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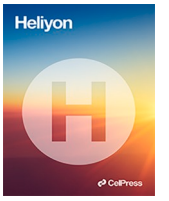

Study of hydrosalpinx on endometrial growth and expression of HOXA10mRNA and related factors

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ABSTRACT

Objective: The objective of the study is to extract *the patient's endometrium* at the time of *proliferative stage using hydrosalpinx in order to* culture the cells and decidualization induction in vitro. Further, the study is also intended at identifying the expression of HOXA10mRNA and related factors and understand the hydrosalpinx's impact upon the working mechanism of endometrial cells.

Methods: Once the extraction of the primary cells is over, the cells are cultured and other activities are performed such as the cell identification, CCK8 assay, cell decidua induction and HE staining. The researchers assessed the expression levels of HOXA10, IGFBP1 and avβ3 in either proliferation or secretion of the endometrium. This was accomplished using Western blot assay and real-time fluorescence quantitative PCR.

Results: The results confirmed that at the time of endometrial proliferation, there was a decline in the expression of HOXA10 *as a result of tubal effusion influence*. This affected its expression in the secretory stage i.e., corresponding function. Further, a significant decline was observed in the levels of HOXA10mRNA of endometrial cells that were subjected to *continuous tubal effusion*, post decidualization. It was found that during decidualization, *if the tubal effusion* is removed, it is possible to restore the expression of HOXA10mRNA to a certain extent, though it is not possible to reach the general endometrial level. So, in terms of clinical aspects, the expression of *HOxa10 mRNA* by the endometrial cells decreases significantly when blocking the hydrosalpinx.

Conclusions: Among hydrosalpinx patients, one of the major mechanisms that damage the endometrium was found to be the abnormal expression of HOXA10 followed by IGFBP1 and avβ3, its downstream genes. This further results in the implantation of the embryo as well. Though it is possible to gradually repair the damage after the removal of hydrosalpinx, the recovery is a time-consuming process.

1. Introduction

As per the literature, hydrosalpinx (HS) generally causes infertility in the range of 10–30% [1]. In spite of treating the patient with IVF-ET, if HS is present, then the success rate of the process gets reduced by 50% [2,3]. At present, the reduced pregnancy rate in

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hydrosalpinx is attributed to a few reasons such as embryo implantation disorder, embryo toxicity, mechanical scouring, alteration of the intrauterine environment [4,5]. Endometrium receptivity denotes the capability of the decidualized uterus to get implanted with an embryo and develop it as a fetus. Some of the common endometrial receptivity indicators are homeobox HOXA10, integrin ($AV\beta3$), leukemia inhibitor (LIF), *mucin* (MUC1), and*air ventilation stomatal homologue 2* (Emx2) [6–8]. In general, it is a monthly process for the endometrium to undergo proliferative to secretory stages so as to prepare for pregnancy. Decidualization corresponds to the transformation of the endometrial stromal fibroblasts as a special category of secretory desidual cells so that the latter can provide the most nutritious and immune-privileged matrix required for the implantation of the embryo and placental development. *On the contrary to most of the mammals, the human endometrium does not need to be decidualized in order to implant the embryo*. Interestingly, this process is promoted by the increase in the levels of progesterone after the ovulation process and high local cAMP production. Such a process demands the mutual regulation of growth factors, steroid hormones, adhesion molecules, cytokines etc., [9].

Having been identified as one of the primary genes for the development of uterine (and endometrium), Homeobox protein A10 (HOXA10) ensures the proper functioning of adult uterine and enacts an important role in the decidualization of the endometrium, implantation of the embryo. Further, it is also required for the endometrial receptivity. Hydrosalpinx fluid tend to reduce the expression of HOXA10. When women are diagnosed with hydrosalpinges, their implantation rates get reduced through a potential molecular mechanism of HOXA10. Thus, *HOXA/HOXA10* gene plays a crucial intrinsic part of the processes such as implantation, decidualization and the immunomodulation in adult uterus. In case of interruption in the expression of HOXA10 gene or its targeted mutation, it results in the development of embryo implantation disorders like *tubal ligation*, HSF etc. This phenomenon impacts the endometrial receptivity as well. As per the immunohistochemical diagnosis of the endometrium among HS patients prior to and post-surgery, it was found that the expression of HOXA10 increases in various places such as endometrial glandular epithelium, luminal epithelium and stroma in the middle luteal period, once the surgery is over [8]. Further, the literature also found that during the implantation window, the expression of LIF was found to be low in the endometrium of the HS patients in comparison with the normal fertile patients *with HS* [10]. In this background, it is crucial to have an in-depth knowledge about the HS patient's endometrium, at the time of proliferative stage, and its association with the expression of HOXA10mRNA and its related factors. This is to understand the impact caused by hydrosalpinx upon the working mechanism of endometrial cells.

2. Material and method

For this study, the researchers collected the data from patients who were in need of surgery due to reproductive surgical diseases (HSF and tubal ligation) in Affiliated Hospital of Guizhou Medical University during the period, March 2021 to March 2022. From the hydrosalpinx patients, the fluid and endometrium in the fallopian tubes were collected. The study group had a total of 10 cases whereas the control group had a total of 8 cases. Average age was varied 32–35 years among groups (Table 1).

2.1. Inclusion criteria

For the study group, the inclusion criteria are as follows; 1) laparoscopy-diagnosed unilateral or bilateral HSF *or ampullary fullness and distension-diagnosed HS at the time of hysterosalpingography, as a result of absence of contrast agent outflow from the tubal end*. From every patient, bilateral or unilateral tubal hydrops were collected separately and the HSF was kept under −20 °C storage. In case of requirement, it is removed. 2) HSF-induced infertility in women.

