Molecular study on neuroinflammation mediated nerve injury and pain hypersensitivity induced in rats(*)

Mahitab Farghaly[**]

Introduction:

NEUROIMMUNOLOGY field is rapidly advancing and there is a growing appreciation for heterogeneity, both in inflammatory composition and region-specific inflammatory responses. This understanding underscores the importance of developing targeted immunomodulatory therapies for treating neurological disorders. The question therefore remains as to whether inflammatory cells responding to spinal cord vs.brain injury adopt similar functions and are therefore amenable to common therapies.

Damage and/or dysfunction of the nervous system occurs quite frequently in our daily life. It can arise from peripheral nerve damage, metabolic endocrine disturbances, infections, toxins/drugs, cancer, primary autoimmune disorders, injury/disease to the CNS. Irrespective of the cause, a major consequence of such neuropathies is the development of neuropathic pain.

Bacterial cell wall endotoxin from Gram-negative bacteria, Lipopolysaccharide (LPS), LPS signal has been identified by Toll-like receptor 4 (TLR4). LPS is a widely used and powerful tool for the activation of microglia and of peripheral immune cells. Although it has no known direct toxic effect on neurons, it only activates microglia to release a host of neurotoxic factors to induce neuronal death.

Binding of LPS to TLR4 leads to the activation of NF-κB through the recruitment and activation of MyD88, IL-1R kinase

(IRAK), TNFR associated factor 6 (TRAF-6), as well as NADPH oxidase (Nox), thereby regulating the expression of inflammatory proteins associated with inflammatory diseasessuch as TNF-α, IL-1β or IL-6.

The spinal cord is the first relay site in the transmission of nociceptive information from the periphery to the brain. Sensory signals are transmitted from the periphery by primary afferent fibres into the dorsal horn of the spinal cord, the output of the spinal cord is dependent on various spinal mechanisms which can either increase or decrease the activity of dorsal horn neurons. these chemical mediators then act to sensitize nociceptors so that afferent activity to a given stimulus is increased.

Altered protein expression is characteristic for chronic pain and contributes to the development of long-term hyperexcitability of nociceptive neurons in the periphery and the central nervous system (peripheral and central sensitization, respectively).

Pain hypersensitivity due to peripheral sensitization has been shown to occur after inflammation, via activation of intracellular signaling pathways such as protein kinase A (PKA) and protein kinase C (PKC). Moreover activation of p38 MAPK in spinal microglia is a critical link in inflammation-induced spinal pain processing.

Epigenetic alterations correspond to changes in DNA methylation, covalent modifications of histones, or altered miRNA expression patterns. Recent evidence provides a strong case for the use of HDAC inhibitors in the treatment of various brain disorders such as bipolar disorder. Among the growing list of HDAC inhibitors we also find the short chain fatty acid valproic acid (VPA, 2-propylpentanoic acid), which is considered primarily a Class I HDAC inhibitor.

HDAC inhibitors promote the acetylation of histones, which are generally associated with transcriptional activation. HDAC inhibitors also increase the acetylation status and modulate the activity of a wide range of non-histone proteins. Included are inflammatory transcription factors, such as NF-κB and signal transducer and activator of transcription (STAT) 3.

2

Dietary components emerged as a promising source of new epigenetically active compounds able to reverse these alterations and to actively regulate gene expression. The polyphenolic compound curcumin (diferuloylmethane), a yellow spice that enters into the composition of curry, already described for its diverse and broad biological activities, is nowadays well described as an inhibitor of DNA methyltransferase so that it is considered as a DNA hypomethylating agent. It reestablishes the balance between HAT and HDAC (1, 3, 4, 5, 8) activity to selectively activate or inactivate the expression of genes implicated in cancer death and progression, respectively.

MiRNAs are important regulators of diverse neurobiological processes such as neurogenesis, neurodifferentiation, growth, proliferation, and apoptosis MicroRNA-124 is one of the most abundant miRNAs in the brain, a neuronal differentiation promoter that also acts as an inhibitor of neuronal stem cells also play multiple roles in the spinal cord. Mouse microRNA-34a (mmu-miR-34a) expression increases in brain of older rodents. MicroRNA-155 (miR-155) has been implicated as a central regulator of the immune system, acts as a broad limiter of pro-inflammatory gene expression once the miR-146 dependent barrier to LPS triggered inflammation has been breached. Also, miR-155 targets Ship-1, a negative modulator of TLR signalling, and thus also increases TLR4 activity. Likewise, miR-155 stabilizes the mRNA of the pro-inflammatory cytokine TNF-a, which will also regulate the NF-kB response.

