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Retinal Digest Procedures for Examining Pericyte and Endothelial On Retinal Vasculature Using Rat Model

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Abstract: Retinal digest procedures is a gold standard method for examine anatomical details of retinal vasculature includes pericyte, endothelial cells, and basement membrane. Knowing the details of retinal vasculature helps us to study and understand more about pathological changes of many retinal vascular disorder, for example diabetic retinopathy. The aimedof this study was to make a modification methodsfrom previously literature. This study using rat model, also give some trick to avoid the fail of making retinal digest in order to make guidance for the next experimental study. The result showed that the pericyte and endothelial cells in retinal vasculature can be seen clearly using this methods.
Keywords : Retinal digest-trypsin digest-retinal vasculature-pericyte-endothel.

Introduction

Examining retinal vasculature is very important to study the mechanism of many retinal vascular disorder such as diabetic retinopathy. In diabetic retinopathy the pathogenesis of capillaropathy first caused by loss of pericyte, followed by loss of endothelial cells.To determine the pericyte, endothelial cells and retinal vasculature retinal digest preparation were used [1,2,3].

There were so many technique for examine retinal vasculature like injection of dye, immunostaining of vascular endothelium, retinal flat mounts staining, and adenosine diphospatase flat embedding technique. Neither of those technique can visualize the detail of retinal vasculature including pericyte, endothelial cells, and basement membrane.Staining technique is nonspecific to retinal vessel because it also stain non vascular tissueso it difficult to differentiate [4-7].

The method for staining retinal vasculature without non-vascular component developed by Kuwabara and Cogan in 1960. This method works by digesting the non-vascular component of the retina using trypsin, so called trypsin digestion technique. Since trypsin digestion was discovered, it become gold standard in evaluating the anatomy of retinal vasculature [8,9].

Trypsin digestion technique is not easy to perform and need a few days process each retina. In this study

we try to make more simple retinal digestion, modified from previous study [8,9]using rat model (*Rattusnovergicus*), to evaluate pericyte and endothelial cells in normal rat and diabetic rat.

Material and Methods

In this study we use retinal tissue isolated from *Rattusnovergicus*from normal rat and diabetic rat (after 1 month). The technique was using a modification of previous methods [8,9]. Three days were needed to make retinal digestion.All experiment using animal in this study were approved by faculty of medicine BrawijayaUniversity ethicscomitee.

Retinal preparation

Fix an enucleated eye with 10% formalin more than 24 h. To dissect out the retina first we have to make circumferential cut of the cornea through the limbus with dissection scissors. After the cornea was opened, remove the lens and vitreous from the eyeball. Two or four side radial incision was made but not to cut the optic nerve side. After the eye was flattened out use straight forceps (Kelman) to separate the retina from choroid and sclera towards the optic nerve, carefully and gently to avoid retinal tear. Put the retina gently into six-welled plate.

Retinal washing and trypsinization

The isolated retina was rinse with 3 mLPBS and put the well plate into shaker for 5 minutes. PBS was removed and added by 3mL trypsin3% (0.3 grams of trypsin in 10 ml PBS), and followed by shake (VRN-200) overnight.

The procedures of retinal preparation, washing and trypsinizations were done in the first day.

Separating retinal vasculature

After the isolated retina has shaked overnight, pipetting (in and out) with blue tip of micropipette, 3-5 times was conductedto break down non vascular tissue. Incubate the vascular network for 60 min at 45° C (Memmert Incubator), followed by shaker overnight.

Staining and visualizing the vasculature

This procedure was done at the third day. Pipette out the trypsin and washed the retinal vasculature with 3 mL aquadest and shake for 5 min. Remove and spray water on retina tissues and wash 3 times.

Place a drop of water into object glass, and retinal vasculature was put into the water on object glass. Make sure the tissue does not fold. Remove water adjacent to the retina using paper towel untill the object glass is completely dry. Stain the tissue with hematoxylin (HE) and let it for 5 min. Remove the hematoxylin and washed 3 times with tap water. Dry the object glass completely, then examine under the microscope (Olympuss cx-31).

Result:

Normal and diabetic retinal vasculature can be seen in figure 2-4. Whole entire network cannot be seen because of over digestion or difficulty on pipetting technique.

We can describe pericyte and endothelial well after trypsin digestion with HE staining. The pericyte looks darker and round, endothelial cells are lighter and spindel shape.

Figure 1.The overview of retinal dissection.

Four radial incision were made so it was is easier to peel the retina from choroid and sclera and keep the central retina intact.

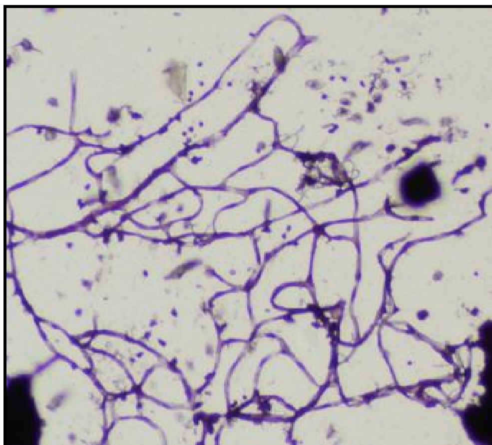

Figure 2.Normal rat retinal vasculature after trypsin digestion with H&E staining (100x). Nonvasculature tissue still seen in certain area due to underdigestion

