THE CRITICAL DAYS IN CHOROIDAL VASCULAR CHANGES IN EMBRYOS FROM DIABETIC RATS

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Summary

Pregnant women who suffer from diabetes are more likely to have a child with more problems; according to a new study. Fetal brain oxigen deficiency occurs in human pregnancies complicated by diabetes mellitus or more common in the offspring of these pregnancies.
In this research, the effect of maternal diabetes on the choroids plexus volume changes and total length of capillaries in different day of gestation in rat's embryos was studied. Diabetes was induced by streptozotocin (55 mg kg^{-1}), given by a single intraperitoneal injection to female Wistar rats (200-250 g). Control animals were given an equivalent amount of citrate buffer saline. In six stage of neonatal life, two embryo of each mother selected and under anesthetized the brain was removed and fixed. Serial cross –sections were cut and stained with toluidine blue. The volume of choroids plexus and total length of capillaries by stereological methods was measured. Statistical analysis's showed significant difference in choroids plexus volume and total length of capillary`s between diabetic and control groups ($p<0.01$) especially in 17/5 and 21/5dg. This study shows that maternal diabetes causes microvascular disorders in choroids plexus system in neonatal life that lead to an increase in volumes of choroids plexus.

Key words: maternal diabetes- choroids plexus- length of capillary- embryo

Running title: Critical days in choroids plexus development

Introduction

Maternal diabetes is associated with an increased risk of several complications in the offspring, such as growth disturbances and congenital malformations (1).Diabetes is a complex metabolic syndrome that increases the risk for vascular dementia and stroke (2). Cerebral micro vascular diseases in diabetes has been attributed to the effects of chronic hyperglycemia on capillary structure, endothelial reactivity and blood-brain barrier permeability, thus affecting regional metabolism and blood flow regulation(3). Major congenital malformations, including those affecting the cardiovascular system, remain the leading cause of mortality and morbidity in infants of diabetic mothers (4). Diabetes angiopathy is characterized by the vessel wall remodeling, media hypertrophy and increased stiffness that may be enhanced by circulating vasoconstrictors and vascular inflammation (5).Diabetes is also associated with a chronic inflammatory state that damage micro vessels, and may impede the growth of new vessels (6). Abnormal angiogenesis in diabetes is most clinically apparent in proliferative diabetic retinopathy and the neovascularization is preceded by the selective destruction of pericytes, capillary failure and hypoxio that leads to the release of pro-angiogenic substances (7). To initate angiogenesis, studies suggest that the metabolic abnormalities of diabetes alter the angiogenic process. There are several mechanisms by which, angiogenesis may be vulnerable to the metabolic abnormalities of diabetes. Although the extent and nature of this vulnerability is not well understood (8).

Offspring of diabetic mothers (both humans and experimental) experience a two-to four fold increase in congenital anomalies (9). Although no particular organ system or tissue seems to be specifically targeted a variety of cardiovascular anomalies are frequently observed (10), arrested development or maldevelopment of this vasculature would lead to fetal demise early resulting in the termination of pregnancy: whereas arrest and/or maldevelopment of vasculature(s) at later times, associated with specific organ or tissue development, would contribute to congenital abnormalities in a wide variety of organs and tissues (4). Several lines of evidence indicate that chronic hyperglycemia is the major cause of vascular endothelial cell injury and that survival/repair mechanism for vascular cell injury are activated in the early stages of the disease (11).

Several theories have been postulated regarding potential mechanisms by which maternal diabetes might induce dymorphogenesis: how ever the pathogenesis remains unknown (4). The aim of present experimental design was to induce maternal diabetes mellitus and to assess the effects of that on choroids plexus capillaries length in the embryos from diabetic and normal mothers in rats. For this aim we estimate the volume of choroids plexus and total length of capillaries in different days of gestation in rats.

Material and methods

All experiment was conducted in faculty of science, Islamic Azad University of Mashhad, Iran (2009). All chemical used in this study were purchased from Sigma (UK).

Animal subjects

Thirty Six female, Wistar rats weighting between 200-250 g served as subjects for these experiments. All animals were housed individually and maintained on a 12/12 light/dark cycle, with lights on at 6.00h. Ambient temperature in the animal facility was kept at 22±2C° .Food and water was given ad libitum.

Groups

(1)Control groups. For baseline measurement in this group salin was injected.
(2)Diabetic groups. For induce diabetes STZ was injected. After one month of inducing diabetes, diabetic female rats and normal male rats were located in a special cage. Choroids plexus was exposed on 15/5 dg. At the different days of gestation two embryos was selected of each mother.

