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Possible Effect of GDF9 Gene Polymorphism on Ovarian Activity of Egyptian Cows

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Abstract: Ovarian inactivity is considered as one of the most important fertility impeding factor in farm animals, causing high economic losses due to drop of calf crop and milk yield. Oocyte-secreted Growth Differentiation factor 9 (GDF9) is one of the transforming growth factor β (TGFβ) super family that plays significant role in the development of ovarian follicular system The current study was carried out to assess the possible relation between mutation of the GDF-9 gene and ovarian inactivity in Egyptian cattle. The work was carried out in veterinary clinics at Benisuef governorate, Upper Egypt. Cows admitted for gynecological examination with owner complain of low fertility, were examined for two successive weeks at least to follow up the reproductive status and/or disorder. The examined cows were categorized into: normal fertile group with normal ovarian structures and infertile group with bilateral smooth ovaries (BSO). Estimation of the incidence of ovarian inactivity, collection of blood samples, assaying plasma progesterone, isolation of DNA for further studying GDF9 gene sequence were done. The amplification of DNA, purification of PCR products and their sequencing were performed. The Sequence analysis, alignment and amino acids translation were performed using suitable bioinformatics software. The current study indicated that the incidence of ovarian inactivity in cattle was 47.69 and 52.3 % of the total examined and total anestrous animals, respectively. Cows having smooth inactive ovaries had very low progesterone level (< 0.08±0.01ng/ml) while animals showed structure on the ovaries and cyclic activity had a level of 0.17±0.01ng/ml (Follicular phase) to > 1ng/ml (Luteal Phase). The sequence alignment of GDF9 gene, between normal and infertile animals, indicated that two out of five infertile cows have nucleotide substitution G→A at nucleotide number 809. The amino acid translation using Fast PCR software showed the replacement of amino acid Glycine by Arginine corresponding to the present SNP. It was concluded that amino acid replacement may confirm the predicted association between polymorphism of GDF9 gene at this position with the ovarian inactivity in the examined caws.

Key word: Ovarian inactivity · Cattle · GDF9 gene · DNA polymorphism

INTRODICTION

Low productive efficiency in Egyptian cows was referred to some reproductive disorders, out of which inactive ovaries, presents 35-47% [1, 2] with a higher incidence in Upper than in Lower Egypt [3]. This reproductive disorder is one of the main reasons of the suboptimal calf crop, low milk production and animal wealth drawbacks [4].

Ovarian follicle is the fundamental unit of female fertility, contains single oocyte, granulose and theca cells. These structures are stimulated to grow, then a single competent oocyte is usually culminating in ovulation in cattle each estrous cycle [5].

Growth Differentiation factor 9 (GDF9) belongs to Transforming Growth Factors β superfamily (TGFβ) that has a significant role in growth and maturation of ovarian follicle [6-8]. It was reported that, GDF9 can

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influence the initiation of primordial follicle [9], inhibits apoptosis of granulosa cell and consequent follicular atresia and keeps on the follicles survived [10] and after ovulation it takes part in luteinization of the follicular wall [11]. Therefore, it was assumed that GDF9 influence oocyte maturation by regulation of proliferation, differentiation and cumulus expansion of granulosa cells [12, 13]. Accordingly, the mutation of GDF9 could lower the ovulation rate and results in inferior fertility, fecundity and litter size [14-16].

It was reported that the SNP is likely to affect the three-dimensional structure of the translated GDF-9 protein [17] and reduce its binding capacity with bone morphogenetic protein 15 (BMP-15) that may influence the phenotype related to fertility performance [18]. Moreover, GDF-9 gene is one of the candidate genes determining the fertility of livestock [19].

The aim of this work was to investigate the presence of SNP in the GDF9 gene and to assess the possible association between the DNA mutation of this gene with ovarian inactivity in Egyptian cattle.

