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Decreased glycodelin A expression in rat endometrium after stimulation with recombinant follicle stimulating hormone (rFSH) recombination affects the number of live births

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ABSTRACT: Glycodelin A (GdA) has been postulated as a marker of endometrial receptivity. However, its expression pattern following ovarian stimulation is conflicting, and its link with implantation and pregnancy is unknown. This study aimed to evaluate the influence of recombinant follicle stimulating hormone (rFSH) on glycodelin expression levels in the endometrium during the secretory phase and how it interacts with live birth. In the amount of 27 female Wistar rats with regular estrus cycles were separated into normal cycle and two stimulated cycle groups of SC1 & SC2 by injecting rFSH doses of 12 IU and 25 IU, respectively, using intraperitoneally (ip) followed by human chorionic gonadotropin (hCG) injection with a dose of 10 IU 48 h later. First, second, and third days after hCG injection, blood and uterine were obtained. GdA expression was examined by immunohistochemistry, while ELISA measured estrogen and progesterone levels. Male rats were mated with female rats from both groups of normal cycle and stimulated cycle. GdA expression in the glandular epithelium significantly decreased ($p < 0.05$) between control and dose 2, and between doses 1 and 2. Estrogen and progesterone levels did not differ between normal and stimulated cycles ($p > 0.05$). Furthermore, there was a significant relationship ($p < 0.05$) between decreased GdA expression in the glands of the SC1 and SC2 groups and the number of live births ($r = 0.68$). In conclusion, a significant decrease in GdA expression of 25 IU rFSH group reduced live births from 10 to 4 individuals.

KEYWORDS: Glycodelin A; recombinant FSH; rat endometrium; live birth.

1. INTRODUCTION

Embryo implantation is a complicated series of occasions main to a successful pregnancy. The process of attachment of the embryo to the luminal epithelium of the endometrium is regulated by a complex system involving the interaction of many factors, such as hormones, adhesion molecules, and extracellular matrix. Two primary components are essential to decide the success rate, particularly embryo high-quality and endometrial receptivity. Failure of the embryo to implant into the endometrium is not unusual in cases of unexplained infertility cases. In the normal cycle, the implantation window opens in the middle of the secretory phase, which is day 20-24 of the menstrual cycle [1-5]. During this period, there were changes in morphology and histology and the expression of several protein molecules as receptivity markers [6]. Glycodelin A (GdA) is a primary biomarker of endometrial function, and its expression will increase through

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the implantation window [7]. GdA synthesis is induced by the ovarian steroid hormones of estrogen and progesterone [8-11]. Changes in steroid hormone secretion due to COH in cycles stimulated in IVF treatment are characterized by abnormal endometrial growth [9]. Reduction of GdA expression all through the receptive duration impairs implantation and pregnancy [12]. Implantation failure and low pregnancy rates in IVF treatment result from impaired reception endometrial [13].

Glycodelin A (GdA) is a particular glycoform of one of the maximum widely secreted glycoproteins inside the endometrium throughout the implantation window [14, 15]. Its secretion is localized to the pinopod surface and secreted from the luminal epithelial gland [13]. Increased GdA expression in the mid-secretory phase suggests its role in facilitating implantation by preventing fetal allograft rejection [16]. Unexplained infertility problems occur in women, while GdA expression is decreased inside the endometrium. It indicates glycodelin may also function in endometrial receptivity throughout implantation [17, 18]. Changes within the period of endometrial receptivity are often the result of increased controlled ovarian hyperstimulation (COH) induced steroid hormone secretion in stimulated cycles in IVF treatment. It is related to the endometrium's development and expansion, which has continued for several days and does not correspond to the menstrual cycle phase. Disruption to endometrial receptivity is characterized by suboptimal expression of several protein molecule markers' receptivity [19-22]. Glycodelin expression increased during the implantation period facilitates embryo attachment, whereas abnormal expression is found in patients with repeated implantation failure [14, 15].

The COH protocol has been shown to affect the expression of many protein molecules, including GdA [23-27]. In this study, the levels of GdA expression and serum levels of the hormones estradiol and progesterone were compared to the number of children born to female rats with normal and stimulated cycles. Furthermore, a correlation test was conducted between GdA expression levels with steroid hormone levels and the number of births in the normal cycle and stimulated cycle groups.

2. RESULTS

2.1. Endometrial dating

Histological dating of the endometrium was assessed based on changes occurring in the endometrial luminal epithelium [9]. Inside the estrus phase, the lumen's epithelium changes from low columnar during metestrus and diestrus to columnar high in proestrus and estrus. A total of 27 endometrial rat samples were assessed, 26 in the secretory phase and one in the proestrus period (Figure 1).