* At the 16.5 days of gestation (dg) (n=6)
* At the 17.5 days of gestation (dg) (n=6)
*At the 18.5 days of gestation (dg) (n=6)
* At the 19.5 days of gestation (dg) (n=6)
* At the 20.5 days of gestation (dg) (n=6)
* At the 21.5 days of gestation (dg) (n=6)

Induce diabetes

Diabetes was induced in overnight-fasted male rats weighting 200-250gr by a single dose of streptozotocin (55mg/kg body weight; Sigma) (12).It was also freshly dissolved in citrate buffer (PH =4.5) and administered via interaperitoneal injection to pentobarbital sodium (50 mg/kg body weight; i.p. Sigma) anaesthetized animals. Control rats received an equivalent amount of the buffer. Diabetes was confirmed by determining glucose levels in blood samples. Only those animals with blood glucose level > 400 mg/dl were regarded as diabetic.

Sampling

Female pregnant rats were anesthetized under interaperitoneal injection of 0.24 cc of a mixture (1:2) of 10% ketamin and 2% xylazine**.** After surgery the uterine was removed of body and two embryo of each mother were selected. Under the pentobarbital anesthesia the embryos brain was rapidly removed and fixed in 10% paraformaldehyde .For histological evaluation samples were placed in same fixative over night and was embedded in paraffin. Serial cross –sections were cut and stained with toluidine blue.

Stereological methods

The length density of capillary by counting the number of capillary particles as described by (13) was estaminated.
The probability that a given structure is hit by a randomly positioned and randomly oriented section is proportional to its linear dimension or length. It follows that if we observe how often a particular type of structure is hit on a section-i.e. count the number of profiles- It has a simple and direct estimator of its total length (if the volume of its containing space is known). It turns out to be as simple as

$$L(struct)=2.Q_A(struct/ref).V(ref)$$

Where the numerical density in the plane QA is estimated as described above for number of profiles in general.

Statistical analysis

Statistical analysis was assessed by a t test and one-way ANOVA.

Student's t test was used for comparison when only two groups were analyzed and a one-way ANOVA followed by a Scheffe' f test when more than two groups were analyzed. Results were expressed as mean ± SEM. A $p<0.05$ was considered significant.

Results

Blood and CSF chemistry

Diabetes were assessed in this study by monitoring the blood glucose levels in both PBS and STZ injected rats (Table 1).There was a significant increase ($p<0.001$) in blood glucose levels from 100±5 mg/dL in control to 470±18 mg/ml in diabetic rats. In addition there was a meaningful increase $p<0.05$ between cholesterol, urea, uric acid, [P] and [Ca] levels of diabetic mothers plasma and control mothers.

Table.1 Concentrations of different metabolites in normal and diabetic rat serum

	P	Cholestrol	Triglycerides	Urea	Uric asid	Cratinin	Glucose	Ca
Control	5/45± 0/4	47/13±2/7	119/8±22	18±1/6	1/91±0/1	0/46±0/03	100± 5	8/2±0/1
Diabetic	7/53±0/5*	87/38±7/6*	216/9±49	28±2*	5/48±0/7*	0/53±0/06	470±18*	8/95±0/2

Values are means± SEM, n=6. $p<0.05$ indicates significant difference from control determined by Student s t test.

Volume of choroids plexus

The volume of choroids plexus in rat's embryos from diabetic mother was increased. The mean of choroids plexus volume in experimental group was (0.0945 ± 0.0172) and in control group (0.0704 ± 0.0130).This increase ($p<0.01$) was meaningful (Fig.1).In all day of gestation (16.5, 17.5, 18.5, 19.5, 20.5, 21.5) in embryo from diabetic mother have increase in volume of choroids plexus.

