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A Randomised Comparative Study of the Modulation of Genetic Expression of TIMP 1 and ERK in Patients with Post-Herpetic Neuralgia

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ABSTRACT

Objectives: To evaluate the efficacy of fixed-dose combination pharmacotherapy of Pregabalin plus Nortryptyline and compare it with Pregabalin monotherapy in patient of post-herpetic neuralgia (PHN) in modulating mRNA expression of TIMP 1 and ERK genes.
Methods: A randomized, controlled study was conducted From November 2016 to April 2018. Forty patients were assessed for pain scores and the patients with NRS ≥5/10 were randomized into two groups (Group A and Group B; each group n=20). GROUP A received fixed-dose combination pharmacotherapy of Pregabalin plus Nortriptyline and Group B received Pregabalin monotherapy for 12 weeks. RT PCR was used for assessing the gene expressions of ERK and TIMP1. The primary outcome measures were changes in ERK and TIMP1expression and secondary outcome measures were change in NRS pain.
Results: The baseline NRS pain score in group A was 7.45 while in group B was 6.8 (p=0.055). During the followup of 12 weeks, group A showed a significantly higher reduction in the pain score (0.8 vs 2.9, p<0.001) Compared to group B, the mRNA expression level of ERK in Group A gene was more down-regulated at 12th week (p<0.001) and for TIMP1,it was more up-regulated (0.5 fold vs 0.4 fold, p=0.046).
Conclusion: The drug combination of Pregabalin plus Nortriptyline holds significant superiority over Pregabalin alone in modulating the genetic expression of TIMP1 in patients with PHN while for ERK, pregabalin alone holds importance.

Key Words: *Herpes, Neuralgia, gene expression*

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INTRODUCTION

Herpes zoster (HZ) or shingles is an acute viral infection caused by reactivation of varicella zoster virus (VZV) that has remained dormant within dorsal root ganglion (DRG) often for decades after patients initial exposure to the virus in the form of varicella (chicken pox)[1].

Its major complication is Post-Herpetic Neuralgia[1] (PHN) which manifests as neuropathic dermatomal pain along the cutaneous nerves located in area of prior HZ rash. PHN most commonly affects the thoracic dermatomes, although ophthalmic, cervical and lumbosacral dermatomes may also be affected. PHN is a typical neuropathic pain which is defined as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system". The neuropathic pain of PHN is characterized by constant or intermittent burning, itching with paroxysmal or lancinating stabbing, shooting pain, and clinically it manifest as "allodynia" and "hyperalgesia"[2,3]. The pain is accompanied by autonomic instability and its intensity is exacerbated by physical and emotional stress and alleviated by relaxation.

Why some patients develop PHN is incompletely understood. There is, for example, no consistent relationship between this type of pain and a loss of large fibres in the damaged nerves, but invitro studies[4] suggest that the virus itself may cause normally silent neurones to produce spontaneous action potentials.

This may be explained at the molecular level where dysregulation of several neurotransmitters, ion channels and proteins are reported to contribute to the development of central and peripheral sensitization which is considered as the

main causes of neuropathic pain. Among the various genes mRNA expression of TIMP1 gene and ERK gene plays a role in the pain processing pathway of PHN[5].

Pain management remains the corner stone for these patients. Various modalities[6,7] used for management of PHN include analgesics, anticonvulsants like Pregabalin/Gabapentin[8] antidepressants, opioids, antiarrhythmics, transcutaneous nerve stimulation, topical preparations, regional sympathetic blocks, intrathecal steroids injection. However no single regimen of treatment is completely effective in neuropathic pain in patients of PHN, so a multimodal approach is required to provide pain relief to the patients.

Moreover if we can know the effects of drugs on the genetic expression, a gene targeted therapy can be designed and provided to the PHN patients, also it can be used as a potential prognostic biomarker for early detection & diagnosis as well as for early management of PHN patients.

Hence the present study was designed to assess the efficacy of two drug choices: (1) Pregabalin and Nortriptyline fixed-dose combination pharmacotherapy and (2) Pregabalin monotherapy; in modulating the TIMP1 and ERK gene expression in cases of PHN.

