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The Deterioration Seen in Myelin Related Morphophysiology in Vanadium Exposed Rats is Partially Protected by Concurrent Iron Deficiency

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Summary: Oligodendrocyte development and myelination occurs vigorously during the early post natal period which coincides with the period of peak mobilization of iron. Oligodendrocyte progenitor cells (OPCs) are easily disturbed by any agent that affects iron homeostasis and its assimilation into these cells. Environmental exposure to vanadium, a transition metal can disrupt this iron homeostasis. We investigated the interaction of iron deficiency and vanadium exposure on the myelination infrastructure and its related neurobehavioural phenotypes, and neurocellular profiles in developing rat brains. Control group (C) dams were fed normal diet while Group 2 (V) dams were fed normal diet and pups were injected with 3mg/kg body weight of sodium metavanadate daily from postnatal day (PND) 1-21. Group 3 (I+V) dams were fed iron deficient diet after delivery and pups injected with 3mg/kg body weight sodium metavanadate from PND1-21. Body and brain weights deteriorated in I+V relative to C and V while neurobehavioral deficit occurred more in V. Whereas immunohistochemical staining shows more astrogliosis and microgliosis indicative of neuroinflammation in I+V, more intense OPCs depletion and hypomyelination were seen in the V, and this was partially protected in I+V. In *in vitro* studies, vanadium induced glial cells toxicity was partially protected only at the LD 50 dose with the iron chelator, desferroxamine. The data indicate that vanadium promotes myelin damage and iron deficiency in combination with vanadium partially protects this neurotoxicological effects of vanadium.

Keywords*:* Vanadium, iron deficiency, hypomyelination, behavioural deficits, neurotoxicity.

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INTRODUCTION

The increase in environmental pollution due to the recent increased exploitation of minerals (Olopade *et al.*, 2005) has suggested a strong link between environmental pollution and the incidence of neuropathologies of the brain (Calderon-Garciduenas *et al*, 2002; Igado *et al.*, 2008). Vanadium (V) is a metallic transition element widely distributed in the environment (Hope, 1994) with atomic number 23. It exists in oxidation states ranging from -1 to +5, and frequently forms polymers (Clark, 1973). Combustion of fossil fuels provides a significant environmental source of vanadium and particulate emission has been estimated to comprise about 53% of the total atmospheric vanadium (Hope, 1994). Environmental contamination also occurs by spilling and burning of vanadium containing crude oil into relatively confined ecosystems (Bycakowski and Kulkarni, 1996). Aside from the acute environmental and occupational exposure to vanadium which is not uncommon (Shrivastava, 2007) the general population is increasingly exposed to this metal mostly as a result of the increased utilization of vanadium containing petroleum fuel (Bycakowski and Kulkarni, 1996). As a vanadate anion, vanadium penetrates the blood-brain barrier and leads to disruption of the ependymal cellular junctions which in turn loose their cilia (Domingo, 1996) as well as central nervous system disorders (Berman, 1980; Garcia *et al.*, 2005), and has been shown to have neurotoxic effects (Garcia *et* al., 2005; Haider *et al.*, 1998).The inhibitory effect of vanadium on the uptake and release of noradrenaline has been demonstrated (Garcia *et al.*, 2005; Haider *et al.*, 1998) and vanadium inhalation shown to produce a time dependent loss of dendritic spines, necrotic-like cell death, notorious alterations of the hippocampus CA1 neuropile which correlate with spatial memory impairment (evaluated by Morris water maze) and significant dopaminergic neuronal loss in the substantia nigra (and corpus striatum) resulting in morphological alterations of the striatum medium sized spiny neurons (Domingo, 1996). Administration of graded doses of vanadium in the form of sodium metavanadate to rats caused lipid peroxidation leading to the alteration of lipid metabolism and

protein concentrations in different regions of the brain (Sasi *et al.,* 1994). In addition, Soazo and Garcia (2007) have also demonstrated significant reductions in both general activity and learning following oral vanadate administration. Recently, Mustapha *et al.,*(2014) showed a progressive reduction in body weight gain, reduction in locomotor activity, graded reactive astrogliosis and hypomyelination attributable to down regulation of cyclic Nucleotide 3'-Phosphohydrolase (CNPase) and Myelin basic Protein (MBP) in mice pups expose to vanadium through lactation. The data of Mustapha *et al.,* (2014) supports the earlier report of Olopade *et al.,* (2011) that showed vanadium induced behavioural deficits, significant reduction in body weight gain and absolute brain weight, and reactive astrogliosis.

It has been proposed that most of the toxic effects of vanadium on the brain are due to the generation of reactive oxygen species and consequent lipid peroxidation (Olopade and Connor, 2011). Todorich *et al.,* (2011) proposed that developmental exposure to vanadium will cause hypomyelination via destruction of oligodendrocyte progenitors in part by increasing iron release from ferritin. The authors thus suggested that the resulting oxidative stress and apoptosis of the oligodendrocyte progenitor cells (OPCs) by vanadium induces further release of iron from ferritin, and that the consequent iron exposure exacerbates its cytotoxic effects.

The brain normally contains a substantially greater concentration of iron than other metals (Yehuda and Youdim, 1988), usually in the form of ferritin (Octave *et al.,* 1983). Connor *et al.,* (2003) have shown iron to be important in the brain as impaired acquisition leads to neurological problems. The homeostasis of brain iron is thought to be necessary for normal brain function, especially in learning and memory (Youdim, 1990). Thus a high content of brain iron may be essential, particularly during development, but its presence means that injury to brain cells may release iron ions that can lead to oxidative stress via formation of oxygen free radicals.

While it is tempting to believe that lowered iron status may be protective against vanadium exposure, iron deficiency itself also carries a neurological burden of hypomyelination. In biological systems generally but specifically in neuronal cultures, vanadium is bound to transferrin, giving the possibility that vanadium could interfere with the uptake, storage and metabolism of iron (and vice versa) (EVM, 2002; Todorich *et al.,* 2011). Whereas iron deficiency is a nutritional problem, vanadium exposure is an environmental concern and the dual effect could be clinically devastating (Todorich *et al.,* 2011). A sizeable population exposed to crude oil burning in Nigeria Niger Delta experience iron deficiency either through nutritional deficits (Ekwochi *et al.,* 2013), hookworm infestation (Ezem *et al.,* 1977) and teen age pregnancy (Oriji *et al.,* 2011) making the study of the interaction of iron deficiency and vanadium exposure one of epidemiological importance. The aim of this work is to establish the interaction between iron deficiency and vanadium exposure on myelination infrastructure and its related neurobehavioural phenotypes.

