Effects of vitamin b12 and omega 3 on ngf, vegf and hif-1

1. Introduction

Maternal micronutrient deficiencies are a cause of concern in low and middle-income countries (Salam et al., 2014) and have been shown to be associated with adverse pregnancy outcomes (Diaz et al., 2003). Reports have also established that maternal nutrition plays a key role in fetal brain development (Oliver et al., 2007; McMillen et al., 2008). In developing countries like India, which has a large population consuming a vegetarian diet suboptimal levels of both vitamin B_{12} (Pawlak et al., 2013) and omega-3 fatty acids (Muthayya et al., 2009) are common. Further reports also indicate that children born to mothers consuming a vegetarian diet may be at an increased risk of neurodevelopmental disorders (Larsen et al., 2014).

Animal studies carried out by us earlier have demonstrated lower levels of neurotrophins, which are important regulators of neural survival, development and function in the brain of the offspring as a consequence of maternal vitamin B_{12} deficiency (Sable et al., 2011, 2012, 2013). Studies indicate that neurotrophins regulate angiogenic markers like vascular endothelial growth factor (VEGF) in the brain (Nico et al., 2007; Turrini et al, 2002; Calza et al., 2001). VEGF is an angiogenic factor and recent studies have demonstrated its role as a stimulator of neurogenesis since vascular and nervous networks share common molecular mechanisms (Galvan et al., 2006). It is suggested that VEGF promotes endothelial cell migration towards a hypoxic area. During hypoxia, hypoxia inducible factor-1 (HIF-1) binds the regulatory region of the VEGF gene, thereby inducing its transcription and forming new blood vessels (reviewed by Ziello et al., 2007).

Apart from vitamin B_{12}, omega-3 fatty acids especially docosahexaenoic acids (DHA) also play a crucial role in brain development and functioning by influencing multiple neuroprotective mechanisms. A recent study reports the protective effects of omega-3 fatty acid supplementation on post-stroke cerebral angiogenesis in transgenic *fat-1* mice. (Wang et al., 2014). Studies carried out in our department in humans and animals have well demonstrated a link between micronutrients (folate, vitamin B_{12}) and omega-3 fatty acids in the one carbon cycle (Khot et al., 2014a; 2014b; Sable et al., 2013; Kulkarni et al., 2011, Kale et al., 2010) (Sable et al., 2011, 2012, 2013). Though several plausible biochemical mechanisms for the individual effects of both vitamin B_{12} and omega-3 fatty acid supplementation are reported, the evidence from observational studies as well as RCTs is generally limited and too inconsistent to draw firm conclusions regarding the use of combined use of vitamin B and omega-3 fatty acids for brain development and functioning reviewed by van de Rest et al., 2012). Further, whether there exist any synergistic or antagonist effects between vitamin B_{12} and omega-3 fatty acids on brain angiogenic markers is unclear.

Therefore, the current study was undertaken to evaluate the effects of maternal vitamin B_{12} and omega-3 fatty acids given either individually or together on the levels of NGF, VEGF and HIF-1 alpha in the pup brain at birth.

2. Materials and methods

2. 1. Animals and study design

The procedures carried out in the present animal study were approved by Institutional Animal Ethics Committee (IAEC) and were performed in accordance with the guidelines provided by the Committee for the Purpose

of Control and Supervision of Experimental Animals, (CPCSEA) Government of India.

The protocol for the study has been recently described by us in detail (Rathod et al., 2014; Khaire et al., 2013). Briefly, the dietary groups were as follows: Control (normal vitamin B_{12} -25µg/Kg diet); vitamin B_{12} deficient group (BD); vitamin B_{12} deficient group supplemented with omega-3 fatty acids (BDO); vitamin B_{12} supplemented group (BS) (vitamin B_{12} -50 µg/Kg diet); vitamin B_{12} group supplemented with omega-3 fatty acids (BSO). These dams were allowed to deliver normally on d22 of gestation and the offspring at birth were dissected to collect brain tissue. The study design is as shown in Fig. 1.