METHODS

A randomized, controlled study was conducted in the Department of Anesthesiology of our institute from November 2016 to April 2018. Patients having a diagnosis of PHN with evident hyperalgesia and allodynia who had pain for at least, 12 weeks after healing of rash and having pain intensity ≥5/10 on Numeric Rating Scale (NRS) were included in the study, while patient with history of Diabetes mellitus, severe renal impairment, neurological intervention for pain relief or with history of Pregabalin in the last 4 weeks were excluded from the study.

Sample size calculation

Evidence suggests an efficacy rate of 40% in PHN patients with the Pregabalin therapy alone. We expect 80% pain relief with this fixed-dose combination pharmacotherapy of Pregabalin plus Nortriptyline. To detect a difference of efficacy rate in the above 2 groups as 40%,the estimated sample size should be 17 patients in each group with 80% power and 5% level of significance (one sided). Adding 10% expected loss to follow-up, the sample size increased to 19 subjects in each group and so we had taken 20 patients in each group. Sample size was calculated using G-Power (version 3.2.1).

A written informed consent was taken from all the participants. The study was conducted after approval from the Institutional Ethical Committee (IEC-Human).

Forty patients were assessed for pain scores and the patients with NRS ≥5/10 were randomized into two groups (Group A and Group B; each group n=20) with the help of computer generated random number list. Figure 1 shows the participant flow diagram.