The aim of our study is to test the therapeutic potential of adding Valproic acid to Curcumin as combined treatment in lipopolysaccaharides induced CNS inflammation and the effect on the spinal cord inflammation that may lead to pain hypersensitivity as a consequence of immune system stimulation and release of inflammatory cytokines, also to study the role of microRNAs in neuroinflammation and their possible role in pain.

MATERIALS AND METHODS:

Animals and establishment of in-vivo neuroinflammation experimental model:

Eighty male adult SpargueDawley rats (80-150 g) were used in the present study. The animals were supplied and maintained at Technology Center for Research and services, Research institute in which the principles of laboratory animal care were followed in all experimental protocols and were approved by ethics committee of animal research.

Two *experimental models* were established to assess the efficacy of curcumin and valproic acid against neuroinflammation and associated pain hypersensitivity conditions as shown in **Figure1**.
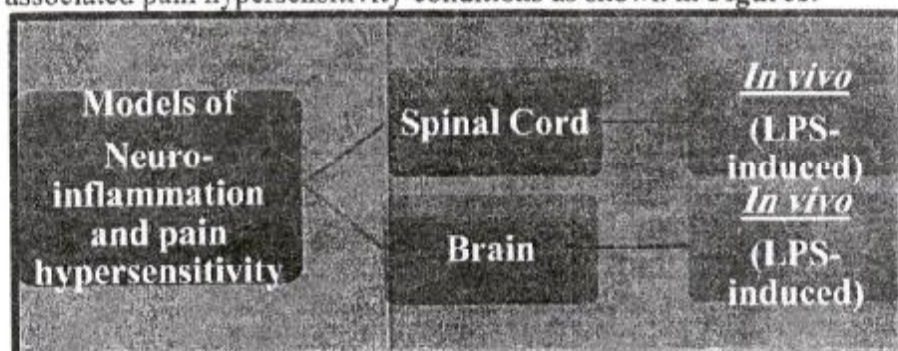
Neuroinflammation induction was established by LPS injection. Experimental designandratsclassificationsare includedin the followinggroups: (1) mock-treatedrats (mock-Trx) thatreceivedemptyvehicle, (2) LPS-inducedratsthat receivedintraperitoneal (IP)injectionof250µg/kg LPS 4 times per week for4weeks,(3)Co-Curratsthatreceivedoral administrationof200mg/kgCurduringLPSinduction,(4)Co-VPA that wereorallyadministered200mg/kgVPA before LPS injection. Treatment was performed using oraladministrationof200mg/kg of Cur(Trx-Cur),VPA(Trx-VPA),ortheircombination(Trx-Cur+VPA)fourtimesperweekfor4weeks.TheCur-VPAtreated ratswereadministeredthetwodosesatthesametime. Also a groupofLPS-inducedratswereleftuntreatedforthe duration of 4

4

weeksinparalleltotreatedonesandreferredasself-recovery
topromoteself-healingmechanism.

DETERMINATION OF OXIDATIVE STRESS MARKERS:

1. *Sera total antioxidant concentration*
2. *Reduced glutathione (GSH) level*
3. *Lipid peroxidation*
4. *Superoxide dismutase (SOD) activity*

TOTAL RNA ISOLATION AND REVERSE TRANSCRIPTASE POLYMERASE REACTION

Total RNA was extracted from frozen sections of brain cortex and spinal cordusing Biozol RNA Isolation Kit.

RNA concentration was determined by measuring the absorbance at 260 nm. The concentration was calculated using the following equation: 1 absorbance unit at 260 nm corresponds to approximate concentration of 40 µg/ml of single-stranded RNA. Purity of the RNA preparation was estimated according to the ratio of absorbance readings at 260 nm and 280 nm [Abs 260/ Abs 280]. Pure preparations of RNA have ratios of 1.8 - 2.0. Alteration in the steady-state mRNA levels of neuroinflammation associated markers were determined using either semi-quantitative reverse- transcriptase PCR (semi-qRT-PCR) or quantitative real time RT-PCR (qRT-PCR).

