LOCALIZATION OF NADPH-DIAPHORASE REACTIVITY IN DEVELOPING RETINAL BLOOD VESSELS AND NEURONS

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INTRODUCTION

The Nitric Oxide (NO) is an essential regulatory molecule in vascular tone and integrity [26]. previous studies has supported the role of NO in remodeling of axons arbors and acts as an activity dependent retrograde signal during the refinement of visual connections [14, 4 & 5].

Recently, it was found that NO has been implicated in retinal pathogenesis of retinal vasculogenesis in development of retinal neovascularization (NV) in ischemic retinopathies [11].

NADPH-diaphorase reaction reflects NOS activity in the retina and neural tissue [3, 17, 1, 2, 13, 6, 16 , 20 & 7]. NADPH-diaphorase staining neurons, have been localized with neurons in the brain and peripheral tissue containing nitric oxide synthase, which generates NO. NADPH- diaphorase staining are localized in the pedunculopontine nucleus and colocalized in amacrine cells of the inner nuclear layer and ganglion cells of the retina [7]. NADPH-diaphorase has been shown to be a reliable marker of nNOS activity in neuronal tissue [16 & 1].

NADPH-diaphorase reaction was, therefore used in this study for localizing the development of retinal vasculature as well as any related neuronal tissue.

MATERIAL AND METHODS

Twenty postnatal mice were used in this study. The mice were maintained on a 12 hours light / 12 hours dark cycle and maintained under pa-

313

thogen-free conditions.

The selected age groups were postnatal day 5 (P5), P7, P9, P14 and P30. Four mice were sacrificed at assigned dates by CO2 inhalation. The eye balls were removed, the cornea were opened and then fixed in 4% paraformaldehyde in 0.1 M PBS pH 7.4 at 4°C. The lens and cornea were removed and the retina was dissected free from retinal pigment epithelium.

NADPH-diaphorase staining :

NADPH-diaphorase (NADPH-d) staining was done on:

A. flat mounted retina:

Mice were sacrificed with CO2 inhalation. Eyeballs were removed, then fixed in 4% paraformaldehyde in 0.1 M Phosphate buffer saline (PBS) pH 7.6 at 4°C. The lens and cornea were removed and the retina was dissected free of retinal pigment epithelium. Retinas were then fixed for 2 hours in 4% paraformaldehyde in 0.1 M. phosphate buffered saline (pH 7.6), washed 3 times 10 minutes each in Tris buffer (pH 7.6). They were then left for 2 hours in 5% Triton X-100 in Tris buffer (pH 7.6). Retinal tissues were then reacted for NADPH-d using 0.25 mg/ml nitroblue

tetrazolium (NBT), 1 mg/ml _-NADPH (Sigma) and 0.5% Triton X-100 in 0.1 MPB at 37°C for 1 hour [23]. Retinas were then mounted on gelatinized slides from 1% gelatin solution and coverslipped with glycerol. After the retina attached to the slides the coverslips were removed by soaking the slides in PBS. Retinas were then dehydrated in ascending grades of alcohol (30%, 50%, 70%, 95%, and 100%) followed by 2 changes of xylene. Thereafter, the tissues were coverslipped with permount. Camera Lucida was used to project the image on the digital planimeter and count the numbers of NADPH-d-positive cells. 4-8 systematically selected 1 mm^2 square areas in each retina beginning at 200 μm from the optic disc and continuing to the ora serrata.Then the NADPH-d-positive amacrine-like cells were counted in each square and calculated cell density / cm2.

b. Retinal cryostat frozen sections:

Frozen sections were done to study the relation between NOS producing cells and developing vessels. The sections of 12 μm were incubated in Tris buffer (pH 7.6) with 2% Triton X-100 for 1 hour. Cryosections were

initially enzymatically processed for NADPH-diaphorase staining using previously described method, and were then incubated with the different antibodies (sst2A, sst2B, TH, PKC, calbindin, and recoverin). The sections were mounted with mounting medium for fluorescence, then dehydrated and coverslipped with permount and examined by light microscopy.