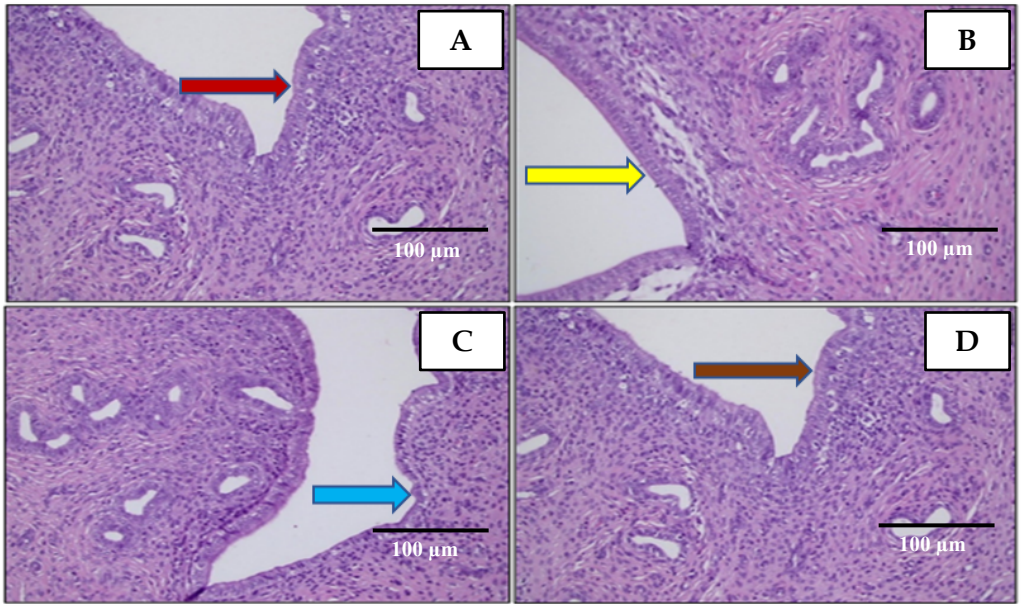

Figure 1. Histological images of rat endometrial tissue throughout the estrus cycle, stained with H-E for histological dating. A, estrus, B met estrus, C diestrus, D proestrus. The optical magnification was 200x. Red arrow = hight columnar luminal epithel; Yellow arrow = low columnar luminal epithelial cells; Blue arrow = low columnar luminal epithelial cells; Brown arrow = hight columnar luminal epithelial cells.

2.2. Glycodelin expression in endometrial gland and stromal compartment

Figure 2 shows the results of immunohistochemical staining of the glandular and stromal compartments of the rat endometrium. The endometrial glandular and stromal compartments showed positive expression for GdA, indicated by brown as depicted in Figure 2A, from the normal cycle group, and Figures 2b and 2C from the stimulated group. A total of 27 samples of the endometrium of female secretory phase rats examined by IHC showed different levels of expression ranging from weak to strong intensity. Figure 2 suggests that the group with the normal cycle produced a stronger brown color intensity compared to the cycles of the stimulated groups 1 and 2.

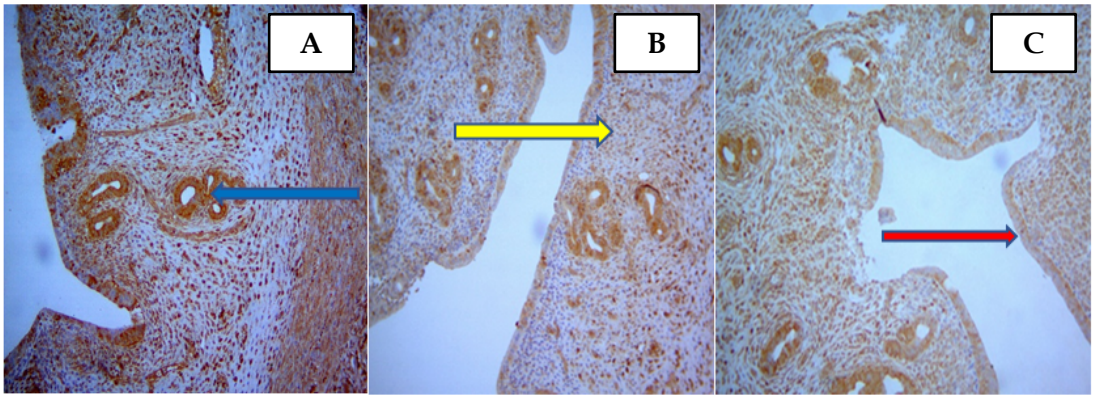

Figure 2. Immunohistochemical staining of GdA in mouse endometrium. A. Nature; B. Stimulated cycle group 1; and C. Stimulated cycle group 2. The optical magnification was 100x. Blue arrow= expression of GdA in gland epithelial cells; Yellow arrow = expression of GdA on stroma cells; Red arrow = expression of GdA in luminal epithelial cells.

