Thesis of the Ph.D. dissertation

GELATINES, ANTIOXIDANTS AND THE EFFECT OF ENVIRONMENTAL FACTORS ON THE ANDROLOGICAL PARAMETER OF RAMS

Author:
Csilla Budai
Ph.D. candidate

Supervisors:
Dr. András Kovács, D.Sc.
Dr. József Rátky, D.Sc.

UNIVERSITY OF DEBRECEN
Doctoral School of Animal Husbandry

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I. BACKGROUND AND OBJECTIVES OF THE DOCTORAL THESIS

In Hungary meat has the greatest economic importance in the sheep industry. Nowadays, when artificial insemination is used, meat production can be increased throughout enhancing the ability of individual meat performance and also by increasing reproductive capacity. In countries where animal breeding has a strong historical bacground artificial insemination become more widely adopted in oder to preserve their competitiveness. By using this biotechnological method, in short time interval a rapid and efficient genetic improvement can be done (PÉCSI, 2007). Sheep with outstanding production could produce more offspring when artificial insemination is used compared to average breeding conditions (VASS et al., 2008). By deviding the ejaculate, the number of breeding rams can be reduced, therefore the selection pressure can be increased in the flock. By using rams with good body conformation, constitution and lifetime production, it allows the breeder to establish a flock with a production that fits to the markets demand (PÉCSI, 2007). Artificial insemination has an important role in intensive animal breeding and also in the protection of the native breeds (SOLTI et al., 2000; CSEH and SOLTI, 2001).

In the past 30 years the usage of biotechnological methods decreased in Hungary among the breeders because of several reasons, although gamet freezing has a history of more than 50 years. In 1960's nearly 60% of the Hungarian sheep flocks was involved in AI programs what nowadays nearly reaches 2% (KUKOVICS and GERGÁTZ, 2009). The use of artificial insemination method would be necessary among the breeds that are used as terminal sires. In cattle breeding the selection of bulls for semen production and semen freezing is a tradition since many generations, while in rams it is still a task to be carried out. The fertility results are still low when cooled/frozen ram semen is used for AI and the reason could be that among breeding rams selection for semen parameters and freezability has not yet been done. In order to improve the reproductive indicators, competitiveness of the sheep sector and also to improve genetic preservation it would be important to have a quality control for semen, and to further develop and improve semen preservation techniques.

II. OBJECTIVES

1. Cold shock has a detrimental effect on ram semen namely cells can loose their acrosome membrane quite easily. The aim of the first study was to examine the effect of gelatine and plant gelatine (sodium-alginate) addition to the extender on ram semen parameters during the short term storage of chilled semen.

2. The purpose of the second study was to investigate the effect of elemental nano-selenium treatment on ram semen quality parameters, blood selenium content and the seminal plasma protein profile in the White Dorper breed.

3. The aim of the third trial was to examine the effect of seasonal changes on Doper and White Doper rams semen parameters and scrotal circumference.

4. The last study was carried out to clear the effect of seminal plasma proteins and curcumin addition to semen extender on the in vitro and in vivo parameters of chilled ram semen during short term storage.

III. MATERIALS AND METHODS

1. The storage of ram semen by using gelatine addition in the semen extender at +5^{o}C and +15^{o}C

   A/ Research carried out in Debrecen

   B/Research carried out in Zaragoza

2. The effect of nano selenium treatment as a feed supplement on ram semen quality parameters, blood selenium content and the profile of seminal plasma proteins.
3. The effect of season on semen parameters and scrotal circumference in Dorper and White Dorper sheep.
4. The effect of curcumin and seminal plasma protein addition to the extender on ram semen quality.

1. table: Experimental design

Number	Location	Breed	Housing	Feeding	Days of experiment
1/A	Debrecen	Tsigai (n=4)	group	ad libitum grass and alfalfa hay, 20 dkg/day/head concentrate (70% corn, 30% barley), mineral lick with selenium	07. Nov. 2011.
1/B	Zaragoza	Rasa Aragonesa (n=6)	individual	ad libitum grass hay, 35 dkg/day/head granulate, mineral lick with selenium	March-May 2013. (4 repetitions) October-December 2013. (4 repetitions)
2	Debrecen	White Dorper (n=12)	group	ad libitum grass and alfalfa hay, 20 dkg/day/head concentrate (70% corn, 20% oat, 10% barley), mineral lick with selenium	September 18-December 8. 2012.
3	Debrecen	Dorper (n=8), White Dorper (n=8)	group	ad libitum grass and alfalfa hay, 20 dkg/day/head concentrate (70% corn, 20% oat, 10% barley), mineral lick	April 16.-December 8. 2012.
4	Zaragoza	Rasa Aragonesa (n=6)	individual	ad libitum grass hay, 35 dkg/day/head granulate, mineral lick with selenium	March-May 2013. (4 repetitions) Oktober-December 2013. (4 repetitions)

The chilled storage at +5°C of ram semen in gelatine supplemented extender, 1/A experiment

