

SIRT1 gene methylation in sperm differs in rams with high and low fertility

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Recently, more evidences of epigenetic impact on the male fertility, particularly on sperm DNA methylation have been reported. Data related to this issue in livestock males is still limited. The present study analyzed the DNA methylation status of the important gene for spermatogenesis, *SIRT1*, in ram sperm and its correspondence with semen quality and fertilizing ability. The ejaculates of 10 rams (5 rams - 1.5 years old, and 5 rams - 4 years old) from Synthetic Population Bulgarian Milk breed were evaluated and used for the artificial insemination of 174 ewes in breeding season. Two semen samples from each animal were used for DNA extraction followed by bisulfite conversion. The DNA methylation status of *SIRT1* was detected through quantitative methylation-specific PCR using two sets of primers designed specifically for bisulfite-converted DNA sequences to attach methylated and unmethylated sites. On the base of age and conception rate the rams were divided in different groups. Data of semen quality, DNA methylation status of *SIRT1* and reproductive performances of each group were statistically processed. Results showed a high average value of DNA methylation of *SIRT1* in ram sperm ($78.5 \pm 23.9\%$) and wide individual variability among investigated animals, with a coefficient of variation of 34.4%. The 1.5 years old animals tended to have a higher level of *SIRT1* methylation than 4 years old animals. The rams in group with high fertilizing ability had significantly higher DNA methylation of *SIRT1* in sperm than those with low fertilizing ability. In conclusion, results of this study provided evidence that the alteration of sperm *SIRT1* methylation is associated with fertility performances of the rams and, probably, with their age.

Keywords: sperm DNA methylation, *SIRT1*, ram fertility

1 Introduction

The common semen evaluation of rams relies on the examination of semen parameters such as ejaculate volume, sperm concentration, sperm motility and survivability. However, the fertilizing ability of sperm does not depend only on these parameters. Recently, more and more researches suggested that the epigenetic effects actively influence male fertility (Molaro et al., 2011, Boissonnas et al., 2013, Urdinguio et al., 2015, Schagdarsurengin and Steger, 2016, McSwiggin and O'Doherty, 2018). Epigenetic events cover the DNA methylation, histone post-translational modification and small RNAs production that independently or in concert can control gene expression, without altering the DNA sequence (Donkin and Barres, 2018). The major mechanisms of the epigenetic regulation of gene expression are DNA methylation and histone acetylation or methylation (Wolffe and Guschin, 2000). Investigations in humans and rodent models showed that alteration in sperm DNA methylation, particular hypermethylation, is associated with poor sperm parameters, idiopathic male infertility, and even pregnancy failure (Aston et al., 2015, Laqqan et al., 2017, Tang et al., 2017). The data related to the sperm DNA methylation patterns in livestock is still limited. Only few reports have dealt with the research on global methylation of DNA in ram sperm (Sharafi et al., 2017, Perrier et al., 2018), but to our knowledge none has demonstrated correspondence between sperm DNA methylation and fertility of rams. Several studies reported results on the global and gene specific DNA methylation in bull and boar sperm, and the relationship with fertility parameters (Congras et al., 2014, Verma et al., 2014, Kropp et al., 2017, Shojaei Saadi et al., 2017, Zhou et al.,

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2018, Ahlawat et al., 2019), while such data for ram is not available. Verma et al. (2014) have shown that high and low fertile buffalo bulls have different methylation on the promoters of 13 genes important to spermatogenesis, sperm maturation and capacitation. Among them, the SIRT1 (silent mating type information regulation 2 homolog 1) gene belongs to the class of NAD-dependent histone deacetylases (Jing and Lin, 2015). SIRT1 can deacetylate a variety of substrates and is involved in a broad range of physiological functions, including control of gene expression, metabolism and aging (Rahman and Islam, 2011). The research in mouse model clearly showed that repression of SIRT1 attenuates spermatogenesis, disturbs sperm morphology and acrosome biogenesis, and leads to male sterility (Coussens et al., 2008, Bell et al., 2014, Liu et al., 2017).

The current study was designed to analyze the methylation status of gene SIRT1 in ram sperm and to determine whether it was related to semen quality, fertilizing ability and fertility parameters.

