,87	10,073	116	32,646	0,39	0,046	0,543	0,035
3	52,8	2,979	32,3	2,998	34,42	0,912	115	3,920	0,298	0,005	0,447	0,005
4	48,14	3,321	27,71	3,498	32,35	1,227	104	6,208	0,318	0,015	0,449	0,011
17	34,33	1,764	26	1,528	51,69	3,931	113	14,214	0,46	0,023	0,55	0,020
21	68,6	4,534	57,4	4,106	47,89	4,085	148	10,556	0,322	0,019	0,442	0,016
22	47,5	4,921	32,25	2,213	44,86	7,292	119	13,004	0,363	0,028	0,465	0,015
23	73,5	1,500	41,5	10,500	29,52	2,365	84,2	5,105	0,345	0,005	0,435	0,025
27	57,5	8,500	25,5	22,500	25,33	8,155	79,2	6,735	0,305	0,075	0,41	0,060
54	55,78	1,115	42,44	2,129	55,73	1,397	123	4,544	0,453	0,019	0,537	0,013
55	55,75	4,802	44,25	4,328	46,41	4,851	126	5,839	0,365	0,030	0,47	0,018
57	74,75	0,750	58	0,913	34,75	0,869	122	4,136	0,28	0,014	0,425	0,012
58	58,33	5,044	40,33	7,881	45,23	4,808	120	7,679	0,37	0,023	0,473	0,013
1215	45,25	4,246	30,88	3,921	42,9	3,358	107	8,899	0,406	0,031	0,496	0,020

Table 1 Motility parameters of frozen/thawed Mangalica semen

Table 2 Boars classified by post-thaw motility

Boar ID	Motility	Class
1	37	weak
17	34,33	weak
1215	45,25	avarage
22	47,5	average
4	48,14	avarage
2	50	avarage
3	52,8	avarage
55	55,75	avarage
54	55,78	avarage
27	57,5	avarage
58	58,33	avarage
21	68,6	top
23	73,5	top
57	74,75	top

Under 45% motility boars were classified as weak, between 45-60% motility they were rated as averagely freezable. Only 4 boars were included in the top freezability group. Investigating seasonal changes in freezability we observed the same tendencies, nevertheless no significant differences in frozen semen quality were noticed in the individual boar, but between boars the differences were significant ($p < 0,05$).

Acrosome status and rate of dead or live cells were determined after Kovács-Foote staining. An important and valuable result for the routine is, that the rate of live motile cells with intact acrosomes is 10% less than the total of motile spermatozoa. Several motile cells had a damaged head, which are incompetent in the fertilization process.

Mean motility parameters after thawing are showed in table 1. 3 individual boars (21, 23, 57), which were characterized by high post-thaw motility (>60%) and the highest progressive motility could be identified, however the rate of live-intact cells was less than in the average freezable group. Considering velocity parameters of motile cells, more the situation was more variable. Some boars, which were classified previously as “top”, were only “average” (57) or “weak” (23). Furthermore if we consider motility and velocity parameters together some average boars graded up to a higher quality group. This seems to be important because several authors report that velocity parameters are related positively to fertility.

In liquid preservation several antioxidants were tested for acrosomal protection. In the freezing experiments the most effective antioxidants in the right concentration were used; 0,5 mg α-tocopherol/$0,2x10^9$ spermatozoa/ml; 1 µg resveretrol / $0,2x10^9$ spermatozoa/ml (R) és 1 µg ubiquinon / $0,2x10^9$ spermatozoa/ml (Q_{10}). R and Q_{10} treatments gave the best solution; samples treated with them had 10% higher motility and live-intact cell rate.

Insemination trial 2

Thirty sows (46.87%) were found pregnant on day 28, however only 29.63% (19) farrowed after 113-116 days pregnancy. Mean litter-size was 6.2 piglets, which is normal in Mangalica.

IMPLICATIONS AND RECOMMENDATIONS FOR THE PRACTICE

- It was found that volume of the ejaculate from Mangalica boars is less (x=177.8 ml, *s=18.92*), the mean concentration ($490x10^6$/ml, *s=*$160x10^6$*/ml*) the total cell number in ejaculate ($894x10^8$, *s=*$308,1x10^8$) are much higher than in commercial pig breeds.

- Six semen extenders were check for liquid preservation of Mangalica semen, from which the best result was obtained with Standard extender. Live motile cells percentage was over 60% after 3 days preservation.

- High genetic value breeding boars (80%) which are mainly used for natural mating semen can be collected on sows in heat for gene banking and conservation breeding purposes.

- A freezing protocol was adapted for Mangalica semen, which can be used in field conditions.

- For cervical insemination of Mangalica sows with frozen/thawed semen it is necessary to use a higher concentration per dose ($5x10^9$ cells) and double insemination after 12 to 24 hours (20 straws/animal total). The sows in heat should be inseminated after ultrasound check approximately 24 and 36 hours after onset of estrus with 2.5×10^9 motile sperm cells /dose.

- Early pregnancy check should be done first after 28 days after conception.