MATERIALS AND METHODS

Reagents

Desferroxamine (Sigma) was dissolved in water and frozen in 50mM aliquots of stock solution and were used for individual experiments. Sodium metavanadate (Sigma) was obtained as powder, diluted with Dulbecco's Phosphate Buffered Saline (DPBS) to a stock solution of 2.5mM which was kept in aliquot at -20^{o}C, and was used as needed.

Animals

Experiments on animals were performed in accordance with Ethical Standards and Institutional Animal Care and Use Committee (IACUC, Protocol Number 43269).

Pregnant Sprague-Dawley rats (CRL SD) were purchased from Charles River Laboratories when they were two weeks pregnant. The rats were housed at Pennsylvania State University College of Medicine Animal Core facilities. Diets used for the research were supplied by Harlan Laboratories, Inc.

Three groups of rat pups with four dams per group were used. Control group (C) dams were fed with normal diet and pups injected with Dulbecco's Phosphate Buffered Saline (DPBS) daily from PND1-21. Group 2 (V) dams were fed with normal diet and pup injected intraperitoneally (IP) with 3mg/kg body weight of sodium metavanadate (Garcia *et* al., 2004; Soazo and Gracia, 2007; Todorich et al 2011) daily from PND1-21. Group 3 (I+V) dams were fed with iron deficient diet after delivery and pups injected with 3mg/kg body weight sodium metavanadate IP from PND1- 21. All litters were culled to eight to ensure standard access to nursing across all groups. The pups were housed with their dams with *ad libitum* access to feed and water. Daily body weight was measured using a benchtop scale.

Behavioural test

At PND 15, rotarod testing of motor function, negative geotaxis, a test of motor coordination and open field test of behaviour was performed across all groups.

Rotarod testing

Rotarod test was as described by Todorich *et al.,* (2011). All animals were taken through a training period on the rotarod, and subsequently tested in three independent trials at a speed of 5rpms. The outcome was measured as the time it took the rat

pups to fall off the rotarod. Two investigators blinded to the experimental condition with good concordance scored the rotarod performance. The times of three trials were averaged across each group and evaluated for statistical significance using two way analysis of variance (ANOVA).

Open field test

For the open field test, we used an open field arena of glossy white plywood cage of 64 cm x 64cm with 30cm high walls. A black marker was used to draw lines on the floor of the field. The lines divided the floor into sixteen 16 x 16 cm squares. A central square (16 cm x 16 cm) was drawn in the middle of the open field (Brown, *et al.*, 1999) with a red marker. A video camcorder, Logitech QuickCam Pro 9000 (Logitech International SA; Newark, California, USA) used for video capturing of behaviour was positioned 65 cm above the centre of the open arena floor. The entire arena in the zone of the camera's view was captured from this position. The video camcorder was connected to a Samsung laptop, (Samsung Electronics, South Korea). The animal's behaviour inside the open field arena was tracked using ANY-maze 4.70 (Stoelting Co.; Wood Dale, Illinois, USA) software. Before commencement of the experiment, the software was used for setting up a tracking protocol. Each rat pup was allowed to explore the open field for five minutes after which they were removed. The open field arena was cleaned with 70% ethanol solution and allowed to dry before the next rat pup was tested.

In our study, parameters measured are as described by Brown *et al.*, (1999) and include:

1. *Line Crossing:* the number of times rat pups crossed one of the grid lines with all four paws.
2. *Center Square Entries:* the number of times rat pups crossed one of the red lines with all four paws into the central square.
3. *Center Square Duration:* the length of time the rat pups spent in the central square.
4. *Rearing:* the number of times rat pups stood on their hind legs in the maze.
5. *Grooming:* the length of time the rat pup spent licking or scratching itself while stationary.
6. *Freezing:* the length of time the rat pup was completely stationary.
7. *Urination:* puddles or streaks of urine number.
8. *Defecation:* fecal boli number.

Negative geotaxis

Negative geotaxis, was used to assess motor coordination of the pups when challenged on a sloped surface (Olopade *et al*., 2011). We used a test apparatus consisting of sloped platform of an angle of 30^{o} from horizontal to the desktop for this test. Rat pups were placed on the paper-towel covered sloped platform facing a downward direction. The latency to turn and orient to face up (180^{o}) the slope was recorded. A delay in ability to reorient was indicative of delays in motor, balance, or vestibular function. All performance was scored by two investigators blinded to the experimental condition with good concordance. Data obtained were subjected to statistical analysis using two way ANOVA (Graph pad prism, version 4.0) considering litter from dams in a group as a single unit.

Immediately after the last behavioural test, four pups from each dam in each group were then sacrificed by lethal injection using a combination of xylazine (10mg/kg) and ketamine (100mg/kg) and the brain harvested over dry ice. The brain weight was taken using a benchtop scale. One half of the brain was immersion fixed in 4% paraformaldehyde (in 0.1M PBS) for 48 hrs and then changed to 0.1M PBS for 48hrs. Subsequently, brain slides were prepared from paraffin blocks by routine histological method. The other hemisphere was frozen for quantitative analysis of markers used. At PND 21, the remaining pups from each group went through the same routine behavioural test and sacrificed as in PND15.

Immunohistochemistry

The prepared brain slides were air dried and labelled with pencil, baked for 20-30mins at 60^{o}C to dewax. They were deparaffinzed in two changes of xylene and hydrated in decreasing percentage of ethanol. Antigen retrieval was done in 10mM citrate buffer (pH=6.0) for 25min, with subsequent peroxidase quenching in 3% H_2O_2/methanol for 20 min. Slides were washed in PBS and sections were circled with PAP pen and blocked in 2% PBS milk for 1hr in humidity chamber (200µl/slide). All sections were probed with anti-Myelin basic protein-MBP (abcam, ab22460), anti-GFAP (Dako, z0334), anti-iNOS (calbiochem, cat. No. 482728)), anti-CNPase (abcam, ab6319), anti-Iba-1(Wako, Japan), anti-NG2 (Millipore, MAB5384), anti- ferritin(abcam ab69090) and anti-transferrin receptor antibody (abcam ab8598) diluted in 1% PBS milk overnight in humidity chamber at 4^{o}C. Detection of bound antibody was done using appropriate HRP-conjugated secondary antibodies in VECTA-STAIN kit (Vector Labs) according to manufacturer's protocol. Reaction product was enhanced with DAB (1:25 dilution) for 5-10min, with subsequent dehydration in ethanol. The slides were mounted with permount, coverslipped and allowed to dry. Images were captured using a light microscope (Bio-microscope, YJ-2005 series) connected to a laptop computer (Hp, China) with TSView 1.0 and AmScope ToupView 3.2 softwares.