Ejaculates of four Tsigai rams were used in this experiment. After collection the volume of semen was recorded, afterwards semen total motility was examined by using OLYMPUS BX64 phasecontrast microscope at 400x magnification. Motility evaluation was based on SALAMON'S (1987) method. The concentration of semen was assessed by using Minitube Photometer SDM. 6µL of fresh semen was diluted in 594Ml 0.9% NaCl buffer in order to evaluate sperm concentration. Afterwards a pool was made from the ejaculates to avoid ram individual effect. The pool was devided into 5-5 groups with the same quantity and stored in a 37°C waterbath till dilution. The concentration for semen dilution was $400x10^6$ sperm/mL, is the same used for cervical insemination. The dilution was made with two different extenders. The first was UHT milk with 1.5% fat content, the other was UHT milk with 1.5% fat content +5% egg yolk. To both diuents 0%; 0.5%; 1.0%; 1.5%; 2.0% Dr. Oetker gelatine (Bloom number = 200) was added. The samples without gelatine supplementation were used as control. After cooling the samples to +5°C the number of live/dead cells and acrosome integrity was assessed by Kovács-Foote staining (KOVÁCS and FOOTE, 1992; NAGY et al., 1999; KÚTVÖLGYI et al., 2006).

Ram semen preservation in plant gelatine based extender at +5°C and +15°C, 1/B experiment

In this experiment semen was collected from six Rasa Aragonesa rams. Only the second ejaculates were used to avoid the individual effect, therefore a pool was made from the samples. To assess motility 10µL of semen was diluted in PBS. From this dilution 50µL was diluted in 450µL MiliQ water to assess sperm concentration. For motility evaluation CASA system (ISAS 1.0.4; Proiser SL, Valencia, Spain) was used. Membrane integrity was assessed by using double fluorescent staining as CFDA (carboxyfluoresceine-diacetate) and PI (propidium-iodide), described by HARRISON and VICKERS (1990). 6 µL of the stained sample was put on the slide and coversliped. The evaluation of the samples was done with Nikon Labophot-2 fluorescent microscpe with 400x magnification by using blue filter (B-2A: 45-490 nm). 200 cells were evalueted in each slide.

Sperm capacitation status was assessed by using chlortetracycline (CTC) staining described by GILLIAN et al. (1997) and combined with $ETDH_1$ stain to evaluate membrane integrity (GRASA et al., 2006). The samples were evaluated by using 1000x

magnification with Nicon Eclipse E-400 microscope and purple filter (V-2A: 380-420 nm) to assess capacitation status and green filter (G-2A: 510-560 nm) to detect dead cells. 200 cells were counted in each slide.

Phosphatidylserine translocation was assessed by using Annexin V-Cy3 Apoptosis Detection Kit. Semen evaluation was made by Nikon Eclipse E-400 microscope using 1000x magnification. Live cells (CFDA+) were detected by using blue filter (B-2A: 450-490 nm) while the cells with phosphatidyltranslocation (AnnV+) were detected with green filter (G-2A: 510-560 nm). In each slide 200 cells were counted.

The effect of selenium as a feed additive on semen production, blood selenium content and the profile of seminal plasma proteins

Subjective motility was assessed by using OLYMPUS BX64 phase contrast microscope by using 400x magnification and evaluation was done based on SALAMON's (1987) method. Sperm concentration was examined by Minitube SDM5 photometer.

The plasma mebrane integrity of the cells was assessed by SYB14/PI fluorescent staining. SYB14 stains live cells, having green fluorescence while PI is to detect dead cells with red flourescence. Membrane integrity was evaluated by flow citometry.

On the 70th day of the experiment seminal plasma from the control and selenium treated group was collected in order to determine the plasma protein profile. For protein identification 2D-PAGE and LC-MS method was used.

The effect of season on Dorper and White Dorper rams semen production and scrotal circumference

The third experiment was carried out at Kismacs Experimental Farm, Debrecen University. Semen collection, evaluation and scrotal circumference measurement was made once per week. After semen collection semen volume, concentration and motility was assessed. Scrotum circumference was measured at the widest part of the testis, with a tape line.

The effect of curcumin and seminal plasma protein supplemented extender on ram semen

For this experiment semen of Rasa Aragonesa rams was used. Afer semen collection a pool was made from the samples to avoid the individual effect. To assess semen motility 10µL semen was diluted in 90µL PBS. From this diution 50µL was

rediluted in 450µL MILIQ water to estimate sperm concentration. For motility evaluation CASA system (ISAS 1.0.4; Proiser SL, Valencia, Spain) was used. For the experiment carried out at +15^{o}C dextran/swim-up method was used to remove seminal plasma from the sample based on the method described by ALVEREZ et al. (1993) and modified by GARCÍA-LÓPEZ et al. (1996).

Membrane integrity and mitochondrial activity was estimated with Mitotracker Deep Red fluorescent staining. After staining the samples were incubated for 15 minutes at room temperature and kept in dark. Afterwards the cells were fixed by adding 5µL formaldehyde 5% (v/v). Evaluation of the samples was done by flow cytometry. The emission of YoPro1 was assessed at 525 nm wave length (FL-1 detector), the emission of Mitotracker Deep Red was evaluated at 755nm (FL-5 detector) to avoid spectral overlap. 20.000 sperm cell/sample was measured and evaluation was done by the method was described by HALLAP et al. (2005).