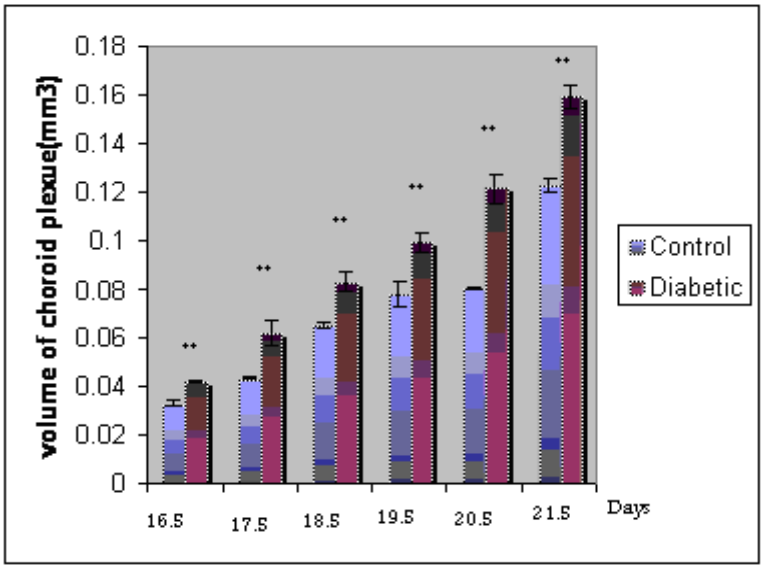

FIG. 1:Comparing volume of choroids plexus in the neonates from diabetic and control rats in different day of gestation.

Length density of capillary in choroids plexus

As expected, the length of choroids plexus capillary in experimental group has increased ($p<0.01$) significantly. The mean of this factor in experimental group was (25.62±4.69mm) and in control (22.38±4.46 mm) (Table.2).

Table.2: **Comparing total length of choroids plexus capillary in the embryo from diabetic(Experimental) and control rats in different days of gestation.**

	Control	Experimental
Mean in 16.5 dg	20.38 ± 1.94	22.56 ± 2.35
Mean in 17.5 dg	9.122 ± 0.187	12.200 ±0.077
Mean in 18.5 dg	14.008 ± 0.98	14.014 ± 0.035
Mean in 19.5 dg	36.25 ± 0.076	37.82 ± 2.47
Mean in 20.5 dg	19.93 ± 0.014	27.98 ± 3.4
Mean in 21.5 dg	34.60 ± 0.017	39.13 ± 2.91
Mean ± SE	22.38 ± 4.46	25.62 ± 4.69

Values are means± SEM, n=6. $p<0.05$ indicates significant difference from control determined by Student s t test.

Although the total length of choroids plexus in all day of gestation in experimental group was larger than control group but as we see in Fig.2, this increase just in 17.5 and 21.5 dg is meaningful (Fig.2).

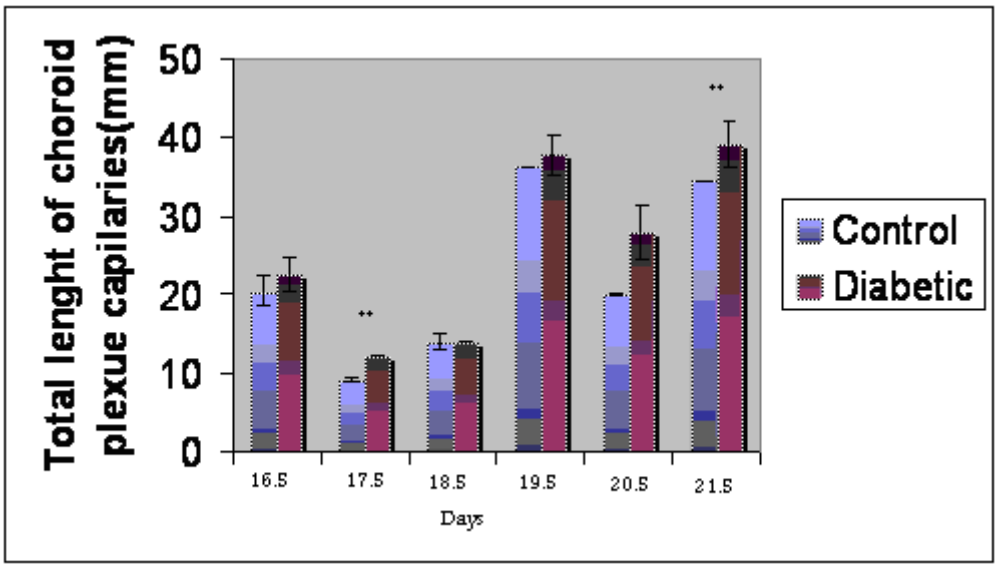

FIG. 2: Comparing theC.P total length capillaries in embryos from diabetic and control rats in different day of gestation.(n=6).

As Photomicrograph of the brain section of embryo show the number of capillary in choroids plexus was increased in embryo from diabetic mothers (Fig.3).

