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On model of angiogenesis and the mechanism in porous silk fibroin films

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Abstract The purpose of this paper is to explore the mechanism of the angiogenesis modes in the biomaterials implanted in vivo. By means of experimental observation and analysis of the capillary growing state in the porous silk fibroin film implanted into rats, we intended to develop a modeling expression on the growth mode of the capillaries in the biomaterials. Additionally, we proposed the response model of endothelial cells (ECs) resulting from vascular endothelial growth factor's concentrations at different stages after the implantation. With the implantation experiment, it was identified that angiogenesis developed in the way of capillary sprouting at the early stage of implantation and of intussusception at the late stage. Based on the response model of ECs, experimental results are explained.

1 Introduction

Functional capillary network system was observed in the biomaterials implanted in the body in 10 days [1]. Elucidation of the mechanism of this process is important in the conformation design of the porous silk fibroin film (PSFF). However, it is unclear how the capillaries grow in the biomaterial and what the growth mode and its mechanism are. Those problems exist on the intersection between physiology and biomaterial science and are given little regard. Up till now we know that there are least two different types of angiogenesis: true sprouting of capillaries from pre-existing vessels and non-sprouting angiogenesis or intussusception [2]. Many reports substantiate these growth modes of the capillary in experiments, whereas the mechanisms of angiogenesis modes are still not clear. The purpose of this paper is to explore the mechanism of the angiogenesis modes in the biomaterials implanted in vivo. By means of experimental observation and analysis of the capillary growing state in the PSFF implanted into rats, we intended to develop a modeling expression on the growth mode of the capillaries in the biomaterials. Additionally, we proposed the response model of endothelial cells (ECs) resulting from vascular endothelial growth factor (VEGF)'s concentrations at different stages after the implantation. With the implantation experiment, it is identified that angiogenesis develops in the way of capillary sprouting at the early stage of implantation and of intussusception at the late stage. Based on the response model of ECs, experimental results are explained.

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2 Materials and methods

2.1 Preparation of porous silk fibroin films (PSFFs)

Silk of Bombyx mori was degummed by cooking for three times with 0.05% (w/w) Na_2CO_3 solution for 30 min. The degummed silk was dried in the air, and then was dissolved in a ternary solvent system of $CaCl_2/CH_3CH_2OH/H_2O$ (1/2/8 mol ratio) at 70 ± 2. During the dissolving process, the

solution was oscillated continuously. The dissolved solution was dialyzed to obtain the silk fibroin solution of about 3.0% (w/w). Then the solution was spread on the plates, and freeze-dried to get the PSFF.

2.2 Animals model

12 male and healthy SD rats (SPF grade) with a weight of 200 ± 30 g were prepared for experiments. Strict aseptic animals were cut open on the skin along the thigh with 2 cm wound. PSFFs with 1 cm × 1 cm were implanted on the injury of thigh. All animals after operation were raised continuously.

2.3 Histological studies and TEM observation

Specimens were harvested at this time points day 5, 7, 10, 13, 17 and 24 after surgery and used for visual observation, Haematoxylin and Eoxin (HE) staining for histological study and TEM for ultrastructural observation. Moreover wound healing of rats and newly formed capillaries were observed too, and the relations of the capillaries and PSFF materials were regarded specially.

2.4 Histological studies

Half of the specimens were fixed in 10% formalin solution at room temperature, embedded in paraffin, sliced, and then stained by HE. The slices were sealed with resin, observed and taken photos with Olympus CX 31 Optical Microscope and JD801 morphological microscope image analysis system.

2.5 Observation of TEM

Another half of the specimens were fixed by glutaraldehyde at 4°C, and stored in refrigerator. Several days later, the specimen were made into super thin biology slices, then stained and dried. They were observed and taken photos with Hitachi H-600 Transmission Electron Microscope (TEM).

3 Results and discussion

3.1 Physical properties of the PSFFs

Figure 1 shows the SEM images of PSFF before implantation. The PSFF is 0.5 mm thick, with a pore diameter of 50–100 μm and a porosity of 70–90%, which have been confirmed to be appropriate for the growth of capillaries [3].