GdA expression was analyzed using image-J application. The results confirmed that glycodelin expression increased in the endometrium glandular compartment compared to the stromal compartment in each normal cycle and stimulated cycle group (Table 1). The common level of GdA expression inside the endometrial glands throughout the secretion phase of the normal cycle and stimulated cycle groups with 12 IU and 25 IU rFSH doses decreased were 203, 181, and 152, respectively. Statistical analysis showed a significant difference between NC and SC2 ($p < 0.05$) and between SC1 and SC2 ($p < 0.05$). In the stromal compartment, there was a decrease in the expression level, but statistically, there was no significant difference in each group. For the number of children born, there has been significant distinction among NC and SC2 and between SC1 and SC2 ($p < 0.05$). Furthermore, the relationship between steroid hormones (progesterone and estradiol) and GdA expression showed insignificant results ($p > 0.05$). Correlation test between GdA expression levels with the number of births, there was a significant relationship ($p < 0.05$) between the decrease in GdA expression in the glands of the SC1 and SC2 groups and the number of births ($r = 0.68$).

Table 1. Mean of GdA expression in endometrial glands and stroma, levels of estradiol, progesterone and number of live births in normal cycle (NC) and stimulated cycles (SC) groups.

Group	NC	SC1	SC2	p-value
Expression GdA:				
In gland cell	203(25.77)	181(27.06)*	152(12.51)*	< 0.05
In stroma cell	181(15.38)	166(9.58)	167(11.27)	> 0.05
mRNA FROK1 (µg/ml)	1.59×10^{-7}	3.88×10^{-7}	2.35×10^{-7}	> 0.05
Estradiol (pg/mL)	55(14.21)	51(15.58)	44(12.07)	> 0.05
Progesterone (ng/mL)	57(28.15)	85(20.81)	61(30.21)	> 0.05
Birth	10 (0.81)	10 (0.81)	10 (0.81)	< 0.05

2.3. The mRNA gene expression analysis

GdA(FROK1) mRNA expression was assessed using quantitative real-time PCR (RT PCR) techniques. All 27 samples examined obtained very small expression results (Table 1). There were differences in expression in the normal cycle and stimulated cycle (1.59×10^{-7}, 3.88×10^{-7}, and 2.35×10^{-7} µg/ml, respectively). However, there were no significant differences ($p < 0.05$) among three cycles.

2.4. Serum levels of the hormones estradiol and progesterone inside the normal cycle group and the stimulated cycle group

Table 1 and Figure 3c and 3d show the average serum estradiol and progesterone levels of rats from the normal and stimulated cycle groups. Serum steroid hormones were taken serially in the secretory phase, the first, second, and third days after the estrus phase. It was found that estradiol hormone levels decreased in the stimulated group. On the contrary, there was an increase in progesterone levels in the stimulated cycle group, but statistical analysis showed no significant difference ($p > 0.05$).

