Histomorphological and Ultrastructural Changes of Retinopathy in Diabetic Rabbit Model

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Abstract: Observation on morphological and ultrastructural changes of retinopathy in diabetic rabbit long been fed with high sucrose and fat fodders. Use alloxan to establish diabetic rabbit model. Feed the rabbits with high sucrose and fat fodders conduct a Fluorescein Fundus Angiography test (FFA) every 3~4 months to observe morphological changes in retina. Conduct Transmission Electron Microscopy (TEM) examination on eyeball of the model 10~12 months later so as to get wise to the ultrastructural changes in the rabbit retina. The FFA indicated that with the protraction of the disease, Fluorescein Isothiocyanate (FITC) leakage and neo-vascularization shadows similar to that of human were seen in the diabetic rabbit model. TEM examination showed that no abnormality was seen in the ultrastructure of the rabbit retina of normal group. The retina of the diabetic rabbit exhibited microfilament and focal dissolving, myelin sheath structure loosening and partial demyelination changes, pericyte mitochondrion tumefaction, endothelial cell layering, space increasing, mitochondrion tumefaction on the nerve fiber layer. Glycogenosome accumulation was seen in the outer nuclear layer retina cells.

Key words: Retinopathy, diabetic model, animal, ultrastructure, accumulation, pericyte

INTRODUCTION

Diabetic Retinopathy (DR) is the most common and serious diabetic complication in eye capillaries. Although, study of retina pathological change through diabetic animal model is an essential way to study DR damaging process and mechanism, research in recent years mainly focuses on prophase damage of retina capillaries in diabetic animal model and some other researches involve prophase changes in retina nerve tissues.

However, little research has been conducted on retina ultrastructural changes in long-lived diabetic animal model. This experiment establishes alloxan-induced diabetic rabbit which has been fed with high sucrose and fat fodders for a long period of time.

Histomorphological change of diabetic rabbit retina is dynamically observed in different periods by Fluorescein Fundus Angiography test (FFA). Ultrastructural change of diabetic rabbit retina is examined by Transmission Electron Microscopy (TEM). These have laid an experimental foundation for research on pathogenesis and further interference therapy of diabetic retinopathy.

MATERIALS AND METHODS

Materials: About 36 healthy grown-up female white rabbits, weighed 2-2.5 kg each provided by Laboratory Animal Center of Xi'an Jiaotong University College of Medicine and used according to Regulations for the Administration of Affairs Concerning Experimental Animals formulated by the State Scientific and Technological Commission. Eyes of all rabbits were examined with slit-lamp and ophthalmoscope to be normal.

Reagent: Alloxan (Sigma). Before application make it up into 5% solution with sterilized normal saline for injection and sterilize and filter by 0.22 µm cellulose membrane.

Instruments: Accu-CHEK Active glucometer manufactured by Germany company Roche, FFA machine manufactured by Germany Heidelberg, Hitachi H-600 TEM.

Animal model: Inject slowly the newly prepared 5% alloxan solution via ear vein at 150 mg kg⁻¹ for 2~3 times every other week. Before administration, all rabbits were

fasted for 12 h and measured 3 times for fasting blood glucose in the morning and the mean value was taken as base value. At least 72 h after alloxan injection, fasting blood glucose in the morning was measured twice a week. If blood glucose could more than triple the base value for 2 continuous weeks and remained stable, the modeling was successful. About 15 were modeled successfully. After the modeling, high sucrose and fat fodders (mixtures of 10% lard, 37% white sucrose and 53% basal feed) were fed. One hutch is for one rabbit and approximately 100 g fodders for a rabbit each day with free supply of water. The experiment lasted for 40-50 weeks and at the end, 8 survived diabetic rabbits were kept as experimental group. Moreover, another 4 normal rabbits were taken as normal group.

Fluorescein fundus angiography test: Examine every 12-16 weeks. Apply Tropicamide eye drops (25 mg/5 mL) for mydriasis. Wrap the heads with cloth to fix, inject 20% fluorescein sodium (10 mg kg^{-1}) via the ear vein and perform FFA shooting immediately after the injection.

TEM observation: At the end of the experiment, rabbits were sacrificed by an overdose of thiamylal sodium and take their eyeballs out immediately. Quickly shear the cornea, lens, vitreous humor and retina above, below and around horizontally moving blood vessels in posterior pole, keep them in 2.5% glutaraldehyde solution for 24 h under 4°C then embathe them in phosphate buffer for 30 min. Keep them in 1% osmium tetroxide for 2 h and then embathe in phosphate buffer for 10 min. Apply ethanol for gradient elution, permeate with Epon812. After embedding, polymerizing, semi-ultrathin sectioning, ultrathin sectioning, uranyl acetate and lead citrate double staining, observe with TEM and shoot pictures.