For the control group, the criteria are as follows; *history of childbirth due to tubal ligation as a result of infertility among women*. The endometrium was divided into two parts in which one is used to extract the primary cells whereas the other one is used for diagnosis; 4) patient excluded fallopian tube and endometrial malignancies; 5) patients who fall under 18–45 years i.e., reproductive age; 6) patients with regular menstrual cycles; 7) No hormone therapy was given 3 months before the operation; *8*. The commonly followed pathological analyses were used to confirm the endometrium involved in this study to be under proliferative stage with no other lesions. Informed consent was obtained from the patients, from whom the specimens were collected. The current study was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University.

Table 1
Analysis of two groups of general data.

	Study group (Group A: 10 cases)	Control group (Group B: 8 cases)	P
age	32.00 ± 5.29	35.25 ± 4.06	0.172
History of pregnancy	4 (40%)	8 (100%)	0.036
History of childbirth	3 (30%)	8 (100%)	0.004
History of adverse pregnancy	3 (30%)	2 (25%)	1
Hydrosalpinx fluid: communicating			0.515
	4 (40%)	0	
Non-communicating	6 (60%)	2 (25%)	
History of cervical infection with Mycoplasma chlamydia	5 (50%)	0	0.036
Bacterial culture of cervical secretions	0	0	NS

2.2. Extraction of primary endometrial cells

The collected samples were placed in sterilized normal saline centrifuge tubes and were stored at a temperature of 4 °C. Then, the samples were cleaned thrice using phosphoric acid buffer salt solution (PBS) and were cut measuring 1 mm3. Then, the samples were centrifuged to a tune of 1000 rpm per min for about 5 min. With the solution, 0.1 mg/ml type I collagenase (C0103, Sigma) was added for digestion and *left undisturbed for about 90 min.* Then, DMEM/F-12 (Gibco) was added to cease the digestion process. Then, the samples were centrifuged again as mentioned above. Afterwards, the samples were cultured in complete culture medium *with 10 extra fetal bovine serum* (South America, BI). The inoculation of the cells was performed in a 25 cm culture flask *and the solution was left undisturbed for 24 h.* Later, the morphology and the growth of the cells were observed using an inverted microscope and the images were captured for preservation.

2.3. Cell identification

From the first-generation endometrial cells, two groups *were considered* and fixed on to a 12-well plate with each well containing 5×10^4 cells and 4% formaldehyde for about 15 min. Then, 0.5%Triton X-100 was used upon the cells for about 20 min to break the cell membrane. The researchers added Rabbit anti-cytokeratin 19 (10712-1-AP, ProteinTech,1:300), *Rabbit anti-cell vimentin 10366-1-AP, ProteinTech,1:300)* and PBS (blank control) to the cells. Then, the cells were kept under incubation overnight at 4°c. The next-day morning, the researchers added green fluorescence of goat and rabbit with secondary antibody (1:200) and second anti - goat anti - rabbit red fluorescence (1:200) and kept it under incubation for about 1 h. The cells were added with a small amount of DAPI stain for 5 min. After washing the wells and drying it at room temperature, the images were captured under fluorescence microscope.

2.4. Grouping and cck8 cell proliferation assay (plot growth curve)

The groups are as follows.

- Under the study group i.e., group A containing the HS patients' endometrial cells, based on decidualization, there are two sub-groups present Aa group: Add *effusion* during decidualization (75%HSF)
- Ab group: No *effusion* was added during decidualization

Under the control group i.e., group B, the endometrial cells from normal patients without HS after tubal ligation are present.

2.5. cck8 cell proliferation experiment

The first-generation endometrial cells were selected from two groups of the well-grown cells. These cells were then inoculated in 12-well plates, in which each well has 5×10^4 cells, and incubation for overnight. In the next day, 10% CCK-8 solution was thawed prior to its usage and each well was supplemented with 10ul of CCK-8. Each group had a total of 6 multiple wells which were calibrated at 450 nm by OD (Optical Density) measurements with enzyme labeled instrument for seven consecutive days.

2.6. In vitro decidualization induction

From the same group, dealt in the previous sub-section, the first-generation endometrial cells were selected once it was found to be in good growth condition. Then, the cells were inoculated in 6-well plates with each well containing 5×10^5 cells. After the cell growth and the fusion accomplishing 60–70%, all the three groups such as Aa, Ab and B were supplemented with decidualization medium i.e., 2% fetal bovine serum medium +1uM progesterone [Progesterone, V900699, Sigma] +10 nM 17-β estradiol [β-Estradiol, E110145, Aladdin] + 0.5 mM 8 -Br-cAMP [8-Bromoadenosine 3′,5′-cyclic monophosphate, B5386, Sigma]. After 72 h of culture, the researchers observed the morphology of the decidual-like cells using an inverted microscope and captured its images for preservation. Then, the cells were made to undergo qRT-PCR and Wb detection.

2.7. Hematoxylin-eosin staining (HE stain)

Prior to staining process, the slip-climbing tablets were utilized to trigger the decidualization process. Then, the cells were washed thrice using the PBS and fixed in 4% paraformaldehyde for about 15 min and again washed thrice using the PBS. Afterwards, the addition of the Mayer's hematoxylin dye solution was performed to stain the cells for about 10 min. For about 30 s, the solution was rinsed in running tap water followed by differentiation using 0.3% acid alcohol. After washing it again with running tap water, then the solution was stained with eosin for about 30 s – 2 min after which washing continued for 30 s using running tap water. Then, the cells were dehydrated using graded alcohols such as 70% alcohol for 30 s, 95% alcohol for 1 min and anhydrous ethanol for 3 min. Then, the stain was cleared using xylene and *mounted onto DPX so as to observe the micrograph.*

2.8. Western blot analysis (WB)

With the approximately-homogenized endometrial cells of volume (5 × 104 cells), 150ul of the pre-cooled RIPA cell lysis buffer, with PMSF

(Beyotime, ST505) (Beyotime, P0013D), was added. Then, it was split was 3 min and the cells were centrifuged for about 12,000 rpm at 4 °C for about 10 min. Then, PBS was used to rinse the tissues after which it was cut into small pieces. Then, to this solution, the precooled RIPA cell lysis buffer, containing PMSF, was added up to ten times of the tissue volume. After having an ice bath for about 30 min, the solution was centrifuged at 12,000 rpm for about 5 min at 4 °C. The supernatant was collected and the BCA method was followed to determine the concentration of protein. Western blot analysis was conducted in line with the traditional methods. The primary antibodies used in the study were GAPDH (1:1000, affinity), HOXA10 (1:1000, affinity), IGFBP1 (1:1000, bioss) while the secondary antibodies include goat against rabbit (1:3000, affinity).