Sperm capacitation status was assessed by using chlorotetracycline (CTC) staining described by GILLIAN et al. (1997) and combined with $ETDH_1$ stain to evaluate membrane integrity (GRASA et al., 2006). The samples were evaluated by using 1000x magnification with Nicon Eclipse E-400 microscope by using purple filter (V-2A: 380-420 nm) to assess capacitation statud and green filter (G-2A: 510-560 nm) was used to detect dead cells. 200 cells were counted in each slide.

Phosphatidylserin translocation was estimated by using Annexin V-FITC Apoptosis Detection Kit and flow citometry. Semen was diluted to 300µL with Annexin buffer to a concentration $4x10^6$ cell/mL. 1mL of Annexin-V stain and 3µL of PI was added to the solution, thereafter the sample was incubated at 37^{o}C for 15 minutes in dark. The cells were fixed using 5µL formaldehyde, afterwards 20.000 events were measured and devided into groupes with flow cytometer.

Caspase activity was determined by using Caspase-3 and-7 Detection Kit and flow cytometer. Semen sample was diluted to 300µL with PBS to a $3x10^6$ cell/mL. 3 mL of FAM-Peptide-FMK reagent was added to the solution then the sample was incubated at 37^{o}C, 5% CO_2 for 60 minutes. Afterwards the sample was washed twice with 100µL washing buffer (the buffer was in FAM Caspase-3 and -7 Assay Kit). Before washing samples were centrifuged at 600rpm for 8 minutes at room temperature. After the second centrifugation the supernatant was removed and the pellet was resuspended with 500µL washing buffer. 2µL $ETDH_1$ was added to the solution to

devide live and dead cells. The sample was incubated for 10 minutes, afterwards 20.000 events were counted by using flow cytometer.

DNA fragmentation was assessed by using Tunel-test (terminal transferase mediated dUDP nick end labeling). Sperm samples were diluted to 500µL ($4x10^7$ sperm/mL) by using 4% PFA solution and incubated for 60 minutes at room temperature. Afterwards samples were washed twice with 100µL PBS, between the washing the solution was centrifuged at 3200 rpm for 8 minutes. After the second centrifugation the supernatant was removed and 100µL of permeabilization solution was added. The sample was incubated with the medium at $+4^oC$ for 2 minutes, afterwards two washing was carried out. Supernatant was removed and replaced with 50µL labeling solution containing TdT and dUTP enzyme. The sample was incubated at 37^oC for 1 hour. After incubation the sample was washed two times in PBS, inbetween a centrifuged at 600 rpm for 10 minutes. After the second washing the supernatant was removed and the pellet was resuspended in 300µL PBS and 2µL ETDH was added. The sample was evaluated with flow cytometry (20.000 cell/ sample).

To estimate the fertilizing potential of sperm cells Zona Binding Assay and In Vitro Fertilization was carried out. The samples were incubated at $+15^oC$ and used for artificial insemination.

Zona Binding Assay is a method used for the functional characterization of spermatozoa, whereby the sperm cells bound to ZP3 proteins on the surface of zona pellucida.

Ovaries were collected from the abbattoir were transported at the laboratory at room temperature and washed in saline solution. Oocytes were collected by slicing and puncture techniques (WANI et al. 2000). Oocytes were kept at 39^oC and 5% CO_2 in a humidified atmosphere until use. Selected spermatozoa were diluted in fertilization medium and added to the oocytes, with a final concentration of $5x\ 10^5$ cells/ml in each well. Wells were covered with mineral oil, and kept under a humidified atmosphere, with 5% CO_2 at 39^oC for 1,5 hour. After incubation oocytes were placed in a Petri dish with Hepes-TCM medium, and washed by gentle pipetting to remove unattached spermatozoa (IVANOVA and MOLLOVA, 1993). Then oocytes were fixed in glutaraldehyde 1.5% for 15 minutes and stained with Hoechst 33342 (1 µg/mL) for another 15 minutes at 37^oC. Groups of four to five oocytes were placed in a slide under a coverslip and examined with a fluorescence microscope at 400x. The number of zona pellucida attached spermatozoa per oocyte was counted and recorded.