3.2 Histological observation

After the initial inflammatory phase, inflammatory cells in the tissues around the PSFFs were seen to decreased by day 5, and almost vanished 7 days later. Histological section results showed that extracellular matrix, collagen, and cells were infiltrated into PSFFs 5 days after the implantation, but only a few capillaries grew in PSFFs. The newly formed tissues and capillaries in PSFFs had grown well by day 7. On day 10, PSFFs were found to have degraded, and many capillaries were infiltrated into PSFFs and cells were adhered to PSFFs closely. By day 13 capillaries had become mature and cells in PSFFs and red blood cells (RBC) in blood vessels had increased. 24 days later, most PSFFs were degraded and newly formed tissues grew well. Even-distributed microvessels were observed and therefore vascular net may be formed. The results of the statistical analysis of the numbers of capillary sprouting and intussusceptive angiogenesis based on the histological images are illustrated in Fig. 2. According to the results, angiogenesis develops in the way of sprouting at the early stage

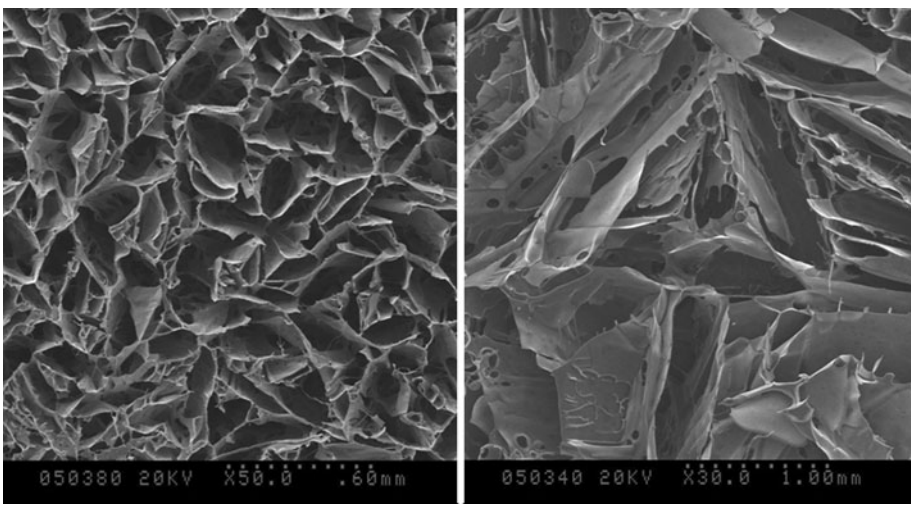

Fig. 1 The photos of PSFFs by scanning electron microscope (SEM)

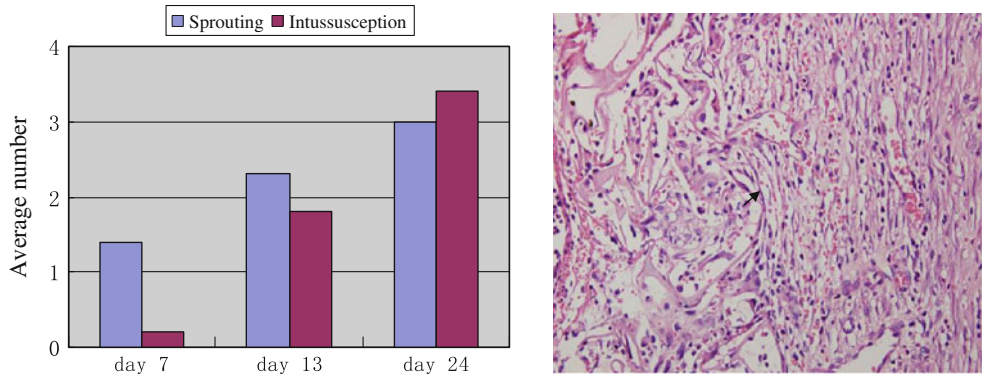

Fig. 2 Statistical results of the numbers of capillary sprouting and intussusceptive angiogenesis and the image on day 7 ↗: A sprouting point

of implantation, and the occurring of intussusception exceeds the sprouting 3 weeks later. The arrow in the left image in Fig. 2 points to a capillary sprouting.

3.3 Ultrastructural observation of ECs

The ultrastructure of ECs was observed by TEM, and the mode of angiogenesis in the implanted materials at different time points was analyzed.