The purified experimental diets were prepared in accordance with the AIN-93 guidelines (Reeves et al., 1993). The source of omega-3 fatty acid supplementation used in the present study was fish oil (MaxEPA, Merck Darmstadt, Germany) and was a combination of DHA (120 mg) and EPA (180 mg).

2. 2. Tissue Homogenization

Brain tissue of the offspring was homogenized in chilled phosphate-buffered saline, at pH of 7. 5 using a Teflon glass homogenizer and the process has been described by us earlier (Rathod et al., 2014). The homogenate was centrifuged at 10, 000 rpm at 4ºC for 20 min after which the supernatant (lysate) was collected and used for total protein estimation. Total protein content of the lysates was estimated by Lowry method (Lowry et al., 1951).

2. 3. VEGF Estimation

VEGF protein levels were measured from the brain homogenate (supernatant) using the VEGF Rat ELISA kit (Abcam, Cambridge Science Park in Cambridge, England). Relative absorbance was measured at 450 nm and the VEGF concentration was calculated using a standard curve. Values of VEGF were expressed as pg/mg protein.

2. 4. HIF-1alpha Estimation

HIF-1 alpha protein levels were measured from the brain homogenate (supernatant) using HIF-1 alpha Rat ELISA kit (Uscn Life Science Inc. Wuhan). HIF-1 alpha protein levels in tissue homogenates were quantitated in the range of 0. 156-10 ng/ml. Relative absorbance was measured at 450 nm. Values of HIF-1 alpha were expressed as ng/mg protein.

2. 5. NGF Estimation

NGF protein levels were measured from the offspring brain homogenates using the NGF Emax immunoassay system (Promega, Madison, WI, USA) as described by us earlier (Sable et al., 2011). NGF levels in tissue homogenates were quantitated in the range of 3. 9-250pg/ml. Values of NGF were expressed as pg/mg protein.

2. 6. RNA isolation and cDNA synthesis

The total RNA from brain tissue was isolated using Trizol reagent (Invitrogen) and was quantified using Biophotometer (Eppendorf, Germany). One microgram of total RNA was reverse transcribed to cDNA using the High-Capacity cDNA reverse transcription Kit (Applied Biosystems, California, USA).

2. 7. Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) Assay

RT-qPCR for VEGF and NGF were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, California, USA) on the Applied Biosystems 7500 Standard Real Time PCR system and the protocol has been described by us earlier (Rathod et al., 2014). The relative expression level of the gene of interest was examined with respect to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalize for variation in the quality of RNA and the amount of cDNA input. ΔCt values corresponded to the difference between the Ct-values of the genes examined and those of the GAPDH (internal control) gene. The RT-PCR reactions for each gene were performed in duplicate. To analyze the RT-PCR results, the average cycle number (Ct) of the reaction when it crossed a threshold valued was determined for each reaction. Relative expression levels of genes were calculated and expressed as $2^{\Delta Ct}$. The following TaqMan ® assays (Applied Biosystems) were used in this study: GAPDH (Rn99999916_s1); VEGF (Rn01511601_m1); NGF (Rn01533872_m1).

2. 8. Statistical Methods

Values are expressed in mean ± SD. The data was analyzed using SPSS/PC package (Version 20. 0, Chicago, IL, USA). Mean values of the estimates of various parameters for the treatment groups were compared with those of control group at conventional levels of significance, using least significance ($p < 0.05$) difference estimated from one way analysis of variance (ANOVA).

3. Results

3. 1 Pup Brain VEGF mRNA Levels

Maternal vitamin B $_{12}$ deficiency showed lower (p <0. 01) VEGF mRNA levels as compared to that of control. Maternal omega-3 fatty acid supplementation to a vitamin B $_{12}$ deficient group normalized VEGF mRNA levels. The VEGF mRNA levels in the maternal vitamin B $_{12}$ supplemented were comparable to that of control group. Similarly, the VEGF mRNA levels did not alter in the vitamin B $_{12}$ with omega-3 fatty acid supplemented group as they remained comparable to that of control group (Fig. 2A).