Primary cell culture

Primary cultures were prepared from newborn rat pups of CRL SD rats according to standard protocols of McCarthy and de Vellis (1980). To purify different types of glia, mixed glial cultures obtained were

subjected to a serial shaking purification protocol (McCarthy and de Vellis 1980). First, the microglia were removed by shaking for 1 hour at 265 rpms. Subsequently, oligodendrocytes progenitor cells were removed by shaking for 18hrs at 265rpm.The remaining astrocytes were allowed to recover for one day, and then collected by trypsinization, counted and plated for experiments in 96 well plates. The oligodendrocytes fraction was subsequently further purified by differential adhesion through incubation in petric dish for 30 mins to remove more adherent astrocytic and microglial contamination (Todorich *et al.*, 2011). These cells were then replated in 96 well plated in N2S or N2B2 media for immature and mature oligodendrocytes respectively and used for experiments.

Cytotoxicity assay

Primary cells (oligodendrocytes and astrocytes) were treated with 0µM, 10µM, 75µM, 100µM and 200µM concentrations of sodium metavanadate with or without the iron chelator, desferroxamine (100qM) for 48 and 96 hrs, unless indicated otherwise. Untreated cells served as control. Cells viability was determined using MTT assay (cell proliferation kit 1, cat. No. 11465007001) according to manufacturer's protocol and absorbance measured on plate reader at 595-650nm.

Western Blot

The frozen sections of the brain were homogenized, sonicated for 30 sec, and centrifuged at 4^{o}C, 8000g for 5mins. Supernatant was collected into a new tube and dilution of 90µl PBS to 10µl sample was done for BCA assay (PierceTM BCA protein Assay Kit No 23225) according to manufacturer's protocol, incubated at 30^{o}C for 30min and read on plate reader at 570nm.

Samples were mixed with sample buffer and boiled for 10min. Samples were transferred to ice and centrifuged for 5 secs then to a TGX 4-20% gel and run at 250V for 25 min using a tris-glycine gel running buffer. It was transferred to a nitrocellulose membrane for 20min at 150 V at 4^{o}C. 5% TBST milk was used to block for 1 hr at room temperature and then probed overnight with anti- GFAP (1:7500) and anti-iBA-1(1:1000) primary antibodies at 4^{o}C. Detection of bound antibody was done using appropriate secondary antibodies for 1 hr at room temperature, washed 3 times for 10min each and ECL for 1min before exposure in Fuji. Multiple replicates of result from a group were presented as a single unit.

Statistical Analysis

All data generated were evaluated for statistical significance using two way ANOVA with Bonferroni's post-test comparison to controls.

RESULTS

The effects of vanadium exposure on the glial cells especially the myelin producing cells in the CNS with iron or iron deficiency was investigated both in vivo and in vitro. Results showed constant daily body weight gain with no difference from PND1-12 across all groups. Differences in body weight gain occurred from PND13-21 in I+V group and PND14-21 in V group with both lower than control. I+V rat pups showed least body weight gain (Fig. Ia). The same pattern was seen in brain weight gain across groups (Fig. Ib) when the pups were sacrificed at PND15 and 21.

The neurobehavioural test detected some impairment of motor function for both of the vanadium treated groups (V and I+V) relative to control at the two time points (PND15 and 21).

Specifically, the rotarod test revealed a significant impairment at PND21 in I+V group (Fig. II a); V group was also significantly impaired relative to control.

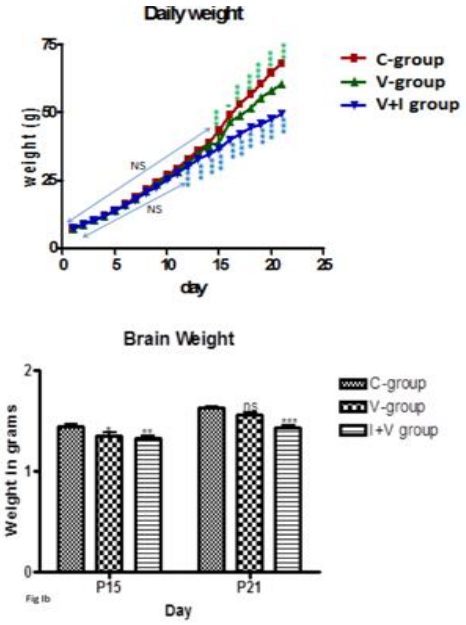

Figure I. Vanadium exposure during early development leads to reduced body and brain weight gain which was aggravated with iron deficiency. Newborn rat pups were injected with 3 mg/kg body weight of Na-metavanadate solution IP once per day for PND1-21 to V and I+V groups. Injection of equal volume of DPBS to littermate siblings served as control. Each day, bodyweights (a) of the pups were measured and at PND15 or 21, the pups were sacrificed and the brain harvested and measured (b) and plotted as means with standard deviation. The mean body and brain weights were evaluated for statistical significance using two way ANOVA with Bonferroni's post-test comparison to control at each time point (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

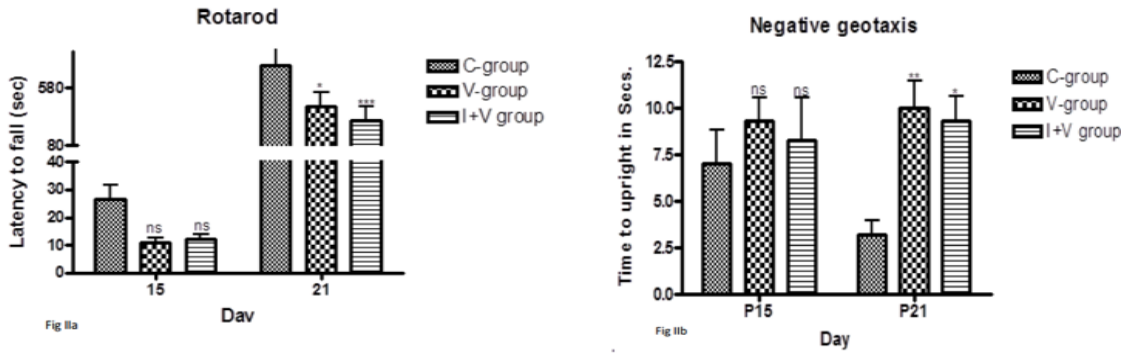

Figure II. Vanadium exposure during early post-natal period leads to impaired motor functioning. At PND15 and 21, groups V, I+V and control rats were subjected to rotarod testing for motor function and negative geotaxis; a test used to assess motor coordination of the pups when challenged on a sloped surface. The time from start of rotarod to the moment that the rat fell of the rotarod (a) and the latency to turn and orient themselves to face up (180^{o}) the slope (b) was recorded by two blinded investigators. The values were averaged and evaluated for significance using two way ANOVA with Bonferroni's post-test comparison to control (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