RESULTS

**A) NADPH-diaphorase stained sections of flat mounted retina : a. At P5 :** There is no obvious NADPH-d activity (Fig. 1). **b. At P7 :** Weak radial NADPH-d reaction in larger vessels and in few small neurons was noticed (Fig. 2). **c. At P9 :** Numerous weak NADPH-d reaction was seen in the blood vessels with positive activity in few neurons (Fig. 3). **d. At P14:** Strong NAPH-d reaction was seen in the large vessels and the nearby neurons. NADPH-d activity was seen in the developing capillaries (Fig. 4). Positive reaction was also seen in many cell bodies and their processes. They varied in shape from rounded to oval bodies. Some of their processes varied from single to multiple processes. Some of the reactive processes were seen to terminate in contact with

the large vessels (Figs 6, 7, 8, 9, 10 & 11). **e. At P30 :** Stronger NADPH-d reaction was seen in the blood vessels and capillaries. Many neurons showed activity, but the cells closely related to the blood vessels were the most reactive to NADPH-d (Fig. 5). Two evident different shapes of NADPH-d reactive cells appeared: cells with round soma (Fig. 12) and cells with oval soma (Fig. 13) with no reaction in their processes.

B) NADPH-diaphorase retinal frozen sections: a. At P5 : An obvious NADPH-d activity was seen in nerve fiber layer, ganglion cells and inner plexiform layers (Fig. 14). **b. At P7:** Some cells showed weak NADPH-d reaction located in the inner border of the nuclear layer, but more evident in inner nuclear cells (Fig. 15). **c. At P9 :** There was an increase in the NADPH-d activity in different layers of the retina especially in neurons. Also these cells showed an increase in their number (Fig. 16). **d. At P14 :** This age showed strong NADPH-d reaction in the retinal vessels, ganglion cells and neuron cells of inner nuclear layer. There was an increase in number of neuron cells and their processes extended to the inner plexiform layer (Fig. 17). **e. At P30 :** Stronger NADPH-d reaction was seen in the blood vessels, but decreased in neuron cells (Fig. 18).

Fig. 1 : NADPH-d stained flat mounted retina from **P5,** there is no obvious NADPH-d activity. (X 50)
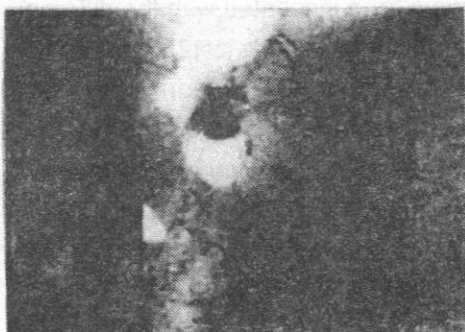
Fig. 3 : NADPH-d stained flat mounted retina from **P9,** notice weak NADPH-d activity in larger vessels (arrowhead) and weak reaction in few small neurons nearby the blood vessels (arrows). (X 50).
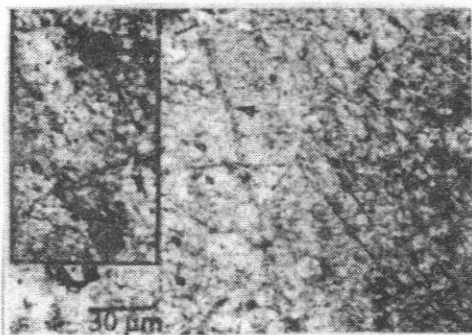
Fig. 2 : NADPH-d stained flat mounted retina from **P7,** shows weak NADPH-d reaction in larger vessels (arrowhead) and in few small neurons (arrows). (X 125)
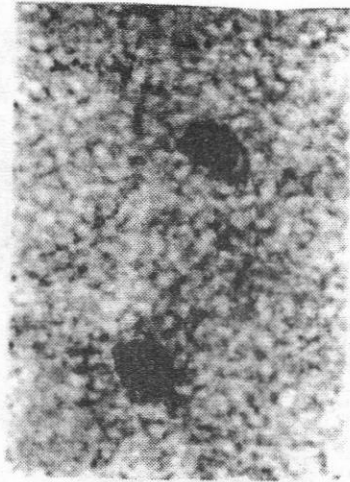

