Chronic Inflammatory Pain Management by BDNF Modulation: A Comparative Study of Gabapentin, Indomethacin and Their Combination on Arthritis Rat Model

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Research Article

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Abstract

Brain derived neurotrophic factors (BDNF) can be secreted either as a pro-BDNF or a mature form (mBDNF) through γ -amino-butyric acid (GABA_A) receptors activation. Depolarization of GABA neurons in spinal cord can be mediated through N-methyl-D-aspartate (NMDA) receptor due to the exogenous secretion of over expressed BDNF. This over expressed BDNF further modulate the excitation and sensitization of nociceptors. We investigated the modulation of BDNF by GABA_A agonist i.e., gabapentin, indomethacin (non-steroidal anti-inflammatory) and their low-dose combination on adjuvant-induced inflammatory arthritis. *Mycobacterium tuberculosis* H37Ra strain, as adjuvant, was injected in the tail base of female Sprague-Dawley rats. Gabapentin (5 mg/kg), indomethacin (5 mg/kg) and low dose combination of gabapentin (1.5 mg/kg) + indomethacin (2.5 mg/kg) were used. Paw edema was measure by plethysmometer and chronic pain was measured by plantar apparatus. Nitric oxide, peroxide and superoxide dismutase levels were also measured to analyze the free radical and antioxidant balance in different treatment groups along with the total protein concentration. Significant reduction of nitric oxide, peroxide as well as total protein concentration was found in low dose combination. Immunohistochemistry data also showed that the low dose combination has more potential in lowering the BDNF expression in cortex as well as in hippocampus region of brain as compared to their mono therapies. Our study suggested that low-dose combination of gabapentin and indomethacin has a significant impact on lowering the chronic and its associated markers as compare to their monotherapy. Thus indicated the additive effects of the combination therapy on neuropathic pain.

1. Introduction

Pain can be characterized as pinching, throbbing, aching, stabbing or steady sensation faces by patients with rheumatoid arthritis (RA) [1–3]. While as a sense of touch, pain can also be a vital parameter to forecast the associated psychological and physiological disability. Although available therapies such as disease modifying anti-arthritic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids showed great potential in reducing the progression of pain. While number of therapies fail to respond in some patients [4], Co-morbidity or advanced disease along with the destruction of joints may provide an explanation for this condition. Nevertheless, failure of pain management therapies cannot be ignored.

Nociceptors are the special subpopulation of peripheral nerve fibers responds to the chemical, mechanical or thermal stimuli [5]. When external stimuli reached above the noxious range, they activate the nociceptors to respond the potentially damaging stimuli. On the basis of type of stimuli and nature of nociceptors [6], pain can be described as (a) acute or short acting pain, and (b) persistent or chronic pain [6]. In acute pain, the short acting secretion of glutamate neurotransmitter further regulates N-methyl-D-aspartic acid (NMDA) receptor activation [7]. Activation of NMDA receptor further facilitated the up-regulation of BDNF in chronic pain [8]. On the other hand, the chronic pain is initiated through any noxious stimulus which is maintained for a longer duration or more than two months [9].

Brain derived neurotrophic factors (BDNF) can be secreted either as a pro-BDNF or a mature form (mBDNF) [10–12] through γ -amino-butyric acid (GABA_A) receptors activation. Depolarization of GABA neurons in spinal cord can be mediated through N-methyl-D-aspartate (NMDA) receptor due to the exogenous secretion of over expressed BDNF. This over expressed BDNF further modulate the excitation and sensitization of nociceptors [13] in chronic pain [12, 14, 15]. In some cases, the nerve injury or inflammation in peripheral nervous system can also causes the excitability of spinal neurons [16–18]. Either due to any reason, chronic pain and its sensitivity have distinguished by two important hallmarks i.e., hyperalgesia and allodynia which are primarily regulated through over expression of BDNF [14, 19–22].