Ovaries of Rasa Aragonesa ewe lambs were collected at the slaughterhouse and transported to the laboratory in a phosphate-buffered saline solution (PBS). Oocytes were collected using a combination of puncture and slicing techniques in a Petri dish, containing handling medium. Afterwards selected cells were transferred into a maturation medium of bicarbonate-buffered TCM-199 supplemented with 10% (v/v) estrus sheep serum, 10μg/mL FSH and LH, 100mM cysteinamine, 0.3mM natrium piruvate, 100IU/ mL penicillin G and 100 μg/ mL streptomycin sulphate covered with mineral oil, and icubated for 24 hour at 39^{o}C under CO_2 and saturated humidity. At the end of incubation, oocytes were freed from the cumulus cells and transferred to a fertilization medium that consisted of synthetic oviductal fluid without glucose (Tervit and Whittingham, 1972), supplemented with 2% (v/v) of estrus sheep serum, 10μg/mL heparin, and 1μg/mL hypotaurine. On the day of fertilization swim up was carried out with the chilled samples and were added to the oocytes in a final concentration of $1x10^6$ cell/mL in 350 μL fertilization medium covered with mineral oil, and incubated for 24 hour at 39^{o}C and 5% CO_2. After 24 and 36 hour presumptive zygotes were assessed for cleavage. Noncleaved oocytes were observed under stereomicroscope to assess for cleavage. Oocytes showing the first polar body were considered matured, and oocytes with two polar bodies were considered fertilized, but not cleaved. Maturation and cleavage rates were calculated over the number of selected oocytes, whereas the fertilization rate was calculated over the number of matured oocytes.

The control and +25μg/mL samples cooled to +15^{o}C were used for artificial insemination three times. In each experminent 30 Rasa Aragonesa ewes were synchronized. Estrus was synchronized using intravaginal Chronogest sponges, containing 40 mg fluorogetone acetate inserted for 14 days. Ovulation was induced with 480 IU of eCG (Folligon, Intervet, Salamanca, Spain) at the time of pessary withdrawal. During estrus synchronization 6 ewes lost their sponge, nevertheless these were also used in the experiment. Artificial inseminations were performed via the cervical route. During the first insemination two, in the third insemination one ewe was not used because the cervical mucosa contained petechial hemorrhaga and the vagina contained white discharge.

Insemination was carried out 48 hours after the Folligon injection. To detect pregnancy 34 days after insemination rectal ultrasound was done.

Statistical analysis of the data was done by using SPSS 13.0 programme.

The experiment carried out with Dr. Oetker gelatine did not requered statistical analysis since the experiment did not had any repetition.

The effect of sodium-alginate concentration on ram semen parameters was estimated by using one-way ANOVA and Tukey post hoc, at 5% significance level. In the experiment when the semen was stored at +5^{o}C GLM model was used to define the effect of gelatine concentration and time of storage on semen motility, membrane integrity and phospatidylserine translocation.

In the second trial statistical analysis of blood selenium content was carried out by GLM using day 0 as a covariate. The effect of selenium treatment on ram semen was also assessed by using GLM modell. As a post hoc test Tukey was used at 5% significance level.

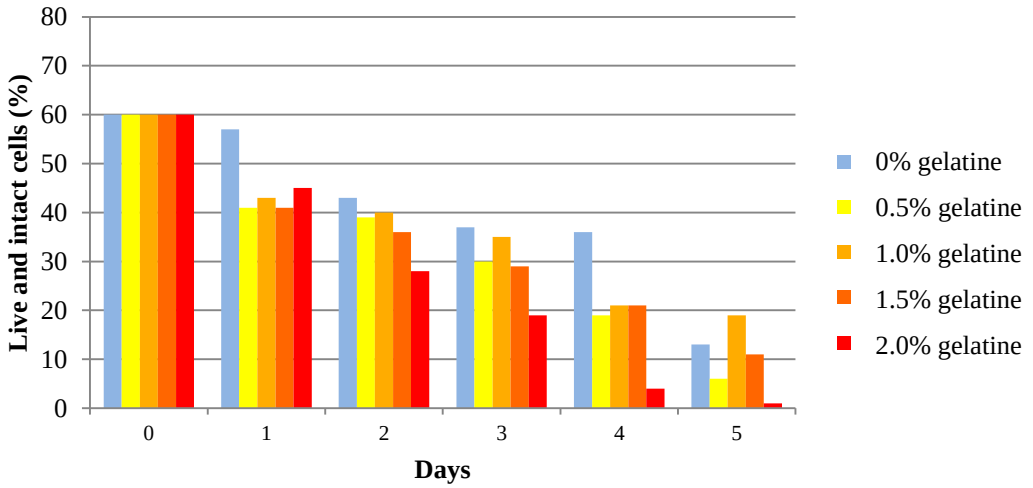
In the third expermiment the effect of season on ram semen parameters was estimated by using one-way ANOVA and Tukey's Test and at overall risk level of 5%.

In the fourth experiment statistical analysis of semen parameters was done by GLM model when the effect of curcumin, seminal plasma protein and time of storage were used as variables. To detect the effect of dilution media on the Zona Binding Assay and In Vitro Fertilization results one-way ANOVA was used and Tukey's Test and at an overall risk level of 5%.

