Research Article

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The spatial and temporal distribution of cationic and anionic radicals in early embryo implantation

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Abstract: The main objective of this study is to obtain some knowledge of cationic and anionic radicals in early embryo implantation in mice. The method used in this study is known as histochemical staining, in which Xylidine ponceau was used at pH 2.5 and toluidine blue was used at pH 4.0. We detected the change in glycosaminoglycans and total proteins in the endometrial stroma during the preimplantation of mice. This study revealed that the distribution patterns of cationic radicals and anionic radicals are similar on days 4 and 5 of pregnancy. However, there was a distinct difference between cationic radicals and anionic radicals on day 8 of pregnancy. The distribution pattern of cationic radicals is more concentrated in the stroma near the conceptus. Laboratory studies on histochemical stain provide more information about early embryo implantation.

Keywords: histochemical stain, toluidine blue, Xylidine ponceau, embryo implantation

1 Introduction

Implantation has been of considerable interest to the pregnancy community in recent years [1]. Great progress has been made in this field; however, endometrial epithelium can not only accept embryos but also control and terminate embryo implantation. The local effect of the embryonic trophoblast on the endometrium cannot be ignored. A large amount of experimental evidence

showed that the surface proteins and polysaccharides played an important role in the implantation process [2,3]. Furthermore, many protein signal molecules have undergone specific changes during the implantation process. Experiments have shown that there is a regular change in the endometrial surface sugar during implantation. Researchers have used histochemical methods to observe regional differential expression of carbohydrate antigens [4]. Studies have shown that the presence or absence of blastocysts in the uterus has a significant effect on endometrial glycoprotein expression. The aforementioned results indicate that the changes of signal molecules during the implantation period are not only affected by hormones but also related to the local effects of blastocysts. The previous study detected the cationic and anionic radicals using Xylidine ponceau (XP) at pH 2.5 and toluidine blue (TB) at pH 4.0 during the early phase of embryo implantation of rats [5]. Although the initial interaction between the concept and the uterus is a complex phenomenon, little research has been done on the spatial and temporal distribution of cationic radicals and anionic radicals during the time of implantation.

On the basis of the existing literature data, we carried out studies in an effort to observe the changes from a global perspective. Our research with histochemical technology is limited. The specific goals are to detect anionic radicals and cationic radicals during the early embryo implantation.

2 Method

2.1 Animal

Northeast Agricultural University's Institutional Animal Care and Use Committee approved all animal procedures. Kunming white mouse was used in this study, which were fed in a room with light control, with a 14 h

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(light):10 h (dark) cycle. To induce pregnancy, the fertile male mice were mated with adult female mice of Kunming White strain by cohabiting (day 1 means day of vaginal plug). From day 1 to 4, gestation was identified by discovering zygotes in the uterus or oviducts. The sites of implantation of day 5 were confirmed by the classical method, i.e., by injecting Chicago blue through tail intravenous. The tissue was collected after the pregnant female mice of various stages were sacrificed by cervical dislocation. The liquid nitrogen was used to freeze uteri quickly, and then, they were stored at -80°C [6].

2.2 Materials

TB is one of the commonly used artificial dyes, which belongs to quinoneimine dyes. The dyes generally contain two chromophores, one is an amine group and the other is a quinone benzene ring to form a chromogen. The molecular formula of TB is $\text{C}_{15}\text{H}_{16}\text{ClN}_3\text{S}$, and its molecular weight is 305.83 (Figure 1). TB is usually prepared for administration at a concentration of 1%. A total of 100 mL of 1% TB consists of 1 g of TB powder, 4 mL of absolute ethanol, 10 mL of 1% acetic acid, and 86 mL of distilled water. The pH is usually adjusted to 4.5 [7].

XP 2R is a solid red power, and its molecular formula is $\text{C}_{18}\text{H}_{14}\text{N}_2\text{O}_7\text{S}_2\cdot 2\text{Na}$ and molecular weight is 480.42 (Figure 2). At pH 2.5, 0.1% xylene butylene (XP) in 3% acetic acid is used for the detection of total cationic radical [8].

2.3 Histochemical workflow

Conventional histological methods were used for analysis. The 3-aminopropyltriethoxy-silane (Sigma)-coated slides were used to mount frozen sections (10 μm). Then, they were fixed using 4% paraformaldehyde solution (in PBS). XP or TB was used to stain the sections.

Figure 1: Chemical formula of TB.

Figure 2: Chemical formula of XP 2R.

3 Results

Our experimental data are briefly summarized as follows:

Day 4: Figure 3a and b shows views of staining with TB and XP on day 4 of gestation. XP staining distinguished the uterine layers: perimetrium, myometrium, and endometrium. As shown in Figure 3a, no significant difference was found between uterine layers with TB staining.

Day 5: Figure 4a and b provides details of the staining results on day 5 of pregnancy. Staining with both TB and XP revealed cytochemical staining close to the sites of implantation.

Day 6: Figure 5a and b shows the results obtained from the staining of day 6 of pregnancy. Interestingly, more intense cytochemical staining of XP around the site of embryo implantation was found, while staining with TB was mild.

Day 8: Figure 6 shows that there were significant differences between staining with XP and staining with TB. There was an evident staining ring around the conceptus (Figure 6b). Histochemical staining with TB was more dispersive in the endometrium.

In the histochemical staining section with TB and XP, we can clearly distinguish the endometrium, myometrium, and perimetrium of uterine layers. Both

Figure 3: Mouse uteri of early pregnancy on day 4 stained with TB (a) and XP (b). 1 = perimetrium, 2 = myometrium, and 3 = endometrium. 100 $\times$ final magnification. Bar = 60 μm .

