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Differences in the histomorphology of the neural retina in *Rattus norvegicus* offspring due to varying maternal perigestational dietary folate levels

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ABSTRACT

Background: Nutrition, particularly folate, is crucial for embryonic development, affecting processes like retinogenesis. However, the impact of varying maternal folate levels on the neural retina's structure remains underexplored. **Objective:** This study aims to describe the histomorphological differences in the neural retina of *Rattus norvegicus* offspring due to varying maternal perigestational dietary folate levels. **Study Design:** The study used primary data from a previous randomized experimental study on 20 rats (40 retinas) divided into four groups: folate-deficient (0 mg/kg), control (2 mg/kg), folate-supplemented (8 mg/kg), and folate-suprasupplemented (40 mg/kg). The morphology of the neural retina was studied across the groups. **Results:** The folate-deficient diet group exhibited significant retinal disruptions, including focal widening of intercellular spaces in the inner nuclear layer, vacuolisation in the ganglion cell layer, and detachment of the internal limiting membrane. In contrast, the folate-supplemented diet group showed preserved retinal structure with an intact internal limiting membrane. However, the folate supra-supplemented diet group displayed retinal abnormalities similar to the deficient group, with focal widening of intercellular spaces, internal limiting membrane detachment, and retinal folding. **Conclusion:** Folate deficiency and suprasupplementation both disrupt retinal morphology, while normal supplementation preserves retinal structure, highlighting the importance of balanced folate intake for maintaining retinal health.

Key words: Folate deficiency; folate supplementation; folate excess; neural retina; maternal folate levels

INTRODUCTION

The retina consists of two major components: the non-neural retinal pigment epithelium and the neural retina. The neural retina is made up of several layers that contain a variety of cells including photoreceptors (rods and cones),

conducting neurons (bipolar and ganglion cells), supporting cells (muller cells), and association neurons (amacrine and horizontal cells) (1).The neural retina originates from the inner layer of the optic cup, which begins as a simple epithelial layer but evolves into a stratified layer as neuroblasts proliferate (2). The nuclear layers are initially dense during development, but they thin out as neuroblasts migrate and differentiate.

Folate is essential for retinogenesis because it promotes DNA synthesis and regulates gene expression (3). Folate works as a one-carbon donor during cell proliferation, generating purines and thymidylate, which are needed for DNA synthesis during the S-phase (4,5). Folate therefore plays an important role in neuroblast proliferation and differentiation during retinogenesis (6). Both folate deficiency and excess during pregnancy impact offspring neurodevelopment, with deficiency impairing neurogenesis and increasing retinal ganglion cell mortality, while excess may disrupt cortical neurodevelopment and raise the risk of neurodevelopmental disorders (7,8). Folate deficiency among women of reproductive age in sub-Saharan Africa is widespread, with efforts like supplementation and fortification in Kenya facing compliance challenges, leading to risks such as neural tube defects (9,10). Since the neural retina develops from the neural tube and is similarly affected by maternal folate levels, this study aims to investigate how different neural retina layers are structurally affected by varying maternal perigestational dietary folate levels.

MATERIALS AND METHODS

Study design: Quasi experimental study using primary data from a previous randomized experimental study.In the parent study, twelve adult female albino rats were randomly assigned to four diet groups: folate-deficient (0 mg/kg), control (2 mg/kg), folate-supplemented (8 mg/kg), and folate-suprasupplemented (40 mg/kg). These diets, based on the American Institute of Nutrition's AIN-93G rodent diet, were started 14 days before mating and continued through gestation and lactation. On postnatal days 1, 7, 21, and 35, five pups from each group were sacrificed. The diets, obtained from Dyets Inc., were formulated to study the exclusive effects of dietary folic acid, with the control diet approximating the human recommended daily allowance and the supplemented diets reflecting recommended intakes for pregnancy and neural tube defect prevention. The offsprings were then preserved in biobanks and the current study obtained eye specimens from the day 35 group.