MATERIALS AND METHODS

Field Study: The present work was carried out on two hundred sixty (N=260) local crossbred cows in veterinary clinics at Beni-Suef governorate at Upper Egypt with history of low fertility. Cows were subjected to clinical and gynecological examinations by ultrasonography with an endorectal array (8.6 M Hertz; PiaMedical Flacse Saote, Netherland) twice for two successive weeks 3-6 months after last calving and the reproductive status and/or disorders were recorded. Cows showing no estrous signs with small non-functioning ovaries were considered suffering from ovarian inactivity. The animals under examination were categorized into two groups. Group (1) includes cows with normal physiological ovarian structures and Group (2) includes cows with bilateral smooth inactive ovaries.

Blood Samples Collection: Duplicate blood samples for each animal were collected on EDTA-containing vacutainer tubes and kept on ice for good storage condition during transportation. One of duplicates was used for separation of plasma and kept at -20°C after centrifugation at 3000 rpm for 15 minutes for progesterone assay and the other one preserved as whole blood at -20°C for DNA isolation.

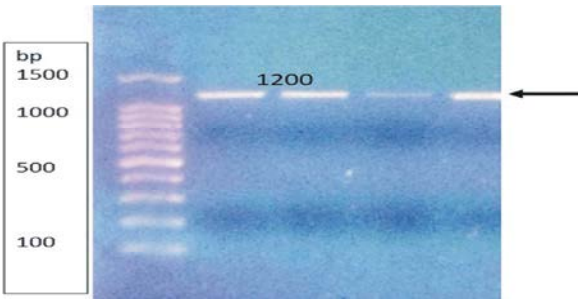

Fig. 1: Showing amplification of GDF9 gene using PCR

Progesterone Assay: Plasma progesterone level was assayed using ELIZA micro wells technique, kits from Novotec, Germany [20] and ELIZA reader (Anthos Zenyth 200rt). The kit had a sensitivity of 2.0pg/ml with inter- and intra-run precision coefficient of variations of 2.9 and 4.85, respectively.

DNA Extraction: DNA was isolated from whole blood samples using DNA extraction kit (Qiagen, Germany) and DNA quality was measured using Nano drop 1000 (Thermo scientific, USA).

PCR Amplification of GDF9 Gene: Nine DNA isolates from 4 normal cyclic group and 5 from smooth inactive ovary group were amplified using PCR. Size of GDF9 gene was amplified using the following primers:

Sense: 5-AAGACTCTCCCTAGAGCTCCATACTC-3 and

Antisense: 5-TAGAACTGCAATTCCACCCAAG-3

The thermal profile of PCR conditions was set up as initial denaturation at 94°C for 5 minutes, 35 cycles ; each consists of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds and elongation at 72°C for 1 minute and final extension step at 72°C step for 5 minutes [21]. The GDF gene was successfully amplified using the previously mentioned GDF sense and anti-sense primers. The amplified samples showing bands at 1200 bp Figure (1)

Purification and Sequencing of GDF9 Gene: The amplified PCR products were purified by universal DNA purification kit and sequenced in LGC, Germany. The amplified fragments were aligned together and matched with published sequences in Gene Bank database. The sequenced segments were matched with

published sequence ID: XM_025292708 (*Bubalus bubalis GDF9*) and transcript variant (X2, mRNA) BioEdit software was used to compare amplified *GDF9* segments between normal and infertile cows. The prediction of translated amino acids of these amplified fragments was done using Fast PCR software.

RESULTS AND DISCUSSION

The reproductive efficiency of the Egyptian cow is declining, with consequent great economic losses. Ovarian inactivity is still a dominant constrain of reproductive potential in this species [22].

The present study revealed that the incidence of ovarian inactivity in cattle was 47.69 and 52.3 % of the total examined and total anestrous animals, respectively. These results were in coincidence with some authors who reported that the incidence of ovarian inactivity ranged from 41-51% from the total number of anestrous cows [22, 23] and the condition was attributed to availability of minerals, vitamins and trace elements in animal feed which is necessary for proper ovarian activity [22].