IV. MAJOR CONCLUSION OF THE THESIS

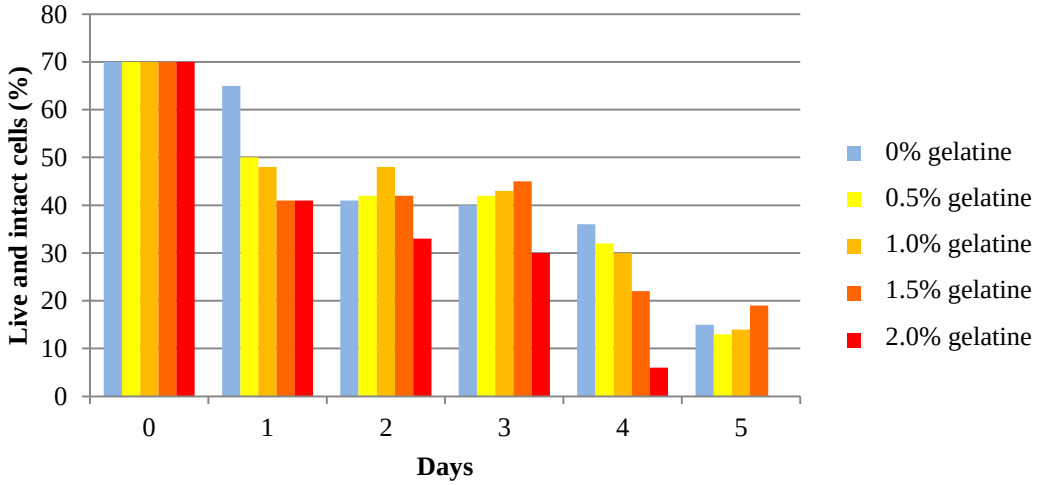
The results of the storage of ram semen in gelatine supplemented extender at +5°C

1/A Experiment carried out in Debrecen



1. figure: Percentage of live and intact cells in 1.5% fat content UHT-milk +gelatine during the five days of storage

Based on the results it can be concluded that the percentage of live and intact cells as the highest in the medium containing 1.0% gelatine (1. figure), therefore UHT milk with 1.0% gelatine addition seems to be a promising extender to ram semen storage at 5°C up to 5 days.



2. figure: Percentage of live and intact cells in 1.5% fat content UHT-milk+5% egg yolk+gelatine during the five days of storage

When 5% egg yolk addtion was used at the fifth day of the experiment the highest number of live and intact cells (%) was recorded when 1.5% gelatine addition was used.

We haven't found live and intact cells at the fifth day in the medium containing 2.0% gelatine (2. figure).

1/B Experimet: Results of ram semen stored in plant gelatine supplemented extender at +5°C and +15°C

The effect of sodium alginate addition on ram semen cooled at +15°C

2. table: The effect of sodium-alginate concentration on ram semen cooled at +15°C

	Sodium-alginate content (%)			
Parameters examined	**0**	**1.0**	**1.5**	**2.0**
(%)	X±SD	X±SD	X±SD	X±SD
Motility	48.75±37.16	50.00±32.81	51.00±31.09	54.50±28.59
Progressive motilility	25.75±22.95	21.50±19.43	25.50±20.87	25.75±13.88
$CFDA^+/PI^-$	47.50±5.06	51.25±2.21	55.00±13.51	50.25±10.34
NCL	21.25±10.30	21.00±8.67	21.50±8.18	17.00±5.35
NCD	13.00±5.35	16.00±5.65	12.75±4.19	11.75±7.54
CL	29.50±11.47	31.75±13.45	26.25±4.11	28.00±12.54
CD	31.00±12.90	28.25±13.22	33.25±5.50	24.50±14.28
R	5.25±3.59	3.00±2.44	6.25±4.03	10.00±8.52
Anx^-/CFDA+	39.25±6.07	42.50±4.65	43.25±10.04	44.25±7.63
$Anx^+/CFDA^+$	24.50±4.65	29.00±2.16	30.50±7.76	26.75±5.12
$Anx^+/CFDA^-$	36.25±4.99	28.50±5.19	26.25±4.99	29.00±3.91

(P>0,05)

Samples supplemented with sodium-alginate had the same motility as the control. The number of membrane intact cells were the highest in the sample containing 1.5% sodium-alginate and the lowest in the control (P>0.05). The number of non-capacitated live cells were mostly tha same in all the samples (2. table).

1/B Experiment: Results of the experiment when ram semen was stored at +5°C up to 48 hours in sodium-alginate supplemented extender

Motility decreased during the period of storage when sodium-alginate addition was used. The lowes motility results were achiveved when 1.5% and 2.0% sodium alginate was used. Sodium-alginate has a high viscosity and also a good water binding capacity. High viscosity has a negative effect on sperm motility, besides it can also be an advantage during the short term storage of ram semen. Sperm cells consume ATP for

their movement out of glucose, lactose is located in the seminal plasma or the diluent. During the process the concentration of free radicals is increasing that impars the membrane structure of the sperm cells thereby reducing the cells fertilizing capacity. By adding gelatine to the media sperm cell motility and the acceleration of glucolysis can be reduced, therefore the sacharides presented in the extender can provide energy for the cells for a longer period (ESBENSHADE and NEBEL, 1990). At 37^{o}C the consistency of sodium-alginate changes to liquid from gel. It also means that after insemination when semen is deposed in the cervix and uterus the viscosity of the media decreases, therefore sperm cells can use the energy sources more easly which will insure the energy for motility. CORCINI et al. (2011) by using boar and LÓPEZ-GATIUS et al. (2005) by using rabbit semen also achieved the same motility results that we obtained in our experiment.