SEMI-QUANTITATIVE RT-PCR

Semi-quantitative RT-PCR was performed using one-step RT-PCR (RT/PCR Master Mix Gold Beads, BIORON) reaction, We investigated the expression of neuroinflammatory markers APP,BACE1, IL-1β, TNF-α, COX-2 and iNOS expressionsusingthefollowingprimerssets:APP; F-AGAGGTCTACCCTGAACTGC-R-ATCGCTTACAAACTCAC-CAAC(154bp),BACE1;F-CGGGAGTGGTATTATGAAGTG- R-AGGATGGTGATGCGGAAG(320bp), IL-1β; F-TGACTCGTGGGATGATGACG- R-CTGGAGACTGCCCATTCTCG (572bp), TNF-α; F-

5

ATGAGCACAGAAAGCATGATCCGCG - R-CCCTTCACAGAGCAATGACTCCAAA , COX-2; F-TGATGACTGCCCAACTCCCATG- R-AATGTTGAAGGTGTCCGGCAGC (702bp), iNOS;F-GTGTTC CACCAGGAGATGTTG-R-CTCCTGCCCACTGAGTTCGTC (576 bp), andforvalidationweused β-actin: F-GGCATC CTGACCCTGAAGTA-R-GCCGATAGTGATGACCTGACC (565 bp).ProductsofRT-PCRwereseparatedonagarosegel, visualizedanddocumentedusingChemiDoc-It®2 Imagerthen analyzedwithVisionWorksLSAcquisitionandAnalysisSoftware for determinationsofrelativebandsintensity.

QUANTITATIVE RT-PCR ASSAY

Quantitative real time RT-PCR was used to measure the mRNA levels of APE1, COX-2, miR 124, miR34a and miR155. Analyses were performed using mi Script II RT Kit (Qiagen) according to the manufacture guidelines. The primers for APE1 were F-GCTTGGATTGGGTAAAGGA- R-TTCTTTGTCTGATGGAGCTG, COX-2; F- AGGCCTCCATTGACCAGA-R-TCATGGTAGAGGGCTTTCAAC, miR124; AGGCACGCGGTGAATGCC, miR34a; AATCAGCAAGTATACTGCCCT, miR155; CCCCTATCACGATTAGCATTAA, β-actin; F-CCGACAGGATGCAGAAGG- R-GGAGTACTTGCGCTCAGGAG, and U6; F-GGAACGATACAGAGAAGATTAGC- R-AAATATGGAACGCTTCACGA.Geneexpressionresultsof indicatedgenesandmiRNAswerenormalizedto β-actin andU6, respectively,folddifferencecalculatedasdescribedbefore(Livak and Schmittgen,2001).

PREPARATION OF CYTOSOLIC AND NUCLEAR EXTRACTS AND WESTERN BLOTTING

The extraction of nuclear proteins was performed by homogenates of brain tissue using hypotonic buffer, the supernatants (cytoplasmic extracts) were stored at -80°C and the pellets were washed in 200µl of

6

hypotonic buffer and re-centrifuged. The pelleted nuclei were re-suspended in 50µl of ice-cold nuclear extract (NE) buffer and incubated on ice for 20 min, with occasional mixing, then centrifuged at 14,000 xg for 15 min at 4°C. APE1 (sc-17774), and COX-2 (sc-7951) immunoblots were performed on prepared cytosolic and total cell extracts respectively were used and equal loading was confirmed by probing with β-actin (sc-81178) monoclonal antibody.

STATISTICAL ANALYSIS

Experiments were repeated two or more times independently and graphs are represented as mean±SD. The difference between groups was analyzed by one-way analysis of variance (ANOVA) and the difference considered significant either at $p < 0.01$ or at $p < 0.001$ when compared to mock-treated group.
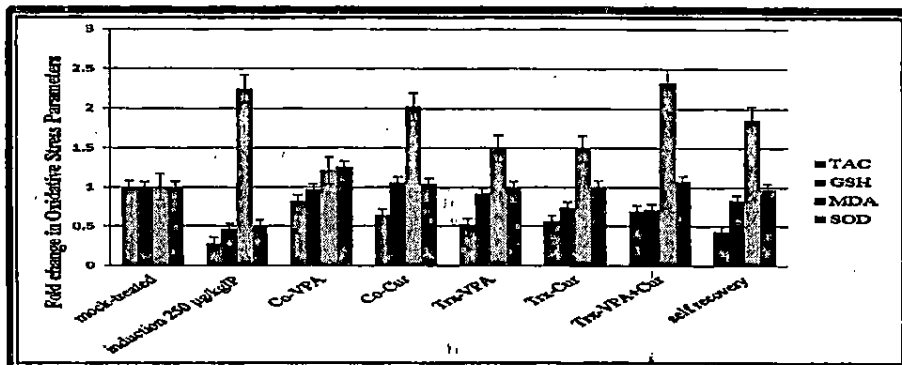
Results:

Figure 2: Fold change of the oxidative stress markers in the different experimental groups calculated in reference to mock-treated rats. Significant reduction in the oxidative stress markers including sera TAC, Tissue GSH and SOD by around 0.5 fold were observed in the induced rats. MDA level was elevated by around 2 folds. Co- treatment with VPA or Cur exerted protective effect and reduced LPS-induced changes by around 50%. Furthermore, treatment of induced rats with Cur, VPA or their combination significantly restored sera and brain tissue antioxidant capacity by around 50 % compared to self-recovery rats.
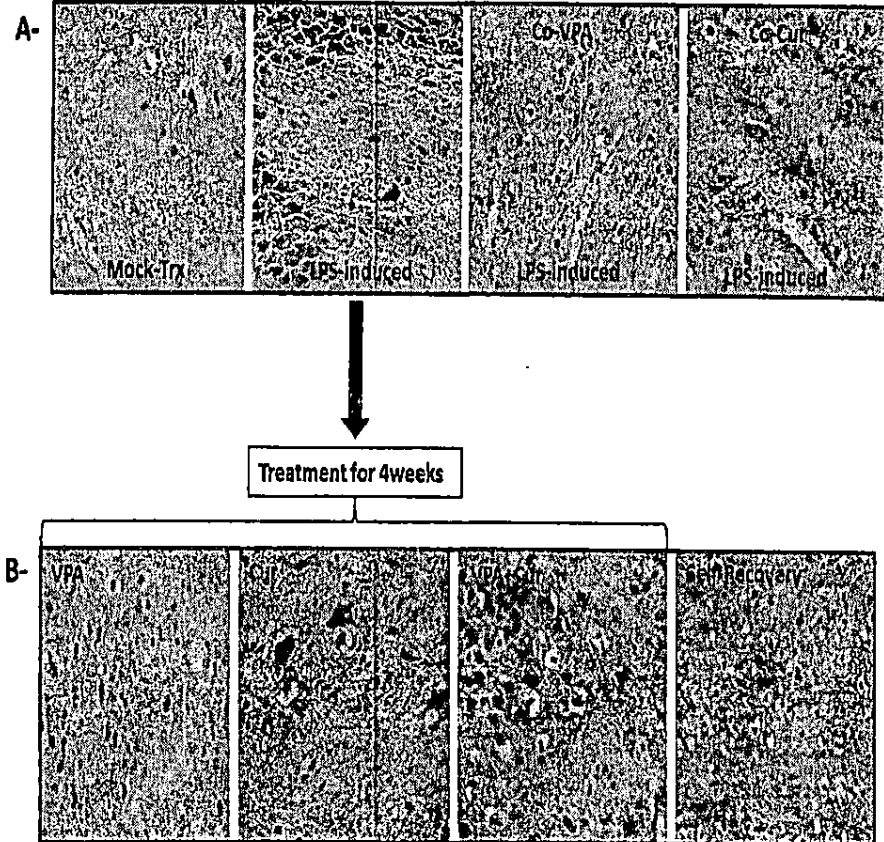
7

Histological Examination:

Figure 3: H & E staining of Cortex sections from different experimental groupsat 400X.

Mock treated animals show normal cell parenchyma and architecture, while **LPS-induced treated** rats show chronic inflammatory cell infiltration, increased vascularity, edema and a collection of degenerated cells. While **Co- treatment,** during LPS induction, with **Curcumin** show less chronic inflammatory cell infiltration than **Valproic** acid which shows significant inflammatory cell infiltration and gliosis, which proves the protective effect of curcumin. Moreover, **4-weeks treatment** of induced rats indicated

that; **Cur -Trx** show less collection of lymphocyte and chronic inflammatory cell infiltration than **VPA-Trx** while their combination **(VPA+Cur)** show normal cell architecture and parenchyma which proves that combined treatment is more effective than treatment with each one alone. **Self-recovery** group, that were induced and left for the duration of treatment to establish self-healing mechanism, show persistent chronic inflammatory cell infiltration and degenerated cells which proves that the treatment is more effective and that the rat needs more time to restore the normal state alone without treatment.

