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# Morphologic Studies of the Retina in a New Diabetic Model; SHR/N:Mcc-cp Rat

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*The pathogenesis of diabetic retinopathy has not been fully explained. The earliest histological lesion is the loss of intramural pericytes and thickening of the basement membrane. Increased activity of the polyol pathway is a probable mechanism for these two abnormalities. Investigations have suffered from the lack of an exact animal model simulating the human condition. Examination of the retina in the spontaneously diabetic SHR/N:Mcc-cp rat demonstrated degeneration and loss of intramural pericytes, a progressive increase in basement membrane thickness, and microinfarctions with an area of non-perfusion. Therefore, this model may be used to clarify the biochemical mechanisms linking the metabolic abnormalities of diabetes and retinopathy.*

**Key Words:** diabetic retinopathy, SHR/N:Mcc-cp rat

Improvement in the treatment of Diabetes Mellitus (DM) has led to an increased life span of the DM patient as well as to complications. Therefore, there has been an increase in DM retinopathy and this is the leading cause of blindness compromising 20% over age 45 (Kahn and Hiller, 1974). The current number of confirmed DM patients are known to be only half of all DM patients, the remainder being unaware of the presence of their disease. Non-Insulin Dependent Diabetes Mellitus (NIDDM) being the majority, 85~90% of DM is usually diagnosed over age 40 while Insulin Dependent Diabetes Mellitus (IDDM) starts its onset before age 30. DM retinopathy needing treatment is mostly in the NIDDM group and is a complication occurring in the capillaries of the retina and is closely related to the duration of the disease. After 20 years of carrying the disease, DM retinopathy can be found in the majority of IDDM and in 60% of NIDDM patients (Klein *et al.* 1984a,b). The pathology of DM retinopathy is in the capillaries of the retina (Robinson *et al.* 1989) causing a loss of intramural pericytes, a progressive increase in basement membrane thickness, increased endothelial cell count, capillary dilation, and microaneurysm (Cogan *et al.* 1961; Kuwabara and Cogan, 1963). The exact mechanisms of DM retinopathy are not known, thus the lack of an absolute method of treatment or prevention and only defending against vision preservation with laser or surgery (Early Treatment Diabetic Retinopathy Study Research Group, 1991).

There is a need for study on animal models since human models are not possible to reveal the mechanisms of DM retinopathy. Recently, DM animal models have been induced by diet (Cohen *et al.* 1972; Rosenman *et al.* 1975; Parachristodoulou *et al.* 1976; Engerman and Kern, 1984; Kador *et al.*

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1988). DM was induced by overloading the polyol pathway with galactose or sucrose. However, the problems with this method were the relatively long length of time for disease onset (24~36 months) and that the occurrence of DM involved other factors than the polyol pathway. Alloxan and streptozotocin were later introduced as a method of changing pancreatic beta cells, but this led to IDDM models but not NIDDM, which was in greater need (Parachristodoulou *et al.* 1976; Kojima *et al.* 1985). There were also genetic methods which have been used such as the Bio Breeding Wister rat (BB Wister rat). This rat has IDDM with disrupted beta cells due to insulinitis and has a low T lymphocyte count making it susceptible to infection. Once this rat is insulin dependent, it then quickly falls into a state of ketosis, thus, the continued administration of insulin is needed (Sima *et al.* 1983, 1985). The Spontaneously Hypertensive Rat-copulent (SHR/N-cp) is another genetic model which is non-insulin dependent. However, the degree of diabetes is mild and must be combined with diet control (Michaelis *et al.* 1986, 1988). The disadvantages of the above mentioned models in studying DM retinopathy indicates the need of a better experimental model.

The ideal experimental DM retinopathy model should spontaneously show NIDDM without any artificial manipulation in a relatively short period of time. In 1988, McCune presented the diabetic model Spontaneously Hypertensive Rat/McCune-copulent (SHR/N:Mcc-cp). This model was attained by breeding the SHR/N-cp rat with the Koletsky copulent (cp) five times. The gene for obesity (copulent gene: cp) follows the Mendalian law of inheritance and thus the homozygous copulent (cp/cp) shows NIDDM. The male rat especially shows the symptoms of diabetes of glucosuria, polyuria, proteinuria, glucose intolerance, and insulin resistence in a short period of time without any manipulation. However, the female only shows insulin resistence. This male rat closely simulates the diabetic state in humans, thus it is used in studies of the heart and kidney (McCune *et al.* 1988, 1990, 1991), and yet it can also be used in the study of DM retinopathy. The purpose of this study was to reveal the usefulness of this model in the evaluation of DM retinopathy.