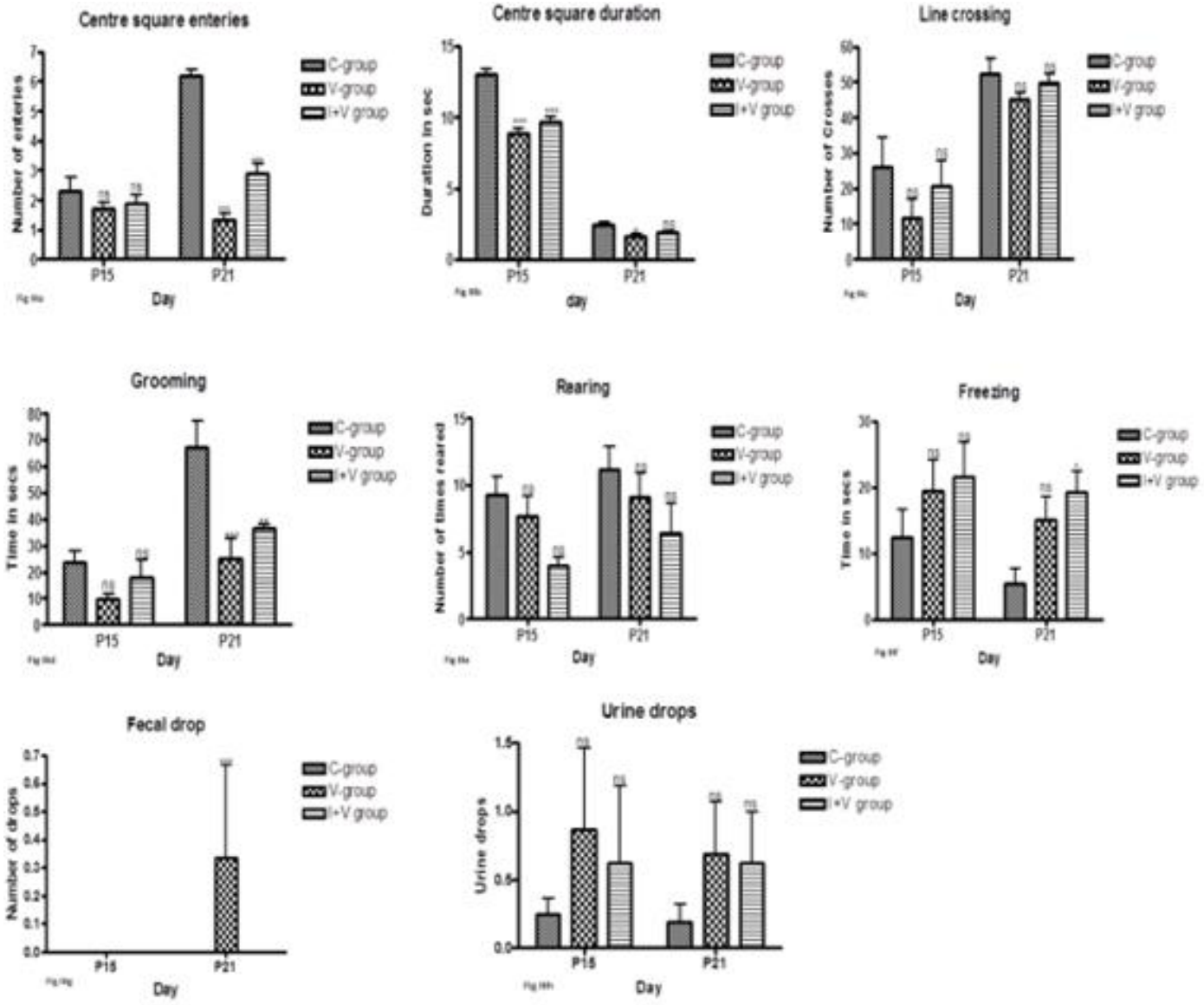

Figure IIIa At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Centre square enteries measures the number of times rat pups crossed one of the red lines with all four paws into the central square. Vanadium treated rat pups (V and I+V) crossed less. Although not statistically significant at P15, centre square enteries test of behaviour was significant at P21. The least cross was seen in V group. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIb At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Centre square duration measures the length of time the rat pups spent in the central square. Vanadium treated rat pups (V and I+V) spent less time both at P15 and 21. The least time was seen in the V group. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIc At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Line crossing measure the number of times rat pups crossed one of the grid lines with all four paws. Although not statistically, Vanadium treated rat pups (V and I+V) crossed less both at P15 and 21. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIId At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Grooming measures the length of time the rat pup spent licking or scratching itself while stationary. Although not statistically at P15 but significant at P21, Vanadium treated rat pups (V and I+V) spent less time grooming. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIe At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Rearing measures the number of times rat pups stood on their hind legs in the maze. Although not statistically significant at both time points, Vanadium treated groups (V and I+V) spent less time rearing. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIf At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Freezing measures the length of time the rat pup was completely stationary. Although not statistically significant at P15, I+V group spent highest time freezing at P21 and this was statistically significant. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIg At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Defecation measures fecal boli number. No fecal bolus was seen in the field in both C and I+V groups at both time points exposed. Only one pup in V group dropped one bolus in the field at PND21

Fig IIIh At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Urination measures puddles or streaks of urine number in the field. Although not statistically significant at both time points, rat pups in V group voided more urine than those in I+V group when compared to control (C) (ns, not significant)