Semi qRT-PCR of neuroinflammation associated markers:

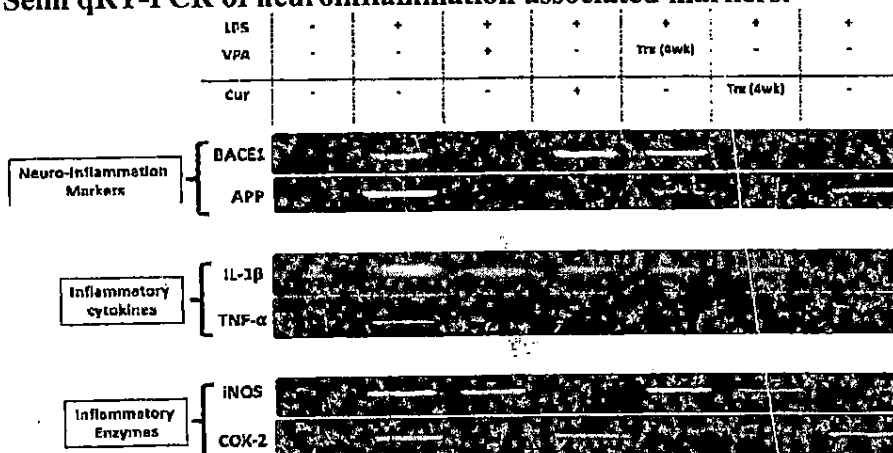|	LPS	.	+	+	+	+	.	+	+
	VPA	.	.	+	-	Trx (8wk)	.	-	-
	Cur	.	.	-	+	-	Trx (4wk)	.	-
Neuro-Inflammation Markers	BACE1								
	APP								
Inflammatory cytokines	IL-1β								
	TNF-α								
Inflammatory Enzymes	iNOS								
	COX-2								

Figure 4: Semi-quantitative analysis (SemiqRT-PCR) of neuroinflammation associated markers. Gene expression analysis of neuroinflammation indices including inflammatory protein (APP), enzymes (BACE1, COX-2 &iNOS) and cytokines (IL-1β, TNFα) were analyzed in the different experimental groups. LPS induced brain inflammation and glial cells activation as indicated by marked expression of inflammatory enzymes and cytokines. Cur or VPA co administration inhibited, in part, glial cells activation and release of IL-1β, TNFα as well as production of inflammatory enzymes and proteins. Our data also indicate that the therapeutic effects of either Cur, VPA or their combination is mediated partially by inhibiting the neuroinflammation markers production in the brain of injured rats compared to self-recovery rats that were left for 4 weeks without treatment.
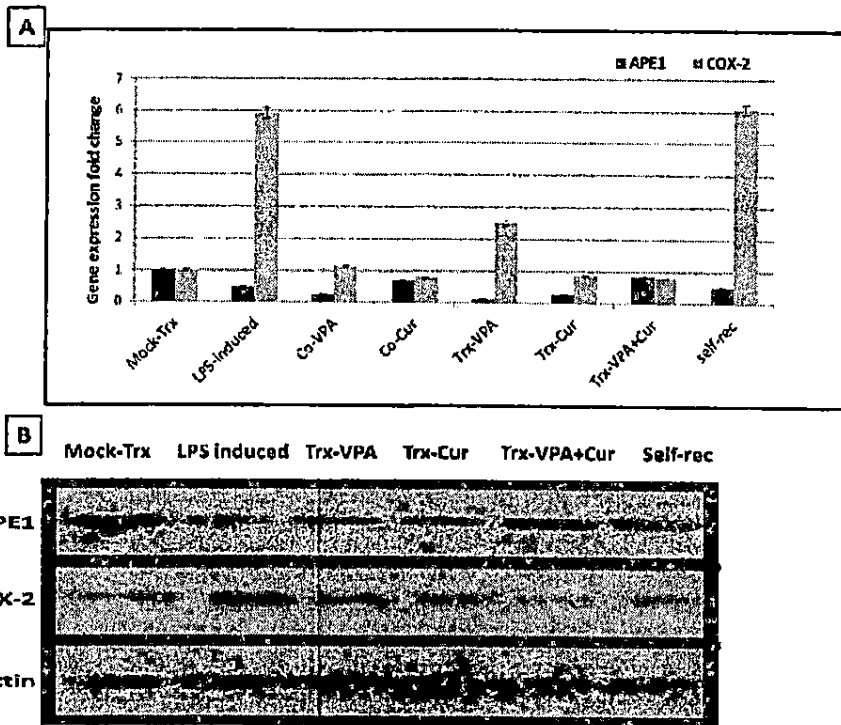
9