The present study emphasis on the GABA and BDNF interplay in the adjuvant-induced chronic inflammatory pain model and modulation of over expressed BDNF by gabapentin, indomethacin and its low dose combination. Gabapentin (2-[1-(amino methyl) cyclohexyl] acetic acid), an analog of γ -amino butyric acid (GABA), clinically it is used for the treatment of peripheral and central neuropathic pain and post-herpetic neuralgia [23]. Studies suggested that the therapeutic efficacy of gabapentin in reducing neuropathic pain is varied due to its less coverage on different pain modulatory pathways [23]. Indomethacin is a non-steroidal anti-inflammatory used to treat inflammatory disease like gout, spondylitis, osteoarthritis and rheumatoid arthritis [24]. Various studies reported that indomethacin is inactive in chronic pain modulation but some studies also suggested that it has an additive effect in pain relieving while use with other anti-inflammatory drugs [24–26].

In this study we used Gabapentin and indomethacin and their low combination for chronic pain management therapy by targeting brain derived neurotrophic factor (BDNF) associated chronic pain pathway. The low doses of gabapentin and indomethacin were selected on the basis of our pilot study where mild symptoms relieving pain plus minimum side effects of gastric ulcer from indomethacin emerged.

2. Methods And Materials

2.1 Animals

Female Sprague-Dawley (SD) rats weighing 180–220 g (6–10 weeks) were used, since female rats are more prone to quick development of arthritis as compare to their male counterpart due to their sex chromosome and estrogen hormone association [27]. The animals were housed at Animal Research Facility, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. The housing facility was controlled at ambient room temperature ($21 \pm 2^\circ\text{C}$), relative humidity of $50\% \pm 10\%$ in a 12-hr light/dark cycle. All procedures were conducted according to the institutional guidelines of Scientific Advisory Committee on Animal Care, Use and Standards by International Center for Chemical and Biological Sciences (ICCBS), University of Karachi (protocol # 2018-0005) and guideline of United States National Institutes of Health guide for the care and use of laboratory animals (NIH Publications, 2011 edition: eight (8)). The group size was determined as the minimum number of rats for valid

statistical analyses based on a pilot study. The group size of 12 has an 80% power to detect differences in the mean. Animals were divided into five different groups (1) normal control (2) arthritis only (3) indomethacin (4) gabapentin and (5) low dose combination of gabapentin + indomethacin treated. All experiments were repeated at least three times independently.

2.2 Induction of arthritis:

Lyophilized *Mycobacterium tuberculosis* H37Ra (MT H37Ra) was purchased from BD™ Difco™ Laboratories (Detroit, USA). A suspension of 10 mg/ml of fresh adjuvant was prepared using mineral oil at the beginning of experiment for the arthritis induction. Intradermal injection of 0.1 ml of suspension was injected at the base of rat tail using a sterile hypodermic needle under a combination of ketamine/xylazine (20 mg/kg/5 mg/kg) anesthesia [28, 29]. Treatment was initiated on the same day of adjuvant injection and count as day 0 and all values were taken as triplicate to get better precision of data set.

2.3 Drugs:

Gabapentin was purchased from Sigma-Aldrich (USA) and indomethacin was obtained from MP Biomedicals (USA). Phosphate-buffered saline (PBS) was used to prepare the suspension of indomethacin (5mg/kg and 2.5 mg/kg) and gabapentin (5mg/kg and 1.5 mg/kg). The gabapentin and indomethacin were injected intra-peritoneally on daily basis, alternatively on the right and left peritoneum region to avoid any bruises and tissue damage. Once the full-blown arthritis was established, the animals were humanely euthanized to collect brain and serum samples for further processing.

2.4 Clinical assessment of the adjuvant- induced arthritis:

The paw volume assessment is a method to quantify the edema of joints, and was done through plethysmometer (Model # 7140; Ugo Basile, Italy). The advantage of using this method over diameter measurements of tibio-tarsal joint is that it can measure the limb in three dimensions and therefore takes into account any variability of the pattern of swelling of individual limbs. The volume of a hind paw is reported as the mean $\pm$ of standard error in milliliters.