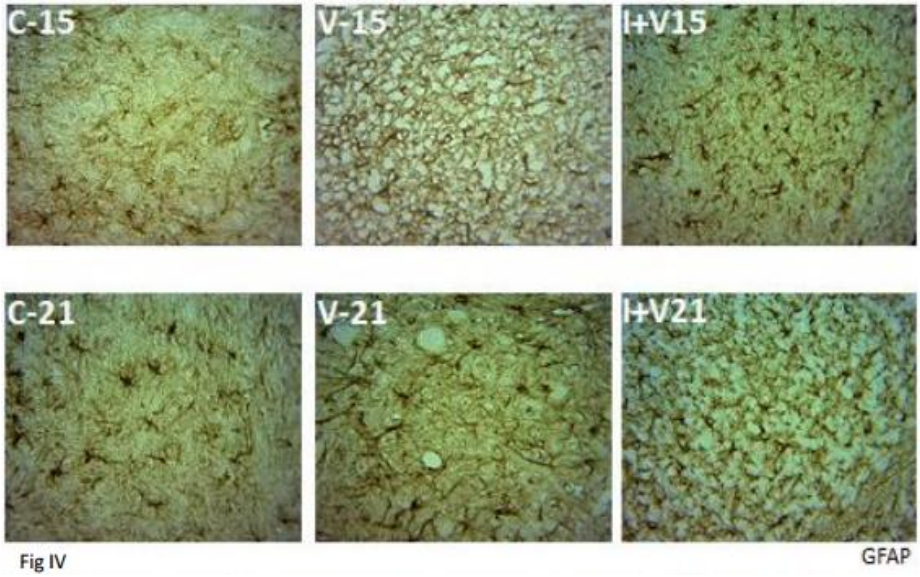

Figure IV. *In vivo* vanadium exposure during early development results in increased GFAP-positive astrocytes in the cerebellum of P15 &21- V (V-15 & V-21)& I+V (I+V15 & I+V21) rat pup when compared with C(C- 15&C-21). Astrogliosis was greater in I+V(I+V15 & I+V21). Cellular vacuolationswere observed both in V and I+V groups but worst in V group.

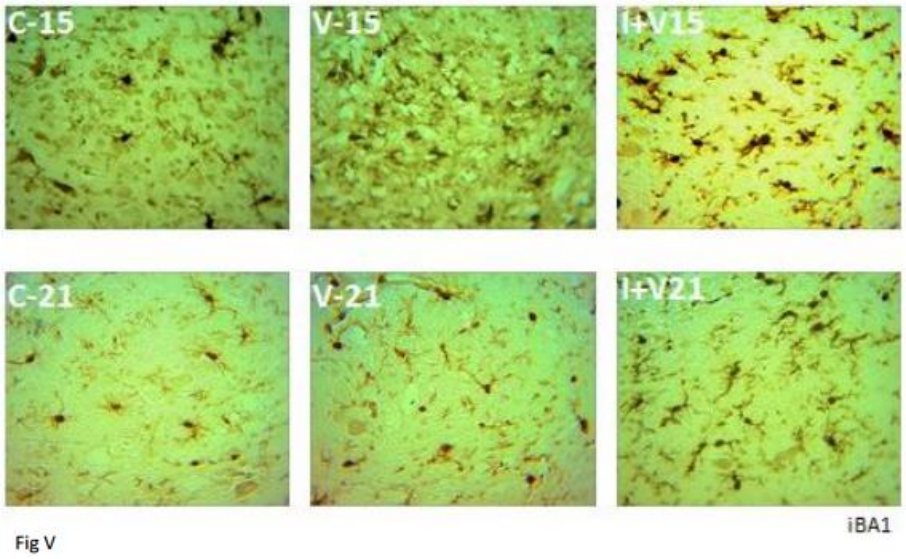

Figure V. *In vivo* vanadium exposure during early development results in increased Iba-1-positive cells in the cerebellum of P15 &21- V (V-15 & V-21)& I+V (I+V15 & I+V21) rat pups when compared with C (C-15 & C-21) group. Microglia activation was more in I+V(I+V15 & I+V21) group

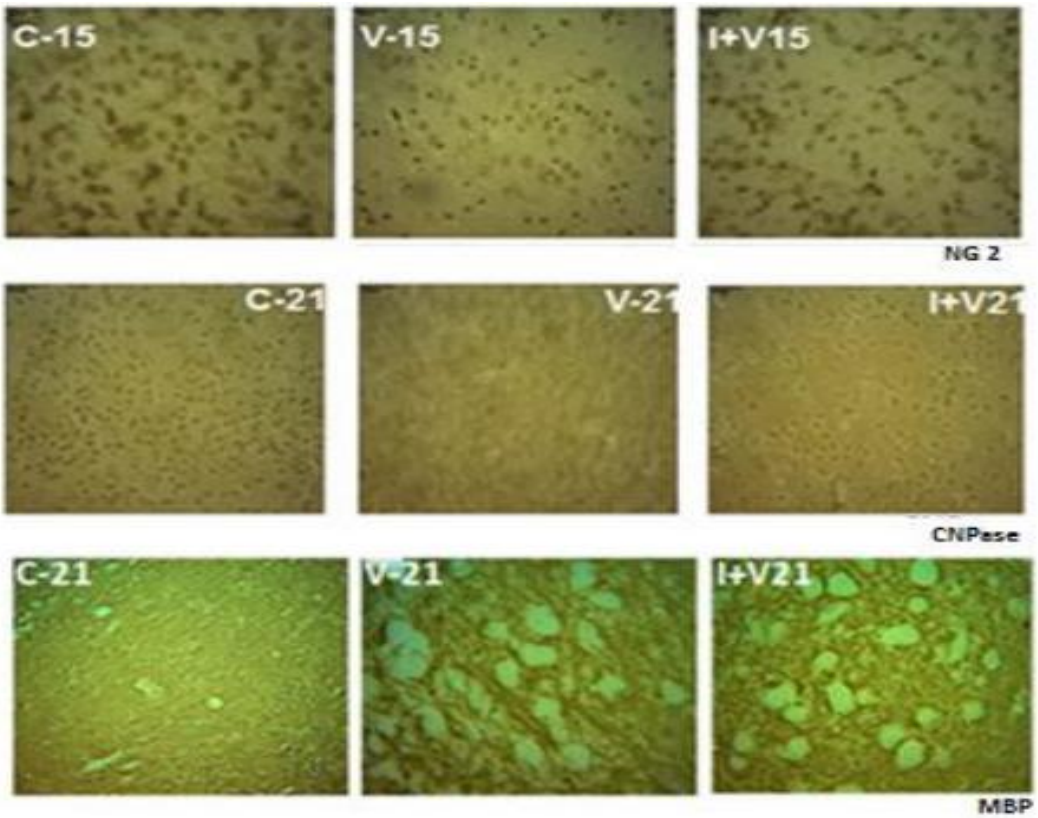

Figure VI. *In vivo* vanadium exposure during early development results in decreased NG-2 positive oligodendrocyte progenitors, P15 (V-15 & I+V15) ,CNPase-positive oligodendrocytes and MBP expression, P21 (V-21 & I+V21) in the cerebellum of rats pups of V & I+V groups. There was a partial protection on pups of I+Vgroup when compared with C (C-15 & C-21). Cellular vacuolations were observed both in V and I+V groups but worst in V group.