Figure 5: **Expression profile of inflammatory associated enzymes** Altered expression of COX-2 as an inflammatory markers has been reported previously. It has been also shown that curcumin is a selective COX-2 inhibitor. Therefore in the current study we determined COX-2 as well as the pro-survival protein APE1 expressions at both mRNA and protein levels. Our data indicated that Induced group shows a significant reduction ($p<0.01$) of APE-1 with significant increase ($p<0.01$) in COX-2 mRNAs expressions at week 4 of LPS administration compared to mock treated rat's cortex. Co-Cur group maintained elevated APE-1 and reduced COX-2 levels (mRNA and proteins) compared to induced rats. Furthermore treatment of induced rats with VPA+Cur combination induced significant elevation ($p<0.01$) in APE-1 mRNA level and protein as well as significant reduction ($p<0.01$) in COX-2 mRNA and proteins as a sign of ameliorated neuroinflammation compared to self-recovery rats. Protein profile showed consistent pattern of expression compared to the alterations in mRNA contents.
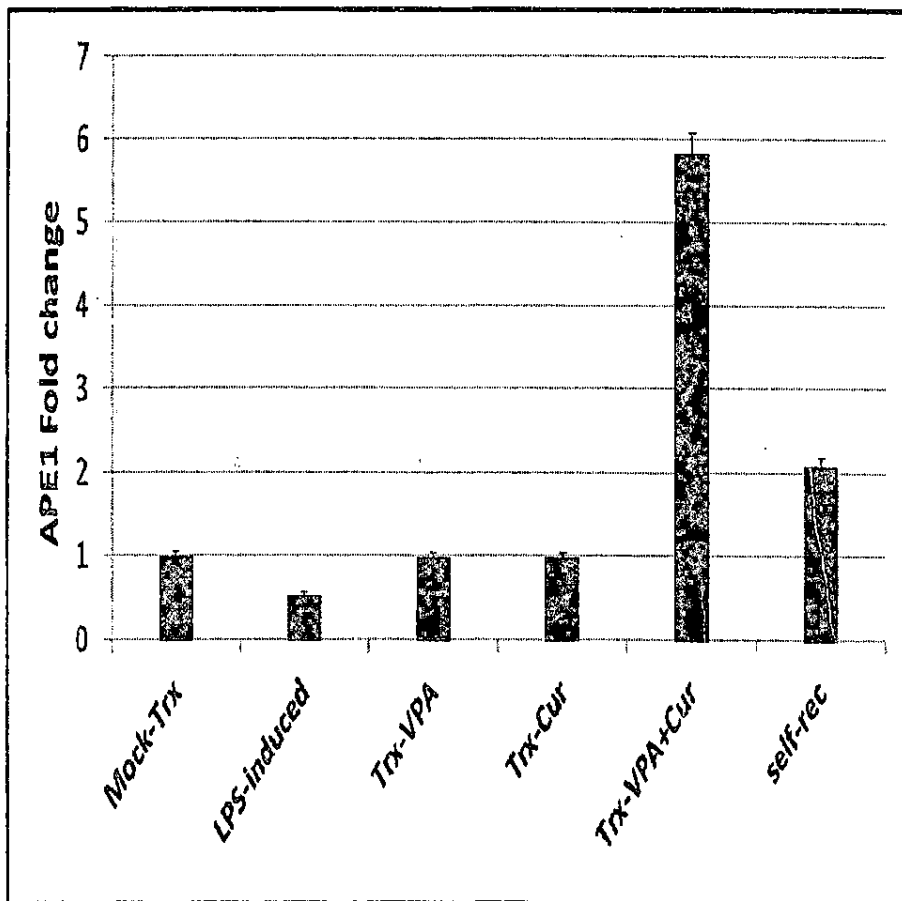
10