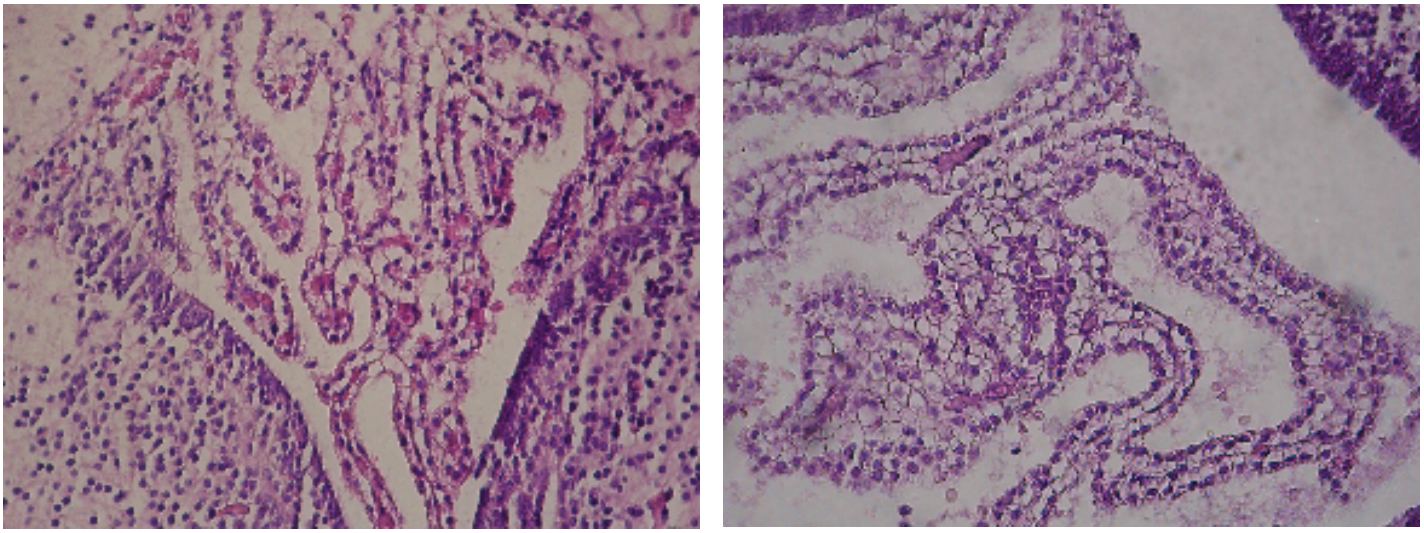

Fig.3 : Photomicrograph of the brain section of embryo at the region of choroids plexus. Right panel: choroids plexus capillary of embryo from diabetic mother. Left panel: choroids plexus capillary of embryo from control mother. (X40).(toluidine blue)

Discussion

Studies suggest that the metabolic abnormalities of diabetes alter the angiogenic process (14). The molecular defects underlying these angiogenic abnormalities have generated much interest but, so far, have remained elusive. Diabetic patients have been reported to have a reduced number of circulating endothelial progenitor cells (15).

The risk of vascular dementia increases greatly with strok, and this may be caused by large and small vessel disease, both of which are associated with diabetes, it is likely that diabetes affects the risk of vascular dementia partly by contributing to dyslipidemia and hypertention (16).

There are several mechanisms by which, angiogenesis may be vulnerable to the metabolic abnormalities of diabetes, although the extent and nature of this vulnerability is not well understood (14).Several stimulators and inhibitors of angiogenesis have identified that modulate the ability of endothelial cells to digest the basement membrane and proliferate, migrate, and/or associate into a new capillary network (17).The balance between naturally occurring inducers and inhibitors of angiogenesis determines the active neovascularization observed during embryogenesis as well as the vascular quiescence maintained by most tissues in adult life (18). For example, angiogenesis in the eye may be the result of an imbalance between stimulatory and inhibitory factors that presumably occurs from the elevated expression of local angiogenic factors induced by ischemia (19). Diabetic condition induces Hypoxia, that effect on developmental blood vessel formation.

Present data implicate raised blood glucose (among the clinical and metabolic variables) is the most important factor that proliferate the choroids plexus capillaries.

Maternal diabetes makes some changes in choroids plexus appearance in embryonic stage (Fig. 3). In addition under hyperglycemic condition in embryo, choroids plexus progress much more than normal condition. The appearance of choroids plexus in embryos is corporate with some disruption that look like angiogenes (Fig. 3).

It is demonstrated that the volume of choroids plexus in embryos from diabetic mothers have show a meaningful ($p<0.001$) increase camper to control in (Fig.1).In addition the total length of capillaries have remarkable increase ($p<0.001$) in all stages of experiment (Fig.2). Although the total length of choroids plexus in all day of gestation in experimental group was larger than control group but as we see in Fig.2, this increase just in 17.5 and 21.5 dg is meaningful. May be these times are very critical for developmental process.