Besides membrane integrity results we also found differences between the control and gelatine treated samples. In each time of the experiment the percentage of membrane intact cells was the highest in the control group. Increasing gelatine concentration had a negative effect on the number of membrane intact cells. COCINI et al. (2011) reported when gelatine was added to boar semen than at the first day of storage the number of membrane intact cells decreased the most severly, afterwards during the four days of storage the number of intact cells had not changed significantly. When motility and membrane integrity was assessed we have found sperm agglutination in the samples contained 1.5% or 2.0% sodium alginate. Sperm agglutination is a problem because the pH decreases arround the cells that are agglutinated. Decrease of pH can be explained with the enriched metabolic products in the environment. High contentration of the metabolits can have a toxic effect on the cells thereby decreasing their viability.

The effect of elemental nano selenium as a feed additive on sperm production, blood selenium content and the profile of seminal plasma proteins

3. table: Changes in the quality of White Dorper semen during selenium treatment

		Time of semen collection (day)						
Examined parameters	**Group**	**0**	**42**	**49**	**56**	**63**	**70**	**86**
		X±SD	X±SD	X±SD	X±SD	X±SD	X±SD	X±SD
Volume (mL)	Se	**1.53±0.57^{a}**	**1.75±0.35^{a}**	1.21±0.60	1.56±0.36	1.61±0.60	1.41±0.51	1.71±0.35
	K	**1.06±0.51^{b}**	**1.43±0.56^{b}**	1.36±0.47	1.36±0.39	1.57±0.55	1.36±0.57	1.75±0.37
Concentration (x10^{9})	Se	4.62±2.03	3.32±1.59	4.04±1.22	3.73±0.74	**1.42±0.73^{a}**	3.66±1.47	2.61±0.78
	K	4.31±1.57	3.41±1.43	4.12±1.47	3.93±0.92	**2.63±1.94^{b}**	3.46±1.46	2.78±0.81
Mass motility (0-5M)	Se	4.40±1.00	4.00±2.00	3.71±1.10	4.10±1.03	**4.54±0.88^{a}**	3.66±1.65	**4.19±2.17^{a}**
	K	3.83±1.00	4.00±1.00	3.47±0.90	4.33±0.81	**3.83±0.92^{b}**	4.09±1.11	**3.26±2.40^{b}**
Progressive motility (%)	Se	66.66±10.32	55.00±20.73	50.83±13.93	56.66±11.69	**71.66±9.83^{a}**	49.16±29.35	**59.16±28.00^{a}**
	K	53.33±20.09	55.83±11.14	48.33±8.75	62.50±7.58	**53.33±18.61^{b}**	55.83±14.97	**44.16±29.90^{b}**
PI-/SYBR14- (%)	Se	78.74±8.67	72.20±6.84	57.38±19.64	67.67±14.25	87.18±8.85	85.05±9.28	89.03±8.22
	K	81.76±7.91	83.40±9.90	59.75±8.42	73.67±8.42	87.25±8.97	80.30±15.82	88.29±3.46
Scrotal circumference (cm)	Se	35.29±1.76	35.41±1.62	32.25±2.42	32.87±1.72	32.87±2.18	32.54±2.18	32.33±2.74
	K	34.58±1.90	35.00±1.26	32.41±2.97	32.41±1.46	32.08±2.20	33.75±1.99	31.08±2.36

We recorded significant differences (P<0.05) in semen volume when control and treated group was compared at the 42nd day of experiment. Selenium treatment did not have a significant effect on semen volume in the other day, nevertheless the volume incresed during the experimental period in both groups. At the 63rd day of the experiment there was a severe increase in the concentration at the control group. We concluded that during the experiment semen concentration decreased in both groupes. In mass and progressive motility we recorded differences between the groups at the 63rd and 86th day of experiment (3. table). In both cases the selenium treated group had better motiliy. In the number of live cells and scrotal circumference there was no significant difference between the groups in neither of the experimental days.

KENDALL et al. (2000) studied the effect of selenium and zinc supplementation on ram lambs semen quality and also on the element content of the blood. The result showed that selenium and zinc supplementation had no effect on spermatokrit, semen

volume, although the number of membrane intact cells increased in the treated group compared to control.

4. table: Blood selenium content (ppb) in different time of the experiment

	Days from bolusing (day)				
	0	**42**	**56**	**70**	**86**
Group	**X±SD**	**X±SD**	**X±SD**	**X±SD**	**X±SD**
Selenium (ppb)	154.66±31.24	168.10±132.09	120.06±52.88	161.25±62.78	171.87±31.32
Control (ppb)	143.16±41.60	129.35±53.07	99.94±6.78	95.038±20.60	113.41±31.16

There was no significant difference ($P>0.05$) in blood selenium content when the the results of the control and selenium treated group were compared (4. table).

KENDALL et al. (2000) made similar experiment using Merino ram lambs. They concluded that the selenium treated group had higher selenium blood content compared to the control group during the whole experiment and selenium content was within the required limit in both groups as well.