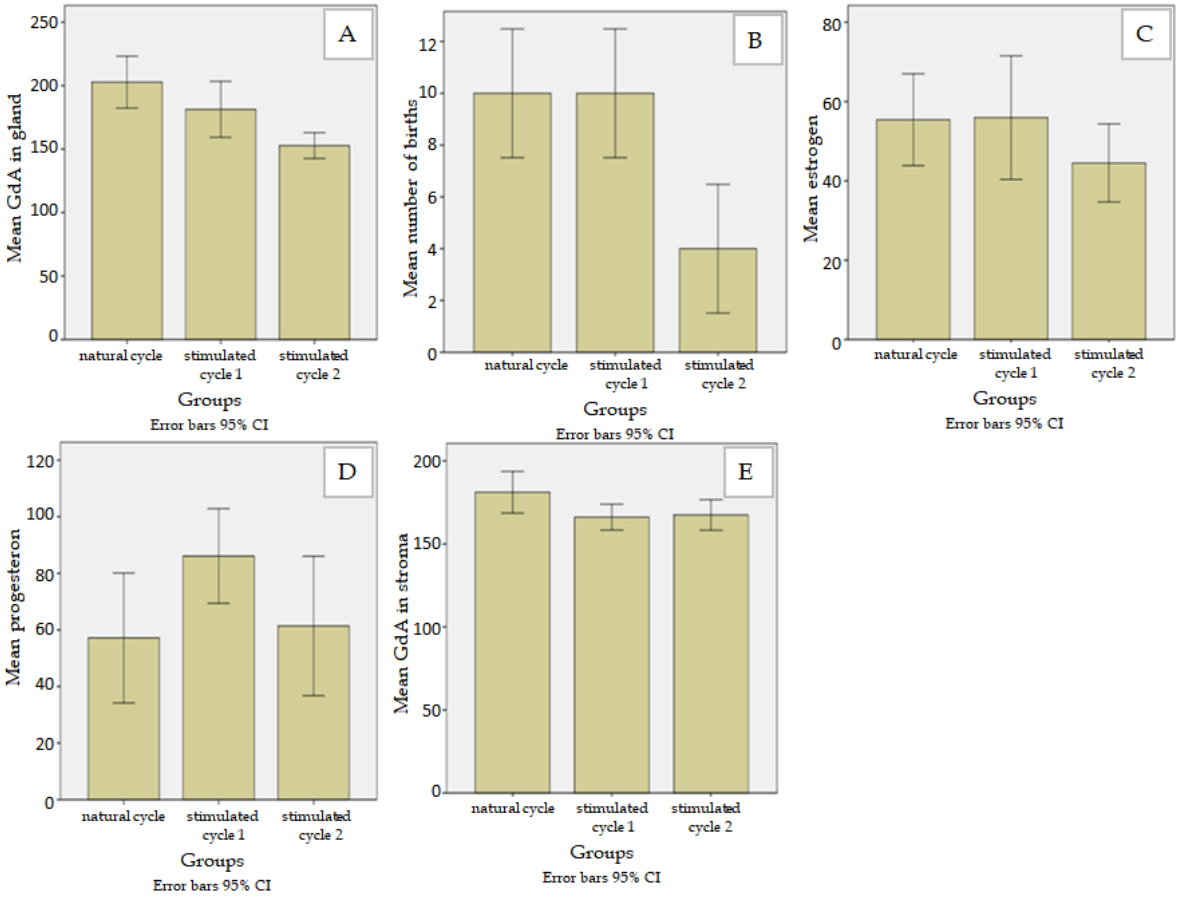

Figure 3. Graphs of GdA expression in gland with $p < 0.05$ (A) and stroma with $p > 0.05$ (B) compartemen, estradiol with $p > 0.05$ (C) and progesterone level with $p > 0.05$ (D) and number of live births GdA with $p < 0.05$S (E) in normal cycle and stimulated groups.

3. DISCUSSION

The increased presence of GdA during the secretory phase and all through the implantation window shows its crucial function within the implantation process. GdA expression inside the endometrium has been validated using various immunohistochemistry techniques [9]. In our observation, GdA expression changed into detected endometrial glands and stroma throughout the secretory phase in the endometrium of female rats in the normal and stimulated cycle groups. GdA expression levels have been better inside the glandular compartment than inside the stromal compartment. It could be because of the origin of the glandular and stromal compartments from epithelial cell lines that produce and secrete higher amounts of GdA than the

endometrial stromal compartments from mesenchymal cell lines. The stromal compartment showing insignificant results may be explained by the low expression of GdA per definition [28].

The presence of GdA during the secretory phase and implantation period suggests that this glycoprotein can also play a role in the early phases of reproduction. Our data suggest that GdA expression inside the gland becomes significantly lower within the endometrium in the cycle group stimulated by the 25 IU rFSH dose compared to the normal cycle group. The decreased expression of GdA in the stimulated cycle group allows endometrial receptivity to be impaired. Current research has proven that the serum GdA/Insulin-like growth factor-binding protein-1 (IGFBP1) ratio is better in women who completed being pregnant than individuals who are no longer pregnant [29]. Lower serum GdA levels were also found in infertile women [18]. Women with idiopathic infertility and women with endometriosis indicate low GdA values [30, 31]. Our statistics recommend that rFSH stimulation harms endometrial receptivity. In contrast, increased GdA expression during the receptive phase has contributed to the attachment of blastocysts to the endometrial epithelium [32]. Our outcomes consider this concept and propose a negative impact of CC or rFSH treatment on endometrial GdA expression, in which GdA functions throughout implantation.