Fig. 4 : NADPH-d stained flat mounted retina from **P14,** shows strong diaphorase reaction in large vessels (arrowheads) and nearby few small neurons (arrows). (X 50)
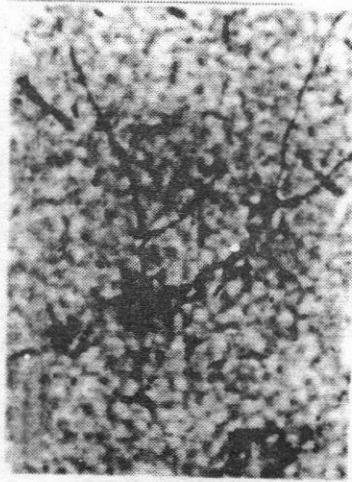
Fig. 5 : NADPH-d stained flat mounted retina from **P30,** showing stronger NADPH-d reaction in large blood vessels and capillaries (arrowheads). Also strong activity in neuron cells closely related to blood vessels (arrows). (X 50)
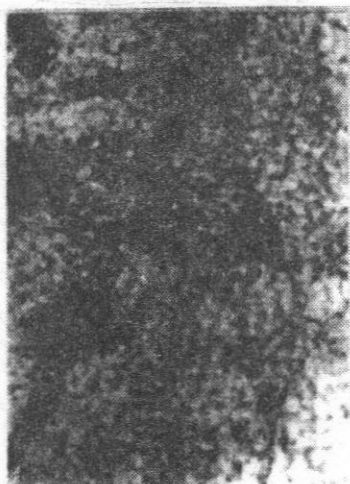

Fig. 7 : NADPH-d stained flat mounted retina from **P14,** shows NADPH-d positive reaction seen in cell bodies and in a single process. (X 500)

Fig. 6 : NADPH-d stained flat mounted retina from **P7,** showing strong NADOH-d positive round cells. (X 500)