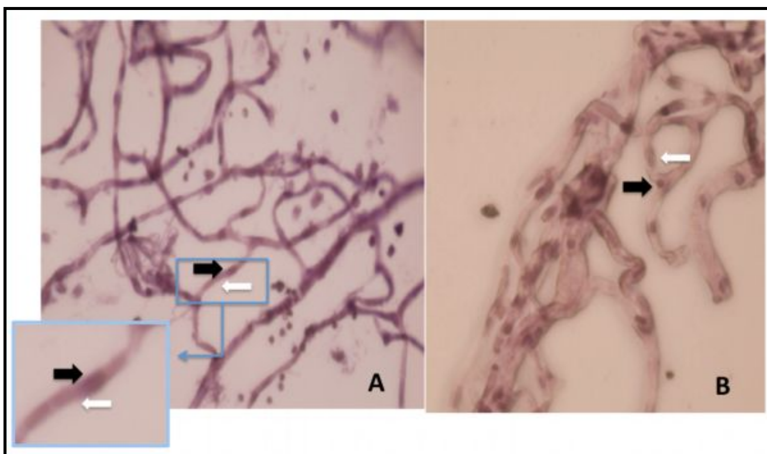

Figure 3.Rat retinal vasculature after trypsin digestion with HE staining (400x)

A. Normal rat :pericytewas darker and round cells (black arrow), endothelwas lighter and spindel shape cell (white arrow) B. Diabetic rat retinal vasculature (1 months). The presence of pericyte less than normal retina.

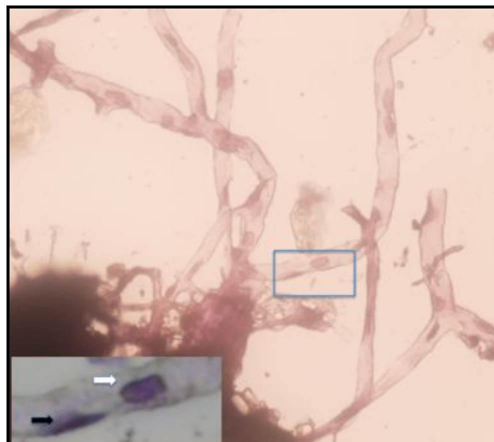

Figure 4.Pericyte and endothel from rat retinal vasculature (800x).

The differentiation between pericyteand endothel. The darker cell and embedded in vascular vessel (black arrow) waspericyte. Endotelwas lighter and spindle (white arrow).

Discussion:

Diabetic retinopathy is one of microvascular complication of both type of Diabetes mellitus. It is a leading cause of irreversible blindness among the people of occupational age worldwide [10].Visual loss in diabetic retinopathy due to macular edema and or retinal neovascularization caused by instability of retinal capillaries. The instability of retinal capillaries starts with pericyte loss, which is the earliest morphological changes in the diabetic retina which will further affect endothelial cells and leaving acelullar capillaries [1-3, 12].

Pericyte is vascular mural cell, embedded in membrane base and make direct contact with endothel. The highest pericyte coverage is observed in the retina, with pericyte :endothel ratio is 1:1. The higher the ratio of pericyte:endothel, the more important its function at the organ. In hyperglicemic condition there are decreases of pericyte :endothel ratio because of pericyte loss. Loss of pericyte in hyperglicemic condition due to alteration of biochemical pathway, and growth factors. Because the retina appears to be the most sensitive site for partial pericyte loss, so the role of the pericyte in early diabetic retinopathy is very important [1,2,12-15].

To study the anatomical condition of retinal vasculature we used trypsin digestion technique. Pericytewere seen as dark an embedded cells into the vessel walls, and endothel that lighter and have spindle nucleus.However Trypsin digestion technique was not easy to perform [8,9,12]. The difficulty includes the dissecting of the retina, pipetting of retinal tissue, and staining.

In dissect out the retina we must be do it gently to avoid retinal tear. In this study we dissected out the retina after the enucleated eye fixed with formalin for more than 24 h. The longer enucleated eye fixed in formalin, it is easier to separate the retina from choroid and sclera. To avoid retinal tear the separation between retina and choroid-sclera started from optic nerve using straight forceps, then widened to retinal periphery.

To separate retinal tissue from adjacent eye tissue like choroid, sclera, and retinal pigmented epithelium is not easy and need skill. In this study we did not peel the internal limiting membrane like previous study because it isdifficult [9].But according to theory the trypsin digestion should digest all tissue except vascular tissue. Based on the experience that's why we shake the tissue overnight and incubated with trypsin.

The pipetting process of the retinal tissue in and out needs special attention in order to avoid the loss of retinal, whether it retinal vasculature or non-vasculature. It is difficult to make the retinal tissue still intact after pipetting in and out, because the contact between tissue and tools. Based on previous study to coat the tools with trypsin, dipping the tools intermitenly and often into trypsin during the procedures are important [9].

Before staining the retinal vasculature with hematoxylin, we had to make sure that the retinal vasculature was already separate with non-vasculature tissue. Because if the separation was poor the staining

will not give a specific result. We have to check whether the retinal vasculature tissue were unfolded, because the folded tissue will not make the good staining result and it is difficult to visualized.

According to previous study the pericyte loss is observed after 3 months hyperglicemic mice [1,2]. In this study we enucleate the rats eye after 1 month hyperglicemic state in order to see pericyte : endothel ratio.In **Fig. 3B** that represent hyperglicemicrat we can see the pericyte is more rare than in normal rat **(Fig. 3A).**

Conclusion

Although it was difficult retinal digestion still showed the best methods to study anatomical details of retinal vasculature. Our method was easier to perform than previously methods described even though the standard technique remain the same. We hope that this can be the guidance for the next experimental study using retinal vasculature.

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