The clinical severity of inflammation through arthritis like symptoms was also determined by quantitating the change in the body weights to assess vulnerability of disease [30]. All measurements were made at the same time of the day. Weight variation and plantar test were performed to analyze the severity and effect of pain on weight gaining and nociception respectively and paw edema volume test is used to confirm the swelling and inflammation of joints.

2.5 Nociceptive assay / Thermal hyperalgesia:

All groups of rats were evaluated for nociception assay [31] by using plantar test apparatus (UGO Basile, Italy). Animals were placed in transparent Lucite cubicle and were acclimatized for at least 20 minutes before taking readings. Sensitivity to noxious heat was assessed by using a plantar stimulator, which was positioned under a glass substrate, directly beneath the hind paws. The time was recorded as paw

withdrawal latency (PWL) from the initiation of the radiant heat until the paw withdrawal. A maximal time of 15 seconds was used to prevent tissue damage. Each paw was tested three times and the mean withdrawal latency was calculated.

2.6 Total protein

Total protein was also estimated in plasma of control and test groups. Various studies suggested that pro-inflammatory markers like tumor necrosis factor alpha (TNF- α) and other markers of inflammation such as leukotriene B₄, prostaglandin E₂ and platelet activating agents may increase the total protein concentration in plasma [32–34] reported that, total concentration of protein in non-painful and effusion free joints is significantly lower than total concentration of protein in painful joints and joints effusion [35–37] due to the fact that healthy synovial membrane is impermeable to the high molecular weight plasma proteins [38]. The permeability of plasma proteins increases with the gradual increase in the inflammation and perforation of synovial membrane [39]. But the content and amount of total protein might differ in each joint due to the degree of inflammation and permeability of blood vessels [33].

2.7 Estimation of Oxidative Stress Parameters in Serum

2.7.1 Nitric Oxide (NO) and Peroxide (PO)

The NO and PO are free radicals normally detoxified by antioxidants in healthy condition, but generated extensively in inflammatory condition like arthritis and other inflammatory diseases. Evidences also suggested that the implication of high level of NO and PO in the development of chronic pain [40]. Estimation of PO and NO was determined by Quantichrome DINO-250 Nitric oxide assay kit and peroxide assay kit (Bioassay systems, USA) according to the user manual. Optical density was taken at 540 nm and data was calculated by standard curve method.

2.7.2 Superoxide Dismutase (SOD) Assay

Superoxide dismutase (SOD) is a part of antioxidant mechanism of biological system in inflammatory condition. Whenever antioxidant level drops, it increases the level of free radicals (NO, PO). For the quantification of SOD, Superoxide Dismutase Assay Kit (Cayman Chemical Company, USA) was used. All experiments were performed according to the manufacturer's protocol. The optical density was taken at 450 nm and results were calculated through standard curve method.

2.8 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis of BDNF mRNA Expression:

Following cardiac perfusion with heparin-PBS solution, animals were decapitated and the brain samples were collected. The region of interests (ROIs) i.e., amygdala, cortex, hippocampus and thalamus were dissected-out and placed in TRIzol™ reagent (Invitrogen, USA) and stored at -80°C for subsequent RNA extraction. Total RNA was purified using TRIzol™ reagent and the manufacturer's protocol was followed. Briefly, frozen tissue sections prefilled with TRIzol according 100 mg: 1 ml ratio at the time of dissection and incubated for few minutes at room temperature and then homogenized. The homogenates were

subjected to phase separation through chloroform, aqueous layer precipitation by propane-2-ol. RNA pellet was washed with 75% absolute ethanol and re-suspended in 30 µl of diethyl pyrocarbonate (DEPC) treated water. The purity and the quantification of the RNA were determined through NANODROP™ 2000 (Thermo fisher Scientific, USA). Samples exhibiting an absorbance ratio (A_{260}/A_{280}) greater than 1.75 or equal to ~ 2.0 were stored at -80°C for cDNA synthesis.