## MATERIALS AND METHODS

### Animals

The rats were obtained from the Veterinary Central Laboratory Animal Resource (Ohio State University, Columbus, Ohio, USA). These SHR/N: Mcc-cp (cp/cp) rats, who rapidly gain subcutaneous fat by week 4 after birth, were separated from the other genetic type (cp/+, +/cp, +/+). Eight male and 8 female rats were then allocated into two groups. Female rats were the control group. The rats were fed unlimited amounts of Purina Laboratory Chow (#5001) and were alternatively exposed to 12 hours of light, 15~25 foot candles in intensity, and 12 hours of darkness.

### Methods

**Measurement of fasting blood sugar:** The 8 hours fasting blood sugar of the rats in both groups were measured by the glucose oxidase method using the Astra (Beckman Instrument Inc. Brea, CA, USA) from blood sampled by a 3~5 mm incision of the tail every month for 6 months. The average value and standard deviation were obtained and observed.
**Trypsin digestion method:** At 6 months of age, both eyes from 3 rats in each group were enucleated after injection with pyrimidinetrione-5-ethyl-5-monosodium salt (Entobar) into the peritoneum for anesthesia. The eyes were fixed in 10% buffered formalin solution for 5 days, then an incision 5mm posterior from the limbus was made and the retina layer was removed and placed in a 3% trypsin (1 : 80) and 0.1 M tris buffer (pH 7.8) solution for 2 hours at 37.5 degrees. The retina was then washed with normal saline and the internal limiting membrane was removed by careful shaking leaving only the vessels. The remaining tissue was dried on a glass slide and observed by light microscope after PAS staining. The abnormalities of the microvasculature and new vessels within the retina were observed first. Then, 5 fields (×250) were randomly selected 4mm within the optic nerve and the pericyte and endothelial cell counts were taken. The endothelial cells/ pericytes count was calculated and statistically analyzed by Mann-Whitney U test.
**Electron microscopic finding:** At 6 months of age,

5 rats from both groups were anesthesized with pyrimidinetrione- 5-ethyl-5-monosodium salt(Entobar) into the peritoneum and both eyes were enucleated. To completely fix the inner layers of the retina, 2.5% gluteraldehyde 0.5 ml was injected with a 26G needle into the eye and then fixed in 2.5% gluteraldehyde for 2 hours. An incision was made along the equator and a 4×4 mm-sized retinal segment was attained in relation to the optic nerve. The 4×4 mm-sized retinal segment was incised horizontally and vertically to obtain four parts. The parts were washed with 0.1M phosphate buffer fixed for 1 hour in 1% OsO4(pH 7.4) buffered in 150 mM Na-K phosphate. Sequential drying was done in 15-minute intervals in 50%, 75%, 90%, 100% ethanol, then converted in ethanol/propylene oxide solution 3 : 1, 1 : 1, 1 : 3 for 20 minutes and when 100% propylene oxide was reached, each part was immersed in propylene oxide/epoxy resin 1 : 1, 1 : 3 solution for 2 hours. The retina was treated in 100% resin for 6 hours and stored at 60 degrees for 48 hours then cut in 1 $\mu$m thicknesses by ultramicrotome(glass) and stained with toluidine blue. The desired section was decided by light microscope then cut by diamond knife at 60~70 nm thickness and double electron stained by uranyl acetate and lead citrate, and then observed by permeative electron microscope (Hitachi-H-500; Hitachi, Tokyo, Japan). The capillaries observed were from the vessel abundant internal granular and external retinal layer. To use that segment which was cut vertically, the maximum internal diameter was within 5~8 um and the basement membrane was observed. The thickness of the basement membrane between the pericytes and endothelial cells from one capillary of each segment was measured by bioquant II digitizing tablet and morphometric analysis system (R&M biometrics, Nashville, TN, USA).

## RESULTS

The blood glucose level was checked in the SHR/N:Mcc-cp rat of each group for the occurrence of diabetes (Table 1). In the case of the male homozygous copulent, the fasting blood sugar level exceeded 140 starting at 3 months of age. However, the female homozygous copulent's sugar level did not reach the diabetic level.