3. 2 Pup Brain VEGF Protein Levels

Maternal vitamin B $_{12}$ deficient group showed lower (p <0. 01) VEGF protein levels in the pup brain as compared to control. Maternal omega-3 fatty acid supplementation to a vitamin B $_{12}$ deficient group also showed lower (p <0. 01) levels of VEGF protein. The protein levels of VEGF in vitamin B $_{12}$ supplemented group were comparable to the control group. Similarly, the group with both vitamin B $_{12}$ and omega-3 fatty acid supplementation did not alter the levels of VEGF protein levels and remained comparable to that of the control group (Fig. 2B).

3. 3 Pup Brain HIF-1 alpha Protein Levels

Maternal vitamin B $_{12}$ deficiency showed higher (p <0. 05) HIF-1 alpha levels as compared to control while maternal omega-3 fatty acid supplementation to a vitamin B $_{12}$ deficient diet normalized the HIF-1 alpha levels in the pup brain. The protein levels of HIF-1 alpha in the maternal vitamin B $_{12}$ supplemented were comparable to control group. Similarly, in the vitamin B

$_{12}$ and omega-3 fatty acid supplementation group, the protein levels of HIF-1 alpha were comparable to control (Fig. 3).

3. 4 Pup Brain NGF mRNA Levels

Maternal vitamin B $_{12}$ deficiency did not alter mRNA levels of NGF. Similarly, it was also comparable to that of control group in maternal omega-3 fatty acid supplementation to a vitamin B $_{12}$ deficient diet. Maternal vitamin B $_{12}$ supplemented group also showed comparable NGF mRNA levels to that of control while omega-3 fatty acid supplementation to the vitamin B $_{12}$ supplemented group showed higher ($p < 0.01$) NGF mRNA levels as compared to control as well as vitamin B $_{12}$ supplemented group (Fig. 4A).

3. 5 Pup Brain NGF Protein Levels

Maternal vitamin B $_{12}$ deficient group showed lower ($p < 0.05$) NGF protein levels in the pup brain as compared to control while omega-3 fatty acid supplemented to vitamin B $_{12}$ deficient diet showed higher NGF protein levels as compared to vitamin B $_{12}$ deficient group. Maternal vitamin B $_{12}$ supplementation did not alter the levels of these proteins as they remained comparable to that of control. Omega-3 fatty acid supplementation to a vitamin B $_{12}$ supplemented group showed higher ($p < 0.01$) NGF protein levels as compared to control and BS group (Fig. 4B).

4. Discussion

The present study reveals several novel and interesting key findings related to maternal vitamin B $_{12}$ and omega-3 fatty acid status on neurovascular unit in the pup brain. The main findings of our study are (1) maternal vitamin B $_{12}$ deficiency showed lower pup brain mRNA and protein levels of VEGF, higher

HIF-1 alpha protein levels, lower NGF protein levels while NGF mRNA levels were not altered (2) maternal omega-3 fatty acid supplementation to a vitamin B_{12} deficient group normalizes VEGF mRNA levels, NGF protein levels and HIF-1 alpha protein levels (3) Levels of NGF, VEGF and HIF-1 alpha in the maternal vitamin B_{12} supplemented group were similar to that of control (4) Omega-3 fatty acid supplementation to vitamin B_{12} supplemented group showed higher NGF protein and mRNA levels while levels of VEGF and HIF-1 alpha protein were comparable to that of control.

The present study reports lower levels of VEGF in the pup brain at birth as a consequence of maternal vitamin B_{12} deficiency. The crucial role of VEGF in vascularization and neuronal cell migration has been well implicated in the developing brain (Acker et al., 2001; Schwarz et al., 2004). It has been reported that the developing brain requires a good vascular system for the delivery of oxygen and nutrients (reviewed by Mackenzie and Ruhrberg, 2012). The lower levels of VEGF in the present study may be indicative of hampered brain vasculature. Reduced VEGF levels are known to be associated with neurodegenerative diseases like amyotrophic lateral sclerosis (ALS) in mice as well as in humans (Lambrechts et al., 2003). We have earlier reported high levels of maternal homocysteine as a consequence of vitamin B_{12} deficiency (Khaire et al., 2013) and likely to influence angiogenesis. It has been demonstrated that homocysteine inhibits angiogenesis through the inhibition of VEGF/VEGFR, Akt, and ERK1/2 mechanisms (Zhang et al., 2012). Hyperhomocysteinemia through the mediation of oxidative stress has been shown to be associated with changes

in the structure and function of cerebral blood vessels (reviewed by Faraci and Lentz, 2004).