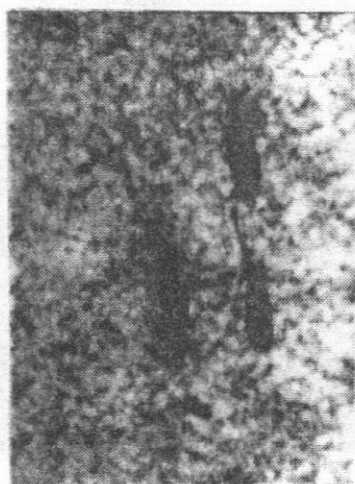

Fig. 8 : NADPH-d stained flat mounted retina from **P14,** showing NADPH-d reactive round cell body with multiple reactive primary processes dividing into reactive secondary processes. (X 500)
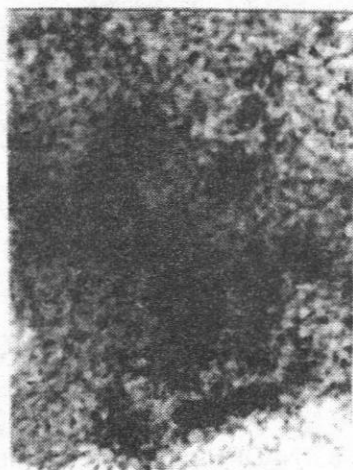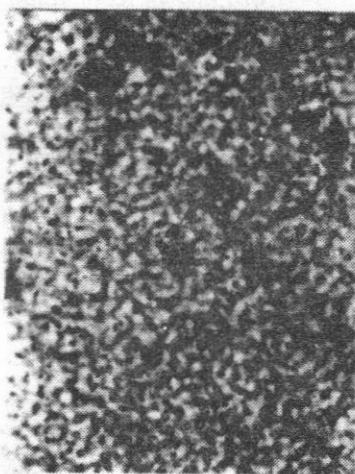
Fig. 9 : NADPH-d stained flat mounted retina from **P14,** showing NADPH-d reactive in some of reactive processes appear to terminate in contact with large vessels.(X 500)
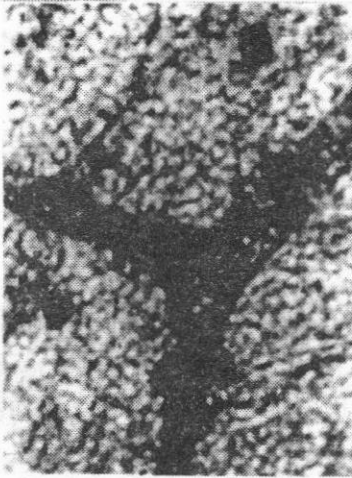

Fig. 11 : NADPH-d stained flat mounted retina from **P14,** showing positive NADPH-d reaction in unipolar reactive process. (X 500)
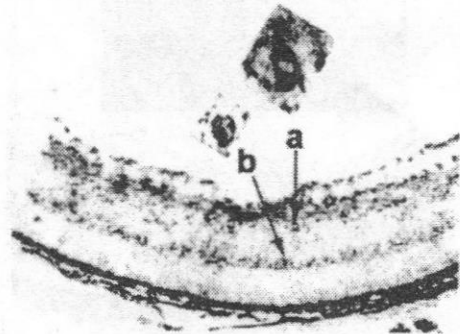

Fig. 10 : NADPH-d stained flat mounted retina from **P14,** showing positive NADPH-d reaction in oval cell body. (X 500)


fig. 12 : NADPH-d stained flat mounted retina from **P30,** note NADPH-d reaction in cells with round soma (Fig. 23, H). (X 500)

Fig. 13 : NADPH-d stained flat mount-
ed retina from **P30,** note
NADPH-d reaction in cell
with oval stoma. (X 500)

Fig. 15 : NADPH-d stained retinal fro-
zen section from **P7,** shows
weak NADPH-d reactive
cells **(a)** located in the inner
nuclear layer, but intense re-
activity is evident in the in-
ner nuclear layer cells **(b).**
(X 400)
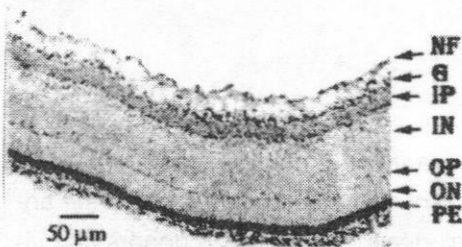
Fig. 14 : NADPH-d stained retinal
frozen section from P5,
shows NADPH-d activity in
nerve fiber layer (NF), gang-
lion cells (G) and inner plexi-
form (IP). (X 100)
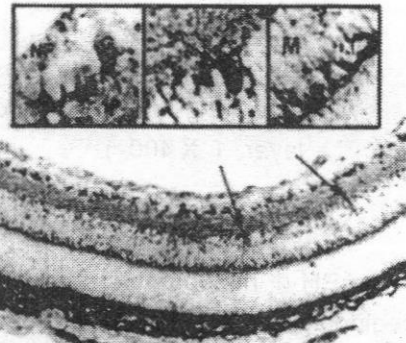
Fig. 16 : NADPH-d stained retinal fro-
zen section from **P9,** shows
NADPH-d activity in different
layers of retina especially
in neuron like-cells (arrows)
and their number increased.
Note also the reactive
Muller cell's processes **(M).**
(X 400)
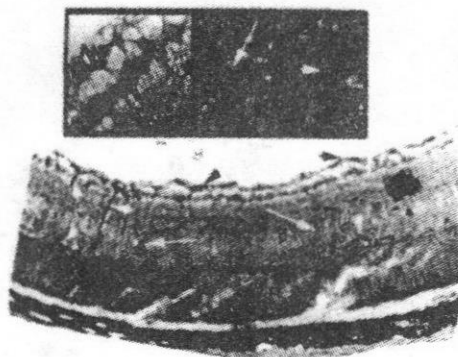
Fig. 17 : NADPH-d stained retinal frozen section from **P 1 4**, shows NADPH-d reaction evident in retinal vessels (arrowheads) in ganglion cells **(G)** and in neuron like-cells (arrows) which increased in numbers. Note also its apparent processes extending to inner plexiform layer. (X 400)
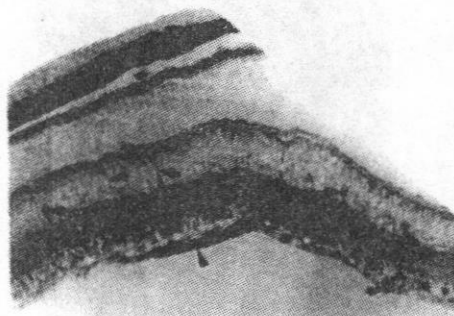