The retinas of the rats aged 6 months in both groups after being treated by trysin were observed by light microscope. The retina of the homozygous copulent male rats (Fig. 1) showed partial capillary obstruction and acellular, tortuous, irregular capillaries. Also, there was a decrease in pericyte count, an increase in endothelial cell count, and remnants of the pericytes surrounded by basement membranes or so-called ghost pericytes. In the female homozygous copulent rats(Fig. 2), consistent-sized capillaries and even-cell distributed capillaries were observed. The nucleus of the pericyte was round and darkly stained while the nucleus of the endothelial cell was oval and lightly stained. However, there were no evidence of neovascularization, or abnormal capillaries. To evaluate the increase of endothelial cells and decrease of pericyte cells, the endothelial/pericyte ratio was evaluated by Mann-Whitney U test (Table 2). The male homozygous copulent showed a significant increase of 3.58±0.19 (mean± standard deviation) compared to the increase of 1.52 ±0.04 of the female homozygous copulent rat (p=0.0495).

The results of the electron microscope findings of rats aged 6 months in each group (Fig. 3) showed thickening and irregularity of the basement membrane along with remnants of pericytes or so-called ghost pericytes. The thickness of the basement

**Table 1. Fasting blood glucose levels(mg/dl) of homozygous copulent male and female rats**

| | 1 | 2 | 3 | 4 | 5 | 6 (months) |
|---|---|---|---|---|---|---|
| male | 80±9.8 | 92±8.1 | 142±11.7 | 196±15.6 | 171±9.4 | 182±10.2 |
| female | 76±7.4 | 78±8.6 | 92±8.2 | 85±9.7 | 89±7.7 | 75±6.7 |
| (P value) | 0.1430 | 0.0969 | 0.0047 | 0.0067 | 0.0050 | 0.0024 |