2 Material and methods

2.1 Animals

The experiment was carried out with 10 rams and 174 ewes of Synthetic Population Bulgarian Milk breed housed in the Agricultural Institute – Shumen (Bulgaria), located at latitude of N 43° 16' N and longitude 26° 55' E. The animals included 5 young rams (1.5 years old, 98.4±4.6 kg body weight) and 5 old rams (4 years old, 115±12.5 kg body weight), while females were between 1.6 and 3 years old and body weight of 65±8.2 kg. All animals were clinically healthy with feeding technology for male and female animals, respectively, and water intake at libitum. Twenty days before the beginning of the experiment a "salt-free-salt" diet was supplied to females. The study was conducted during the breeding season. The experimental design was approved by the National Ethics commission for animals on the base of the Bulgarian Veterinary Law (25/01/2011) regarding the life conditions and welfare of livestock animals used for the experimental purpose - AF 9747A-0002 / N1430 from 05.04. 2018

2.2 Semen collection, evaluation and handling

From each ram 15 ejaculates were collected by the artificial vagina method in presence of sheep in estrus. Immediately after collection the ejaculates were placed in a water bath at 37°C and submitted to a primary assessment. The volume was measured by graduated pipette, the sperm concentration ($\times 10^9$ /ml) was determined by Thoma counting chamber and motility (%) was evaluated under a light microscope through the method described by Ax et al. (2000). Only ejaculates with a normal reference for ram (normal color and transparency, volume > 1 ml, concentration > 1×10^9 /ml and motility > 70%) were used. Semen dilution before insemination was made by Tris-glucose-citrate semen extender for ram until adjustment of the sperm concentration to 800×10^6 cells/ml.

2.3 Estrus detection and artificial insemination

The ewes were tested for standing estrus with a trained ram-teaser introduced into the flock daily early morning (6.00 am) and late afternoon (6.00 pm). Each ewe was submitted to a double artificial insemination (AI) twelve hours apart. The fresh diluted semen of 0.5 ml with 400×10^6 motile sperm cells was deposited intra-cervically in a depth of 0.5-1 cm. The number of inseminated ewes per ram was in accordance with the individual breeding plan developed on the base of the genealogical origin of animals and productivity parameters. Fourteen days after the first AI a ram-teaser was introduced again into the group of inseminated animals for one week. A daily observation for detection of ewes in estrus was provided and animals with no expressed standing estrus were accepted as pregnant after the AI.

2.4 Reproductive parameters recording and calculation

The data obtained at lambing was arranged in subgroups according to used ram. For each subgroup lambed ewes after AI, ewes with a single fetus, twins or triplets and total lambs born were recorded. After lambing of all animals an average gestational period (AGP) of 148 ± 3.2 days was calculated. The information for individuals was compared with the records for AI, repeated expression of standing estrus after AI and pregnancy termination. During the experimental period abortions were not registered. The values of AGP were used in additional retrospective analysis to verify the correct conception data for each animal and calculate the conception rate at lambing. Conception rate at lambing, lambing percentage and fecundity were calculated according to Oliver (2014). The conception rate was used as an indicator of ram fertility. The conception rate of 90% was defined as an average value of this parameter in sheep inseminated with fresh semen during the breeding season (Kennedy, 2012). On this basis, the rams were divided in two groups: rams with high fertility (conception rate > 90%, n=5) and rams with low fertility (conception rate \leq 90%, n=5).

2.5 Analysis of DNA methylation of SIRT1 in sperm

Two semen samples from each ram were used for analyzing the methylation status of gene *SIRT1*. The spermatozoa were separated by centrifugation, mixed with somatic cell lysis buffer to avoid DNA contamination, and washed twice with PBS. Genomic DNA was extracted from the spermatozoa pellets using Qiazol Lysis Reagent (Qiagen), following the manufacturer's instructions. Spectrophotometric control of the quantity and purity of genomic DNA was done by NanoDrop 1000 (Thermo Scientific, USA). The extracted DNA samples were treated with sodium bisulfite using the EpiTectBisulfite Kit (Qiagen) in accordance with producer's

recommendations. DNA methylation of the *SIRT1* gene was analyzed using SYBR green-based quantitative methylation-specific PCR (Tolić et al., 2018). Two sets of primers, designed specifically for bisulfite-converted DNA sequences to attach the methylated and unmethylated sites, were used as follow: *SIRT1* Ram MSP Methylated, forward GTATGTTGTAGTTTATGGGGTCG and reverse TACTCCTTTAATCTTAAATTCGCT-3, and *SIRT1* Ram MSP Unmethylated forward GGTATGTTGTAGTTTATGGGGTTGT and reverse ACTCCTTTAATCTTAAATTCACT (Nayak et al., 2016).