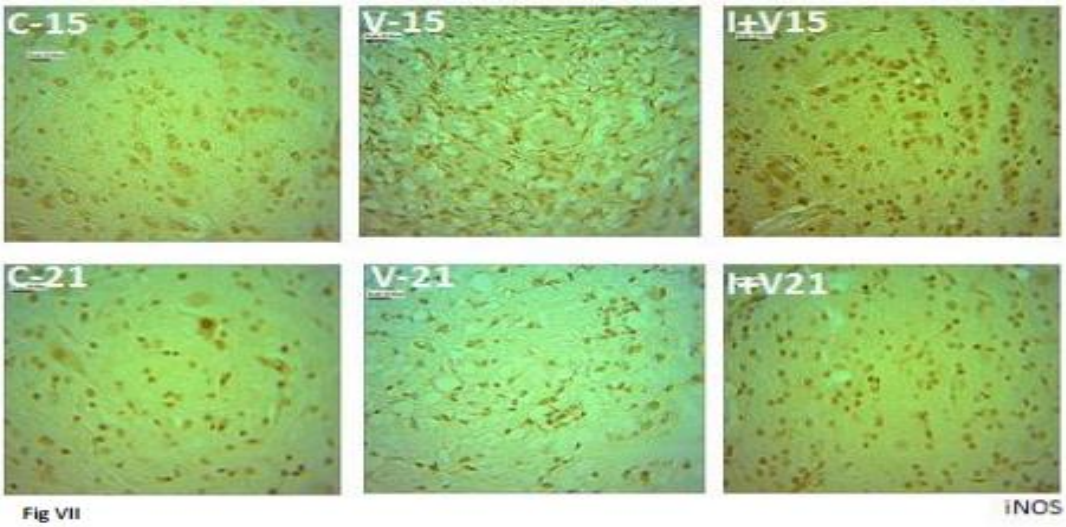

Figure VII. *In vivo* vanadium exposure during early development results in increased iNOS-positive staining in the cerebellum of P15 &21- V (V-15 &V-21) & I+V (I+V15 & I+V21) rat pups when compared with C (C15 & C- 21). Staining was more in I +V (I+V15 & I+V21)

For negative geotaxis, vanadium treated groups (V and I+V) took longer to reorient themselves to face up when challenged with a sloped platform both at PND15 and 21. The longest time was seen in V group (Fig. II b) and this was statistically significant.

When rat pups from all groups were exposed to five minutes exploration of the open field, behavioural deficits occurred in V and I+V rat pups on all parameters taken at the two time points. Deficits on centre square entries, centre square duration, line crossing, and grooming were observed more in V group (Fig. III a, b, c, and d) while rearing and freezing were more in I+V group (Fig. III e and f). No faecal bolus was seen in the field in both C and I+V at both time points exposed. Only one pup in V group dropped one bolus in the field at PND21 (Fig. III g). Although not statistically significant at both time points, rat pups in V group voided more

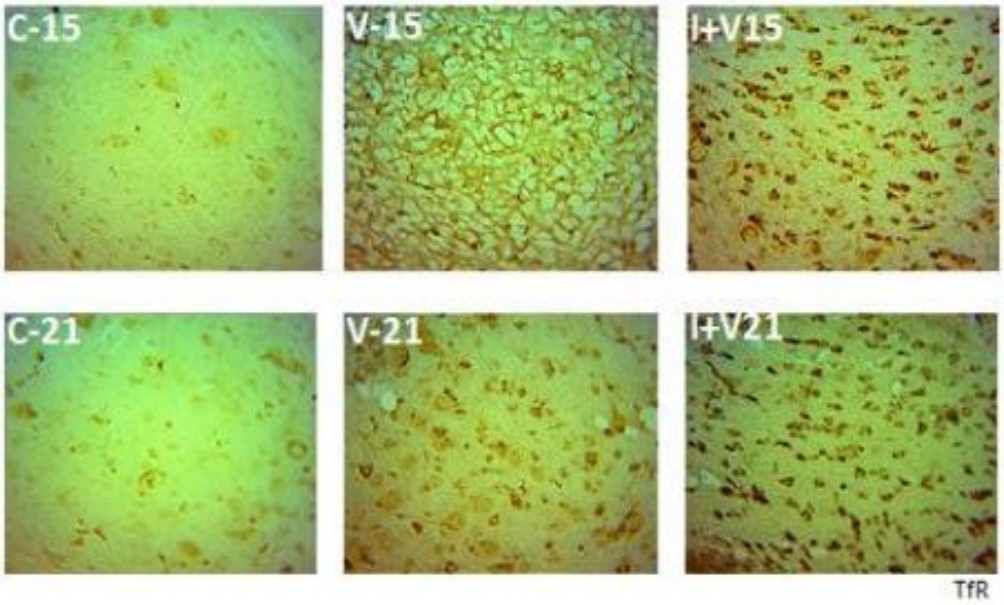

Figure VIII. *In vivo* exposure to iron deficiency to pups through lactation from dams on iron deficient diet and concurrent vanadium intraperitoneal injections during early development results in increased TfR positive staining at P15 and P21(I+V15 & I+V21) in the cerebellum of I+V rat pups when compared with C (C15 & C- 21).