The numbers refer to the mean±standard deviation.
Mann-Whitney U test

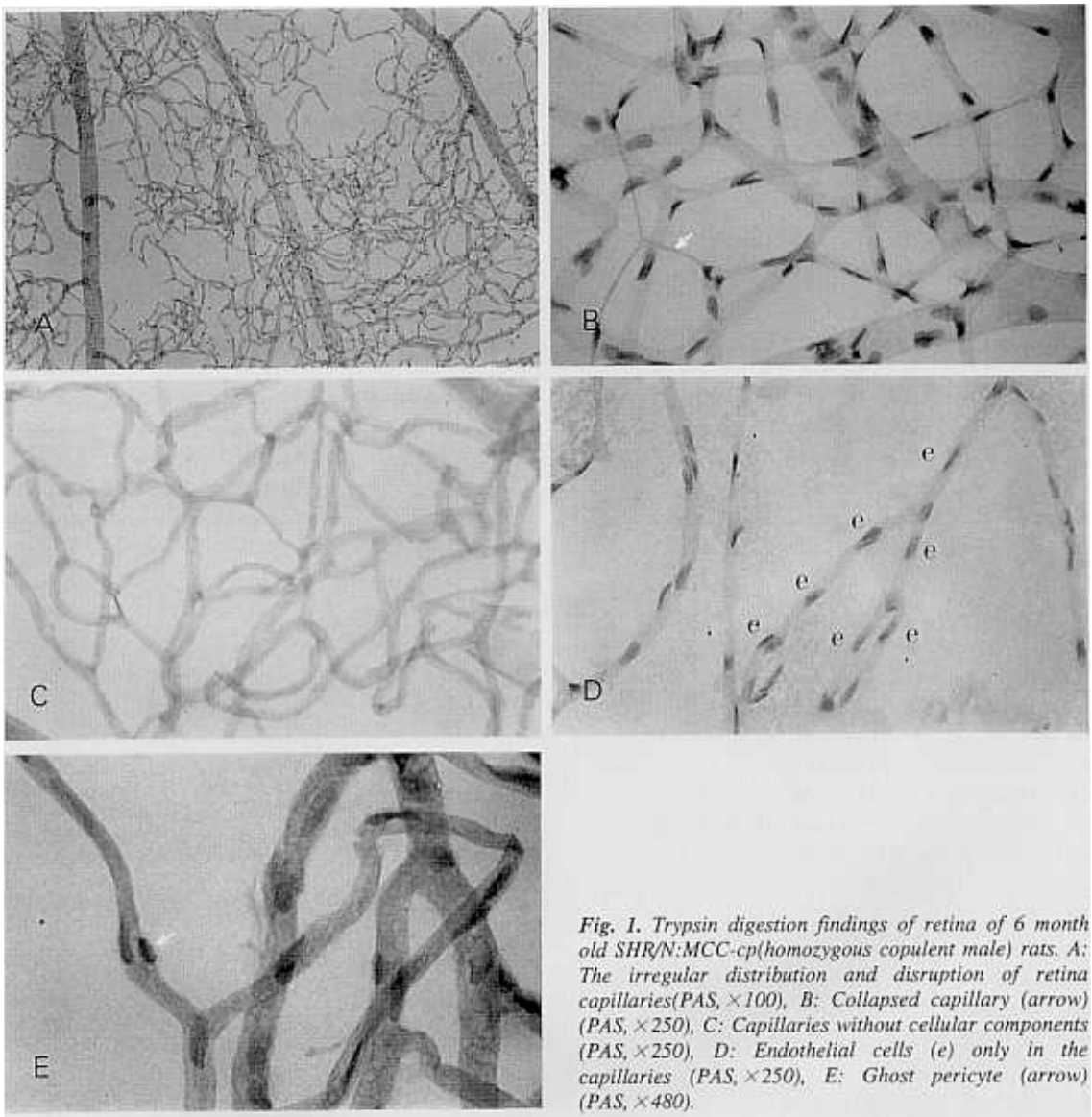


*Fig. 1. Trypsin digestion findings of retina of 6 month old SHR/N:MCC-cp(homozygous copulent male) rats. A: The irregular distribution and disruption of retina capillaries(PAS, ×100), B: Collapsed capillary (arrow) (PAS, ×250), C: Capillaries without cellular components (PAS, ×250), D: Endothelial cells (e) only in the capillaries (PAS, ×250), E: Ghost pericyte (arrow) (PAS, ×480).*

**Table 2. Endothelial cells/pericytes of retinal capillaries of homozygous copulent male and female rats**

| | Endothelial cells/pericytes(mean±S.D.) | |
|---|---|---|
| male | 3.58±0.19 | |
| female | 1.52±0.04 | p=0.0495 |

Mann-Whitney U test

**Table 3. The basement membrane thickness between endothelial cell and pericyte in homozygous copulent male and female rats**

| | The basement membrane thickness(mean±S.D.) | |
|---|---|---|
| male | 99.9±0.6 μm | |
| female | 85.9±4.4 μm | p=0.0472 |

Mann-Whitney U test

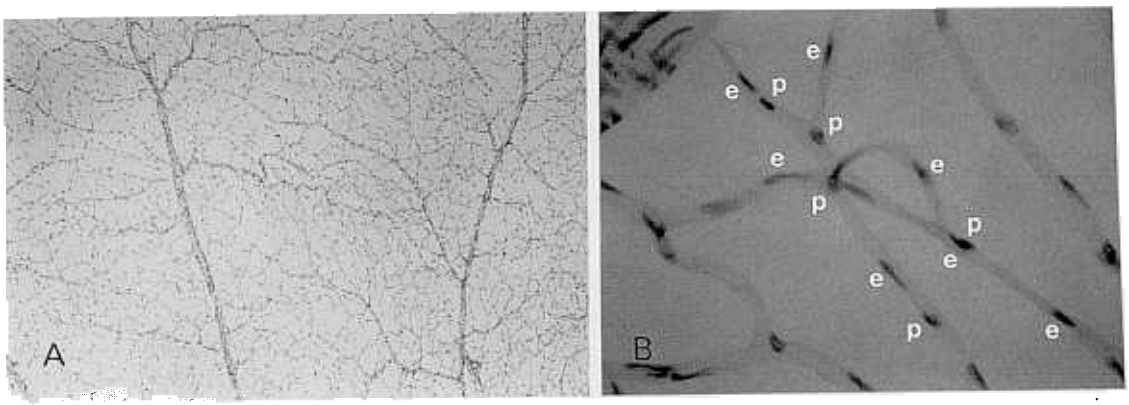


*Fig. 2. Trypsin digestion findings of retina of 6 month old SHR/N:MCC-cp (homozygous copulent female) rats. A: Regular distribution of capillaries (PAS, ×25), B: Endothelial cells (e) and pericytes (p) in the capillaries (PAS, ×250).*

