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Evaluation of morphology, morphometry and follicular dynamics in FecGE genotyped ewes

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ABSTRACT

This study aimed to evaluate the effect of $FecG^E$ mutation on the development of ovarian follicles. To this end, 42 Santa Inês ewes were genotyped for $FecG^E$ mutation and classified as wild-type ($FecG^{+/+}$), heterozygous ($FecG^{+/E}$) or mutant homozygous ($FecG^{E/E}$). Ovarian fragments were processed, and the follicles were analyzed with regard to the morphology and morphometry using classical histology. For the evaluation of follicular dynamics, ewes underwent oestrous synchronization and were monitored throughout an interovulatory period. A higher ($P < 0.05$) percentage of morphologically normal follicles in the primordial stage was identified in $FecG^{E/E}$ (90.0%) and $FecG^{+/E}$ (88.1%) ewes than in the $FecG^{+/+}$ (73.0%) ewes. There was also a significantly greater ($P < 0.05$) number of morphologically normal follicles in the $FecG^{E/E}$ (87.3%) and $FecG^{+/E}$ (83.3%) ewes than in $FecG^{+/+}$ (76.8%) ewes in the transitional stage. A smaller ($P < 0.05$) diameter was observed in the secondary follicles in $FecG^{E/E}$ (93.8 μm) ewes than in $FecG^{+/E}$ (171.8 μm) ewes. Regarding follicular dynamics, $FecG^{E/E}$ ewes showed a greater ($P < 0.05$) number of ovulations (2.5 ± 0.2) than $FecG^{+/+}$ ewes (1.5 ± 0.3) ewes. Ovulatory follicles were smaller ($P < 0.05$) in the $FecG^{E/E}$ (5.1 mm) and $FecG^{+/E}$ (5.2 mm) ewes than in $FecG^{+/+}$ (5.8 mm) ewes. Santa Inês nulliparous ewes carrying the $FecG^E$ mutation showed a greater proportion of morphologically normal follicles in the primordial and transitional stages than those not carrying the mutation. $FecG^{E/E}$ ewes demonstrated a higher number of ovulated follicles and that $FecG^{E/E}$ and $FecG^{+/E}$ ewes presented ovulatory follicles with a smaller diameter.

1. Introduction

The mechanism of ovarian folliculogenesis is a consequence of a complex endocrine interaction between the central nervous system and the ovary as well as the paracrine and autocrine function of intra-ovarian factors [1]. Among the mechanisms affecting follicular development, the performance of a set of genes called the fecundity genes (*Fec*) [2,3] has been highlighted. In ewes, natural genetic mutations are related to either anovulatory cycles or cycles with a 20% and 40% or >100% prolificacy rate [1]. These changes support the importance of some members of the transforming growth factor beta superfamily and its receptors as regulators of ovarian follicular development and ovulation rate (OR) [4–6].

Ewes carrying mutations in the Fec genes, in addition to showing differences in OR, ovulate from smaller follicles [7,8]. These mutations also affect the response of follicles to hormonal stimuli, regardless of their diameter [9], as well as the development of the uterus and the ovaries causing sterility [10–12].

A specific mutation in the growth differentiation factor 9 (*GDF9*) gene, called $FecG^E$, expresses a phenotypic behavior contrary to other mutations [13]. In this mutation, homozygous ewes showed an increased OR (82%) and prolificacy (50%) [14].

Some studies described the interplay between $FecG^E$ mutations

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and animal production [15,16], pointing out some ovarian and follicular features that differ between these mutant ewes (Chaves et al., 2018, unpublished data). However, to our knowledge, there are no reports describing the effects of $FecG^{E}$ effects on follicle growth until their ovulation. Therefore, this study aimed to evaluate the influence of the $FecG^{E}$ mutation in nulliparous Santa Inês ewes, on both follicle morphometry and morphological features, in the early stages of follicular dynamics and development.

2. Material and methods

2.1. Place and experimental animals

This research was conducted after evaluation and approval from the Committee of Ethics in Animal Use, Brazilian Agricultural Research Company - Embrapa, Tabuleiros Costeiros Unit, Aracaju-SE, Brazil (License: 13072016.006).