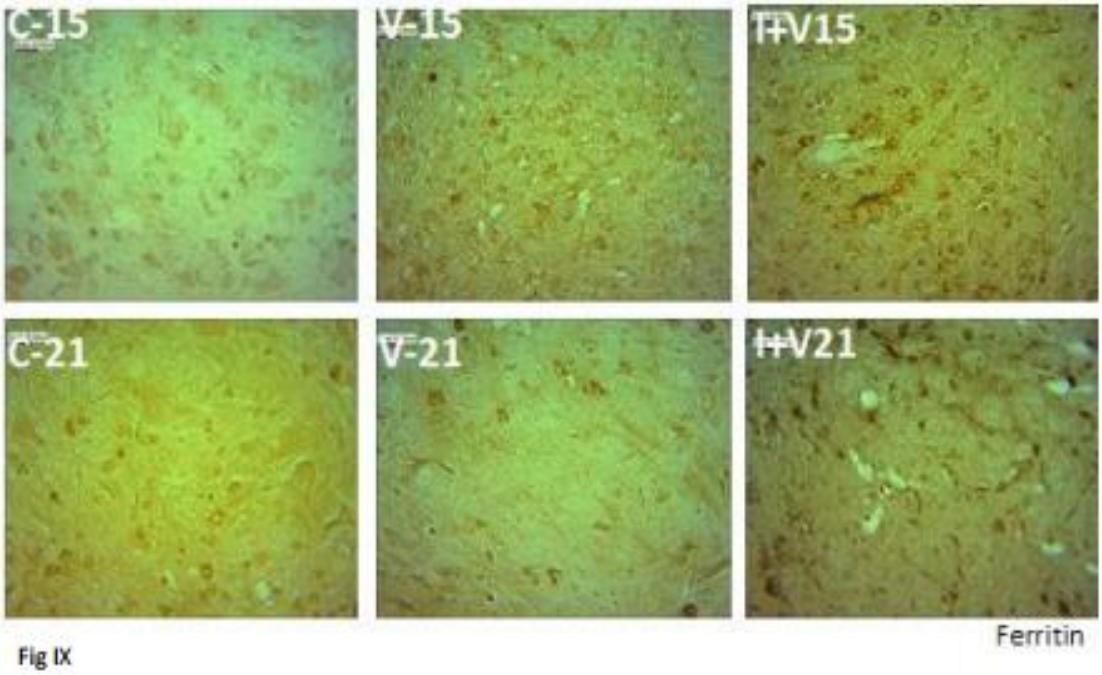

Figure IX. *In vivo* exposure to iron deficiency to pups through lactation from dams on iron deficient diet and concurrent vanadium intraperitoneal injections during early development results in increased ferritin positive staining at P15 and P21 (I+V15 & I+V21) in the cerebellum of I+V rat pups when compared with C (C15 & C- 21).

urine than those in I+V group when compared to control (C) (Fig. III h).

Upon immunohistochemical examination of the brains of the rat pups at PND15 and 21, we examined the genu of the corpus callosum and the arbor vitae of the cerebellum and observed that vanadium exposure was associated with increased number of astrocytes (Fig. IV) and microglia (Fig. V) in both organs. These were observed more in I+V group. Conversely, we observed a decrease in the same regions of the brain in the number of NG-2 positive oligodendrocyte progenitor cells, mature myelinating oligodendrocytes, decreased myelination and cellular and tissue vacuolation (Fig. VI) in V and I+V groups, which were however worst in V group. There was increased staining for iNOS (Fig. VII) positive cells in V and I+V groups but was more intense in I+V group. To determine the pathophysiologic status of iron in the brain, we stained for transferrin receptors nd ferritin (Fig. VIII and Fig. IX) and our result showed an increase in I+V group.

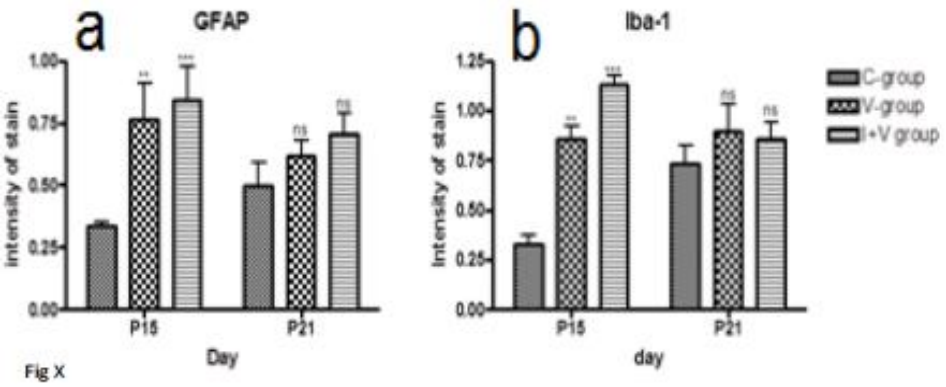

Fig X

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igure X. *In vivo* vanadium exposure during early development results in increased GFAP and Iba1 -positive expression exacerbated with iron deficiency. At PND15 and 21, brains of V, I+V and control rat pups were harvested after lethal injects and astrogliosis (a) and microgliosis (b) quantify using western blot. Data of multiple experiments were pooled together and means evaluated for significance using two-way ANOVA with Bonferroni's post-test comparison to control (ns, not significant; *P<0.05;**P<0.01;***P<0.0001).

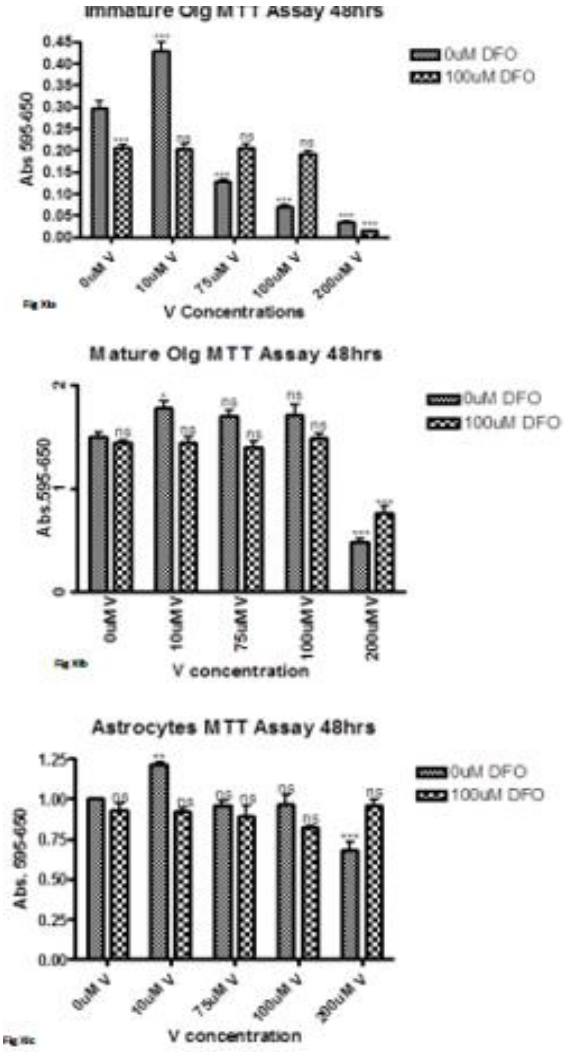

Figure XI. *In vitro,* primary oligodendrocyte progenitors (OPCs) (Fig XIa) are more vulnerable to cytotoxic effects of vanadium than mature oligodendrocytes (Fig XIIb) or astrocytes (Fig XIIc). Primary astrocytes, OPCs and mature oligodendrocytes were exposed to increasing concentrations of Na-metavanadate for 48 h. MTT reagent was added for last 4 h of treatment, cells solubilized and absorbances measured at 595-650 nm. Data of at least four experiments were pooled together and means evaluated for significance using two-way ANOVA with Bonferroni's post-test comparison to control (ns, not significant; *P<0.05;**P<0.01;***P<0.0001).