2.9. Real-time quantitative PCR(qRT-PCR)

The extraction of the total RNA was performed using Trizol (TAKARA, 9109) whereas M-MLV reverse transcriptase first strand cDNA synthesis Kit (Promega) was utilized for the synthesis of cDNA.

The study used the following HOXA10 primer sequences; 5′-AAGGTGAAAACGCAGCCAAC-3′,5′-CTAATCTCTAGGCGCCGCTC-3’。IGFBP1 primer sequence 5′-TGAAAGCCCAGAGAGCA-3′,5′-TACTGATGGCGTCCCAA-3’.avβ3 primer sequence 5′-TCTTTGGGGCTGATGACT-3′,5′-CAACAATGAGCTGGAGGAC-3’. GAPDH primer sequence5′-GGGGCTCTCCAGAACATC-3′,5′-TGACACGTTGGCAGTGG-3’.

Then, RT-qPCR was conducted in a 10 μL reaction system (40 reaction cycles) in line with the instructions from the manufacturer. The quantitative PCR procedure was conducted as given herewith; 95 °C for 5min; 40 cycles were performed at 95 °C for 10s and 60 °C for 30min.

mRNA expression levels were normalized as per GAPDH and were determined using $2^{-\triangle\triangle Ct}$. All the measurements were calculated thrice separately for every experimental condition.

2.10. Statistical analysis

In this study, the data analysis was conducted using the GraphPad prism and SPSS software (version 25.0). Both the groups were differentiated using double-tailed student T test for continuous variables. Further, Fisher’s exact test was used to analyze the categorical variables. On the other hand, the measurement data is expressed as mean ± standard deviation. T-test was used to statistically analyze the mean between the groups under study. ANOA was used to analyze the repetitive measurement data while $P < 0.05$ was considered to be statistically significant.

3. Result

3.1. Morphology of endometrial cells

The group B endometrium cells exhibited the same morphology alike the primary endometrium cells (Fig. 1A), when the endometrium of both the groups got passed onto the first generation in equal amounts. However, the group A endometrial cells exhibited a long spindle shape with enlargement in its cell mass and also a round nucleus with parallel growth, as shown in Fig. 1B.

3.2. Cell identification

Immunofluorescence was used to identify the first-generation endometrial cells from both the groups. The results show that the epithelial cells are labeled with keratin antibodies *whereas vimentin antibodies label the mesenchymal cells.* As per the outcomes, *decreased*

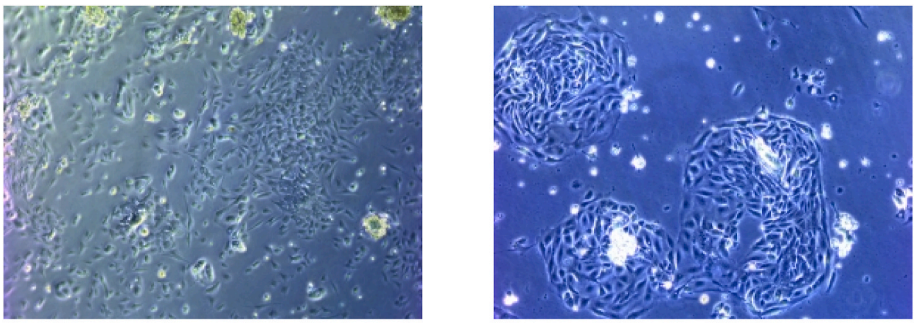

Fig. 1. Observation of primary endometrial cells in two groups under inverted microscope. The endometrial cells were fully adherent within 24 h, and the two types of cells were polygonal, tadpole and spindle shaped cells in primary culture and at the early stage of passage. The nucleus is centered and round, and the cells are clustered and grow in an island shape. Figure A (100×) shows primary endometrial cells of hydrosalpinx patients in group A. Figure B (100×) shows primary endometrial cells of women with tubal ligation in group B.

epithelial cytoplasm and interstitial cytoplasm of the endometrium were stained with green and red fluorescence. When observed under microscope, the epithelial cells were found to be polygonal and also spindle-shaped. Fig. 2A and B shows the spindle-shaped and polygonal shaped *mesenchymal cells*.

3.3. Growth curve

As per the grouping method mentioned earlier, the researchers measured the growth curves of the endometrial cells during 7-day proliferation period on a continuous basis. It can be observed that the group A exhibited a slow growth rate of the endometrial cells in comparison with group B, at the time of proliferation period and *once the tubal effusion operation got over*. This phenomenon can also be said as an S-shaped one containing three stages such as the slow growth, logarithmic growth and plateau. On the other hand, the study group recorded a slow growth rate of the endometrial cells during the logarithmic growth period. Fig. 3 shows the comparison outcomes between the groups that achieved a statistically-significant difference ($P < 0.05$).

3.4. In vitro decidualization induction

After 72 h of decidualization induction in the same group as discussed earlier, the group-B contained decidualization-like cells in abundant number with an enlarged cell volume, transparent cytoplasm and oval or round nuclei with irregular polygonal shape while the nucleus was located at the center of the cells. Every cell was found to be connected with each other with blurred boundaries. *Once the decidualization started*, the Aa group exhibited no alterations in the deciduate-like cells. These cells were found to be spindle-shaped, slender, though a few are polygonal shaped. With small cell volume, low cytoplasm, the cells has fuzzy nucleus with a clear interconnecting cell boundary between the cells. *Once the decidualization started*, changes were observed in a few deciduate-cells in Ab group that were either spindle or polygonal-shaped. In comparison with group B, the cell volume was found to be lesser with less cytoplasm. Further, with blurred nuclei, the cells also had their interconnections with boundaries to be blurred as shown in Fig. 4.