Nulliparous ewes (n = 42) belonging to the in situ Conservation Center of Santa Inês Ovines of Embrapa Tabuleiros Costeiros located in Pedro Arle Experimental Field (Frei Paulo-SE, Brazil) were used. This experimental field is geographically situated at Latitude 10°36′15″ South and Longitude 37°38′29″ West.

The ewes were genotyped for $FecG^{E}$ in the Laboratory of Animal Genetics of Embrapa Genetic Resources and Biotechnology (Brasília-DF, Brazil) using the PCR/RFLP technique as reported by Silva et al. [14]. According to the genotyping, the ewes were distributed into the following three groups: wild-type ($FecG^{+/+}$), heterozygous ($FecG^{+/E}$), and mutant homozygous ($FecG^{E/E}$).

2.2. Experiment 1: morphometry and follicular morphology

For this evaluation, ewes (n = 21) with a mean age of 16.0 ± 1.4 months and an average mean body weight of 38.6 ± 5.7 kg, belonging to the $FecG^{+/+}$ genotypes (n = 5), $FecG^{+/E}$ (n = 10), and $FecG^{E/E}$ (n = 6) were used. The ewes were slaughtered, and their ovaries were aseptically collected and transported in buffered medium (DPBS) containing antibiotics (gentamicin) to the Animal Reproduction Biotechnology Laboratory in the Embrapa Tabuleiros Costeiros (Aracaju-SE, Brazil).

Morphometry and follicular morphology were evaluated using classical histology. Tissue fragments (3 × 3 × 1 mm) were removed at random from the right and left ovarian cortex of each ewe to be processed histologically according to Luz et al. [17]. After fixing these fragments into paraffin, 7-μm-thick serial cuts were made with a microtome (Leica RM 2125RT, Heidelberg, Germany) to prepare the microscope slides. After staining with hematoxylin and eosin, as proposed by Behmer et al. [18], the slides were screened every 28 μm under an optical microscope (Olympus BX41TF, Tokyo, Japan) with a magnification of 100 or 400x, depending on folicular size.

Follicles were classified as per their developmental stage by considering their morphology and the amount of granulosa cell layers surrounding the oocyte. Based on the report by Silva et al. [19], follicles (n = 4323) were classified as primordial, transitional, primary, secondary, or antral. Follicles were classified as degenerate when they showed a retracted oocyte, with a pyknotic nucleus and disorganized granulosa cells detached from the basement membrane [20].

Ovarian follicle images were captured using a digital camera linked to an optical microscope (Olympus, Tokyo, Japan). Follicles were measured using a micrometric caliper rule and the ZEN software, version 2011. Two diameter readings were recorded for each follicle, and the average of these two values was calculated as per the method by Lundy et al. [21].

2.3. Experiment 2: follicular dynamics

2.3.1. Estrous cycle synchronization

In order to evaluate follicular dynamics, ewes (n = 21) that were equally distributed into the genotypes: $FecG^{+/+}$, $FecG^{+/E}$, and $FecG^{E/E}$, had a mean age of 30.4 ± 5.8 months, and had a mean body weight of 53.1 ± 5.3 kg were used.

Ewes were submitted to an the estrous synchronization protocol adapted from Lima et al. [22]. On day zero (D0), vaginal sponges impregnated with 60 mg of medroxyprogesterone acetate (Progespon® - Syntex - Buenos Aires, Argentina) were inserted, and 125 μg of Cloprostenol (Prolise®-Tecnopec - Buenos Aires, Argentina) were injected intramuscularly. On day nine (D9), the sponges were removed, and the ewes were monitored for estrus for 30 min every 12 h (6:00 and 18:00) by a trained practitioner assisted by a vasectomized ram.

2.3.2. Ultrasonography evaluation

Follicular dynamics, during one interovulatory period, were evaluated by a single professional using an ultrasound machine (MINDRAY DP2200Vet, Nanshan, China) coupled with a 7.5 -MHz rectal linear transducer. The examinations were performed from the first acceptance of mounting after the synchronization. During the metaestrus, diestrus, and proestrus, ultrasonography was carried out every 24 h, [23]. From estrous detection to ovulation, ultrasonography evaluation was performed every 12 h (6:00 and 18:00) [24]. The time of ovulation was considered as corresponded to the mean time between the examination that detected the preovulatory follicle and the one wherein the follicle was no longer visualized, as described by Tenório Filho et al. [25].