For the PCR, the bisulfite-treated DNA template was mixed with the SYBR qPCR Kit (Genaxxon Bioscience, Germany) and a pair of primers. The PCR conditions included initial denaturation at 95°C for 10 min and 40 cycles of two-step PCR at 95°C for 15 s and 60°C for 60 s. The PCR reactions were performed using a cycler real-time PCR instrument (Agilent Stratagene Mx3005P). After PCR amplification, a dissociation curve was generated to confirm the PCR methylated and unmethylated products. The percentage of DNA methylation in a sample was estimated using the following formula: Methylated DNA (%) = $1/(1+2^{-\Delta Ct}) * 100$, where $\Delta Ct = CtU - CtM$, CtU being the threshold cycle for the unmethylated primers and CtM being the threshold cycle for the methylated primers (Islam et al., 2020). Each measurement was performed in triplicate.

2.6 Statistical analysis

The data were processed by StatSoft v.10. (Tulsa, OK, USA) and presented as mean±SD. Means were statistically compared using Student's t-test. Pearson's correlation coefficients between sperm DNA methylation of *SIRT1* and fertility parameters of rams were estimated. For this purpose, the average percentage of *SIRT1* methylation of each ram was compared with the average semen parameters and percent of fertility. Statistical significance was set at $P \leq 0.05$.

3 Results and discussion

The investigated rams demonstrated active sexual behavior during the breeding season. The collected ejaculates had high spermatozoa concentration and motility, which corresponded to requirements of the qualitative ram semen (Tibary et al., 2018). Significant differences in semen parameters such as spermatozoa concentration and motility between 1.5 years old and 4 years old rams were observed (Table 1).

Table 1 Semen characteristics of the ejaculates (n=15 per animal) according to age of the rams

Age of rams	Volume (ml)	Concentration (10 ⁹ /ml)	Motility (%)
	Mean±SD	Mean±SD	Mean±SD
1.5 years			
R1	2.31±0.24	2.28±0.42	86.91±4.70
R2	1.82±0.42	2.25±0.45	89.25±2.50
R3	2.40±0.46	2.30±0.41	88.72±3.52
R4	2.25±0.14	2.50±0.12	82.51±5.14
R5	1.61±0.48	1.83±0.47	85.00±6.82
Total	2.07±0.36 ^a	2.23±0.37 ^a	86.48±2.65 ^a
4 years			
R6	1.81±0.40	2.46±0.27	83.15±10.46
R7	1.90±0.53	2.39±0.21	87.11±4.54
R8	2.65±0.41	2.48±0.22	88.55±5.16
R9	1.62±0.44	2.62±0.17	82.13±11.34
R10	1.71±0.78	2.58±0.11	82.00±4.21
Total	1.93±0.44 ^a	2.51±0.19 ^b	84.58±2.94 ^b

^{a,b} Means with different superscripts within the same column differ at $P < 0.05$; SD – standard deviation

The classification of the rams according to their fertility and the reproductive parameters based on the lambing results are presented in Table 2. Each group included approximately an equal number of rams with the same age and inseminated ewes. The average values of the evaluated semen parameters differed significantly between rams with high and low fertility (Table 3). Analysis of DNA methylation status of gene *SIRT1* in ram sperm showed that average percent of methylation in whole group was relatively high (78.5±23.9%). This result agrees with data on the higher global DNA methylation in ram's sperm compared to sperm of other species, in particular bulls (Perrier et al., 2018). The values of *SIRT1* methylation in two samples of each animal analyzed in triplicate were very close. The coefficient of variation (CV) varied between 1.5 and 2% in the samples with high level of methylation and between 5 and 8% in the samples with low level of methylation. Similar results for double semen samples from one bull were reported by Zhou et al. (2018). However, the variability between animals was much higher. In young rams the methylation level of the *SIRT1* varied between 49% and 99% (CV of 23.6%), and in the

mature group between 20.5% and 99% (CV of 44.8%). Similar interindividual variations in DNA methylation patterns of sperm were reported for bulls, including the sperm of monozygotic twin bulls (Shojaei Saadi et al., 2017).