3.5. HE staining

The same group was again stained with HE once the decidualization was initiated. The cell nuclei were found to be purple-blue in color with red-colored cytoplasm and*ECM* whereas the cell morphology of all the three groups showed significant alterations. Group B cells were polygonal in shape whereas the group Aa exhibited spindle or polygonal shape, with latter being lesser in nature. The cells in group Ab were either spindle or polygonal as shown in Fig. 5A-C.

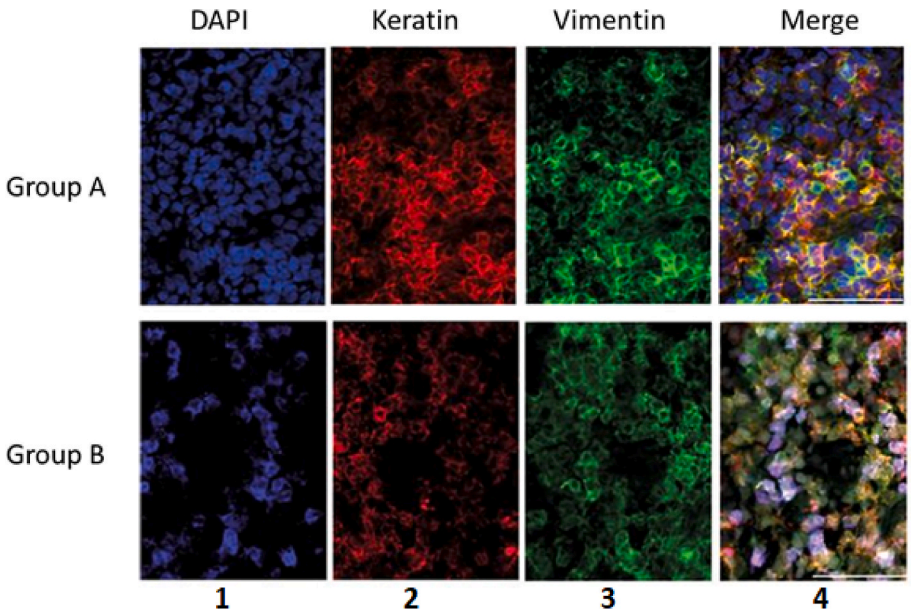

Fig. 2. The endometrial cells and mesenchymal cells were identified by immunofluorescence assay using epithelial labeled keratin antibodies and mesenchymal labeled vimentin antibodies, respectively. (A1 100 ×) Group A endometrial cells (DAPI). (A2 100 ×) Group A endometrial cells (CK19). (A3 100 ×) Group A endometrial cells (Vimentin). (A4 100 ×) Group A endometrial cells (merge). (B1 100 ×) Group B endometrial cells (DAPI). (B2 100 ×) Group B endometrial cells (CK19). (B3 100 ×) Group B endometrial cells (Vimentin). (B4 100 ×) Group B endometrial cells (merge). Scale bars = 50 μm.

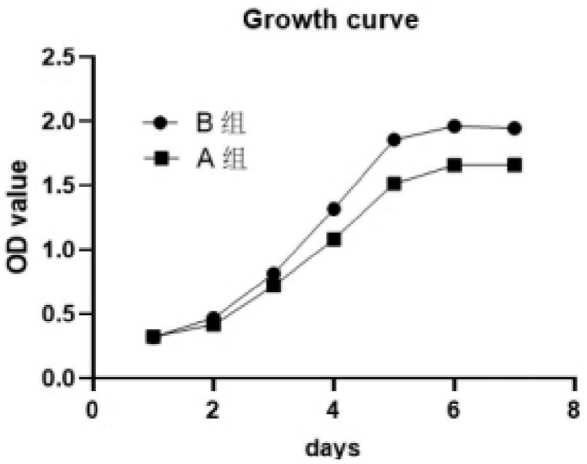

Fig. 3. According to the above grouping method, the growth curve of endometrial cells during the 7-day proliferation period was measured continuously. The growth curve showed an "S" shape, experiencing slow growth period, log growth period and plateau period. Group B: oviduct followed by proliferative endometrial cells; Group A: proliferative endometrial cells of hydrosalpinx patients; There was statistical significance between the two groups ($P < 0.05$).

3.6. Changes of HOXA10 expression in proliferative endometrium

As shown in Fig. 6, the authors conducted the RT-qPCR and WB analysis and found a low expression of HOXA10 in the endometrial cells of HS patients during proliferative stage, *then the group B*.

3.7. Expression changes of HOXA10, IGFBP1 and avβ3 in endometrial cells after decidualization induction

There was a remarkable decline observed in the expression of HOXA10 (Fig. 7A), IGFBP1 (Fig. 7B) and avβ3 (Fig. 7C) in endometrium, in comparison with group B, by decidualization. The group Ab recorded higher HOXA10 and IGFBP1 expression levels in comparison with the group Aa, though it was still lower than the group B as by qPCR (Fig. 7) and Western Blot (Fig. 8).