3.4 Angiogenesis at the early stage of repair process

Figure 3 shows the photos of ultrathin histological sections at day 7 and day 10 after the implantation. At day 7, the broken basement membranes of the capillaries which located about two microns away from the edge of SPFFs could be observed in Fig. 3a. The ECs which seemed to be in mitosis appeared to move along the arrow to outside of the lumen through the broken basement membrane. This indicated the symptoms of sprouting. At day 10, Two cell nuclei (N) closed to a peripheral blood vessel cells (PC) and the broken basement membranes were seen in Fig. 3b. On the left of the figure, the ECs were in mitosis and stretched towards the outside of the lumen. This showed the initial trait of sprouting. The ultrastructure photos showed that ECs were in the complicated forms in the early stage of post-operation. This was a multiple period of capillary sprouting. According to Makanya et al. [4], it is known that strong VEGF expression appears in the stage of the sprouting.

3.5 Angiogenesis in the latter stage of repair process

Figure 4 shows the TEM photos of ultrathin histological sections at day 10, 13, 17 and 24 after the implantation. The photo at day 10 showed the endothelial cytoplasm was in the column state, which entered into the lumen and metamorphosed the RBCs. The endothelial cytoplasm was rich in mitochondria and plasma membrane vesicles, and the vessel wall became bulgy and thick. These traits corresponded with intussusception. The photo at day 13 showed the front stage of the process in which the vessel lumen divided into of two lumens. The cytoplasm entered into the indentation of the lumen (arrows). It is presumed that the blood vessels were divided into two independent vessels finally. This was non-sprouting (intussusceptive) angiogenesis. The photo at day 17 could also be seen that the plasma membrane vesicles and mitochondrias were rich in the cytoplasm of ECs. The wall of lumen seemed to be bulgy and the cytoplasm formed the cylindrical structure towards the lumen. This showed an intussusceptive angiogenesis process. In the image at day 24 the arrow showed

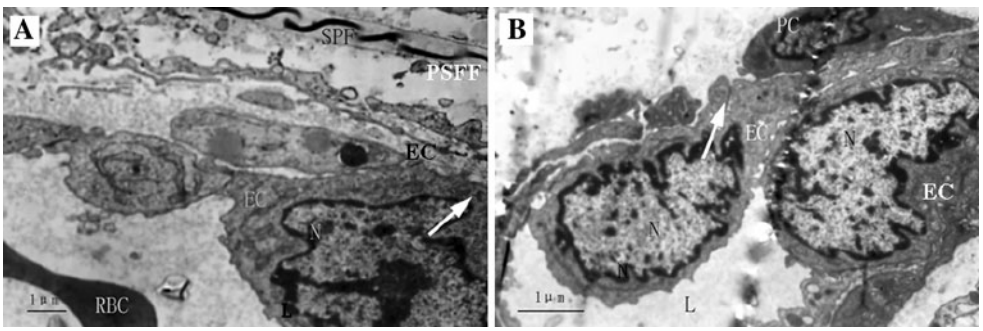

Fig. 3 The TEM photos at day 7, 10 after implantation PSFF : Material; *EC* endothelial cells; *N* nucleus of EC; *PC* peripheral blood vessel cells; *L* vessel lumen; *RBC* red blood cell