Ten protein spots were identified with LC-MS method and showed significant difference in the expression level ($P<0.05$) between the groupes. Matrix metallopeptidase 2 belongs to the matrix metalloproteinase (MMP) family. This enzyme plays an important role in embryo development, morphogenesis and also in the implantation of the blastocyst (WOESSNER, 1994). This protein was already identified in the epididymal secretion of ram, boar and stallion (MÉTAYER et al., 2002). Actually there is lack of information about the exact function of this protein, but it can be assumed that it plays a role in the transportation of spermatides in the seminiferous tubules (WOLFSBERG, 1995). Serum albumin plays an important role in zinc transport. HARRISON et al. (1978) reported that serum albumins are important to maintain motility, but also increase agglutination in boar semen. Phosphoglycerate kinase 2 (PGK2) protein is expressed during spermiogenesis and is involved in glycolysis, what ensures the ATP as an energy source for sperm movement (DANSHINA, 2010). Not much is known about the role of isocitrate dehydrogenase protein in male fertility. In Holstein-Friesian bulls it was described that isocitrate dehydrogenase expression increased in the bulls considered to have low fertility (RODRIGUES et al., 2012). In Santa Ines breed the expression of Ram Seminal Vesicle

Protein (RSVP) and Bodhesin-2 protein was the main when seminal plasma protein profile of the breed was assessed. RSVP belongs to Binder of Sperm Protein (BSP) family, while bodhesin-2 belongs to the spermadhesin protein family. BSP proteins are in an interaction with the phospolipids of the sperm membrane and stimulating cholesterol efflux, membrane permeabilization and the presence is also linked with capacitation (THÉRIEN et al., 1998). RSVP14 and RSVP20 proteins are considered to have a decapacitating effect in several species (SOUZA et al., 2012). DRUART et al. (2013) examined the seminal plasma profile of sheep, cattle, horse, camel and alpaca and they got the conclusion that RSVP14 protein is presented in all species seminal plasma.

The effect of season on semen quality and scrotal circumference of Dorper and White Dorper breed

5. table: The semen quality and scrotal circumference of Dorper ram in the different seasons

Examined parameters	**Season**		
	spring (May) (n=8)	summer (June-August) (n=71)	autumn (September-Október) (n=45)
	X±SD	X±SD	X±SD
Volume (mL)	1.3±04[a]	1.2±0.4[a]	1.4±0.5[a]
Concentration (x10^9/mL)	2.7±1.6[a]	3.3±1.5[a]	4.1±1.1[b]
Mass motility (0-5)	4.0±1.1[a]	3.5±1.4[a]	3.7±1.4[a]
Progressive motility (%)	71.3±21.0[a]	65.3±21.0[a]	68.7±26.3[a]
Scrotal circumference (cm)	30.1±1.2[a]	34.9±2.6[b]	36.2±1.3[c]

[a, b, c]- averages with different letters show significant differences (P<0.05) for the same examined parameters

There was a significant difference (P<0.05) in sperm concentration when spring and autumn seasons were compared. In scrotum circumference we obtained significant difference in all three seasons (P<0.05) (5. table).

6. table: Changes in the semen quality and scrotal circumference of White Dorper rams

Parameters	Season			
	spring (May) (n=7)	summer (June-August) (n=69)	autumn (September-October) (n=74)	winter (December) (n=24)
	X±SD	X±SD	X±SD	X±SD
Volume (mL)	1.6±1.3[a]	1.2±0.4[a]	1.3±0.5[a]	1.6±0.4[b]
Concentration ($x10^9$/mL)	3.9±1.3[a]	3.7±1.8[a]	3.4±1.7[a]	3.1±1.2[a]
Mass motility (0-5 M)	4.0±1.1[a]	3.7±1.2[a]	3.6±1.4[a]	3.5±1.2[a]
Progressive motility (%)	72.1±24.1[a]	69.5±24.1[a]	67.1±26.2[a]	64.3±26.7[a]
Scrotal circumference (cm)	31.4±2.9[a]	34.7±2.2[b]	34.8±2.0[b]	32.3±2.4[a]

[a, b, c]- averages with different letters show significant differences (P<0.05) for the same examined parameters

The volume of White Dorper ram semen was within the required limit 0.5-2.0 mL (Evans and Maxwell, 1987; Gergátz, 2007). In the examined period semen volume was the lowest in summer (1.2 mL) and the highest in winter (1.6mL). The differences were significant (P<0.05) (6. table).

The effect of curcumin and seminal plasma addition on ram semen parameters

Results of the ram semen quality stored at +5^{o}C stored up to 48 hours

We concluded that when semen was stored at +5^{o}C there was no main difference between the curcumin and seminal plasma protein treated groups semen quality. 1.0nM curcumin and 25mg/mL seminal plasma protein addition had a positive effect on embrional development. Some of the seminal plasma proteins could protect sperm membrane from cold shock, therefore it could be interesting to do further investigations in order to identify the proteins that have protective effect on sperm membrane.