Since our *in vivo* data showed astrogliosis and microglia activation in the vanadium treated (V and I+V) groups, we quantified these with brain samples of all groups using the same antibodies used for immunostainning. Our results were consistent with the immunostain results with astrogliosis (Fig. X a) and microglia activation (Fig. X b).

Furthermore, because our *in vivo* data showed astrogliosis, and depletion of oligodendrocyte, we investigated if these changes were due to the differences in the relative sensitivity of the glial cells to the cytotoxic effects of vanadium and if iron deficiency can protect the cells from these cytotoxic effects *in vitro.* Enriched cultures of astrocytes, immature and mature oligodendrocytes were raised. We demonstrated that vanadium was cytotoxic to all the cells types. Immature oligodendrocytes were more sensitive to the cytotoxic effects of vanadium with LD50 of approximately 75µM than mature oligodendrocytes and astrocytes which have LD50 of around 200µM. Our results also showed that these cells were protected partially from vanadium toxicity by the iron chelator, desferroxamine only at the LD50 dose for the three cell types (Fig. XI a, b and c).

DISCUSSION

We showed in this study that vanadium exposure caused reduction in body and brain weight gain which was exacerbated with iron deficiency. Our findings are consistent with previous reports of decreased body and brain weights of vanadium exposed animals when compared to controls (Altamirano *et al.,* 1993; Olopade *et al.,* 2011 Sanchez *et al.,* 1991, and Todorich *et al.,* 2011).

Our findings contrast those of Garcia *et al.,* (2004; 2005) who reported that body and brain weight of vanadium exposed rats did not differ from control rats, and physical conditions were almost normal in young adult mice. Thus we can attribute the reason

for the differences in our study to be the age difference in the rats and duration of vanadium exposure used for the experiments. While three month old rats were used for their studies, we exposed our rats from PND1, and noticed weight differences from PND 13-21. Our data in combination with those of Garcia *et al*., (2004; 2005) strongly argue for a developmental effect. Moreover our data on the oligodendrocytes cultures are consistent with the developmental effect concept because the immature oligodendrocytes were more vulnerable than mature. Vanadium is a potent inhibitor of DNA and protein synthesis and affects several metabolic processes (Leonardo and Geber, 1994; Roldan and Altimirano, 1990). Vanadium has more effect on growth when exposed to tissue and animals that are undergoing a high degree of cellular proliferation (Todorich *et al.,* 2011). The reduced brain weight from vanadium exposure is thus most likely the product ofcellular and tissue (Avila-Costa *et al.,* 2004, 2006, Olopade *et al.,* 2011) loss and vacuolation (Igado *et al*.,2012) in the brain.

Our *in vivo* experiments showed that vanadium induced neurobehavioural deficits. Deficits on rotarod, rearing and freezing were worse in I+V group. However, deficits on negative geotaxis, centre square entries, centre square duration, line crossing, grooming, faecal bolus drops and urination was more in V group. In summary, iron deficient and vanadium exposed rat pups fared better in neurobehavioural responses compared to rat pups exposed to vanadium alone. We hypothesize that myelination plays a role in locomotor activities and because in iron deficiency, OPCs and mature oligodendrocytes were partially protected rescuing the myelin, neurobehavioural deficits were relatively less in the iron deficient and vanadium treated (I+V group).

We demonstrated *in vivo* vanadium induced OPCs and mature oligodendrocytes depletion (NG2 and CNPase stains) at PND 15 and 21, similar to the earlier report of Todorich *et al.,* (2011). Also, because iron deficiency confer a partial protection to the OPCs both *in vivo* and *in vitro,* our immunohistochemical staining with MBP at PND 21showed more myelination in I+V group compared to V group suggesting that the iron deficient status minimized the loss of the myelin producing oligodendrocytes. It is likely that though vanadium induced reactive oxygen species affected oligodendrocytes in I+V and V groups, intracellular iron overload which is an additional apoptotic pathway in vanadium induced OPC depletion (Todorich *et al.,* 2011) was relatively spared in I+V group. It had been shown that astrocytes transfected with ferritin constructs rich in intracellular iron were more vulnerable to vanadium induced cell death than those with constructs having minimal iron concentrations (Todorich *et al.,* 2011).

Microglial activation and astrogliosis are commonly observed during the neuroinflammation associated with brain injury, infection, and neurodegenerative diseases (Sunyach *et al.,* 2012). Our results with GFAP, Iba1, and iNOS showed oxidative stress and neuroinflamation induced by vanadium exposure was enhanced by iron deficiency. This enhancing effect of iron deficiency can be attributed to the increased expression of transferrin receptors in I+Vrat pups as a response to increased need for iron in the brains. Incidentally however, this increased transferrin receptors will lead to increased uptake of vanadium through the blood brain barrier (Nagaoka et al., 2004). While iron deficiency induced more astrogliosis and microglia activation after vanadium exposure, it confers a partial protection to OPCs. This consequently protects against hypomyelination in part due to reduced OPC and mature oligodendrocytes depletion resulting from a minimized vanadium induced iron overload in these cells.

In our *in vitro* studies, we manipulated the availability of iron to oligodendrocytes in the presence of vanadium due to the fact that iron is a key component of oligodendrocyte maturation and vanadium's binding to transferrin protein (EVM 2002; Monteiro *et al,* 1991). Interestingly, we show that the partial protection of not only the OPCs but the glia cells in general by the iron chelator, desferroxamine is only at the LD 50 of vanadium. These data are consistent with the earlier report of Todorich *et al.,* (2011). Interestingly, we show that at doses above or below the LD50, iron deficiency exacerbates the toxic effects of the glia cells to vanadium exposure. This will warrant further investigation. In addition however, we observed that vanadium at low doses led to proliferation of astrocytes, mature and immature oligodendrocytes. This was an unexpected effect but may be due to the ability of vanadium to modulate growth-factor-mediated signal transduction pathways and promote cell transformation. This explanation is consistent with vanadium's mitogenic action and its capacity to mimic mitogenic growth factors, and stimulate expression of proto-oncogenes (Stern et al., 1993).

In conclusion, we showed that vanadium induced OPCs depletion, hypomyelination and neurobehavioural deficits were partially protected with iron deficiency. However, vanadium induced reduced body and brain weight gain as well as astrogliosis and microgliosis was exacerbated with iron deficiency. Iron deficiency therefore has a partial protection effect on the myelin profile, and thus suggests that limited iron exposure may be helpful during acute vanadium toxicity.

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