Table 2 Reproductive performances of rams based on the lambing results

Group of rams	Rams	Inseminated ewes	Lambd ewes	Single	Twins	Triplets	Total lambs	Reproductive performance		
								Conception rate at lambing (%)	Lambing percentage	Fecundity
High fertility (conception rate > 90%)										
	R1	13	13	4	9	0	22	100	169.2	1.69
	R3	15	15	10	4	1	21	100	140	140
	R5	40	37	17	20	0	57	92.5	142.5	1.54
	R8	17	16	7	9	0	25	94.1	147.1	1.56
	R10	3	3	0	3	0	6	100	200	2.00
	Total	88	84	38	45	1	131			
	Mean±SD							97.3±3.7 ^a	159.8±25.3 ^a	1.64±0.23 ^a
	CV (%)							3	16	14
Low fertility (conception rate ≤ 90%)										
	R2	9	8	4	3	0	10	88.9	111.1	1.25
	R4	2	1	0	1	0	2	50	100	2.00
	R6	20	18	7	11	0	29	90	145	1.61
	R7	33	29	7	21	1	52	87.8	157.6	1.79
	R9	22	19	6	12	1	33	86.4	150	1.74
	Total	86	74	25	48	2	126			
	Mean±SD							80.6±17.1 ^b	138.2±25.5 ^b	1.68±0.28 ^a
	CV (%)							21	18	17

^{a,b} Means with different superscripts within the same column differ at P<0.05; SD – standard deviation; CV – coefficient of variation

Table 3 Semen parameters of the ejaculates (n=75 per group) in high and low fertile rams

Group of rams	Volume (ml)	Concentration (10 ⁹ /ml)	Motility (%)
	Mean±SD	Mean±SD	Mean±SD
High fertility	2.05±0.35 ^a	2.29±0.28 ^a	86.34±2.45 ^a
Low fertility	1.88±0.20 ^b	2.44±0.13 ^b	84.75±2.81 ^b

^{a,b} Means with different superscripts within the same column differ at P<0.05; SD – standard deviation

Despite the lack of statistical significance, results indicated a lower SIRT1 methylation in the 4 years old rams compared to the young ones (P = 0.071; Figure 1). In support of this finding, there is evidence that the age-associated changes of the total DNA methylation in different tissues may occur in both ways, i.e. decrease of the global methylation and hyper methylation of the specific genes (Marttila, 2016, Jenkins et al., 2019). Other investigations underlined that the total sperm DNA methylation in bulls increases with age (Lambert et al., 2018, Takeda et al., 2019). However, the existed inter species differences in DNA sperm methylation should be considered (Perrier et al, 2018).

The present data demonstrated a significant correspondence between DNA methylation of SIRT1 and conception rate at lambing (Table 2). The reproductive performance on flock level showed higher (P < 0.05) means for conception rate (97.3±3.7%) and lambing percentage (159.8±25.3%) in rams with high fertilizing ability compared to males with low fertilizing ability (80.6±17.1% and 138.2±25.5%, respectively). Fecundity did not differ significantly between high fertile and low fertile rams (1.64±0.23 and 1.68±0.28, respectively; Table 2). Whereas sperm quality has significant influence on the conception rate, fecundity is mainly attributed to ovulation number in the female animals. The aforementioned information can support the hypothesis of a relationship between sperm

DNA methylation and fertilizing capacity of ram spermatozoa. The DNA methylation of SIRT1 in sperm of high fertile rams was significantly higher than in low fertile rams (Figure 2).