RESULTS

FFA observation: As the FFA fundus picture of diabetic rabbit fed with high-sucrose and fat fodders for 14 weeks, Fig. 1a shows that retina vessels in posterior pole are basically normal without any sign of FITC leakage or neo-vascularization. Figure 1b is the FFA fundus picture of this animal in the 28th week showing tortuous retina vessel and hemangiectasis in posterior pole as well as a sign of FITC leakage at the ends of the vessels. Figure 1c is the FFA fundus picture of the animal in the 40th week showing serious FITC leakage in the retina vessels beside posterior pole.

As the FFA fundus picture of another diabetic rabbit fed with high-sucrose and fat fodders for 15 weeks, Fig. 1d shows that retina vessels in posterior pole are basically normal without any sign of FITC leakage or neo-vascularization.

Figure 1e is the FFA fundus picture of this animal in the 28th week showing un conspicuous tortuous retina vessel and hemangiectasis in posterior pole as well as a sign of FITC leakage at the ends of the vessels. Figure 1f is the FFA fundus picture of this animal in the 50th week showing tortuous retina vessel and hemangiectasis, visible FITC leakage in retina vessel beside posterior pole and obvious neo-vascularization.

TEM observation: The retina capillaries of normal rabbit are composed of endothelial cell, pericyte and basilar membrane. Endothelial nucleus and heterochromatin distribution are normal. The pericyte are between two basal membranes with normal nucleus. As shown in Fig. 2a, the basal membrane is a model structure with continuous and uniform electron density surrounding

Fig. 1: FFA observation

Fig. 2: TEM observation

endothelial cells and pericyte. About 40 weeks after the diabetic rabbits were fed with high-sucrose and fat fodders, endothelial cell layering could be seen in retina capillaries. The space became wider and bigger vacuoles were seen in cytoplasm. The basal membrane became thicker, obvious mitochondria tumefaction was seen in endothelial cells. Mitochondria tumefaction in pericyte was seen. Crista disorganization or varnishment happened (Fig. 2b-d). Cytoplasm of nerve cell axon on the retinal nerve fiber layer of normal rabbits is even and organelles

such as canaliculus, microfilament, mitochondria, etc., are normal. The somas are ovate with big and clear nucleus (Fig. 2e-g).

About 40 weeks after the diabetic rabbits were fed with high-sucrose and fat fodders, the somas deformed and vacuoles of various sizes were seen in cytoplasm. Obvious nucleus pyknosis was seen. Some nucleuses dissolved and mitochondria tumefaction happened (Fig. 2f). Myelinoclasia of different degrees happened in medullated nerve fibers. Medullary sheath structure

became thinner. Microfilament dissolved in neuraxon, vesicular changes appeared, mitochondria tumefaction denatured, neurogliocyte hyperplasia appeared around the degenerated neuraxon and organelle of gliocyte denatured (Fig. 2h-j). Glycogenosome accumulation was seen in diabetic rabbit fed with high-sucrose and fat fodders for >40 weeks (as shown by arrow in Fig. 2k).

Figure 2a normal group: normal retina capillaries and endothelia cells have clear nucleus and uniform cytoplasm. No tumefaction is seen in the cell structure ($\times 4,000$). Figure 2b-d are diabetic rabbits fed with high sucrose and fat fodders. In Fig. 2b shows endothelial cell layering and increase of space, basilar membrane becoming thicker ($\times 15,000$). Figure 2c shows mitochondria tumefaction of endothelial cells, obvious confusion of cristas ($\times 30,000$). Figure 2d shows mitochondria tumefaction of pericyte and cristas missing ($\times 30,000$). Figure 2e shows the normal group: the normal cross section of retinal nerve fiber layer ($\times 10,000$). Figure 2f is for the diabetic rabbit model group: the nerve fiber layer is loose and separated ($\times 10,000$). Figure 2g is the normal group: the nerve fiber is normal and there is no sign of degeneration. Figure 2h-j are diabetic rabbit model group: Fig. 2h shows partial nerve fiber demyelination changes ($\times 10,000$), Fig. 2i shows degeneration of nerve fiber, microfilament and focal dissolving ($\times 10,000$). Figure 2j shows Wallerian degeneration ($\times 10,000$) in nerve fiber. Figure 2k shows glycogen storage ($\times 5,000$) in the outer nuclear layer of retinal of diabetic rabbits fed with high-sucrose and fat fodders.