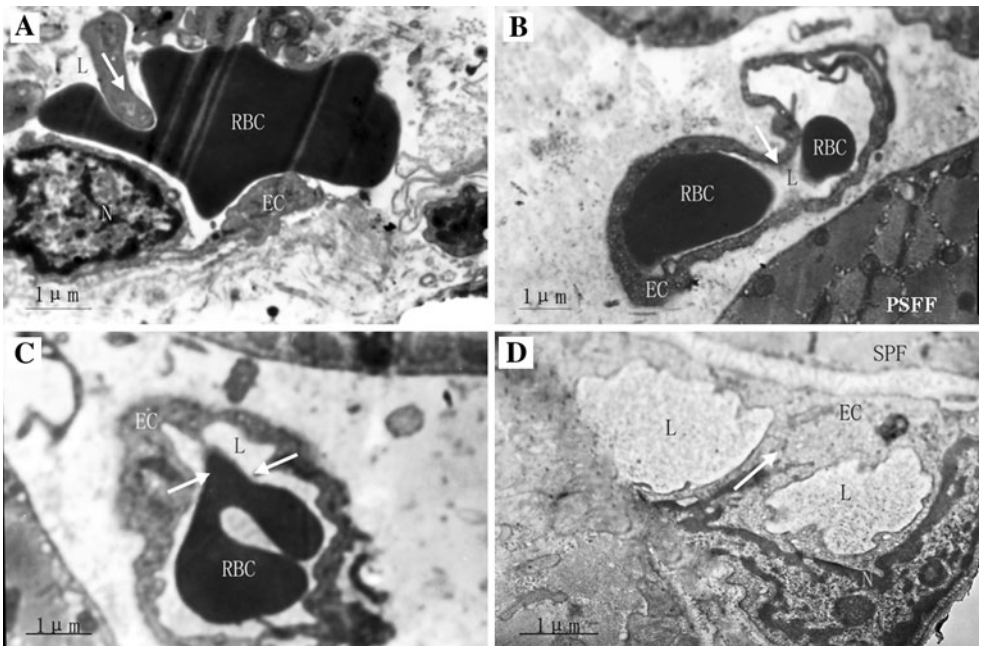

Fig. 4 The TEM photos at day 10, 13, 17, 24 after implantation PSFF: Material; *EC* endothelial cells; *N* nucleus of EC; *L* vessel lumen; *RBC* red blood cell

the lumen was divided into two lumen by the cytoplasm with cylindrical structure. This was the later stage of intussusceptive angiogenesis. These photos showed that intussusceptive angiogenesis appeared more frequently in the area of implanted SPFFs in the latter stage of the repair process. In this case, it was shown that the basic fibroblast growth factor declines in the stage of the intussusception [4].

4 Growth model of the capillaries in PSFFs

After PSFFs were implanted into the body, a series of complex reactions including inflammation [5, 6] were triggered to response injured tissue firstly. Then the extracellular matrix and the fibroblasts infiltrated into the materials and grew at once. As the number of cells within materials increased gradually, hypoxia and an insufficiency of nutrition became more and more manifest [7], which required the formation of new blood vessels. Angiogenesis observed in the experiment happened with the mode of sprouting or intussusception. Based on the experimental results, we will discuss on the modes and the mechanism of angiogenesis in the biomaterial that implanted into the body.

4.1 Modes of angiogenesis in PSFFs after the implantation

The experimental results showed sprouting was the main mode of angiogenesis at the early stage of wound healing. The similar results also were showed by Joanne [8]. However, intussusception was dominant at the middle and late stages of the repair process. These results should be paid more attention to the investigation of the angiogenesis mechanisms.

After the implantation, inflammation response and immunoreaction occurred immediately, angiogenesis began to occur with the infiltration of the extracellular matrix, collagenous fibers, cells and so on. In order to identify different circumstance conditions of various angiogenesis modes, we divided the process of the angiogenesis into two stages.

The first stage was the early stage of the repair process, which was the first week after the implantation in our experiments. In this stage, a lot of oxygen and nutrients were required for acute local response and cell viability in the PSFFs. In a short period of the early stage of the repair process, quite a few oxygen and nutrients from damaged microcirculation were supplied to the wound tissues. However, hypoxia reactions quickly occurred with closing of the capillary vessels, and the reactions rapidly became acute before new capillary vessels form. Acute hypoxia occurred first in the joints between the materials and the tissues, and then entered into cell tissues within the materials. The characteristic of this stage was acute hypoxia. The experimental results showed that the mode of angiogenesis was sprouting mainly in this stage.

The second stage was the slight hypoxia stage after the acute hypoxia stage. In this stage, extent of hypoxia in new tissues within the materials relieved because lots of

capillaries formed in previous stage. However, in the new tissues within the materials, the hypoxia state still existed before the capillary networks completely formed and maturated. The characteristic of this stage was slight hypoxia. The experimental results showed that the intussusception mode was dominant and the blood vessels were regulated and remodeled at this stage.

4.2 Response model of ECs to VEGF's concentrations

VEGF was the most effective specific stimulating factor of mitosis of ECs in the process of angiogenesis, and it also played a major role in proliferation and migration of ECs and in vessels formation [9]. The extent of hypoxia was directly related to the expression quantity of VEGF and its receptors in ECs [10, 11]. Therefore, in the process of the tissue regeneration, the main difference between the acute hypoxia stage and the slight hypoxia stage was the different concentrations of vascular growth factors such as VEGF in the damaged region. However, little was known of the ECs response in different modes of angiogenesis under different concentrations of VEGF. Therefore, we try to explore its solutions by next response model of ECs to the concentrations of VEGF.