Fig. 18 : NADPH-d stained retinal frozen section from **P30**, shows stronger NADPH-d reaction in the retinal vessels (arrowheads) and decrease in neuron like-cells (arrows). (X 100)

DISCUSSION

NAPH-d histochemistry showed beginning of NADPH-d activity in the retinal vessels and few neurons at P7. By P9, numerous diaphorase – positive cells with neuron like morphology were evident. This is concomitant with studies of on developing lung[10]. They noticed that endothelial NOS (eNOS) is present very early in developing capillaries but decreased postnatally. Also, it was demonstrated that the angiogenic effect of NO- donors on cultured endothelial cells in vivo[22 & 27]. These results suggested that NOS activity is upregulated in the blood vessels and neurons of retina during the critical period of angiogenesis (P7 – P9), where the deep retinal vessels begin to sprout from superficial ves-

sels [28] & [15]. However, it was found that, NO didn't involve or establish any role in retinal angiogenesis[25].

At P14, stronger NADPH-d reaction was noticed in the larger vessels, the nearby neurons and in the developing capillaries. This activity increased and became stronger at P30. Many of NADPH-d neuronal activity increased in nerve cells closely related to blood vessels. Also, increased in numbers of NADPH-d reactive cells in retina at P14 – P30. It is suggested that the activity of nNOS is important for normal vascular development in the mouse retina and for maintaining adequate oxygen delivery during retinal metabolic activity. Expression of the VEGF receptors VEGFR-1 and VEGFR-2 has been demonstrated within extravascular neural cells of the developing mouse retina and in cultured Muller glial cells [18]. Thus, VEGF could induce nNOS activation and NO production in the retinas via activation of VEGFR-2 in perivascular neurons and/or glia.

It is demonstrated that strong NADPH-d activity up-regulated in larger vessels and begins to be evident at P14, which is about the same time when the visual activity begins in mice, this is because of increased vascular NOS activity [21]. This correlation between the vascular NOS activity and visual activity when photoreceptors differentiation and activity begin suggests that. NO produced by eNOS is involved in maintaining retinal blood flow to supply the metabolically active photoreceptors [12] & [24].

The results of this study indicate that there is a close relation between the NOS active neurons and the developing vessels. Some of these neurons send NOS active processes into close proximity with blood vessels. So, any involvement of both endothelial and neural NOS in the angiogenic stage of vascular development, will involve the other in regulation of the blood flow[18] & [9].