DISCUSSION

Alloxan is the most commonly used chemical drug in building diabetic animal model of type 1 but due to high death rate of animal models, some hyperglycemia will naturally release. Research shows that small doses of STZ injections accompanied with high sucrose and high fat food can achieve good diabetic animal model of type 2 (Masiello *et al.*, 1998; Shi *et al.*, 2004). In this research, small doses of alloxan intravenous injections are used together with high sucrose and high fat food ensuring a stable and moderate-level hyperglycemia of the animals to the largest extent which is similar to symptoms of type 2 diabetic patients who usually have decreased carbohydrate tolerance raised fasting blood glucose and hyperlipemia. With long-term feeding, the acquired animal models are more similar to human diabetes of type 2. Thus, it has high value in terms of research and application. Neo-vacularizaion is often seen in the proliferative phase of human diabetic retinopathy. FFA shows angiotelectasis and neo-vacularizaion as well as

surrounding fluorescein leakage. Hammes found that when induced by STZ for 24 weeks, the number of blood capillaries obviously increased in diabetic rats while the number of pericyte decreased by 47% compared with that of normal mice. The earliest hemangioma happened in the 32nd week and the capillary basement membrane obviously became thicker in the 56th week (Hammes, 1995). In this research, no characteristic changes of FFA were seen in the diabetic rabbit fed with high-sucrose and fat fodders for 14 weeks. Angiotelectasis and a small amount of fluorescein leakage were seen during the 28-30th week but no neo-vacularizaion angiogenesis was seen. This indicates that at least during the 28 weeks of diabetes, no characteristic changes or neo-vacularizaion will take place in diabetic retinal lesion in diabetic rabbit models. From the 40-50th week of the feeding, clear fluorescein leakage and focal blood shadow were seen in diabetic rabbit models but no widespread neo-vacularizaion or proliferative lesion was seen. This indicates that retinal retinopathy in the diabetic rabbit models happens after the 40th week but complete retinal lesion in proliferative phase still needs more feeding time and observation.

FFA shows that focal neo-vacularizaion shadows happen in the same period of time with fluorescein leakage. TEM shows retinal capillaries endothelial cell layering, space increase and relatively big vacuoles in cytoplasm, mitochondria tumefaction, mitochondria tumefaction of pericyte, confusion or missing of cristas, basilar membrane becoming thicker, etc. Under long-term hyperglycemia, aldose reductase becomes more active. Extra glucose is transferred into sorbite through aldose reductase which accumulates in cells and leads to high osmotic pressure and further results in histiocyte edema or breakage, damage of endothelial cell and pericyte in retinal capillaries, basilar membrane becoming thicker and capillaries shutting (Zhao and Jin, 1999). The retina runs short of blood and oxygen resulting in increase of angiogenesis factors like VEGF. Finally, this will lead to retinal neo-vacularizaion. Hyperglycemia can also result in poor convergent force of retinal capillaries, poor self-regulation ability of vessels and often causes higher capillary pressure which is an essential cause of diabetic microangiopathy (Hao *et al.*, 2001; Jiang *et al.*, 2001).

Martin *et al.* (2004) found with morphology that in the 14th week, retinal gangliocyte of STZ diabetic rat models decreased by 20-25%. Park *et al.* (2003) found that in the 4th week, retinal gangliocyte of rat models began to die and photoreceptor cells started to die. In the 12th week, some amacrine cells and horizontal cells also exhibited necrosis signs. Lu Yan believe that damage has already been caused in retinal ganglion cells before

abnormal signs appear in retinal endothelial cells and basilar membrane of the diabetic rat models of early phase (Lu *et al.*, 2002). Therefore, retinal ganglion cells become one of the most vulnerable cells in diabetic retina. To distinguish this from the early changes, this research mainly focuses on long-term changes of diabetic retinal lesion on the nerve fiber layer. TEM shows that after 40 weeks of feeding, the high-sucrose and fat fodders fed rabbits show a shortage of blood and oxygen. Myelinoclasia of different degrees happen in medullated fibers. Medullary sheath structure becomes thinner. Microfilament is seen in neuraxon. Vacuole changes and degeneration of mitochondria tumefaction are seen. Neurogliocyte gliosis is seen around regressed neuraxon and neurogliocyte organelle degenerates, leading to axoplasmic flow difficulty on the nerve fiber layer so that the neurotrophic factor can not reach the neuron somas through normal axoplasmic transportation and fail to maintain normal physiological function of the neuron. This is also an essential factor causing regression and death of neuron (Yu and Jian, 2010).

The experiment shows glycogen storage in the outer nuclear layer of retinal of the diabetic rabbits fed with high-sucrose and fat fodders for a long period of time. This needs further research and testifying. More observation and researches are still needed in terms of ultrastructural changes in retinal capillaries and retinal nerve tissues when a great amount of diabetic retinal proliferative lesions including neo-vascularization appear.

CONCLUSION

Retina FFA showed FITC leakage and neo-vascularization shadows in diabetic rabbits fed in a long term with high sucrose and fat fodder. Besides ultrastructural changes on retinal nerve fiber layer, obvious changes were also seen in capillaries of pericyte, endothelial cells, etc. These provide experimental basis for further interference therapy.

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