In order to research the responses of ECs to the VEGF's concentrations, we took notice on the process, in which the concentrations of VEGF in the wounded tissues and within the materials changed from the early stage of the highest intensity of hypoxia to the stage declining gradually with the repair of blood vessels and angiogenesis. In this process, ECs produced different responses to the different concentrations of VEGF (C_{VEGF}). We suppose Q_1, Q_2 ($Q_1 > Q_2$) as the limits of the concentrations of VEGF [12]. And the responses of ECs could be classified as three categories.

Strong response : $C_{VEGF} \geq Q_1$

Moderate response : $Q_1 > C_{VEGF} \geq Q_2$

Weak response : $C_{VEGF} < Q_2$

In the stage of strong response, by the stimulating of a mass of the VEGFs, the ECs quickly grow and proliferate with the highest activation energy, and some of the ECs leave from the parent capillaries to the intercellular substance through the basement membrane.

In the stage of moderate response, the ECs quickly grow and proliferate without being dissociated from the parent capillaries with the secondary activation energy. Finally, sprouting is formed.

In the weak response stage, the ECs become enlarged and thickened with lower activation energy, and the opposite cell walls adhere to each other before the mitosis of the deformed cells. Finally, intussusception is formed.

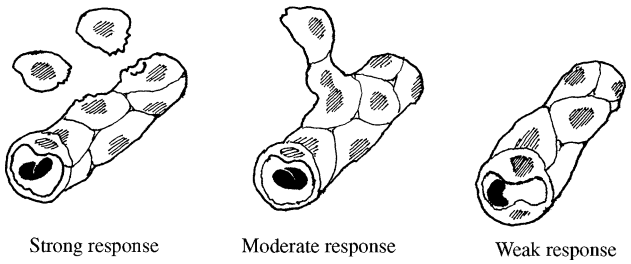

Fig. 5 Response model of ECs

This response model of ECs to the concentration of VEGF can be described as a sketch in Fig. 5, which shall be helpful to explain this mechanism occurring during the angiogenesis. On the other hand, the response model of ECs was enlightened from above experimental results, while it needs to be supported by further study. Besides we will ascertain the values of limits Q_1, Q_2 of the concentration of VEGF in the further work.

4.3 Sprouting at the early stage of implantation

Based on the model of EC response, we discussed the models of angiogenesis in the silk fibroin materials at the early stage, middle and late stages of wound healing, respectively. The results of in vivo study showed that the model of angiogenesis at the early stage was mainly sprouting, which means the strong response and moderate response occurred. Herein, we divided the process into 6 steps as follows:

(1) Acute hypoxia in the wounded tissues appeared immediately after the implantation because of the operating trauma and the damaged blood circulation, which induced inflammatory reaction. With body fluid and cells infiltrated into the materials, lots of growth factors such as VEGF are expressed. Accordingly, at adjacent capillaries around the trauma area, the ECs up-regulate the expression of VEGF receptors.
(2) Subsequently, the strong response of the adjacent ECs was inspired by the VEGFs with high concentration, namely ECs proliferated quickly, left from the parent blood vessels and move according to the VEGF gradient [13, 14].
(3) These separate ECs then assembled and formed cord-like cell lines at the verge of the temporary matrix [15] which contained the body fluid and the exuded plasma and acted as a niche for cell migration.
(4) In adjacent tissues around the trauma, the moderate response was evoked by the lower concentration of VEGFs, and the ECs quickly proliferated. So the sprouts formed which oriented to the higher concentration of VEGF.