Ethical Consideration: This study was approved by the Biosafety, Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Nairobi (ethical approval number: FVM BAUEC/2017/133).

Sample size: 5 rats per group were used. A total of 40 retinas were therefore studied.

Study methodology: The eyes were enucleated with part of the optic nerve intact and bisected horizontally along the optic nerve. The vitreous humor and optic lenses were removed to facilitate the penetration of the fixing medium into the retina. The tissues were dehydrated in ascending grades of isopropyl alcohol, followed by clearing in toluene solution. The hemispheres were embedded in paraffin blocks, sectioned into 3-micrometer-thick sections using a rotary microtome, and mounted on glass slides with egg albumin adhesive. Systematic uniform random sampling method was used to select sections for staining. One ribbon of 15 sections was obtained from each block, and every third section was selected, yielding five sections per block. This process was repeated for all blocks, resulting in 20 slides per rat. Selected sections were stained with

hematoxylin and eosin, and examined under a light microscope to assess the retinal structure.

Data collection: Photomicrographs were taken at x400 magnification using a Richter OpticaTM (model UX1) digital photomicroscope connected to Motic Images 3.0 software. Histomorphological observations were done in the area of the central retina (0-1000 um from the optic disc) as shown in Figure 1.

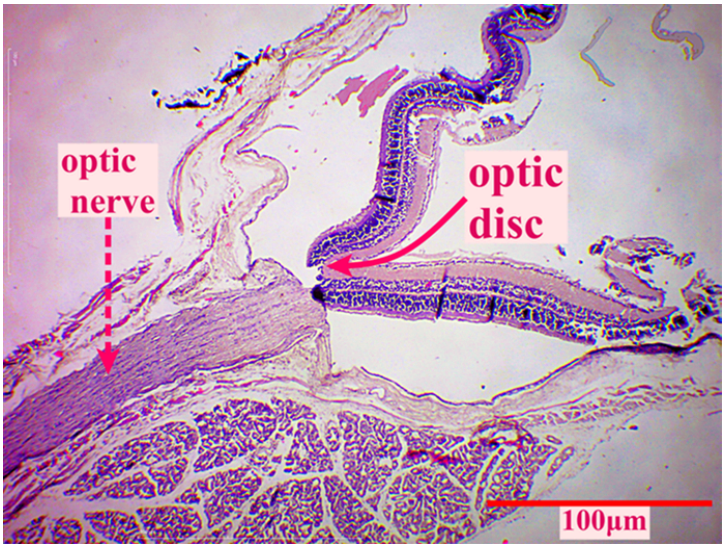

Figure 1: Photomicrograph of the eye hemisection depicting the neural retina, optic disc, and optic nerve. Hematoxylin and Eosin stain, Magnification X40.

RESULTS

a) **Controls**

Retina of the control group had a well-preserved laminated structure, including distinguishable nuclear and plexiform layers. The photoreceptor layer (RC) was additionally identified. The internal limiting membrane (ILM) was intact (Figure 2a).

b) Folate deficient diet group

The neural retina was disrupted, evidenced by focal widening of intercellular spaces within the inner nuclear layer (Figure 2c) and vacuolization within the ganglion cell layer (Figure 2c). Detachment of the internal limiting membrane was also seen (Figure 2c).

c) Folate-supplemented diet group

The normal retinal structure was preserved with the internal limiting membrane (ILM) being intact (Figure 2b).

d) Folate suprasupplemented diet group

Offspring of the folate supra supplemented diet group showed disruption of the retinal morphology characterized by focal widening of intercellular spaces in the inner nuclear layer (Figure 2d), detachment of the inner limiting membrane (Figure 2d,3a), and retinal folding (Figure 3a).