4. Discussion

Hydrosalpinx is characterized by fallopian tube getting distended and obstructed due to acute and chronic pelvic inflammatory disease that results in dilation and effusion of the fallopian tubes. This leads to 50% mitigation in the success rate of implantation and the pregnancy rate among the patients. Further, it also increases the chances of abortion up to *two times* [11]. When the fallopian tube contains fluids, it tends to reduce the success rate of*IVD-ET procedures to a remarkable rate*. As per the literature, it is possible to enhance the pregnancy outcomes among the IVF-ET patients, when hydrosalpinx is cured [12]. From the literature, it can be inferred that the patients with hydrosalpinx have significantly low endometrial receptivity. In this background, the current study cultured the endometrial cells of those patients with hydrosalpinx in order to understand the growth of cells, decidualization and the HOXA 10 and its downstream genes' expression. In this way, the researchers attempted to analyze the damage caused by hydrosalpinx upon the endometrium of the patients. Here arises the question of the timeline at when the fluid in oviduct to endometrium gets damaged. The current study results infer that the cell morphologies of normal endometrium and the proliferative endometrium of hydrosalpinx patients are highly similar. However, there was a significant decline observed in the growth rate from the CCK8 assay. The endometrial cells tend to undergo periodic alterations from the proliferative stage to secretory stage, *as a result of trigger by sex hormones and by a few fluid components present in the fallopian tube.*

The hydrosalpinx patients exhibited a low growth rate of the endometrial cells. In spite of the insignificant morphological changes observed under the optical microscope, *the literature inferred that the presence of large irregular gaps corresponds to the destruction of the organelles*. Further, the membranous whorls have an association with the degradation of the organelles too. In addition to these, surface epithelium thinning, reduction of microvilli and spinous pedis in the microvilli cells were also observed among the hydrosalpinx patients during the late proliferative or early secretory stage, when analyzing the ultrastructure of cells in endometrium.

When the fluid gets accumulated in the fallopian lumen, it tends to negatively impact the minor changes in nucleus, proliferation of the intraepithelial macrophages and lymphocytes, increased heterochromatin, multiple functions of endometrial cells and the vacuolation of the apical endoplasmic reticulum cisterns [13]. This may be attributed to the occurrence of low or reduced cell division.

HOXA10, the transcription factor, is a crucial component for the differentiation and receptivity of the endometrial cells [14]. This transcription factor further controls the expression of implantation target genes like avβ3 and IGFBP1. The function of HOXA10 is to ensure the receptivity of the endometrium and to keep the implantation and decidualization of the embryo under regulation [15–17]. In normal female endometrial epithelium and stroma, the HOXA10mRNA is expressed and regulated in a cyclic manner, in the

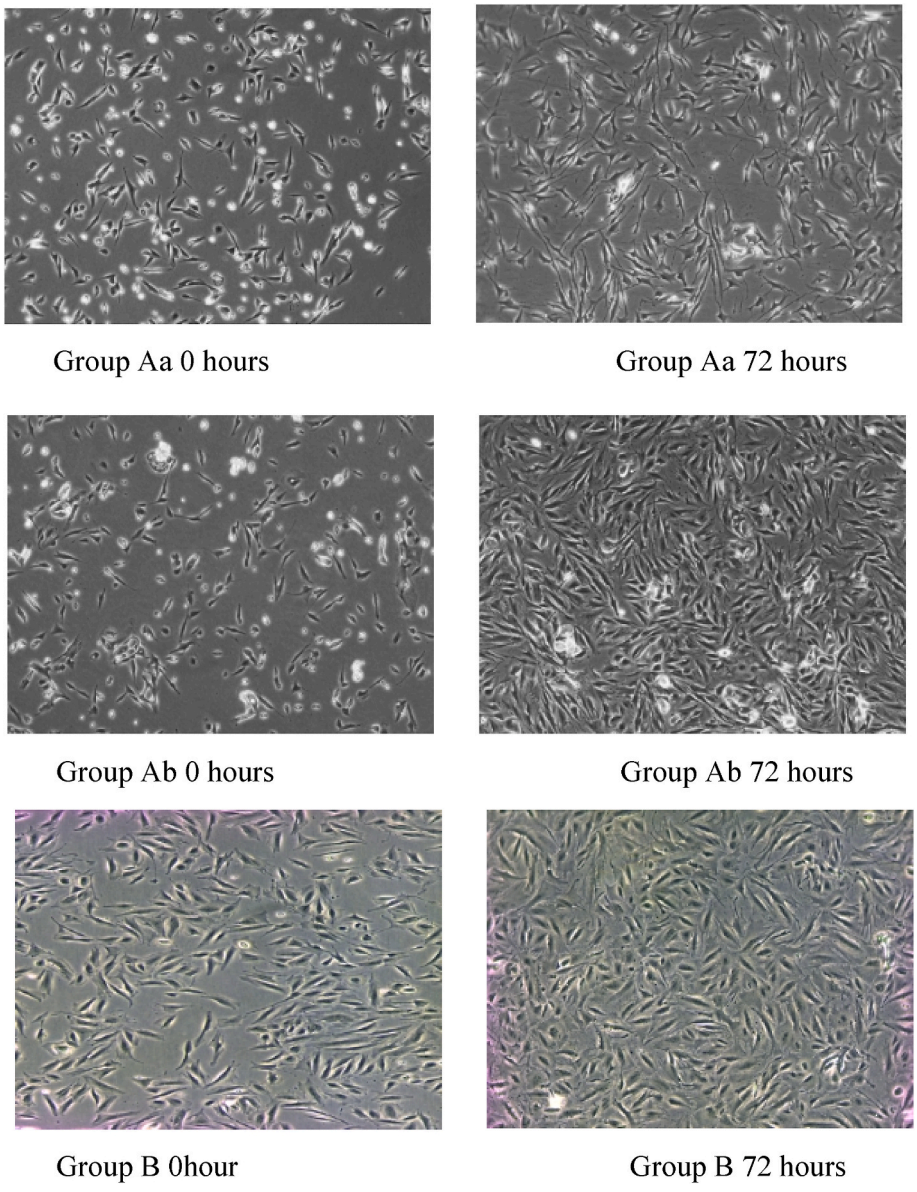

Fig. 4. 72 h after decidualization induction in the same group as above, the Aa group showed fewer Deciduating cells changes, smaller cell volume, less cytoplasm, blurred nucleus, and clear cell boundaries between some cells. Some decidual-like cells in group Ab were spindle-shaped or polygonal. Compared with group B, the cell volume was slightly smaller and the cytoplasm was less. The nuclei became blurred and the boundaries between cells became blurred. In group B, decidual-like cells were changed, the cell volume was enlarged, the cytoplasm was rich and transparent, the nuclei were enlarged and oval or round, located in the center of the cells, and the cells were connected with each other and the boundaries became blurred. (Fig. 4 is 100 ×).

supervision of ovarian steroids. So, *HOxA10mrna's expression* concentration is spatio-temporal in nature i.e., low expression during the proliferation stage of endometrial cells and high expression at both middle and late secretory stages [18]. At the time of embryo implantation, there is a significant increase observed in the expression of HOXA10, thus establishing the crucial role played by HOXA10 in this process. When the HOXA10 expression becomes defective, it can result in abnormal implantation [15,19]. So, it can be said as a crucial regulator to ensure the pregnancy is successful.