So, these findings suggest that there is a compensatory activity of neuronal and endothelial NOS, is important for normal vascular development in mouse retina and for maintaining the adequate oxygen supplying during retinal metabolic activity. Thus NO production in mice retinas as noticed from increased NAPH-d reaction indicates activation of perivascular NADPH-d reactivity was associated with larger vessels of

retina. The number of neuron like-cells showing NADPH-d reaction increased from P9 – P14. Moreover the processes of these NADPH-d reactive neurons like- cells were often close to the blood vessels [21, 19 & 18]. The physiological significance of this difference is unclear. Recent studies suggest that both superficial and deep vascular networks are formed by the same mechanism of angiogenic sprouting [8 & 9]. So, Further investigation is required to specify the cells responsible for the vascular-associated nNOS expression in the retina and also to show whether or not endothelial cells in mice are able to express nNOS. Further studies are needed to investigate this issue.

SUMMARY

This work was done to localize NADPH- diaphorase distribution / activity in the retinal blood vessels and developing neurons. NADPH-d has been shown to be reliable marker of nNOS activity in neuronal tissue. The mice were sacrificed at postnatal days 5 (P5), P7, P9, P14 and P30. Their eyes were thoroughly dissected out and their retinas were prepared for NADPH-d stained flat mounted retina and for NADPH-d stained retinal frozen sections (to follow NOS activity in different layers and structures of retina).

NADPH-d stained flat mounted retinas of mice showed diaphorase activity beginning at P7. Weak activity was seen in large vessels and in few neurons. By P9, numerous diaphorase positive cells with neuron- like morphology were evident. At P14 and P30, the reaction in the larger vessels was stronger and diaphorase activity was also evident within the microvasculature. Many of diaphorase- positive cells were closely related to the blood vessels.

The frozen sections showed similar distributions of NADPH-d activity in the retinas in the nerve fiber layer, inner nuclear layer and both inner and outer plexiform layers.

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تحديد تفاعل النيكوتين أميد أدنين ثنائى النيكلوتيد فوسفات (النوع المختزل) فى نشوء الأوعية الدموية والخلايا العصبية بشبكية العين

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قسم التشريح بكلية طب جررجيا الولايات المتحدة الأمريكية*

تم إجراء هذا البحث لتحديد إنزيم النيكوتين أميد أدنين ثنائى النيكلوتيد فوسفات (النوع المختزل) إنتشاراً وفاعلية وذلك لدراسة نمو الأعصاب والأوعية الدموية لشبكية الفأر. حيث أن الأنزيم المحفز لـ (النيكوتين أميد أدنين ثنائى النيكلوتيد فوسفات (النوع المختزل) يعتبر مرتبط ومحدد لنشاط أكسيد النيتريك فى الخلايا العصبية).

لقد تم التضحية بالفئران بعد الولادة فى الأيام : ٥،٧،٩،١٤،٣٠ يوما. وتم تشريح العين وحضرت لعمل شرائح مصبوغة[النيكوتين أميد أدنين ثنائى النيكلوتيد فوسفات (النوع المختزل)] لشبكية العين وكذلك عمل شرائح مجمدة وبنفس الصبغة.

أظهرت شرائح الشبكية فى الفئران المصبوغة [النيكوتين أميد أدنين ثنائى النيكلوتيد فوسفات (النوع المختزل)] بداية ظهور نشاط الأنزيم فى اليوم السابع. لكن فى اليوم التاسع ظهر عديد من خلايا شبيهة العصبية وتفاعل أكبر . وفى اليوم الرابع عشر واليوم الثلاثين كان التفاعل أقوى فى الأوعية الدموية الكبيرة وكذلك بشبكة الأوعية الدموية. كما ظهرت عديد من الخلايا الشبيهة بالعصبية ملاصقة للأوعية الدموية.

أما فى الشرائح المجهزة بالتبريد فلقد ظهر إنتشار نفس الأنزيم وتفاعله فى شبكية الفئران فى طبقة الألياف العصبية والطبقة العصبية الداخلية والطبقتين المحببتين الداخلية والخارجية.