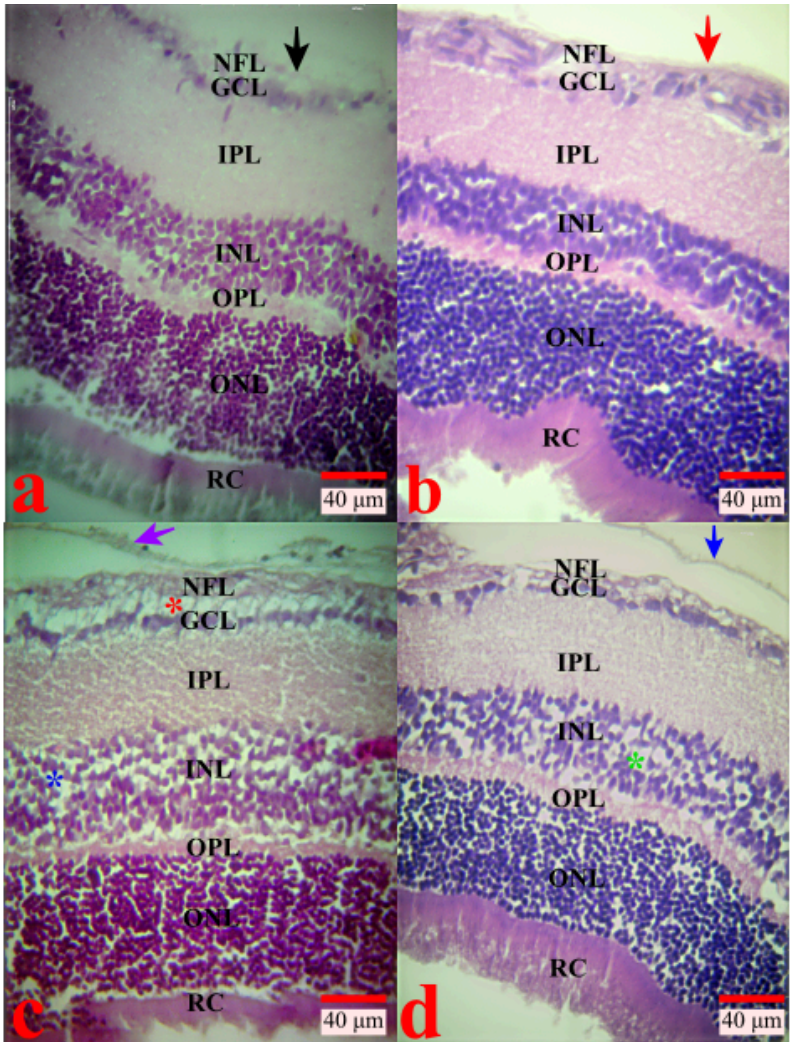

Figure 2: Photomicrograph showing the histomorphological differences across the four groups.
2a: In the control group, the retina displayed distinct layers, including the layer of rods and cones (RC), the outer nuclear layer (ONL), which contained highly pigmented nuclei of rods and cones, the outer plexiform layer (OPL) which appeared as a narrow pale region, and the inner nuclear layer (INL) which contained large and pale nuclei. The nerve fiber layer (NFL) and internal limiting membrane (pointed by the black arrow) are both observable. **2b:** In the folate-supplemented group, the retina displayed distinct layers, including the photoreceptor layer (RC), the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), inner plexiform layer (INL), ganglion cell layer (GCL), nerve fiber layer (NFL) and internal limiting membrane (red arrow). **2c:** In the folate-deficient group, vacuolations were seen in the ganglion cell layer (red asterisk). Focal enlargement of intercellular spaces were seen in the inner nuclear layer (blue asterisk). The inner limiting membrane was also detached (purple arrow). All the layers of the neural retina were present. **2d:** In the folate suprasupplemented group, focal enlargement of the intercellular spaces within the inner nuclear layer (green asterisk), was observed. The internal limiting was detached, as shown by the blue arrow. All layers of the neural retina were, however, present. *Hematoxylin and Eosin stain, x400.*