In the study conducted by Daftary GS et al. [8], the endometrial cells collected from the hydrosalpinx patients were analyzed at the time of middle lutel stage. The authors found a low HOXA10mRNA expression in the endometrial cells of the HS patinets in comparison with that of the fertile group. The study suggested that the HOXA10 gene expression must be altered in order to ensure that the hydrosalpinx does not affect the pregnancy rate. So, it is important to keep the expression levels so as to achieve successful implantation [15]. The current study did a comparative analysis of the endometrial cells from the hydrosalpinx patients and the tubal ligation ones. Both the samples were found to have a clinical association with the obstruction of the tubes. However, in case of tubal ligation

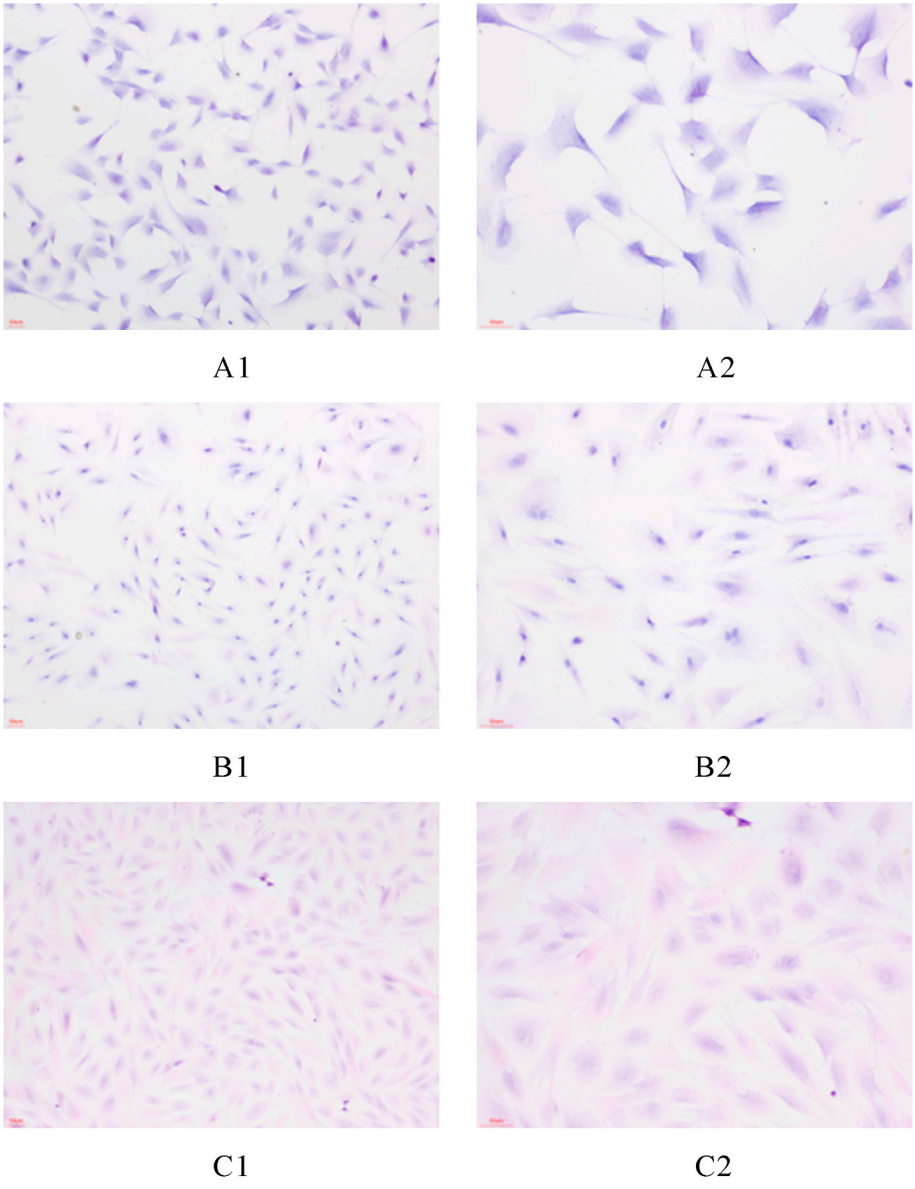

Fig. 5. HE staining was performed in each group after decidualization induction. The nuclei were purplish blue, and the cytoplasm and extracellular matrix were red. The cell morphology of A1 (100 ×) and A2 (200 ×) group Aa was spindle or polygonal, and a very small number of cells were polygonal. B1 (100 ×) and B2 (200 ×) showed that cell morphology of group Ab was spindle or polygon. In the figure C1 (100 ×) and C2 (200 ×), the cell morphology of group B was polygonal.

patients, there was no fluid formed in the tubal lumen. The current study outcomes confirmed a low HOXA10mRNA expression in the endometrium of the hydrosalpinx patients in comparison with the tubal ligation patients during both proliferative and secretory stages. However, *when the endometrium of the hydrosalpinx patients was get rid of*, from the HS at the time of decidualization, it certainly exhibited a recovery of HOXA10mRNA. So, the current study infers that the reduced expression of HOXA10mRNA in the endometrial cells of patients with hydrosalpinx is primarily due to effusion in the tubal cavity of the patients. In general, the fallopian tube mucosal layer secretes the mucosa and when the patient ovulates, its secretion tends to increase in a gradual manner. This is because the peristaltic direction of the fallopian tube, before ovulation, is from proximal end to the distal end. So, less fluid flow occurs from the fallopian tube to the uterine cavity. Once the ovulation is over, at the fallopian tube peristaltic direction from the distal end to the proximal end so as to transport the fertilized eggs into the uterine cavity. During this phase, a huge quantity of the fluid tends to flow to the uterine cavity that tend to negative impact the decidualization of the endometrium.