Uterine development is controlled by the presence of the hormones estrogen and progesterone. We serially measured levels of the hormone estradiol, progesterone and GdA expression on the first, second and third days of the estrus phase. It was done because the implantation period in female mice throughout the secretory phase was difficult to determine. Our data show varying values. There is no conclusive pattern in the normal cycle and stimulated cycle groups. In the group stimulated with doses of rFSH 12.5 IU and 25 IU, estradiol levels were lower than in the natural cyclic group.

In contrast to the hormone progesterone, there was an increase in the stimulated group, the highest at the rFSH dose of 12.5 IU. Van der Gaast et al. study showed no distinction in steroid hormone levels during the luteal phase in each normal cycle and stimulated cycle groups [33]. It could be because exposure to extra hormones that affect P4 production by the corpus luteum did no longer go through functional luteolysis and may have retained some of its ability to produce P4 in the body under stimulation by endogenous and exogenous gonadotropins in the stimulated cycle [34]. Variations between low E2 and high P4 can occur in cycles given ovarian stimulation [12, 35].

This study found a significant association between decreased GdA expression levels and live birth rates. The mean live births in the normal cycle group and the stimulated cycle at 12.5 IU of rFSH decreased significantly to the group stimulated by rFSH at 25 IU from 10 to 4 live births. Our findings advise that abnormal GdA expression after stimulation with 25 IU of rFSH reduces the live birth rate. Our findings correlate with the study from Skrzypczak et al. (2005), who obtained the lowest GdA values in women with idiopathic infertility and infertile women with endometriosis [18]. GdA expression decreases drastically in women with repeated implantation failure than in fertile women throughout the implantation window [17]. The role of GdA in the implantation process has been widely reported. Because of its immunosuppressive properties, GdA can adjust the mechanisms of fertilization, implantation, and embryonic development. The authors assessed GdA concentrations throughout the implantation period of the nonconception cycle in women with reproductive disorders [17]. While glycodelin expression became high within the polycystic ovary syndrome (PCOS) group, the being pregnant rate became 60%, and all pregnancies led to live births. However, inside the vulnerable expression group, three out of four pregnancies ended as early miscarriages [36].

The limitation of this study is that the period of implantation cannot be known with certainty during the secretory phase, so the parameters were measured serially on the first, second, and third days after the estrus phase and after the administration of hCG in the stimulated cycle. Furthermore, other receptivity markers, such as Mucin-1 and pinopod, have not been tested, although each sign is associated with GdA.

4. CONCLUSION

There was a significant decrease in GdA expression in the group that was stimulated by 25 IU of rFSH, which resulted in a reduction in the live birth rate from an average of 10 to only 4 individuals. Therefore, it proved that there is a correlation between lower levels of GdA expression and lower fertility in women as well as lower levels of cell proliferation in the uterus.

5. MATERIALS AND METHODS

5.1. Preparation of rat experiment

The ethics committee has approved Wistar female rats that have been used as experimental animals of the Faculty of Medicine, University of Indonesia (Number: KET-721/UN2.F1/ETIK/PPM.00.02.2020). Handling procedures followed the recommendations outlined in the guidelines for the care and use of experimental animals. A total of 36 adult female rats, aged 3.5 months, and 5 male rats, weighing 150-200 g, were acclimatized for 1 week under 12 h of light; 12 h dark cycle. Animals were kept in the Biomedical Center for Basic Health Technology (PBTDK) unit at a temperature of 22^{o}C and provided with drinking and standard feeding pellet. Vaginal cytology was completed every day to decide the estrus cycle of each animal for 3-5 cycle periods. Female mice without normal estrous cycle periods were excluded from the study, and only female mice with regular cycles were used. After three or more cycles of normal estrus in a row, the animals were randomly divided into three groups: the control group and two treatment groups.

The treatment group was injected with recombinant FSH (clone) at a dose of 12.5 IU and a dose of 25 IU intraperitoneally (ip) at the beginning of the diestrus phase and continued with injection of human chorionic gonadotropin (hCG), a dose of 10 IU, 48 h later. Uterine removal was performed on day 1, day 2, and day 3 after hCG administration, and the same pattern was also performed in the control group. Some of the female rats in the control and treatment groups were mated with male rats after administering hCG (two males with 3 females in one cage). Successful mating is indicated by the presence of a vaginal plug the next day.