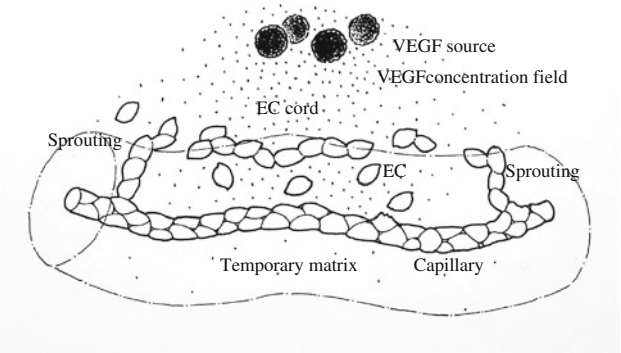

Fig. 6 The model of a new capillary formation with sprouting

(5) With the extension of EC cords, once the tip cells at both ends of the cords connected with the tips of the sprouts, a new loop of the capillary formed.
(6) When the lumens of cell cords formed, the new capillaries became functional.

Based on the steps proposed above, a sketch map of the sprouting model of angiogenesis in the silk fibroin materials is created as Fig. 6. The early angiogenesis being due to the sprouting and the cords of ECs, was induced by acute hypoxia, which was characterized with swift formation of blood vessels loops in order to meet the need of cellular metabolism and biochemical reactions. Based on the original capillaries, the first layer [16] of the new capillary looped to the wounded center or implanted biomaterials. Therefore, the sprouting mode has been a feasible mode of angiogenesis in the silk fibroin materials before the materials degradation because either the cell cords or new capillaries could extend through the pores without any obstacles. Furthermore, the rational, efficient blood vessels networks with an economy of ECs could be established. Contrarily, only by the sprouting with haploid direction to form capillaries, the tips of the sprouts shall extend up against the walls of the PSFF, thus capillaries are formed, however, it can not through the obstacles of walls. Accordingly, in this case the capillary networks would be deformed and consuming more ECs.

4.4 Intussusceptive angiogenesis at the middle and late stage of wound healing

Because of the first layer of blood vessels formed by the sprouting and cell cords, the wounded tissues gained oxygen and blood supply to a certain extent. Therefore, at this time, the hypoxia of the cells was alleviated compared to that at the early stage, so the cells down-regulated the expression of growth factors such as VEGF and its receptors accordingly. However, the increasing cells in the biomaterials were still in hypoxia because of the limitation of oxygen and blood supply. So the weak response of the ECs to VEGF occurred. In other words, despite of stimulation of VEGF, it was so weak that ECs could not depart from the parent blood vessels but expand with thickened cytoplasm. Consequently, the lumens of the blood vessels enlarged without broken basement membrane [17]. When the lumens enlarged to a certain extent, they could not keep a round shape, and the cytoplasm of ECs projected to the lumens. Once the opposite walls of the vessel anastomosed with each other, then a pillar spanning the lumens of vessel formed, and the lumen was divided into two parts. Furthermore, with the growth of cells and the extension of the pillar, the vessel caved in [18]. The pillars were shown as arrows in the Fig. 3 a, c and d. Furthermore, an abundance of plasmalemmal vesicles could be found in the ECs, which caused the increase of permeability of cells. With the extension of the pillars, the plasmalemmal vesicles amalgamated and caused the perforation of the vessel walls, which facilitated the formation of the lumen intussusceptions. In our study, the histological sections at the latter stage of repair process showed the intussusceptions within the silk fibroin materials.

Due to the trait of the intussusceptions, the new capillaries could not bestride the walls of the silk fibroin materials to form capillary network. Therefore, the intussusceptions should occur at the later stage after the degradation of biomaterials. In the present study, the results showed that obvious degradation of the silk fibroin materials could be observed at 10 days after the implantation, so this provided basis for the intussusceptions. With the increase of the blood vessels, further degradation of the materials and tissues regeneration, the intussusceptions have also played a key role in regulating and remodeling the networks of blood vessels in order to adapt the metabolism and attain the needful density of capillaries [19].

4.5 Further studies

Based on the results of implantation experiment, a model of angiogenesis in PSFFs was proposed and a model of ECs response to VEGF's concentrations was advanced to explain the mechanism occurring during the angiogenesis. A further study needs to be conducted to confirm the ECs response model, which will be of great significance. The research group will continue to clarify the model using various technical methods.

5 Conclusion

In this study, it is identified that angiogenesis develops in the way of capillary sprouting at the early stage of implantation and of intussusception at the late stage. A

response model of ECs to VEGF's concentrations was advanced to explain the experimental results. However, the importance of the theoretical results is to lay a foundation for exploring the formation of the capillary network. That is next subject to be solved.

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