The patients with hydrosalpinx tend to have fluid in their fallopian tubes which is not its usual secretion, but with a complex composition, because of the obstruction of the fallopian tubes itself. According to Bao H et al. who analyzed the fallopian fluid's biochemical composition and its different parameters such as pH value, osmotic pressure, and the rest of the physical characteristics. The

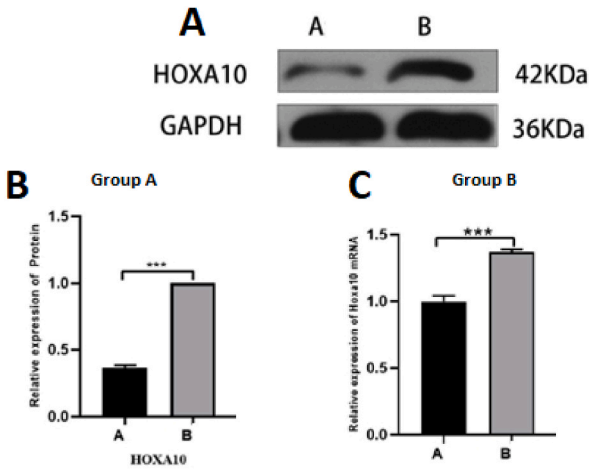

Fig. 6. Representative images of HOXA10 expression detected by WB (Fig. 6A) and qRT-PCR (Fig. 6B and C) in the proliferative endometrial tissue of hydrosalpinx patients. Group A: proliferative endometrial tissue of hydrosalpinx patients; Group B: Proliferative endometrial tissue of patients with tubal ligation. There was a significant difference between the two groups, ***P < 0.001 (bar chart shows mean ± standard deviation).

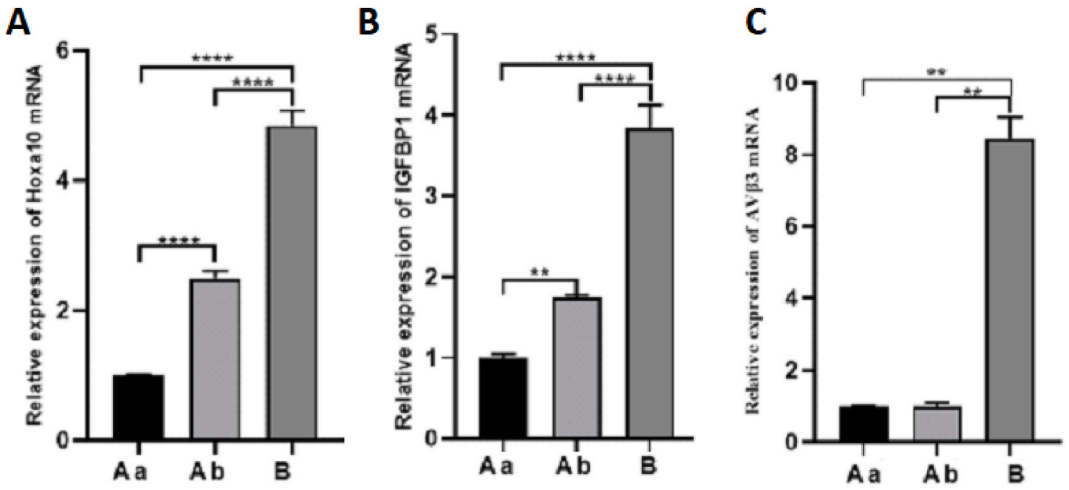

Fig. 7. The expression levels of HOXA10 (Fig. 7A), IGFBP1 (Fig. 7B) and avβ3 (Fig. 7C) in each group after in vitro decidualization induction were detected by qRT-PCR. Aa group: endometrial cells of patients with hydrosalpinx during decidualization induced by adding 75% HSF; *Ab group: No fluid* was added into endometrial cells of patients with hydrosalpinx during decidualization. Group B: Endometrial cells from patients without HS after tubal ligation. There were significant differences between group Aa and group Ab compared with group B, and there were significant differences between group Aa and group Ab, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (bar chart shows mean ± standard deviation).

study inferred that both the groups exhibited no significant difference. *In comparison with the colored tubal effusion, the amount of glucose in the uncolored effusion was high [20]. The tubal effusion collected* in this study had different colors such as yellow, white, thin dark red, etc., and every element had a notable influence on HOXA10mRNA expression in the endometrium. The component that is attributed to the low HOXA10mRNA expression should be investigated in detail. Though it is an established fact that the expression of HOXA10mRNA is negative impacted by sex hormones, the component in tubal effusion too affects it.