Follicles were classified according to diameter. However, only those structures ≥2 mm in diameter were considered. Follicles were classified as recruited (≥2 mm to ≤ 4 mm), selected (>4 mm to ≤ 5 mm), dominant (>5 mm), or ovulatory (>5 mm and present in the last follicular wave) [26].

Follicular development, ovulation number, ovulatory follicle diameter, estrous duration, and the time interval between the onset of estrus and ovulation were analyzed.

2.4. Statistical analysis

Initially, the variables were tested for normal distribution based on the Lilliefors test and for homoscedasticity using the Bartlett test. When necessary, the data were transformed using the Box-Cox methodology [27]. Further, the parametric variables were submitted to ANOVA with Tukey's post-test. For the evaluation of non-parametric variables, the Kruskal–Wallis test was used with Dunn's post-test. The difference between the genotypes for the observed and expected of morphologically normal follicular frequencies was evaluated using the Chi-square test.

Statistical tests were performed using the IBM SPSS Statistic software, version 20. A significance level of 5% was considered.

3. Results

3.1. Morphometry and follicular morphology

The distribution of the evaluated follicles, considering both the developmental stages and the genotype groups, is shown in Fig. 1.

Although the $FecG^{E}$ genotype did not influence the total number of follicles in the ovary ($P > 0.05$), the number of follicles per stage of development was influenced ($P < 0.05$), the number of follicles per development stage was affected ($P < 0.05$). $FecGE^{E/E}$ ewes showed a higher percentage ($P < 0.05$) of primordial follicles and lower percentage of ($P < 0.05$) transitional follicles than those from

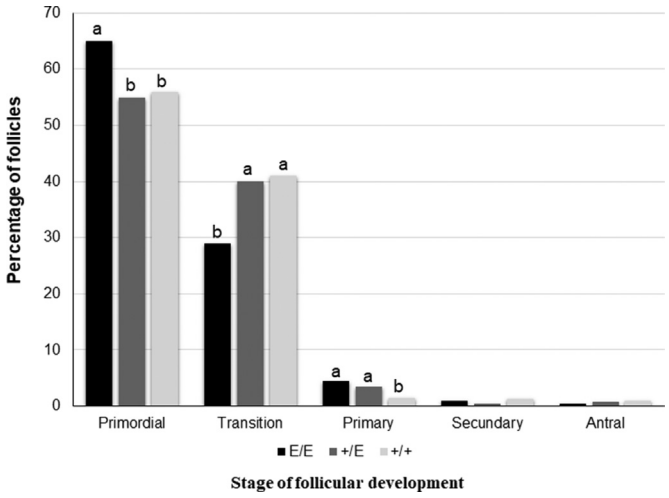

Fig. 1. +/+: homozygous wild-type genotype; +/E: mutant heterozygous genotype; E/E: mutant homozygous genotype.

other genotypes. Regarding the percentage of primary follicles, $FecG^{+/+}$ ewes presented a lower value than the other genotype groups ($P < 0.05$).

Concerning the follicular morphology, a higher percentage ($P < 0.05$) of normal follicles was observed in the primordial follicles in $FecG^{E/E}$ (90.0%) and $FecG^{+/E}$ (88.1%) ewes as compared to that in the $FecG^{+/+}$ (73.0%) ewes. With respect to the transitional follicles, ewes carrying the FecG mutation had a higher percentage ($P < 0.05$) of normal follicles ($FecG^{E/E}$ - 87.3%, $FecG^{+/E}$ - 83.3%) than the non-carriers ($FecG^{+/+}$ - 76.8%). There was no influence of the genotype ($P > 0.05$) on the morphology of primary ($FecG^{E/E}$ - 87.0%, $FecG^{+/E}$ - 80.6%, $FecG^{+/+}$ - 66.7%), secondary ($FecG^{E/E}$ – 78.6%, $FecG^{+/E}$ - 100.0%, $FecG^{+/+}$ - 80.0%), or antral follicles ($FecG^{E/E}$ – 75.0%, $FecG^{+/E}$ – 78.6%, $FecG^{+/+}$ - 100.0%).