For cDNA synthesis, reverse transcription of RNA samples was performed using Superscriptase III First-Strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (product size: 450bp) gene was used as an internal standard. The cDNA was then amplified through PCR GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) with BDNF primer sequence of (F: 5'-GGGTGAAACAAAGTGGCTGT-3') (R: 5'-ATGTTGTCAAACGGCAGCAAA-3'); and GAPDH (F: 5'-GGAAAGCTGTGGCGTGATTGG-3') (R: 5'-GTAGGCCATGAGGTCCACCA-3') (product size: 175).

The amplified PCR products were resolved in 1% agarose gel with ethidium bromide as a DNA intercalating dye. Samples were carefully loaded to avoid the cross mixing between the wells and the electrophoresis was run for 70 minutes at 70 mV. Along with samples, ladder was also run to confirm the product size. The gel bands were visualized through CCD under the UV Gel-Doc System (Alpha Innotech) and quantified through NIH ImageJ software.

2.9 Immunohistochemistry of BDNF on Brain Sections

At the end of each experiment, animals were trans-cardially perfused under anesthesia with cold phosphate-buffered saline (PBS) containing a 100 U/ml of heparin. Once animals were decapitated, brain samples were removed and post fixed in 4% paraformaldehyde (PFA) and then cryoprotected overnight in 30% sucrose in PBS at 4°C. Optimal cutting temperature (OCT) media was used to make blocks for cryo-sectioning and placed in the -20°C until the OCT media was frozen. The sample blocks were placed in the cryostat to equilibrate at -20°C prior to cryo-sectioning for at least one hour. The coronal sections of 35 µm were cut and directly transferred onto the poly-L-lysine coated slides and stored overnight in a humid chamber at ambient temperature. Next day, the sections were re-hydrated by rinsing with PBS followed by incubation in blocking solution containing 2% BSA, 2% normal goat plasma and 0.1% Tween-20 for 1 hr at 37°C. The immunostaining was performed by incubating sections with purified BDNF (N-20) rabbit polyclonal IgG primary antibody (Santa Cruz, USA) for 2 hr at 37°C. After washing with PBS, the tissue sections were incubated with secondary antibody i.e., Cy3® anti-rabbit (IgG ABCAM) for 1 hr. at room temperature. The slides were examined by fluorescence microscope (Nikon 90i, Japan). Images were taken through CCD camera using NIS Element AR 3.2 software and further processed through NIH ImageJ and adobe Photoshop software.

2.10 Statistical Analysis

The data was analyzed by using SPSS 20 and represented as a mean $\pm$ standard error of mean (SEM) of multiple experiments ($n = 3$). Each group was compared by using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test analysis. The p -value of ≤ 0.05 was considered as significant for comparing all groups.

3. Results

3.1 Effects of Gabapentin, Indomethacin and their Low Dose Combination on Body Weight, Paw Volume and Plantar Test

Total body weight was calculated for each rat on day 0 of the study and thereafter on every alternate day until the end of the experiment. The increase in the mean body weights of the non-arthritic rats was observed as compared to arthritic rats which shows gradual decrease in body weights after day 8 onwards ($^{\#}p < 0.04$) and becomes persistent and severe at day 14 ($^{###}p < 0.001$). Arthritic rats given gabapentin and indomethacin either as a single agent therapy or in combination also showed a gradual increase in the body weights over a period of 14 days unlike to arthritic rats but this increase was statistically non-significant (Fig. 1a).

Figure 1b shows that there was a significant difference in the paw edema of normal control rats and arthritic control ($^{\#}p < 0.04$). On the last day of treatment i.e., day 14, a chronic inflammation was observed with maximum thickening of the joints ($^{###}p < 0.001$) in rats injected with adjuvant with no other treatment given (arthritis only). Inter-comparison between arthritic control group and low dose combination therapy of gabapentin (1.5 mg/kg/s.c.) + indomethacin (2.5 mg/kg/i.p.) shows promising reduction of joints thickening and inflammation in right paw from day 12 and on day 14 in left paw ($^{**}p < 0.005$). However, no significant differences were found between arthritis only and monotherapy groups.