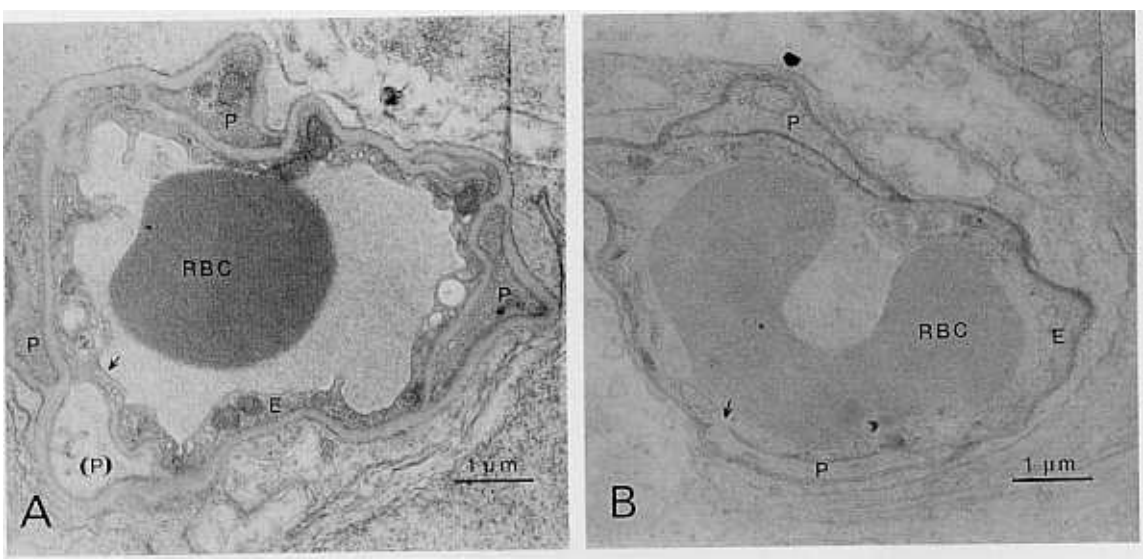


*Fig. 3. Electron microscopic findings of retinal capillary of 6 month old SHR/N:MCC-cp rats. A: Homozygous copulent male rat. Endothelial cell (E) and pericyte (P), ghost pericyte ((P)), irregular and thickened basement membrane (B), contact area between pericyte and endothelial cell (×18000), B: Homozygous copulent female rat.Endothelial cell (E) and pericyte (P), consistent thickness of basement membrane (B), contact area between pericyte and endothelial cell (×18000).*

membrane between the pericytes and endothelial cells and the portion which forms the outer wall was analyzed. The inter-pericyte and endothelial thickness (Table 3) was significantly greater in the male homozygous copulent rat (99.9±0.6 um) than in the female homozygous copulent rat (85.9±4.4 um) (p=0.0472). The outer basement thickness (Table 4) was significantly greater in the male homozygous

**Table 4. The thickness of the outer basement membrane of capillaries in homozygous copulent male and female rats**

| | The basement membrane thickness(mean±S.D.) | |
|---|---|---|
| male rats | 187.7±4.8 μm | |
| female rats | 124.0±6.4 μm | p=0.0009 |

Mann-Whitney U test

copulent (187.7±4.8 um) than in the female homozygous copulent rat (124.0±6.4 um) (p=0.0009).

## DISCUSSION

The changes which occur in diabetic retinopathy are deeply related to the type and duration of diabetes. Diabetic retinopathy does not occur in IDDM of 3~5 years. The duration of NIDDM is difficult to know due to uncertainty about the exact time of occurrence. There is a higher incidence of DM retinopathy in IDDM, which is suspected to be due to higher glucose levels and metabolic disabilities. Macular edema which causes a decrease in vision in DM retinopathy is more frequent in NIDDM. Although the mechanism is not known, pregnancy (Moloney and Drury, 1982; Phelps *et al.* 1986; Serup, 1986), chronic high glucose levels (The Kroc Colaborative Study Group, 1984; Brinchman-Hansen *et al.* 1985; Grunwald *et al.* 1987), hypertension(Krolewski *et al.* 1988), hyperlipidemia (Stern *et al.* 1989), and renal diseases (Chase *et al.* 1989) contribute to the occurrence and exacerbation of DM retinopathy.