The results of the morphometric evaluation of the follicles are presented in Table 1. It can be observed that the secondary follicle diameter from $FecG^{E/E}$ ewes was lower ($P < 0.05$) than that of the $FecG^{+/E}$ ewes, but no different ($P > 0.05$) to that of $FecG^{+/+}$ ewes. The effect of genotype on the diameter of the antral follicles was not evaluated because of the low number of ewes with follicles in this development stage.

3.2. Follicular dynamics

The follicular wave pattern ranged from three (n = 15) to four (n = 6) waves in the different genotype groups. There was a reduced number of animals presenting four follicular waves per genotype (3 +/+, 2 in +/E, and 1 in E/E); therefore, the statistical analysis of follicular dynamics was conducted using only data from ewes that presented three follicular waves (Table 2).

Estrous duration did not differ ($P > 0.05$) among the ewes, regardless of the genotype group (34.3 ± 3.1 h in +/+, 39.4 ± 4.3 in +/E and 39.4 ± 2.2 in E/E). Similarly, the interval between estrus onset and ovulation did not differ ($P > 0.05$) among ewes, regardless of genotype group (21.3 ± 2.3 h in +/+, 28.0 ± 4, 1 in +/E and 26.3 ± 2.6 in E/E).

4. Discussion

Ewes carrying mutations in the *Fec* genes have specific traits in both morphology and follicular dynamics [28–30]. These facts support the importance of *Fec* genes over folliculogenesis and allow a better understanding of the aspects associated with reproductive biology [31,32].

Thus, we hypothesized that the mutation in the *GDF9* gene, $FecG^{E}$, would also show changes in the follicle morphology, either in the earlier or in the more advanced follicular development stages. It was also believed that these changes would elucidate some of the morphophysiological mechanisms responsible for the higher OR and prolificacy in $FecG^{E}$ ewes. The results indicated that some follicular parameters differed between genotypes, making it possible to describe the effect of $FecG^{E}$ on the development of the follicles.

Although a higher percentage of primordial follicles were observed in mutant homozygous ewes ($FecG^{E/E}$), the number of follicles recruited during the ovulatory wave by ewes from this group was not higher. This is contrary to the reports by Silva et al. [33] and Gutiérrez [34] who found that the number of recruited follicles is related to the size of the pool of primordial follicles.

Even considering primordial and transitional follicles in the same follicular development stage group [35] that would increase the number of primordial follicles, the follicular pool vs. recruitment relation does not occur in $FecG^{E/E}$ because these ewes did not present a greater number of follicles recruited in the ovulatory wave.

The higher percentage of morphologically normal follicles in the primordial and transitional stages in $FecG^{E}$ ewes can be hypothesized as an outcome of the increased *GDF9* expression by these ewes. The importance of GDF9 protein in follicle development during the early developmental stages [4,36] and higher expression of this protein in the preantral follicles when compared to the other stages of development is known [37]. Similarly, the importance of *GDF9* was also identified in the preantral follicles of goats via the stimulation of the transition from primordial follicles to primary follicles and the consequent progression to secondary follicles [38] as well as the anti-apoptotic action of *GDF9* in the granulosa cells of the preantral follicles [39].

Smaller diameters of the secondary follicles in $FecG^{E/E}$ and $FecG^{+/}$

Table 1
Mean diameter (μm) with standard error of the Santa Inês nulliparous ewe follicles genotyped for $FecG^{E}$.