The latent time to stay at the base i.e., paw with-drawl latency time (PWL) for arthritis only group shows that a gradual decreasing from day 8 to day 12 as compare to normal control ($^{\#}p < 0.02$) until day 14 of immunization where it achieves maximum significant reduction in PWL time ($^{###}p < 0.0007$). In the treatment group of gabapentin monotherapy, there is a significant increase in PWL time when compare to the arthritis only from day 4 ($^{***}p < 0.001$), day 6 ($^{****}p < 0.0001$), day 8 ($^{****}p < 0.0001$), day 10 ($^{***}p < 0.007$), day 12 ($^{****}p < 0.0001$) and day 14 ($^{***}p < 0.0003$). GBP-Indo combination therapy group also shows significant increase in the PWL time from day 4 ($^{**}p < 0.005$), day 6 and day 8 ($^{****}p < 0.0001$), day 10 ($^{***}p < 0.008$), day 12 ($^{***}p < 0.004$) and day 14 ($^{****}p < 0.0001$). While indomethacin monotherapy group shows no significant increase in PWL time when compare to arthritis only (Fig. 1c).

3.2 Measurement of Reactive Oxygen Species

3.2.1 Peroxide and Nitric Oxide

Peroxide and nitric oxide analysis were performed on serum and plasma samples respectively from control and test groups. A significantly increased concentrations of PO ($^{##}p < 0.01$) and NO ($^{###}p < 0.001$) was observed in the arthritis only group as compared to the normal control group (Fig. 2a & 2b).

Next, we compared the treatment groups with the arthritic control animals. It was observed that indomethacin only group significantly reduced the PO level ($^{*}p < 0.02$) as well as the low dose combination of gabapentin and indomethacin ($^{*}p < 0.05$ for PO $^{**}p < 0.01$ for NO).

3.2.2 Superoxide Dismutase (SOD)

For SOD, inter-comparison of arthritis only and normal group and different treated groups with arthritis only did not show any statistically significant results (Fig. 2c).

3.2.3 Total Protein Estimation

In total plasma protein estimation, arthritis only group shows significant increase in total protein as compared to the normal ($^{##}p < 0.01$). While in treatment groups indomethacin only and low dose combination shows significant decrease as compare to the normal ($^{**}p < 0.005$; $^{*}p < 0.01$ respectively) (Fig. 3).

3.3 Reverse Transcriptase Polymerase Chain Reactions (RT-PCR) Analysis for BDNF Gene Expression

To measure the BDNF gene expression in different groups, four ROIs of brain samples were processed for RNA isolation in order to locate the region, which showed pronounce expression of BDNF in chronic pain condition. The results showed basal level of BDNF expression in amygdala, cortex, hypothalamus, hippocampus regions of the normal brain samples. Unlike normal control, the arthritic control group demonstrated significantly high expression of BDNF in amygdala, cortex, and hypothalamus ($^{####}p < 0.0001$). However, in hippocampus the BDNF expression was found to be significantly reduced ($^{####}p < 0.0001$) (Fig. 4). The comparison between different treatments groups and arthritic only group revealed a highly significant reduction in BDNF expression in amygdala and cortex ($^{****}p < 0.0001$; Fig. 4a & 4b). However, the expression pattern was different in hypothalamus and hippocampus. Indomethacin and low dose indomethacin and gabapentin combination demonstrated significantly reduced expression of BDNF ($^{****}p < 0.0001$) in hypothalamus, whereas, gabapentin monotherapy significantly increased the BDNF expression in hypothalamus as compared to the arthritis only ($^{****}p < 0.0001$; Fig. 4c). Samples from hippocampus shows reduced level of BDNF expression in arthritis only while all three treatment groups show relatively high expression of BDNF as compared to arthritis only ($^{****}p < 0.0001$; Fig. 4d).