The mechanism of DM retinopathy is not exactly known but the morphologic characters are revealed. The changes in DM retinopathy first start at the capillaries with a decrease of pericytes, an increase of endothelial cells, basement membrane thickening, vessel dilation, and microaneuryms (Cogan *et al.* 1961; Kuwabara and Cogan, 1963; Cogan and Kuwabara, 1967; Kohner and Henkind, 1970; de Venecia *et al.* 1976; Ashton *et al.* 1983). The decrease of pericytes and vessel dilation are among the first changes of DM retinopathy and they occur throughout the retina while the increase of endothelial cells and microaneuryms is located in the central retina (Robinson *et al.* 1989). The decrease of pericytes lessens the support of the vessels causing chronic dilation (Cogan *et al.* 1961), microaneuryms (Yanoff, 1969), and complications in blood flow (Frank, 1984). Other early changes are capillary basement membrane thickening and increase in collagen (type IV) and laminin (Das *et al.* 1990). The thickening of the basement membrane is involved in capillary permeability and obstruction. Skovberg's photos of the retina in 1969 showed that the retinal vessels were dilated 10% more in DM retinopathy patients and the degree of dilation was directly related to the level of blood glucose returning to normal with the control of sugar levels (Skovberg *et al.* 1969). Sims reported in 1986 that the basement membrane between the pericytes and endothelial cells were perforated allowing direct contact between the cells and found diverse junctional complexes (Edelman and Thiery, 1985; Sims, 1986). Following many studies on the junctional complexes, however, their function was not revealed (Hogan and Feeny, 1963; Bruns and Palade, 1968; Leeson, 1979). In 1970, Crocker reported that one of the functions of the pericytes was to control the proliferation of endothelial cells and in 1987, Orlidge and D'Amore experimently proved the pericyte control of the endothelial cell (Crocker *et al.* 1970; Orlidge and D'Amore, 1987). Thus, in DM retinopathy, the lose of junctional complexes leads to the lose of endothelial control and proliferation.

To reveal the mechanisms of the above DM retinopathy changes, experimental studies are needed. However, due to limitations in human models, animal substitutes were needed. The animals that have been used were diet induced models. The diet method consists of giving galactose or sucrose to dogs or rats (Cohen *et al.* 1972; Rosenman *et al.* 1975; Parachristodoulou *et al.* 1976; Engerman and Kern, 1984; Kador *et al.* 1988; Robinson *et al.* 1989) and overloading the polyol pathway, one of the mechanisms of DM retinopathy, thus inducing diabetes. The limitations of this method are the long DM induction time (24~36 months) and the fact that the natural occurrence of DM involves more than the polyol pathway. Alloxan and streptozotocin were later introduced as a method of changing pancreatic beta cells, but this led to IDDM models and not the NIDDM which was in greater need (Arison *et al.* 1967; Cameron *et al.* 1971; Leuenberger *et al.* 1971; Sosula *et al.* 1972; von Sallman *et al.* 1972; Cameron *et al.* 1973; Watanabe, 1973; Babel and Leuenberger, 1974; Leuenberger *et al.* 1974). There are also genetic methods which have been used such as the Bio Breeding Wister rat (BB Wister rat). This rat has IDDM at 7~21 days of age with disrupted beta cells due to insulinitis. The large consumption of muscle protein and fat and the

accompanying state of ketosis calls for the continued administration of insulin. Also, the decrease of T cells and lymphocytes causing infection causes many difficulties (Sima *et al.* 1983, 1985). The Spontaneously Hypertensive Rat-copulent (SHR/N-cp) is another genetic model which is non-insulin dependent. However, the degree of diabetes is mild and must be combined with diet control such as sucrose (Michaelis *et al.* 1986, 1988). The disadvantages of the above mentioned models in studying DM retinopathy bring limitations in the usage for the DM retinopathy model.