5.2. Uterine retrieval and endometrial dating

Uterine organs were taken on day 1, day 2, and day 3 after hCG administration. Before the surgical operation, animals were sedated with ketamine 0.1 mL/kg BW. The left and right uterine organs were cut and stored in 10% formalin normal buffer solution (BNF). Eventually, the samples were dehydrated in a graded alcohol series, continued in xylene solution, and embedded in paraffin blocks. All sample pieces embedded in paraffin were stained with hematoxylin & eosin (H&E) staining for pathological diagnosis and histological dating. The histology of endometrial tissue was analyzed by observing the development of the endometrial luminal epithelium [37]. In the metestrus phase, the luminal epithelium has a columnar height and was low in the diestrus phase. During the proestrus and estrus phases, thickening and forming a pseudostratified layer, the luminal epithelium reaches its maximum height, and apoptosis increases.

5.3. Immunohistochemistry staining of glycodelin A

The tissues retrieved from paraffin blocks were then stained using the immunohistochemistry method. The tissues were cut with a thickness of 3.5 µm. Afterward, antigen retrieval was conducted in a container using Tris EDTA (pH = 9). The container was heated in Retrieval Generation One BioGear with a temperature of 98^{o}C for 15 min. The tissues then undergo incubation with glycodelin A monoclonal antibody obtained from rabbit. The incubation was performed overnight (24 h) at 4^{o}C. We measured the immunoreactivity of three components from the tissue, which might be the glandular, stromal, and luminal components. The images of the tissue on the objective glass were taken by Olympus Microscope confocal camera CX23 with 40x objective. The images were then exported from the camera using IndoMicroView software as TIF images. The images were analyzed by using the Immunohistochemistry (IHC) profiler plugin of ImageJ software [38].

5.4. Measurement of steroid hormone levels

Hormone levels in rat serum were examined by the enzyme-linked immunosorbent assay (ELISA) method, using BIOENZY reagent kit. No. BZ 08181470-EB (Indonesia), standard curve in the range within 2-600 ng/mL. The steroid polyclonal antibody bound in the well reacted with the progesterone antigen in the mouse serum, and a second antibody labeled with biotin was added. In the binding process of antigen-antibody labeled with biotin, streptavidin-horseradish peroxidase (HRP) was added and incubated. Unbound streptavidin-HRP is wasted during washing. The color that appears after the substrate's addition is measured by its absorbance value at 450 nm. The sensitivity value for measuring the hormone estradiol was 15 pg/mL, and the coefficient between examinations was 6%. The sensitivity value for progesterone was 1.046 ng/mL, and the coefficient between measurements was < 10%. Prior to the examination, serum and reagents were stored at -20^{o}C.

5.5. Gene expression analysis

Gene expression was analyzed using quantitative real-time quantitative PCR (qPCR). Total RNA was extracted from all samples using a total RNA mini Kit for tissue samples (Geneaid, Taipei, Taiwan). Both the

quality and quantity of the RNA were determined using a Nanophotometer implen. cDNA was synthesized from 5 µg of total RNA using Toyobo (Japan) according to the manufacturer's protocol. The sequences of specific primers of PROK1 gene are listed in Table 2. All reactions were performed using 20 ng cDNA, 10 µl of SensiFAST™ SYBR® No-Rox (Bioline, Meridian Bioscience, Ohio, USA), and sets of primers at the optimized concentrations. Final reaction volumes were made up to a total volume of 20 ul with RNAse-free water. The cycling conditions were 95°C for 5 min, 40 cycles with 95°C for 5 seconds, 57°C for 10 sec, and 72°C for 15 sec. The qPCR was carried out on the Prime Pro 48 Real-Time PCR (Techne Cole-Parmer, Staffordshire, United Kingdom). Quantification was performed using an external standard curve with five serial dilutions between 10 and 10^{-3} ng/µL.

Table 2. Primer sequence of PROK1 gene.

Gene	Primer Sequence	
	Forward	**Reverse**
PROK1	AAG TCT TCA TCA TGC TCC TTC T	ACA CTG GAC ATC TCG TTC AC

5.6. Statistical analysis

Statistical analysis was conducted using SPSS release 22. Step one is to conduct the normality and homogeneity test. If the data are not normal and not homogenous, nonparametric tests using Kruskal-Wallis were then conducted. We used One-way ANOVA to find any correlation between the histological score of three designated groups (one control and two intervention groups) and the protocol of the groups for each of the different compartments (stromal and gland epithelium). Post hoc analysis was then used, using the Tukey HSD (Honestly Significant Difference) test.

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