Figure 6: Quantitative analysis of APE1 expression in spinal cord tissues of induced and treated rats. QRT-PCR results showed significant reduction (p<0.01) in APE-1 expression in LPS induced group at week-4 of induction compared to mock treated rats. Treatment with VPA+Cur combination, induced significant expression (p<0.01) of APE1 compared to groups received either Cur or VPA alone, which indicate that adding VPA exerts synergistic effect on curcumin in enhancing tissue recovery and increasing capacity for DNA repair mechanism by inducing APE1. Moreover elevation of APE1 level was also observed in self-recovery group which confirm APE1 role in tissue healing mechanism.
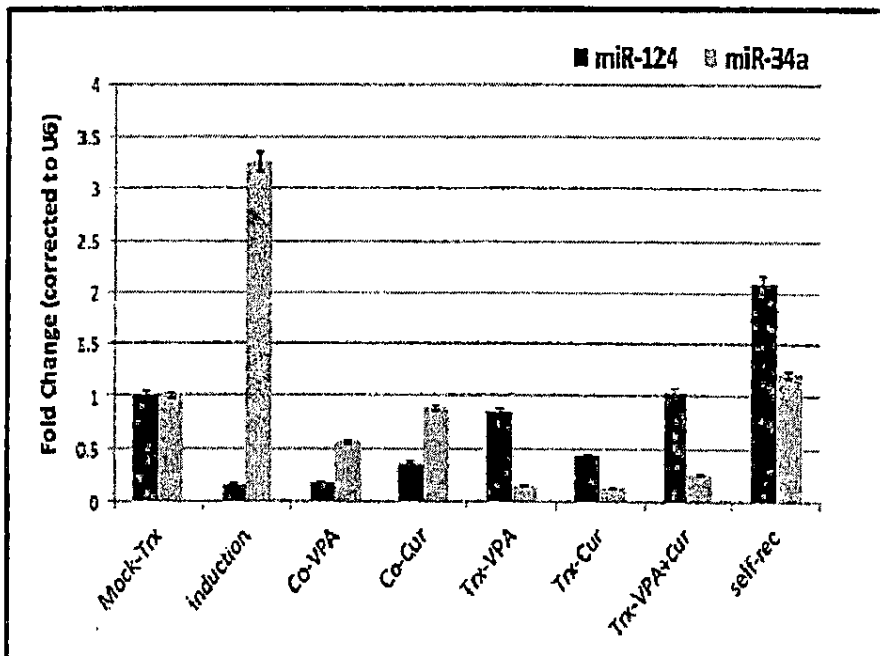
11

Figure 7: Altered profile of miRNA biogenesis in the cortex tissues of the different experimental groups.MiRNAs expressions were detected using quantitative real time PCR (qRT-PCR). Our data indicate that miRNA 124 is centrally down-regulated by 0.75 fold in LPS induced rats with concomitant increase in miRNA 34a (by 2.25 fold) versus mock-treated rats. Co administration of Cur or VPA during the course of induction restored partially and preferentially the expression levels of both miRNA towards normal profile. Moreover treatment of induced rats with VPA+Cur combination induced significant (p<0.01) restoration of miRNA-124 and miRNA-34a levels compared to the groups treated with either Cur or VPA alone for 4 weeks. Self-recovery group showed significantly higher expression levels of both indicated miRNAs which imply that adding adjuvants to inflamed tissues affect gene expression and enhance tissue recovery by different mechanism for normal wound healing.
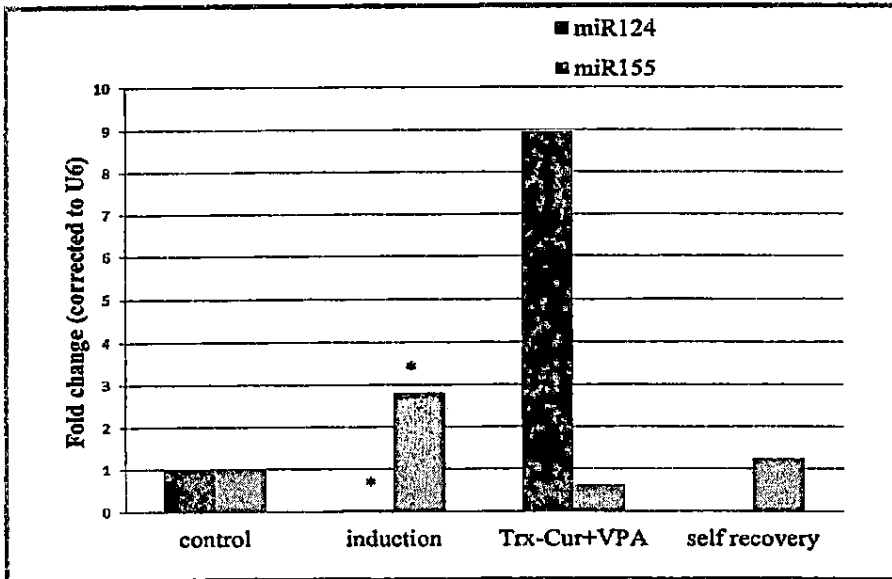
12

Figure 8: Expression levels of miR-124 and miR-155 in induced versus treated groups. Our data in consistent with previous reports indicated significant (p<0.01) reduction in miR-124 while miR-155 induced significantly (p<0.01) upon LPS induction. Treatment with VPA and Cur combination for 4 weeks restored normal expression profile of both indicated miRNAs compared to the Self-recovery group, that showed altered profile during the healing mechanism that follow chronic LPS-induced neural toxicity.