The ideal characteristics of the DM retinopathy model are the ability to become NIDDM in a short period of time without any artificial intervention. In 1988, McCune introduced a DM rat model SHR/N:Mcc-cp which is the result of breeding the SHR/N-cp and Koletsky copulent (cp) 5 times. The gene for obesity (copulent gene:cp) is inherited recessively and the homozygous copulent (cp/cp) becomes NIDDM. The female rat merely becomes glucose intolerant unlike the male rat which shows characteristics of NIDDM such as glucosuria, polyuria, proteinuria, glucose intolerance, and insulin resistence. Severe glucosuria is observed during 2 to 6 months of age, with some cases of glucosuria reaching levels of 8000 mg/dl. Polyuria and proteinuria also reach their maximum at 14 weeks of age and decrease thereafter. The resistence to insulin was 6 times greater without regard to sex (McCune *et al.* 1990, 1991). This male rat model showed well the characteristics of DM, therefore, it has been used in cardiology and nephrology research but has never been used in ophthalmology. To determine whether this model is fit for DM retinopathy research, it must show the findings of DM retinopathy in humans. This study used the male homozygous copulent rat and the female homozygous copulent rat as the control. The rats in both groups fasted for 8 hours and the glucose levels were checked every month and at 6 months the retina was observed by electron microscope. The progress of DM retinopathy was followed by the trypsin digestion method. The results of fasting glucose were high for the male homozygous copulent beginning at 3 months of age, but were normal for the female.

The trypsin digestion method resulted in the male homozygous copulent rat showing partial capillary obstruction, acellular capillaries, and dilated, tortuous capillaries along with a decrease in pericyte count, an increase in endothelial cell count, and ghost pericytes. The capillaries of the male homozygous copulent rat were weak and easily destroyed making it difficult to use. The female homozygous copulent rat showed evenly-sized capillaries with even-cell distributed capillaries. The nucleus of the pericyte was round and darkly stained while the nucleus of the endothelial cell was oval and lightly stained. However, neovascularization or abnormal capillaries were not observed in any groups. The reason for the absence of microaneurysm was that although the central retina was used, the most common location for microaneuryms is around the optic nerve and this was difficult to observe by the trypsin digestion method.

To compare the decrease of pericytes and increase of endothelial cells, the endothelial cells/pericytes ratio was statistically analyzed. The endothelial cells/pericytes ratio being 1.5/1 in the normal rat, the male homozygous copulent rat was $3.58 \pm 0.19$, which was significantly higher than the female rat at $1.52 \pm 0.04$.

The electron microscope findings showed thickening and irregularity of the basement membrane with the presence of ghost pericytes. The basement membrane of female rats was consistent and the cells were evenly distributed.

The basement membrane situated between the pericytes and endothelial cells and a portion of the external layer were analyzed. It was thought that the decrease of pericytes preceded the thickening of the basement membrane and after the regression of the pericytes, the two basement membranes surrounding the pericytes became one and resulted in thickening. However, the basement membrane between the cells was much thicker in the male homozygous copulent rat ($99.9 \pm 0.6$ um) compared to in the female ($92.7 \pm 4.4$ um). Also, the basement membrane of the external layer was thicker in the male homozygous copulent rat ($187.7 \pm 4.8$ um) compared to the female ($124.9 \pm 6.4$ um). The results prove the thickening of the basement membrane, but it was not directly related to the regression of the pericytes. Thus, the thickening of the basement membrane rather precedes the regression of the pericytes.

Therefore, the retina findings of the SHR/N:

Mcc-cp rat that McCune presented at the NIH workshop in 1988 well reflected those of the human DM retina and this male homozygous copulent rat was ideal in becoming NIDDM in a relatively short period of time without artificial intervention. This study found the model used to be invaluable for further DM retinopathy research.

In summary, the SHR/N : Mcc-cp (male homozygous copulent) rat was tested to see if it was useful in the research of DM retinopathy by measuring fasting blood sugar, observing the electron microscopic retina findings, and by trypsin digestion methods in 6-month-old homozygous males and females to observe the progression of DM retinopathy. The results are as follows:

1) The male homozygous copulent showed elevated fasting blood sugar levels from the age of 3 months

2) Trysin digestion method showed male homozygous copulents to have partial capillary obstruction with acellular, dilated, and irregular capillaries. Also, a decrease in the pericyte count, an increase in the endothelial cell count, and ghost pericytes were found. However, neovascularization or abnormal retina capillaries were not found in any groups. The endothelial cells/pericytes ratio was $3.58 \pm 0.19$ in the male homozygous copulent, showing a significant increase.

3) The thickness of the basement membrane located between the endothelial cells and pericytes was greater in the male homozygous copulent ($99.9 \pm 0.6$ um) than in the female homozygous copulent ($92.7 \pm 4.4$ um). The basement membrane comprising the outer wall was also significantly higher in the male homozygous copulent ($187.7 \pm 4.8$ um) than in the female homozygous copulent ($124.9 \pm 6.4$ um).

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