Figure 4: Mouse uteri of early pregnancy on day 5 stained with TB (a) and XP (b). * = embryo. 100× final magnification. Bar = 60 μ m.

Figure 5: Mouse uteri of early pregnancy on day 6 stained with TB (a) and XP (b). * = embryo. 100× final magnification. Bar = 60 μ m.

Figure 6: Mouse uteri of early pregnancy on day 8 stained with TB (a) and XP (b). * = embryo. 100× final magnification. Bar = 60 μ m.

TB and XP showed more dense staining in the lining epithelium from day 4 to 5. XP staining showed more concentrated staining in the stroma near the embryo in days 6 and 8 than TB staining.

4 Discussion

Implantation is a complex process and a key to pregnancy. During implantation, the endometrial epithelium and the embryonic trophoblast are compatible and synchronized with each other. However, the mutual recognition, adhesion, and initiation mechanisms are still being explored. Usually uterine epithelial cells are nonadhesive. During the implantation process of blastocysts and endometrium, they undergo a synchronous change from a nonadhesive state to an adherent state,

that is, the endometrium enters the receiving state during implantation. The blastocyst enters the implanted state and uterine epithelial cells are polarized tissues, and there are a variety of glycoproteins that act as adhesion molecules on the basement membrane, thereby mediating cell-to-cell and cell-to-matrix adhesion. Under the action of steroid hormones, the polarity of uterine epithelial cells changes [9]. The experiments showed that the negative charge of the endometrium of mice on days 4 and 5 of gestation decreased. These changes can reduce the repulsion of the surface charge between the endometrium and blastocyst, which is conducive to implantation. Our results also validate this phase-specific change.

Currently, a large amount of experimental evidence showed that the glycoconjugates on the two surfaces play a vital role during the preimplantation process [10]. The research we have done showed that the glycosaminoglycans (GAGs) were present in tissues by staining with TB, a basic dye, which is capable of forming electrostatic bonds with acidic radicals. GAG is a polysaccharide chain consisting of repeating disaccharide units. One of the disaccharide units is a glycosamine, such as glucosamine and galactosamine; the other is uronic acid, such as glucuronic acid [11]. Important GAGs in the body are heparin, heparan sulfate, dermatan sulfate, hyaluronic acid, chondroitin sulfate, and keratan sulfate. As the sugar chain components of proteoglycans in the body, they are the main components of extracellular tissue matrix, cell surface, and cell membrane basal layer [12–14]. The histochemical staining of GAGs was less intense after implantation in this study. These findings are understandable because hyaluronic acid also shows an unusually important role in controlling the invasion of the trophoblast. The synthesis of hyaluronic acid increased significantly in mouse endometrium during blastocyst implantation, while the synthesis of hyaluronic acid did not change in nonpregnant mice after mating. After implantation, there is a regional selective loss of hyaluronic acid, that is, the extracellular matrix of the decidua on the opposite side of the mesentery. On the 6th day of mice pregnancy, hyaluronic acid began to disappear from the stromal cells close to the implantation site and was more pronounced on the 7th day.

The myometrium and endometrial epithelium showed intense staining with XP in this study. In particular, we observed marked staining ring in the endometrial stroma around the conceptus. The data obtained differ from the results reported earlier [15]. The endometrial tissue is not a fibrous tissue and should

therefore not show distinct intense staining. But XP belongs to an acidophilic dye that can stain total proteins at pH 2.5. Therefore, these experimental results indicated that there is a large amount of proteins in the regions. Pregnancy is a kind of allogeneic transplantation. Normal pregnancy is regulated by systemic and local immune regulation. The immune regulatory network at the fetomaternal interface is particularly important. The maternal–fetal interface cell composition is quite complex. Maternal-derived cells, fetal-derived trophoblasts, and various proteins produced by these cells together form a distinctive immune microenvironment at the maternal–fetal junction to maintain a successful pregnancy.

Embryo implantation is a multilevel process that involves a series of biochemical and immunological concepts. The uterine microenvironment shows a decisive role during the implantation process [16]. Therefore, monitoring the microenvironment in the early stages of pregnancy allows characterization of events about maternal and infant activity to better understand these biochemical events.

In recent years, the study of various factors in the “implantation window period” forming the “implantation regulation network” has become a hot topic and a frontier topic for exploring the mechanism of embryo implantation regulation. The effects of protein factors and/or GAGs on implantation in the blastocyst and endometrium were observed *in vivo* and *in vitro* implantation models in the early life. The present histochemical study demonstrated the distribution of GAGs and proteins during the early embryo implantation by the exploration of anionic and cationic radicals.

5 Conclusion

For each time point, slides were stained with TB and XP for morphological evaluation. The reason we can observe these changes is that the matrix changes in these areas are due to the secretion of new components by endometrial cells and the cleavage of the extracellular matrix by embryonic enzymes. There are a large number of COO^- , SO_4^- , and PO_4^- groups in GAGs, RNA, and DNA [17]. TB bonds with those groups in tissues. It is well known that NH_3^+ radical groups are present in total proteins when solution is at pH 2.5. We also know that XP is an acidophilic stain that can bind to the NH_3^+ group. A large number of proteins participate in the interaction between the endometrium and embryo as

signaling molecules. Therefore, the distribution characteristics we detected are the changes of proteins by XP at pH 2.5. From the results of our experiments, we concluded that a large number of DNA, RNA, GAGs, and proteins participated in the interwork between the embryos and endometrial epithelial cells, and their distribution appeared spatiotemporal during the early embryo implantation of mice.

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Conflict of interest: The authors declare no conflict of interest.

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