Discussion

The results of the present study show that ip LPS injection indeed induced brain inflammation. Microglia became morphologically activated; neutrophils infiltrated the brain; and inflammatory mediators, including interleukin-1β **(IL-1β)**, tumor necrosis-alpha **(TNF-α)**, IL-6, and iNOS were synthesized (Figure 4). However, no neuronal death was evident (Figure 3). These results indicate that systemic inflammation indeed causes brain inflammation, but to only a mild extent and does not result in neuronal death.

Histological analyses indicated that co-administration of either VPA or Cur inhibited LPS-induced lymphocyte infiltrations in the cortex tissues. However treatment of induced rats with VPA, Cur or

13

their combination for four weeks post LPS-induction, exerted neuronal recovery, but the most significant improvement was observed upon treatment with VPA-Cur combination as confirmed by H & E staining (Figure 3).

We investigated the anti-oxidant effect of VPA, Cur and VPA-Cur combination by measuring tissues MDA and GSH levels as well as SOD activities. Our results clearly demonstrate that Co-Cur administration inhibited significantly LPS-induced brain toxicity and oxidative stress compared to Co-VPA group. In addition to, treatment with Cur alone or in combination with VPA restored oxidative stress balance significantly (close to normalization) compared to both VPA-treated and self-recovery groups (Figure 2).

Consistently in the present study, by investigating APE1 expression in LPS-neuroinflammation established model, the results revealed significant reduction of both APE1 mRNA and protein levels compared to mock treated rats. Furthermore, we show that Cur co-administration maintained significantly elevated APE1 level during the course of LPS-induction compared to induced rats. Furthermore we found that VPA-Cur treatment was more effective than either Cur or VPA treatments alone in restoring high APE1 expression profile (Figures 5, 6). This suggests that Cur and VPA-Cur combination actions are mediated, in part, by maintaining elevated APE1 level.

We also extended our study to test both COX-2 and iNOS expressions as strongly inhibited targets by Cur-induced anti-inflammatory effects. Our data established that co-expression of inflammatory proteins COX-2,iNOS and amyloid peptides were higher in the LPS-treated rats. However, curcumin/valproic acid decreased the LPS-induced expressions of COX-2, iNOS and amyloid peptides. Curcumin administered rats either alone or in combination with VPA exhibited the most significant inhibition levels on COX-2 and iNOS expressions in their brain tissues extracts (Figure 5).

In order to investigate the role of miR-155 in inflammation-induced neurovascular dysfunction with leucocyte-triggered immune responses such as TNFα secretion, we determined miR-155 level in our acute inflammation model induced by intraperitoneal injection of

14

lipopolysaccharide (LPS). LPS administration induced a 2-fold increase of miR-155 rats compared to PBS-treated miR-155 rats (Figure 8).

Among the tissues and organs examined in normal rat, brain expressed relatively high levels of miR-124 (Figure7). In the presence of Curcumin&valproic acid combination (potentiated effect), which effectively decreased LPS-induced serum IL-6 and TNF-α levels, the miR-124 level was further increased by 2-fold in the brain. As previously reported and shown in Figure 7, miR-124 is highly expressed in the central nervous system and microglia, which function as macrophages in the CNS.

Our miRNAs profile showed altered expression during induction with downregulated expression of miR-124 by 1 fold accompanied with upregulated expression of miR-34a by 2 folds compared with mock treated group, a modified expression was observed in Co-Cur than Co-VPA group for miR-124 due to transcriptional regulation by NF-κB, while VPA has more effect on miR-34a expression (figure 7).

While combined treatment of Cur-VPA showed that expression of miR-124 was upregulated while miR-34a was down regulated compared to self-recovery group which confirm their involvement in the restoration of normal state of quiescent resting microglia cells and attenuation of pain with restoration of normal brain state (figure7).

Our data demonstrate the critical role of miR-155 in regulating neuropathic pain, and suggest that miR-155 may be an important and potential target in preventing neuropathic pain development. We analyzed the *in vivo* effect of miRNA-124 on spinal cord injury (SCI) in rats. In agreement with Dr Yuji Nakamachi research (just published on line Jan. 16, 2015) we found that miR-124 ameliorated rat pain by suppressing critical prerequisites for spinal cord, such as cytokines and NF-kappa B. Thus, miR-124a is a candidate for therapeutic use for human SCI (Figure 8)