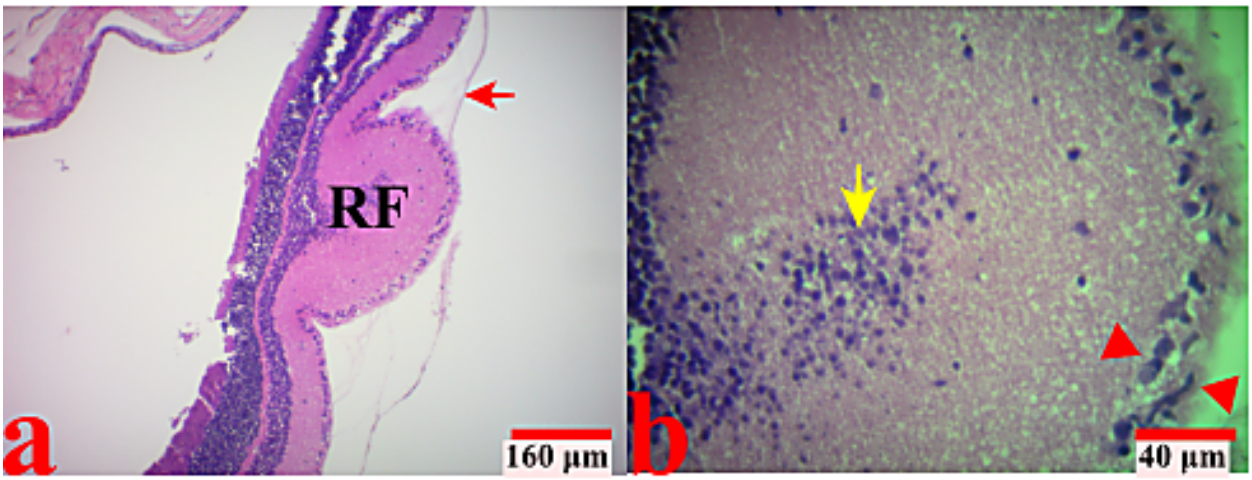

Figure 3: Photomicrograph showing retinal folding in the folate suprasupplemented group. **a:** In the folate suprasupplemented group, retinal folding (RF) was seen. The inner limiting membrane was also detached, as indicated by the red arrow. All layers of the neural retina were present. (Hematoxylin and Eosin stain, x100). **b:** At a higher magnification of the retinal fold, the inner plexiform layer was seen to have cell nuclei, indicated by a yellow arrow, embedded within it. The ganglion cells were seen overlying the fold, as shown by the red arrowheads. Note that there is a loss of single cellularity of the ganglion cell layer at this point, and it occurs in two rows. (Hematoxylin and Eosin stain, x400).

DISCUSSION

The results of the present study showed that the development of the neural retina is affected by varying levels of perigestational maternal folate levels

Folate deficient diet group

Maternal consumption of a folate-deficient diet was associated with detachment of internal limiting membrane in offspring. The internal limiting membrane is the basement membrane and consists of intricate networks of secretory extracellular matrix (ECM) proteins such as collagen IV, laminins, nidogens, and heparan sulfate proteoglycans (11). Previous studies have illustrated that folate deficiency affects the expression of collagen IV and laminin-1 in the eye, both of which are essential components of the basement membrane, by inducing overexpression of collagen IV and underexpression of laminin-1 leading to detachment (12).

Vacuolations were seen in the inner nuclear layer and the ganglion cell layer. The neural retina develops from the proliferation of the inner layer of the optic cup. Cell division requires an adequate supply of folate essential for DNA replication. In folate-deficient states, cell division is impaired, leading to the formation of a few bipolar cells. The association between folate deficiency and elevated homocysteine levels within circulation is also well-established. Folate is required for the conversion of homocysteine to methionine. Folate deficiency results in accumulation of homocysteine in the bloodstream (12). Hyperhomocysteinemia induces retinal ganglion cell death by inducing apoptosis. Hyperhomocysteinemia also causes retinal vascular diseases, including central retinal vein occlusion. This causes hypoxic injury to the retinal neuronal cells, leading to ischemic necrosis (13). As a result, the number of bipolar and retinal ganglion cells decreased, leading to focal enlargement of

intercellular spaces, creating a vacuolated appearance.