In the present study, pup brain HIF-1 alpha levels were higher in the maternal vitamin B_{12} deficient group. It has been demonstrated that higher HIF-1 alpha levels may be responsible for hypoxia-induced growth arrest and apoptosis (Goda et al., 2003). It has also been shown to regulating local brain hypoxia and influence and brain vascularization (reviewed by Giordano and Johnson, 2001). In the current study, VEGF levels were low although HIF-1 alpha levels were high suggesting that neuroprotective genes like VEGF may also be regulated by another transcription factor, independent of HIF-1alpha (Benderro et al., 2012). In the present study, omega-3 fatty acid supplementation to a vitamin B_{12} deficient group was able to restore HIF-1 alpha protein levels. Our results are in accordance with another recent study which demonstrates that omega-3 fatty acid consumption decreases the protein levels of HIF-1 alpha in subcutaneous adipose tissue of obese adolescents (Mejía-Barradas et al., 2014). However, the underlying mechanisms are not fully understood and needs to be explored.

In the current study, maternal vitamin B_{12} supplementation maintained the levels of VEGF, HIF-1 alpha and NGF in the pup brain as comparable to that of the control group. Similarly, the combined supplementation of vitamin B_{12} and omega-3 fatty acid group showed comparable levels of VEGF and HIF-1 alpha to that of control while NGF mRNA and protein levels were significantly higher as compared to control group. NGF is essential for the development and maintenance of sensory neurons and survival of neurons (reviewed by

Berry, 2012). Apart from neurotrophic properties, recently NGF has been described as an important angiogenic molecule (Lazarovici et al., 2006), it is known to have a cross-talk with VEGF (Calza et al., 2001; Hansen-Algenstaedt et al., 2006) and is also known to promote endothelial cell proliferation and migration (Dolle et al., 2005; Moser et al., 2004).

We have recently reported that maternal vitamin B_{12} and omega-3 fatty acid supplementation increases brain derived neurotrophic factor (BDNF) and DHA levels in the brain of the offspring and also improves cognitive performance (Rathod et al., 2014). Vitamin B_{12} and omega-3 fatty acids may be involved in the regulation of neurovascular unit which requires proper network of molecules like angiogenic factors and neurotrophins which are involved in path finding, growth, migration and differentiation of neurons (Lee et al., 2009). Few other studies have also analyzed the effect of omega-3 polyunsaturated fatty acids on cerebral angiogenesis in several stroke models and shown to be protective against ischemic brain injury (Wang et al., 2014; Belayev et al., 2009; Zhang et al., 2010). Reports indicate that omega-3 fatty acid supplementation in transgenic mice has been associated with neurogenesis and oligodendrogenesis to improve post-stroke brain repair and long-term functional recovery (Hu et al., 2013). The process of neurogenesis has been shown to be associated with angiogenic microenvironment in the brain (Palmer et al., 2000),

5. Conclusion

In conclusion, this is the first study which demonstrates the effect of maternal omega-3 fatty acids on a vitamin B_{12} deficient diet in influencing

angiogenesis in the pup brain. Further it also demonstrates the effect of combined supplementation of vitamin B_{12} and omega 3 fatty acids on the pup brain NGF levels indicating the need for a combined maternal supplementation of these vital nutrients in improving the brain development and function in the offspring. The role of maternal nutrition in influencing the levels of neurotrophins and angiogenic factors will help to understand the putative mechanisms involved and may provide important clues to prevent early cognitive deficits and later neurobehavioral disorders in the offspring. Further investigation is required for better understanding the effect of vitamin B_{12} and omega-3 fatty acids on other molecules involved in the process of angiogenesis in the brain.