Follicular stage	*FecGE* genotypes		
	+/+	+/E	E/E
Primordial	23.6 ± 1.6	20.6 ± 0.8	23.6 ± 0.8
Transitional	26.9 ± 3.2	24.9 ± 1.2	26.5 ± 1.2
Primary	37.0 ± 6.2	43.3 ± 3.4	43.7 ± 2.0
Secondary	129.7 ± 13.1^{b}	171.8 ± 6.5^{a}	93.8 ± 6.6^{b}
Antral$^{(*)}$	429.0 ± 59.4	201.0 ± 22.3	250.7 ± 57.5

+/+: non-mutant homozygous genotype; **+/E:** mutant heterozygous genotype; **E/E:** mutant homozygous genotype.
(*)Follicular stage with an insufficient number of replicates for statistical analysis. Different letters on the same line mean significant difference ($P < 0.05$).

Table 2
Follicular dynamics parameters (mean ± standard error) of the ovulatory wave of nulliparous Santa Inês ewes genotyped for $FecG^{E}$ mutation.

Parameters	$FecG^{E}$ genotypes		
	+/+	+/E	E/E
Recruited follicles (≥2 to ≤ 4 mm)	4.0 ± 0.4^{ab}	5.6 ± 0.4^{a}	3.7 ± 0.4^{b}
Selected follicles (>4 to ≤ 5 mm)	2.7 ± 0.2	3.2 ± 0.2	2.5 ± 0.2
Dominant follicles (>5 mm)	1.7 ± 0.4	2.0 ± 0.0	1.7 ± 0.4
Ovulatory follicles (mm)	5.8 ± 0.2^{a}	5.2 ± 0.1^{b}	5.1 ± 0.2^{b}
Ovulated follicles (n°)	1.5 ± 0.3^{b}	1.8 ± 0.2^{ab}	2.5 ± 0.2^{b}

+/+: non-mutant homozygous genotype; **+/E:** mutant heterozygous genotype; **E/E:** mutant homozygous genotype. Different letters on the same line mean significant difference ($P < 0.05$).

$^{+}$ ewes was also observed by Chaves et al. (unpublished data).

Despite the smaller number of follicles recruited in ewes from the E/E genotype when compared to +/E and similar to +/+, more follicles ovulated in ewes from the E/E genotype than in those from/+ and similar to +/E. Driancourt et al. [40] reported that in ewes carrying the Booroola polymorphism, a higher number of ovulated follicles may occur due to increased recruitment of follicles and/or increased resistance to atresia. Thus, based on the obtained results, we can suggest that the higher number of ovulated follicles observed in *FecG*$^{E/E}$ ewes is not a result of a higher follicular recruitment by these females, as hypothesized and suggested by Chaves et al. It may happen that in mutant homozygous ewes, the follicles are more resistant to atresia, as observed in Finn ewes that had high rates of ovulated follicles [29]. This occurrence may be favored by the early differentiation of the granulosa cells that promote earlier onset of LH receptors [41]. Additional studies analyzing the anti-apoptotic role of *GDF9* in the granulosa cells of *FecG*^{E} antral follicles during the recruitment phase will be important to elucidate the mechanisms responsible for increased number of ovulated follicles in ewes carrying this mutation.

Here, ewes carrying *FecG*^{E} had ovulatory follicles with a smaller diameter than that in ewes that did not carry the mutation. This result is similar to other studies that have revealed ewes carrying mutations associated with greater prolificacy ovulated from smaller follicles [30,41]. The GDF9 protein, which is involved in some aspects that precede and are necessary for ovulation may also be responsible for the smaller diameter of ovulatory follicles.

With respect to its functions, GDF9 is responsible for increasing the proliferation of the granulosa cells [42,43] that increases the concentration of estradiol by sensitizing the somatic cells to the action of gonadotrophins [44]. In addition, the GDF9 protein is involved with the nuclear maturation of oocytes [45] and increased progesterone production by the granulosa cells [42].

5. Conclusions

Santa Inês nulliparous ewes carrying the *FecG*^{E} mutation showed a greater proportion of morphologically normal follicles in the primordial and transitional stages than those not carrying the mutation. We can also conclude that *FecG*$^{E/E}$ ewes demonstrated a higher number of ovulated follicles and that *FecG*$^{E/E}$ and *FecG*$^{+/E}$ ewes presented ovulatory follicles with a smaller diameter.

Conflicts of interest

The authors declare no conflict of interest and are available to provide clarification.

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