Folate Supplemented diet group

The folate-supplemented group displayed normal retinal morphology. This is because folate is required for optimum neuronal cell proliferation and neuronal cell differentiation (14). Folate supplementation is associated with decreased homocysteine levels in circulation. This reduces the risk of retinal vasculature injury, hence adequate vascularization, which reduces ganglion cell death (13).

Folate suprasupplemented diet group

In this group, the internal limiting membrane was detached. Excess folic acid activates STAT3 through folic acid receptor alpha (FRα) leading to increased deposition of collagen IV in renal mesangial cells (15).

Overexpression of collagen IV in the internal limiting membrane leads to a disequilibrium in the combination and concentration of the components that define the internal limiting membrane as a basement membrane, causing detachment (16). The internal limiting membrane initiates a signaling cascade during retinogenesis that controls retinal ganglion cell migration, lamination, and axon orientation. Disruption of this signaling cascade has been shown to cause loss of single cellularity in the retinal ganglion cell layer (16). The current study proposes that detachment of the inner limiting membrane disrupts the signaling cascade, leading to disorganization of the retinal ganglion cell layer. Excess folic acid has been shown to disrupt the normal folate metabolism in the body, leading to the accumulation of homocysteine that causes retinal vascular injury (13) leading to insufficient retinal vascular development which is associated with congenital retinal folds (17). Other studies have observed retinal folds under folate-deficient states (18).

Vacuolations were also seen in the inner nuclear layer for the folate suprasupplemented group. In vitro, studies have demonstrated that excess folic acid effectively inhibits the function of methionine synthase, which plays a crucial role in the metabolic processes of folate and methionine. Consequently, these metabolic abnormalities cause an accumulation of homocysteine, resulting in significant oxidative stress (19). The present study proposes that the exact mechanism could occur here, causing cell death of the bipolar neurons. Excess folate has also been shown to impair neuronal sodium-potassium pumps in the hippocampus, leading to short-term memory loss (20). Retinal bipolar cells possess sodium-potassium pumps, essential for maintaining an ion gradient across the cell membrane to conduct nerve impulses. Inhibition of these pumps causes intracellular sodium accumulation, leading to cell swelling and burst (21).

Excess folic acid intake has also been shown to increase DNA de novo point mutations, causing genome and epigenome instability by downregulating DNA repair gene expression. Genome instability causes activation of the intrinsic apoptotic pathway via the p53 molecule (22). The current study proposes these could have occurred for some bipolar cells causing the vacuolations in the inner nuclear layer.

Study limitations: Storage in 10% formalin decreased the tissue integrity, making the eye specimens friable and cutting difficult and may have resulted in tissue shrinkage which could have altered the morphology.

Strengths: Tissue blocks were stored in a freezer at 4°C before cutting, which made cutting easier. Shrinkage occurred uniformly since all the parts of the eyes were exposed to the preservative (10% formalin); hence, the histomorphometric parameters were all affected uniformly.

CONCLUSION

Maternal consumption of a folate-deficient diet severely affects the morphology of the offspring's neural retina by causing thinning of the plexiform layers and detachment of the internal limiting membrane. Folate supplementation promotes the development of neural retinae in the offspring. Consumption of excess folate levels, on the other hand, affects the morphology of the neural retina but not as severely as folate deficiency. The research findings highlight the importance of balanced folate intake during pregnancy to support healthy neural retina development in offspring. Clinicians can use these findings to counsel expectant mothers on adequate folate consumption, and policymakers could advocate for folate fortification in foods, particularly in regions with limited access to nutrient-rich diets, to prevent retina-related developmental issues.

SUGGESTIONS FOR FUTURE STUDIES

Additional research using specific immunohistochemical methods should be conducted to investigate how varying maternal perigestational levels affect the various cell types of the neural retina. Further research should be conducted to investigate how varying maternal perigestational folate levels affect the development of the neural retina at various stages of development, both prenatally and postnatally.

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