Results of ram semen quality with the semen stored at +15^{o}C

The progressive motility of the fresh semen was 34.0%, decreased to 20.0% after cooling. When seminal plasma addition was used motility maintained after cooling and curcumin also had a positive effect on motility results, although there was no significant difference between the results (P>0.05). The number of membrane intact cells was the highest in the protein treated group (77.16%), while the other treatments didn't had a

significant effect on sperm cell membrane integrity (P>0.05). Mitochondral activity did not differed from the contol group in neither cases.

When capacitation of the cells examined only 30.25% of the cells were non capacitated live in fresh semen, which is extremly low. MENDOSA et al. (2013) reported 61.3% non capacitated live cell number in fresh semen, decreased to 26.6% when the sample was cooled to +15^{o}C and with seminal plasma addition to 33.2%, that was 51.66% in our experiment. The number of non capacitated live cells achived by using milk and curcumin based extender did not differed significantly (P>0.05) compared to fresh semen.

When caspase activity was determined the number of Casp+/Eth- cells in fresh semen was 5.15%, that did not decreased severly after collong (5.45%). The addition of seminal plasma protein (5.66%), 1.0nM curcumin (5.00%), 2.0nM curcumin (5.26%) did not increased the number of caspase positve cells. After cooling in seminal plasma based extender the number of non apoptotic cells (52.33%) increased compared to fresh semen (31.27%) and also to the control (30.55%). Curcumin was also able to reduce the intensity of apoptosis regardless of the curcumin concentration was used.

In fresh semen the number of Anx+ cells was 39.87%, similar to the result 34.00% that MENDOZA et al. (2012) reported. After cooling the number of Anx+ cells nearly doubled in the control group (63.4%), counter to the protein treated group, whre the number of Anx+ cells was 25.97%. The number of Anx-/PI- cells in fresh and control sample showed significant difference (P<0.05).

The number of cells with fragmented DNA was high in the fresh sample, resulting in 25.60%. After cooling the samples to +15^{o}C we obtained the highest number of DNA fragmented cells in swim-up media based samples (69.87%) and the same result occured when protein addition was used (68.80%). When curcumin supplementation was able to decrease the number of DNA fragmented cells. In 1.0nM curcumin supplemented sample 61.42% in 2.0nM curcumin 57.50% was the number of DNA fragmented cells. We achieved the lowest number of DNA intact cells when milk besed extender was used (56.77%), but there was no significant difference between the effect of the different media on DNA fragmentation result (P>0.05).

Fertility of ram semen was assessed by using Zona Binding Assay. Regarding the results seminal plasma protein supplementation had a positive effect on sperm ferility, because 8.2 sperm binded to one oocyte, while in 1.0nM curcumin extender 6.4 sperm/oocyte at 2.0nM curcumin 6.2 sperm/oocyte was the binding ratio. We achieved

the lowest result in the control sample where the number of binding spermatozoa was 4.8 to one oocyte.

With the control and seminal plasma treated samples we carried out transcervical artificial insemination in three repetitons. To assess the effect of protein treatment 87 ewes were involved in the experiment. 34 days after insemination to detect pregnancy rectal ultrasound exemination was used. After the first isemination the number of pregnant ewes was 6.7% in both groups. After the second insemination in the control group the number of pregnant ewes was 35.7% while in the protein treated group was less, only 28.5%. After the third insemination the results were nearly the same (control: 31.3%, seminal plasma treated group: 30.8%). The pregnancy results we achieved could be affected by the ewes as well. Rasa aragonesa breed is considered to be strongly seasonal, therfore the results obtained in non season are lower than it can be expected during the season. The pregnancy result were not effected by the type of extender ($P>0.01$).

V. NEW SCIENTIFIC RESULTS OF THE THESIS

Based on the results obtained during the experiments the following new scientific result can be concluded:

1. It was prooved that the addition of gelatin and plant gelatin to milk based extender does not have a negative effect on semen quality, therefore they can be suggested to use a sextender additives. The advantage of using plant gelatin is that it does not contains pathogens (virus, prion).
2. We concluded that the nano selenium feed supplementation does not have an effect on ram semen quality, blood selenium content, but has an effect on ram seminal plasma protein expression
3. It was also prooved that season has an effect on Dorper and White Dorper semen quality and circumference as well, nevertheless less fluctuation in the traits was assessed compared to the European breeds.
4. We concluded that seminal plasma protein and cucrumin addtion does not have an effect on ram semen quality paramaters, neither on pregnancy results when trascervical insemination was used.

VI. PRACTICAL USE OF THE RESULTS

1. Cervical insemination remains one of the most effective method to use milk based ram semen cooled to +15^{o}C and +5^{o}C.
2. Elemental nanoselenium is a suitable feed additive for breeding rams
3. Dorper and White Dorper rams are able to produce good quality semen all year arround, therefore it's recommended to use them all year arround
4. Seminal plasma proteins and curcumin are able to decrase the effect of cold shock, therefore they are suitable as extender additives

VII. LIST OF PUBLICATIONS

Publications related to the dissertation

Chapter in book

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