It is postulated that a kind of angiogenesis process happen when blood glucose rose. Considerable experimental evidence inculpates Vascular Endothelial Growth Factor (VEGF) as one of the candidate factors providing a mechanistic link between hyperglycemia and diabetic complications (20). When the vascular endothelial growth factor (VEGF) was identified, it became clear that this was the candidate paracrin factor specifically stimulating endothelial proliferation and sprouting via high affinity receptors VEGF survival as well as for vascular remodeling during embryonic perivascular cells. The vascular endothelial growth factor (VEGF) and the high affinity VEGF receptor (VEGFR) comprise a key signaling system that regulates vasculogenesis.

Various angiogenic factors have been proposed to mediate vasoproliferative eye diseases, including bFGF, insulin-like growth factor-1, and, most important, vascular endothelial growth factor (21). However, the imbalance responsible for the pathologic angiogenesis may also result from down regulation of inhibitors of blood vessel growth (22).

Presence of hyperglycemia and/or insulin deficiency in children with new-onset of diabetes is associated with plasma VEGF elevation, even at the outset of disease and this can be mitigated by insulin therapy (23). It is the primary determinant of circulation VEGF and Ang-2 levels (24). Raised blood glucose in diabetes exerts toxic effects on the endothelium through a number of mechanisms by increasing substrate flux through the sorbitol pathway, elevated blood glucose can induce a hyperglycemic pseudo-hypoxic state may induce VEGF production(9).The accelerated formation and accumulation of glycotion products associated with raised blood glucose may also up regulate VEGF and Ang-2 mRNA *in vitro*(25).

Present findings are consistent with these clinical observations and it is tempting to speculate that improvements in cardiovascular outcomes may be related to reductions in these angiogenic growth factors and associated improvement in endothelial abnormalities (26). In other report demonstrate that hyperglycemic insults in reduced levels of VEGF- A which in turn, leads to abnormal VEGFR signaling, resulting in embryonic vasculopathy (27). Vascular endothelial growth factor (VEGF) and the angiopoietins are two families of growth factors believed to act predominant on vascular endothelial cells. VEGF is mitogenic for endothelial cells, acting early and at most points in the angiogenic cascade (9). In creasing evidence suggests a role for VEGF in the pathophysiology of cardiovascular disease. Elevated plasma VEGF has been shown in patients with hypertension and diabetes with levels correlating with

measures of endothelial damage/dysfunction and overall cardiovascular risk in hypertensive patients(s) (28).

There is accumulating evidence that enhanced formation of Advanced Glycation End products (AGE_s) and activation of AGF receptors in the diabetic state may contribute to impaired angiogenic potential (25). Recently discovered vasoconstrictors and angiogenesis regulators such as endothelin (ET) and vascular endothelial growth factor (VEGF) have been intensely studied for possible pathogenic roles in diabetic vascular complications (29). Thus, poor glycemic control causes increased levels of plasma VEGF, which may result in hypertension and vascular complications in diabetes. Short term treatment resulting in good glycemic control can improve levels of VEGF and may provide beneficial effects on diabetic vascular complication (9).

New findings indicate that the commen pathophysiologic mechanism linking chronic hyperglycemia to vascular pathology in diabetes is the mitochondrial overproduction of Reactive Oxygen Species (ROS) leading to the increased formation of AGE_s, activation of protein kinase C, activation of the aldose reductase and deliberation of active nuclear factor kB-mechanisms that have been correlated with the pathogenesis of diabetic microangiopathy (11). Others have suggested that maternal diabetes may also have lasting adverse consequences on cardiovascular function of the next generation, particular because offspring of diabetic pregnant rats demonstrate overt insulin resistance in adulthood (30). These finding and our observation that addition of exogenous VEGF-A blunts the hyperglycemia-induced vasculopathy may ultimately lead to novel therapeutic approaches for the prevention and treatment of congenital abnormalities associated with diabetes (4). Further investigation is needed to clarify the mechanisms involved and lead to a new understanding of the importance of nutrition during pregnancy. This will provide an important approach to the primary prevention of diabetes and chronic degenerative diseases (31).

Present study high lights the importance of the cross-talk between maternal hyperglycemia and proliferation of choroids plexus capillary`s in all stages of embryonic life. May be a kind of angiogenesis process will be take place that proliferate the capillaries and increase the choroids plexus volume. It opens new insights into necessary of treatment the raised blood glucose in pregnant women.

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