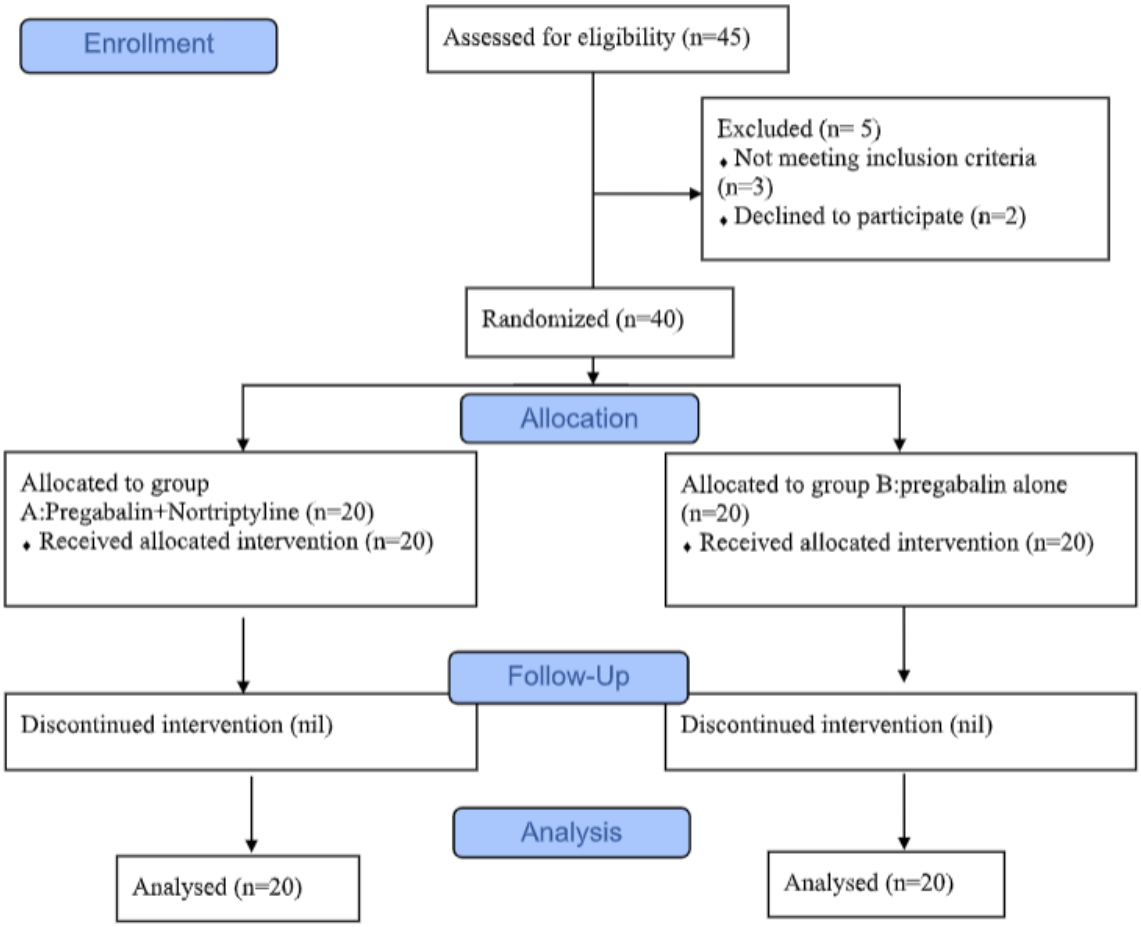

Figure 1: Participant flow algorithm

Group A: PHN patients received fixed-dose combination pharmacotherapy of Pregabalin plus Nortriptyline for total of 12 weeks, administered orally (Pregabalin 75 mg plus Nortriptyline 10 mg for patients <80 kg body weight and Pregabalin 150 mg plus Nortriptyline 20 mg for patient >80 kg body weight) on a twice daily basis to all PHN patients.

Group B: PHN patients received Pregabalin monotherapy for 12 weeks administered orally as 75 mg bid (for patients <80 kg body wt.) and 150 mg bid (for patients >80 kg body wt.) on a twice daily basis to all PHN patients.

A detailed evaluation was carried out in each patient which included baseline assessment of pain intensity and severity by utilizing Numeric rating scale.

Analysis of blood samples for mRNA expression of TIMP-I and ERK genes was done using Real Time PCR (RT-PCR) which included the following steps:

a) Sample collection: A total of 2 ml of venous blood sample was withdrawn from patients of PHN and collected in EDTA vial.
b) Storage: Sample was stored at -80 degree Celsius after fixing in TRIzol reagent for further expression studies.
c) RNA isolation: Total RNA isolation was done by TRIzol method.
d) cDNA synthesis of mRNA: Complementary DNA synthesis was done by using commercially available kit .
e) Final expression studies: Gene expression was done using real time PCR for mRNA expression of TIMP1 and ERK genes.

Step 1: Extraction of RNA from blood sample

RNA was isolated from whole blood using TRIzol reagent. The concentration and purity of samples was determined using spectrophotometer (Nanodrop1000; Thermofisher, USA). All the samples had ratio of absorbance at 260/280 between 1.8 and 2.0. Quality of isolated RNA was checked by agarose gel electrophoresis. RNA loading dye and isolated RNA were mixed properly in a ratio of 1:1. The mixture was kept at 800C for 10 minutes and then immediately placed on chilled ice and loaded on 1.5% agarose gel.

Step 2: Synthesis of complementary DNA (cDNA)

From the total RNA extracted, mRNA was used as a template for the synthesis of cDNA. On the same day preferably, total RNA (1µg) was converted into first strand cDNA using Maxima first strand cDNA synthesis kit (Thermo USA) according to manufacturer's protocol. Equal amount of total RNA were calculated for conversion of RNA into cDNA. The product of the first strand cDNA synthesis reaction was diluted four times in nuclease free water and used in qPCR. The volume of first strand cDNA synthesis reaction mixture did not comprise more than 1/10 of the total PCR reaction volume. cDNA was diluted four times and stored at -800C until expressional quantification using Real time PCR.

Reverse transcription was carried out using Thermo Scientific Verso cDNA synthesis kit). From each RNA sample, 1 µg of total RNA was used in a volume of 20 µl reaction containing 4µl of 5X cDNA synthesis buffer, 2 µl of 5 mM dNTPs, 1 µl RT enhancer (added to remove contaminating DNA, eliminates need of DNAse treatment), 1 µl of verso enzyme mix (contains RNAse inhibitor), 1 µl of random hexamer and nuclease free water added to volume and briefly centrifuged to collect the volume to the bottom. Reverse transcription was carried out in a thermal cycler (BioRad, USA) at 42°C for 30 min followed by 95°C for 2 min (inactivation of reverse transcriptase).

Step 3: Quantification Of TIMP 1 and ERK gene expression by using Real Time PCR.

A real time quantitative polymerase chain reaction (RTqPCR) experiment was conducted to measure expression of TIMP1 and ERK gene in blood samples of subjects. The qPCR reactions were performed on Real Time PCR.

Briefly, the PCR amplification master mix of 45 µl contained 6.75 µl of diluted cDNA, 10µl of Eva green master mix, 10p mole of each forward and reverse specific primer pairs and 4µl of nuclease free water and finally 20µl of master mix were dispensed into two PCR tube. Duplicate of each samples along with no template control wells were placed into the chamber and kept into the machine.