The current study showed that cows having smooth inactive ovaries had very low progesterone level (0.08±0.01ng/ml) while those showed structure on the ovaries and cyclic activity have plasma progesterone concentration of (0.17±0.01 ng/ml) during the follicular phase. Greater than 1ng/ml was reported as indicator of corpus luteum activity during the follicular phase. Plenty of studies indicated that, serum progesterone profile is a good indicator for diagnosing ovarian activity and the animal fertility [1].

In the current study the *GDF9* gene was successfully amplified at 1200 bp as shown in Figure 1. The amplified fragments were purified and sequenced to identify any nucleotide mutations that may be responsible for the smooth inactive ovary status as compared to the normal cyclic cows. The sequence alignment indicated that two out of five infertile cows have nucleotide substitution (G→A) on nucleotide number 809 (Figs 2, 3).

The amino acid translation using FastPCR software showed the replacement of amino acid Glycine (G) by Arginine (R) corresponding to the present SNP. This amino acid replacement may confirm the predicted association between polymorphism of *GDF9* gene at this position with the ovarian inactivity in the examined caws.

As previously mentioned that GDF9 and BMP15 cooperate to initiate the oocyte development and maturation in mammals [24, 25] but the specific participation of BMP15 and GDF9 in this process differ among species [26]. It has been found that GDF9 and BMP15 are not like most TGFβ members, which are bound by the intermolecular disulfide bond using cysteine ligands monomers, as the GDF9 and BMP15 can bind each other by chemical cross linking and immune precipitation. The GDF9 protein acts as a dimer and forms either self homodimers or unites with BMP15 forming heterodimers [27]. The previous studies reported that malfunction of

Fig. 2: Shows the substitution (G A) on nucleotide number 809

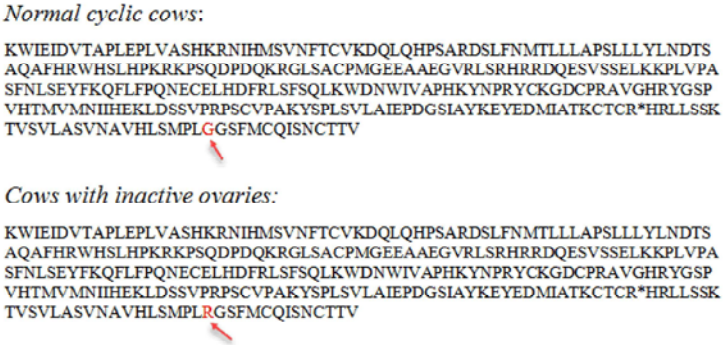

Fig. 3: Predicted amino acid for normal cyclic and infertile cows

this protein hinders the development of ovarian secondary follicles and lowers the number and decrease the size of antral follicles in cattle [28]. The amino acid substitution(Ala/Glu) resulted from SNP in GDF9 was associated with lower number of viable oocytes [29]. Other study reported that Arginine defines high binding to its receptor in the prehelix loop of GDF9 and the bioactive mouse GDF9 share the conserved arginine with human BMP15 in the pre-helix loop. On the other side. their low-activity counterparts (mouse BMP15 and human GDF9) share a conserved glycine or proline instead [30]. On the other side, mutations in BMP15 associated with low synergy with GDF9, reduced mature protein production and most likely underlie the physiological changes in ovary and lower ovulation [31].

Recent study reported that the residues Gly^{391}, Ser^{412} and Lys^{450} might be important for human GDF9 bioactivity as it may affect the stability of the pro-mature complex or may change the receptor binding efficiency of mature GDF9 [32].

It can be concluded that signaling from the GDF9 dimer may be partially impaired by the amino acid substitution in one of the GDF9 proteins [21]. The difference in fertility status among the examined Egyptian cattle in the current study may be referred to the substitution of Glycine in GDF9 gene by Arginine in low fertile cows.

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