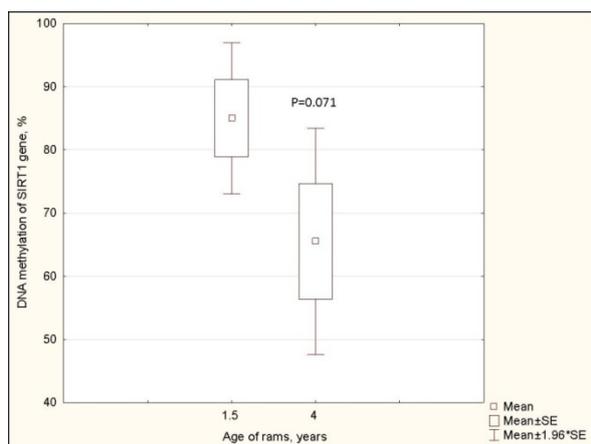


Figure 1 Age-dependent changes of the DNA methylation of *SIRT1* in ram sperm

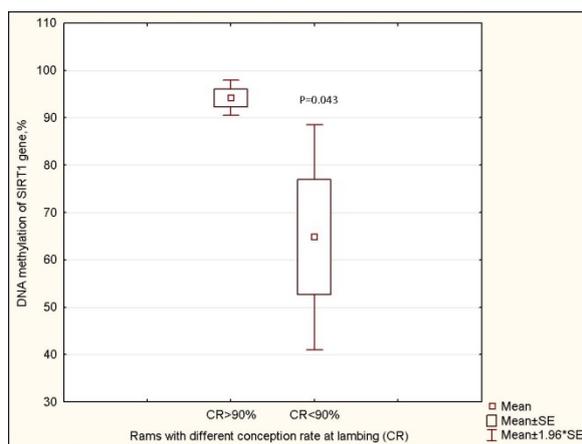


Figure 2 DNA methylation of *SIRT1* gene in sperm of rams with high and low fertilizing ability

This finding disagrees with data of Verma et al. (2014), who established hyper methylated status of SIRT1 in sperm of sub-fertile buffalo bulls. Some authors showed SIRT1 as a gene related to the properties of sperm maturation and capacitation that constitute important attributes of fertilizing sperm (Rahman and Islam, 2011, Liu et al., 2017), and therefore the inhibition of its transcription due to methylation might lead to worsen the semen quality. However, Bell et al (2014), studying the SIRT1 role in the spermatogenesis on mouse model, detected that deletion of SIRT1 in the pre-meiotic germ cells results in multiple defects of spermatozoa and whole male fertility, while deleting SIRT1 in the late stage of post-meiotic cells has no effect. They confirmed a lack of the SIRT1 mRNA expression in the late round and early elongated spermatids. As SIRT1 is regulated epigenetically by DNA methylation signaling mechanism (Islam et al., 2020), this silence in the late post-meiotic cells may be likely related to methylation of the SIRT1. It is known that the genome-wide DNA methylation occurs prior to meiosis in male germ cells (Oakes et al., 2007). In addition, SIRT1 plays a key role in regulation of the transcription of many genes that is stopped in mature spermatozoa. In this case the high methylation level of SIRT1 in the ejaculated spermatozoa may be a result of the successful maturation and properly epigenetically reprogramming of germ cells during the spermatogenesis. Significant correlations between DNA methylation level of SIRT1 and sperm parameters was not recorded. However, the ram separation according to their semen fertility showed high methylation of SIRT1 in sperm collected from high fertile rams. It was in correspondence with higher sperm motility and lower sperm concentration (Table 3). Bell et al. (2014) reported that in mouse the SIRT1 deficiency decreases sperm count.

4 Conclusions

The current study provided the first results on the DNA methylation status of gene SIRT1 in ram sperm and its relationship with fertility parameters. In addition, we established a clear tendency for alteration of sperm SIRT1 methylation with the ram age. The sperm DNA methylation status at global and gene specific level as an epigenetic modification that regulates the gene expression, can play important role in the prediction of male fertility. The limited data on this issue in rams calls further research, including the effect of breed as well as the environmental conditions and nutrition on the methylation status of ram sperm. The accumulated results will allow the development of new tools for the evaluation and prediction of fertility parameters of rams.

Acknowledgments

The research was implemented under National Scientific Programme “REPROBIOTEH” (grant 0406-105) funded by Ministry of education and science of Bulgaria.

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