3.4 Immunohistochemistry for Brain Derived Neurotrophic Factor

To further confirm the transcriptional expression of mRNA, translational product i.e., protein expression was analyzed through immunohistochemistry (IHC) in the brain samples of rats from treated and untreated control groups. A basal level of BDNF was found in the brain sections of normal control,

expressing its behavior in normal physiological condition. In disease condition, significantly high expression of BDNF was found in both cortex and hippocampus region (#### $p < 0.0001$; ## $p < 0.005$ respectively; Fig. 5). In treated groups, gabapentin monotherapy and low dose combination therapy has significantly reduced the BDNF expression only in cortex (*** $p < 0.0004$; **** $p < 0.0001$ respectively). However, in hippocampus only low dose combination of GBP + Indo shows significantly reduced BDNF expression as compared to the arthritis only (** $p < 0.039$).

4. Discussion

Pain is an unusual condition caused by the persistent stimulus from the sensory neurons, and often caused immobility in patients and economic burden. Most frequently, chronic pain occurs after any tissue injury which results in partial or complete loss of patient's functionality and also affects quality of life [41].

BDNF is responsible for the modulation of neuronal activity and neuronal plasticity in pain amplification pathway through NMDA receptor [42, 43]. This provides a base to study the role of BDNF in chronic pain [44]. Previously, the BDNF expression has been shown to be modulated by additive effects of commercially available drugs specially gabapentin and indomethacin [45], but specifically focused on monotherapy or inter-class drug comparison in pain relieving [46–49]. Keeping this in mind, the present study was designed to study the role of gabapentin, indomethacin and its low-dose combination on chronic pain via BDNF pathway. In our study we found that in non-treated arthritis only group, PCR expression of BDNF is significantly decreased as compared to their healthy counterpart. But it is significantly increase in the cortex, amygdala and hypothalamus region of brain. Further, suggesting that the sensitivity of BDNF expression in different regions of brain during chronic pain. On the other hand, protein expression of BDNF in hippocampus and cortex is significantly very high in arthritis only then the normal control. This further cemented the hypothesis that, during chronic pain BDNF expression in hippocampus is pulsating in nature with respect to the time [50, 51]. Though the possible explanation is, in immunohistochemistry the BDNF expression shows that peak translation phase of mRNA and PCR expression shows the BDNF expression during basal phase due to its pulsating nature [50]. In treatment groups, low dose combination of gabapentin and indomethacin significantly inhibits the BDNF level in different regions of brain as compared to their monotherapy. Previously, only the role of gabapentin and indomethacin monotherapy in chronic pain has been described [47, 48]. Our study reported that a low dose combination of gabapentin and indomethacin have significant effects in modulating pain and BDNF expression as compared to the monotherapy of either drug.

Various studies reported that total protein concentration is also increases in the chronic pain whether it is due to the metabolic protein or immune system protein high titer value, pointing out the potential of increase total protein concentration as a major hallmark of chronic pain [52–55]. We also found high titer value of total protein concentration in arthritis only groups as compare to the normal control. Our study also reported that low dose combination of gabapentin and indomethacin as well as indomethacin

monotherapy significantly reduced the total protein concentration suggesting that they decrease the tissue perfusion of protein by decreasing the permeability of protein in tissue [32–34].

Pain can be studied by injecting complete Freund's adjuvant (CFA) in the tail base of rodents subcutaneously. After reaching into the lymphatic system, the adjuvant acts like an immunogen resulting in the excessive release of free radicals (NO and PO) into the blood stream further activating the inflammatory process in the joints. [56] reported that high titer value of NO⁺ modulates the level of pain mediator called bradykinin. Previous studies suggested that in inflammatory arthritis, level of reactive oxygen species become altered [52, 57].

Our study also supports the evidence of high level of NO and PO in chronic pain condition with concomitant low level of antioxidants (although non-significant) suggesting that imbalance of free radicals and antioxidant levels in the pathology of pain and reported that low dose combination of gabapentin and indomethacin decreases the level of free radicals. The evidence for the link between BDNF activity and free radicals, specifically nitric oxide production, is not clear yet. Biojone et al, [58] reported that there is interplay between NO level and BDNF high expression as they modulate each other with respect to the region of brain and conditions in which they tested. Some evidences supported the fact that high NO level linked with gradual increase in BDNF expression in neuronal cortex primary culture cells [59]. This might be possible that they modulate each other in either way in the presence of another factor or other regulatory signals which are yet to be studied. Nevertheless, our study supports the hypothesis that both BDNF and NO have high activity in chronic pain condition and both are modulated simultaneously with low dose combination of gabapentin and indomethacin as compare to their monotherapy.