The mRNA expression of these genes were evaluated at the baseline, at the end of 2nd week and at the end of 12th week of follow up. The fold change (FC) analysis for evaluating the gene expression was done.
ΔCt = Average Ct_Target- average Ct_Normalizer.

Then the difference in the mean Ct values of two groups were assessed which is delta delta Ct: $\Delta\Delta Ct = \Delta Ct\_case - \Delta Ct$

A true FC was calculated by $FC = 2^{-\Delta\Delta Ct}$ where if FC >1, true fold change = FC and if FC <1, true fold change = $-1/FC$.
GAPDH was taken as the reference gene for comparison.

The primary outcome measures were change in ERK and TIMP1expression with the drug therapy and secondary outcome measures were change in NRS pain.

Statistical Analysis

The quantitative data were presented as the means ± SD. The following statistical tests were applied for the results:
1. The comparison of the variables which were quantitative in nature like age, weight, duration, NRS scores at different time intervals were analysed using Independent t test. Paired t test was used for comparison NRS scores across follow up, True Fold change difference in mRNA expression of TIMP 1 and ERK genes.
2. Pearson correlation coefficient was used to correlate NRS pain score and delta Ct values of mRNA of ERK gene and TIMP1 gene.

The data entry was done in the Microsoft EXCEL spreadsheet and the final analysis was done with the use of Statistical Package for Social Sciences (SPSS) software, IBM manufacturer, Chicago, USA, ver 21.0.

For statistical significance, p value of less than 0.05 was considered statistically significant.

Results

Table 1 shows the demographic profile of the patients. Both the groups of PHN patients were statistically comparable with respect to age, gender ratio, duration of pain and body weight which had no obvious effect on the outcome. The mean age was 52.40 ± 8.57 (in years) in patients of group A and was 54.45±11.01 (in years) in patients of group B. The gender ratio (M:F) in both group A and B was 9:11. The mean body weight was 61.80 ± 9.73 (in kg) in group A and 62.10 ± 10.47 (in kg) in group B. The mean duration of pain in patients of group A was 3.88±1.12 months and in group B it was 3.75±1.07 months.

Table 1:- Demographic profile of both the groups.

Demographic profile	Group A	Group B	P value
Age (years)	52.4±8.57	54.45±11.01	0.515*
Weight (kg)	61.8±9.73	62.1±10.47	0.926*
Duration (months)	3.88±1.12	3.75±1.07	0.709*

***Independent t test**

The baseline NRS pain score in group A was 7.45 while in group B was 6.8 (p=0.055). The NRS scores (mean± SD) on intra group comparison showed a decreasing scores at the end of 1st 2nd 4th 8th and 12th week and a statistically significant difference (p <0.001) was observed as compared to the baseline scores. During the follow-up of 12 weeks, there was a significant reduction in both the groups with values reaching to 0.8 in group A and 2.9 in group B. On comparison group A showed a significantly higher reduction in the pain score (p<0.001). (Table 2)

Table 2:- NRS scores at different time intervals in group A&B.

NRS	Group A	Group B	P value
Baseline	7.45±1.28	6.8±1.24	0.055*
End of wk1	5.35±1.35†	4.85±0.93†	0.09*
End of wk2	4.3±1.49†	4.15±1.04†	0.357*
End of wk4	3.2±1.24†	3.45±0.89†	0.234*
End of wk8	1.75±0.91†	3.15±0.59†	<0.001*
End of wk12	0.8±1.06†	2.9±0.64†	<0.001*

***Independent t test, † p-value <0.001-Significant on intragroup comparison**

The mRNA expression level of ERK in Group A gene was down-regulated (2.4 fold) at end of 2nd week and (3.54 fold) at 12th week (p<0.001) when compared with baseline data while in Group B the down regulation was 3.66 folds at 2nd week and 4.99 folds at 12th week (p<0.0001), the values being significantly higher than Group A (p=0.046).(Table 3)

Table 3:- True Fold change mRNA expression of ERKin the two study groups

ERK	Group A	Group B	P value
Baseline			
Expression	28.56±1.55	28.47±1.6	0.858*
DELTA CT	4.49±1.81	5.18±2.05	0.265*
2 Weeks			
Expression	27.83±1.17	27.5±1.51	0.443*
DELTA CT	3.76±1.52	4.21±1.82	0.403*
ΔΔ CT	-0.73±1.23	-0.97±1.56	0.589*
Fold change	2.4±2.86	3.66±5.32	0.354*
12 Weeks			
Expression	27.13±1.55	26.95±1.27	0.693*
DELTA CT	3.06±0.85	3.66±0.98	0.045*
ΔΔ CT	-1.44±0.12	-1.52±0.08	0.018*
Fold change	3.54±1.67	4.99±2.66	0.046*
Intra group p value (baseline vs 12 weeks)			
Expression	<0.001†	<0.001†	-
DELTA CT	<0.001†	<0.001†	-