With a decline in the expression of HOXA10mRNA, it consequently affects the working mechanisms of the downstream genes as well. The decidual tissue can be seen with the expression of large number of IGFBP1 genes while IGFBP1 gene is present near the HOXA gene cluster on chromosome 7. This phenomenon suggests that both IGFBP1 and HOX gene families are linked with one another through evolution and HOXA10mRNA regulates the IGFBP1 promoter. This further acts as a signature secreted proteins in the decidualization process and also enacts a crucial role in the implantation of the blastocysts [21]. Integrin avβ3 remains a crucial marker for endometrial receptivity and is also an important factor for the implantation of the embryo. Being a transmembrane glycoprotein, it exists on the surface of the cell and plays a role in cell adhesion process as well as cell surface-mediated signal transduction process. Several studies showed that the direct regulation of avβ3 by HOXA10 in endometrial cells [22]. *The current study found the IGFBP1 and avβ3 expressions in endometrial cells after the decidualization got over. On the other hand, there was a decline observed in the expression levels of IGFBP1 and avβ3 in the endometrial cells of the patients with hydrosalpinx.* When the hydrosalpinx got released, there was an increase

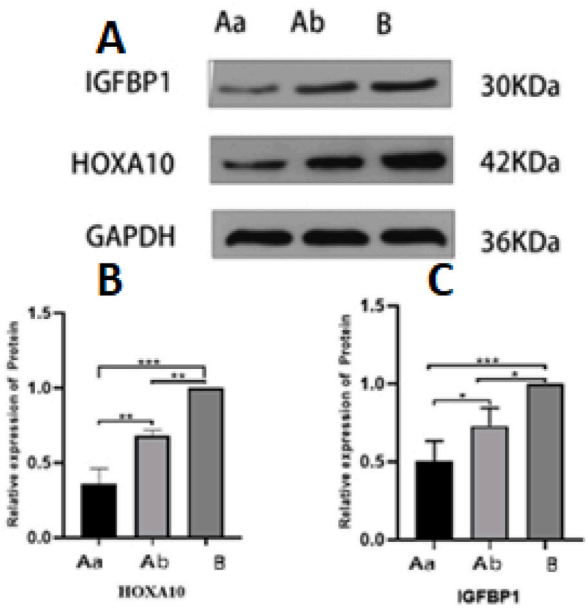

Fig. 8. The expression levels of HOXA10, IGFBP1 and avβ3 in each group after in vitro decidualization induction were detected by Western Blot. Aa group: endometrial cells of patients with hydrosalpinx during decidualization induced by adding 75% HSF; Ab group: No fluid was added into endometrial cells of patients with hydrosalpinx during decidualization. Group B: Endometrial cells from patients without HS after tubal ligation. There were significant differences between group Aa and group Ab compared with group B, and there were significant differences between group Aa and group Ab, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (bar chart shows mean ± standard deviation).

observed in the expression of both IGFBP1 and avβ3, though it was unable to achieve the normal level. This scenario establishes that once the clinical hydrosalpinx, a fertility barrier, is removed either through salpingostomy drainage, or salpingectomy or ligation and until the fluid no longer flows from the fallopian cavity to the uterine cavity, it is possible to ensure the endometrial receptibility. However, it is not possible to retain it to the normal state. So, it is suggested that the fallopian tube obstruction should not be removed, immediately after the transfer of embryo or natural conception. This is because, the endometrial receptivity should be provided sufficient time to recover after multiple menstrual cycles. As per the literature, there is a significant reduction in the expression of endometrial avβ3 among the patients with hydrosalpinx, at the time of implantation window period, then their fertility controls.

In the in vitro 2D cell culture, no significant morphological changes were observed in the endometrial cells that were subjected to effusion in the fallopian tube, during the proliferation period. In other words, it can be said that the effusion in the fallopian tube showed no impact upon the cell morphology at the time of proliferation stage. There was a slow growth recorded among the endometrial cells during the proliferative period due to the effusion in the oviduct. This may have resulted in the shortage of total cells at the time of decidualization. Once this process was initiated, it was obvious for the endometrial cells to exhibit abnormal morphology since they were subjected to effusion in the fallopian tubes in in vitro culture. But the group of endometrial cells, without any effusion from the fallopian tube, also exhibited abnormal morphology of the decidualization cells. This infers that the effusion in fallopian tube tend to negatively impact of the basal endometrial layer cells. This phenomenon results in abnormal decidualization of the functional layer cells in the next cycle. During the proliferation stage of the endometrial cells, there was a decline observed in the expression of HOXA10 as a result of tubal effusion. This may have impacted the HOXA10 expression negatively in the secretory stage i.e., the corresponding function. There was a significant reduction in the expression of HOXA10mRNA of endometrial cells, when the latter was subjected to continuous tubal effusion. This may be due to decidualization. When the tubal effusion was removed at the time of decidualization, the process restored the expression of HOXA10mRNA to a certain stage though it did not accomplish the actual endometrial level. So, in terms of clinical aspects, *the expression of HOxa10 mRNA* by the endometrial cells decreases significantly, when hydrosalpinx is blocked. During such instances, it is not appropriate to transfer the embryo immediately since the endometrium requires sufficient time to recover. Both IGFBP1 and avβ3 expression levels get affected by HOXA10 whereas the decline of these genes' expression levels are established earlier. In other terms, one of the endometrial receptivity mechanisms got reduced as a result of tubal effusion and due to the decreased expression of HOXA10. This scenario further denotes that HOXA10 expression is not only affected by the estrogen and progesterone.

5. Conclusion

Hydrosalpinx tend to damage the endometrial receptivity. The effusion causes damage not only to the functional endometrial cells, but also to the basal layer cells that lead to reduction in the expression of HOXA10 during proliferative and decidualized endometrium. The decline in the expression levels of IGFBP1 and avβ3 recommend the implantation of the embryos to be impaired. The current study

outcomes exhibit a consistent expression pattern IGFBP1 and avβ3 in decidualized cells of hydrosalpinx patients with that of the HOXA10. So, the abnormal expression of HOXA10 and its downstream genes such as IGFBP1 and avβ3 can be considered as one of the solid and promising mechanisms of endometrial damage among hydrosalpinx patients, which results in the failure of embryo implantation. Though it is possible to gradually repair this through the removal of hydrosalpinx, the recovery process is a lengthy and time-consuming one.

Author contribution statement

Taolan Li, Dixian CAI, Yonghun Zeng, Hualei CAI: Conceived and designed the experiments; Wrote the paper.

Fan Lu, Cheng Zhou, Chengrong Wu, Yi ling CAI, La Yang: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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