5. Conclusion

Different approaches are being used for symptomatic relief of chronic pain by targeting BDNF. Our study successfully reassesses the severity of BDNF in chronic inflammatory model and its modulation through commercially available drugs i.e., gabapentin, indomethacin and their low dose combination. Based on our observations, consistently low dose combination of gabapentin and indomethacin has an upper-hand in reducing the BDNF expression and other markers of chronic pain. Further suggesting that either they have the additive or supra-additive effect while using in combination. We affirm that gabapentin and indomethacin low dose combination has better affect in lowering BDNF associated chronic pain as compare to their monotherapy.

Declarations

Ethical Acknowledgements

Compliance with Competing Interest

The authors have no conflict of interest to declare.

Ethical Approval

The animal study protocol was approved and according to the Institutional guidelines of Scientific Advisory Committee on Animal Care, Use and Standards of International Center for Chemical and Biological Sciences (ICCBS), University of Karachi (protocol # 2018-0005) and according with the guideline of United States National Institutes of Health guide for the care and use of laboratory animals (NIH Publications 2011 edition eight (8)).

Contributions of Author

SK performed all experimental work including preparation of samples, ZAR actively involved in data interpretation, manuscript writing, and reviewing. SUS as PI proposed the whole study plan, and also provided her technical and theoretical guidance in the writing of the manuscript. All authors have read and approved the final manuscript.

Availability of Data and Materials

All the data generated or analyzed, supporting the findings of the study are included within the article.

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Figures

A

B

C

Figure 1

a: Effect of gabapentin (GBP), Indomethacin (Indo) and GBP+Indo combination therapy on the mean body weight of rats with post induction of arthritis. Each value in the graph is shown as Mean $\pm$ SD (n=12/each group), over the period of 14 days of immunization with adjuvant. Arthritic control group shows a significant difference in the body weight from day 8 onward (#p < 0.04) and this difference was persistent until day 14 (####p < 0.001). Where; # shows level of significant difference between normal and

arthritis only group; * represents difference between arthritis only and treatment groups. Arth+Indo represents animals receiving indomethacin after induction of arthritis, Arth+GBP represents arthritic animals receiving gabapentin treatment, Arth+Combo represents the arthritic group of animals receiving low dose of indomethacin and gabapentin. b: Time course of the change in the right and left hind paw volume, post-induction of arthritis. Each value in both graphs is shown as Mean $\pm$ SD (n=12/each group), over the course of 14 days of immunization. Arthritic control compared to the normal control showed a significant increase in the hind paw volumes of both right and left paw from day 12 (#p < 0.04). This condition severely persists till the day 14 in both hind paws(###p< 0.001) While only group receiving GBP+Indo combination shows significance reduction in paw edema volume in right paw from day 12 and in left paw on day 14 of immunization (**p < 0.005) (not shown on graph). c: Paw withdrawal latency (PWL)time measured from the thermal stimulus induced by radiant heat in arthritic and non-arthritic rats due to inflammation. All values in the graph are shown as Mean $\pm$ SD (n=12/ per group), over the 14 days of immunization period. Arthritis only group shows gradual decreasing of paw withdraw latency time, from day 8 to day 12 it declines significantly as compare to normal control (#p< 0.02) until day 14 where it achieve maximum reduction in PWL time (###p<0.0007). In treatment group of gabapentin monotherapy, there is significant increase in PWL time from day 4 (**p< 0.001), day 6 (****p< 0.0001), day 8 (****p< 0.0001), day 10 (**p<0.007), day 12 (****p< 0.0001), day 14 (**p<0.0003) while indomethacin monotherapy group shows no significant increase in PWL. GBP-Indo combination therapy group also show significant increase in the PWL time from day 4 (**p< 0.005), day 6 and day 8 (****p< 0.0001), day 10 (**p<0.008), day 12 (**p<0.004), day 14 (****p< 0.0001).

Figure 2

Serum peroxide PO(ng/ml) and plasma nitric oxide NO(mg/dl), superoxide dismutase SOD activity. Each bar on each graph showed Mean $\pm$ SD (n=12/each group), during the course of immunization. (a) For PO-activity, arthritis only exhibited a significant increase in comparison to the normal (##p< 0.01). Only indomethacin and combination of GBP-Indo showed a significant reduction of PO(*p< 0.02; *p< 0.05) compared to arthritis only, (b) For Plasma NO, significant increase in the nitric oxide level was observed in the arthritis only (###p< 0.001) compared to normal group. In treatment groups, only GBP-Indo showed a

significant reduction in the nitric oxide levels compare to the arthritis only (**p< 0.01).(c) No significant difference was found between arthritis and normal groups and arthritis only and treatment groups for SOD estimation. Where; # shows level of significant difference between normal and arthritis only group; * represents difference between arthritis only and treatment groups.

Figure 3

Total protein concentration (mg/50 µl) estimation in plasma. Each bar represents means $\pm$ SD of n = 12 animals/ group. The total protein in plasma samples of arthritis only animals showed a significant increase compared to the normal (###p < 0.01). Among treatment groups, indomethacin significantly reduced the total protein level (**p < 0.005) as compare to arthritis only. While GBP+Indo combination also showed (**p < 0.01) significant reduction of the total plasma protein concentration vs arthritis only. Where; # shows level of significant difference between normal and arthritis only group; * represents difference between arthritis only and treatment groups.

Figure 4

RT-PCR analysis for BDNF gene expression from brain samples. Normal (A), Arthritis (B), Indomethacin (C), Gabapentin (D), Low doseGBP+Indo combination (E). In amygdala, significant upregulation of BDNF gene observe in arthritis only as compare to the normal (#### $p < 0.0001$) while treatment groups of GBP, indomethacin and GBP+indo also shown significance decrease in BDNF expression (**** $p < 0.0001$) when compare to arthritis only. In cortex, arthritis only shown significance increase of BDNF expression

(####p< 0.0001) with normal, while all treated groups decrease the BDNF expression (****p< 0.0001) as compare to arthritis only. In hypothalamus, same pattern was found between inter-comparison of arthritis only with normal groups and Indomethacin and GBP+Indo treatment groups vs. arthritis only (####p< 0.0001 and ****p< 0.0001 respectively) but gabapentin only shows contrasting significant increase in the BDNF level as compare to arthritis only (****p< 0.0001). In hippocampus, arthritis only shows very low level of BDNF expression (####p< 0.0001 significant) as compare to normal, interestingly, all three treatment groups show significantly high BDNF expression as compare to the arthritis only (****p< 0.0001). Where; # shows level of significant difference between normal and arthritis only group; *represents difference between arthritis only and treatment groups. BDNF product size: 175 bp (near at 200 bp band of ladder), GAPDH product size: 450bp

Figure 5

Immunohistochemistry of brain derived neurotrophic factor (BDNF) in normal and arthritis and other treatment groups.(10x magnification at 50 μ m scale bar of fluorescence micrograph). In cortex, significantly high expression of BDNF was detected in the arthritis only (B) as compare to normal (#### $p < 0.0001$). While treatment groups of (D) and (E) shows significant decrease in the BDNF expression (*** $p < 0.0004$) and (**** $p < 0.0001$) respectively as compare to the arthritis only. In

hippocampus significant expression of BDNF is detected in arthritis only group as compare to normal ($##p < 0.005$). While in treatment groups, combination therapy of gabapentin and indomethacin only shows significant decrease in the BDNF expression as compare to the arthritis only ($**p < 0.039$). Normal (A), arthritis control (B), indomethacin (C), gabapentin (D), low-dose combination of GBP-into(E). Where; # shows level of significant difference between normal and arthritis only group; * represents difference between arthritis only and treatment groups.