***Independent t test, † Paired t test**

The mRNA expression level of TIMP1 in Group A gene was up-regulated (1.15 fold) at end of 2nd week and (0.5 fold) at 12th week (p<0.001) when compared with baseline data while in Group B the down regulation was 0.85 folds at 2nd week and 0.4 folds at 12th week (p<0.0001), the values being significantly lower than Group A (p=0.046).(Table 4)

Table 4:- True Fold change difference in mRNA expression of TIMP1 genes in the study groups

TIMP1	Group A	Group B	P value
Baseline			
Expression	26.94±1.74	26.62±1.69	0.563*
DELTA CT	2.87±1.75	3.34±2.1	0.453*
2 Weeks			
Expression	27.45±1.28	27.25±1.51	0.654*
DELTA CT	3.38±1.48	3.96±1.94	0.294*
ΔΔ CT	0.51±1.7	0.62±1.13	0.799*
Fold change	1.15±1..05	0.85±0.64	0.278*
12 Weeks			
Expression	28.45±1.47	27.97±1.66	0.34*
DELTA CT	4.43±0.34	4.69±0.38	0.028*
ΔΔ CT	1.56±0.28	1.35±0.32	0.033*
Fold change	0.5±0.1	0.4±0.15	0.018*
Intra group p value (baseline vs 12 weeks)			
Expression	<0.001†	<0.001†	-
DELTA CT	<0.001†	<0.001†	-

***Independent t test, †Paired t test**

DISCUSSION

Till date no study has been conducted, as confirmed by our extensive search on the modulation of mRNA expression of TIMP1 and ERK gene following treatment of PHN using a fixed-dose combination of Pregabalin plus Nortriptyline & pregabalin alone in patients suffering from PHN. The majority of gene expression studies have focused solely on animal models of neuropathic pain, ranging from chronic constriction injury and spinal nerve ligation to spared nerve injury and drug-induced neuropathy models[5,9,10].

ERK is one of the members of Mitogen Activating Protein Kinase (MAPKs) family consisting of three major members Extracellular signal-regulated kinase (ERK), P38 and JNK kinase. MAPKs are important for intracellular signal transduction and play critical roles in regulating neural plasticity & inflammatory responses[9].

In one of the study conducted by Wei et al using adult rats for chronic neuropathic pain because of limb amputation, it was demonstrated that ERK activity during different phases of chronic pain was significantly up regulated and they concluded that ERK contributes to both induction and expression of chronic pain[11].

In another study done by Ji RR et al[9] on different animal models they demonstrated that there was an activation of ERK and other MAPKs following nerve injury in spinal glial cells, leading to the synthesis of pronociceptive mediators, thereby enhancing and prolonging pain, while the inhibition of MAPKs has been shown to attenuate neuropathic pain. In our study, we used drug combinations to relieve the chronic neuropathic pain, whose effect is also reflected at the molecular level in down regulation of the ERK gene expression.

Study done by Ma W et al[10] also demonstrated that following nerve injury – induced phosphorylation of ERK occurs in DRG which leads to neuropathic pain. Inhibitors of ERK, proved to be effective in alleviating pain, thus proving that the regulation of ERK/MAPKs can be considered as a potential therapeutic target for treatment of neuropathic pain.

In accordance with this, we observed that in both the groups A & B there was a significant down regulation of the ERK gene and pain scores, thereby showing a direct correlation between down regulation of ERK and pain sensations in PHN. In comparison we found that the combination therapy was better than Pregabalin alone.

TIMP1 is an inducible, soluble and secreted protein with cytokine-like properties. Its function is to inhibit MMPS, involved in extracellular matrix degradation, with several consequential roles in cell-cell interactions, migration and cell proliferation. Such inhibitions has been shown to reverse allodynia[12].

Buckley et al[5] carried out animal study on adult male Sprague-Dawley rats which demonstrated significant ($p<0.05$) upregulation of TIMP1 gene in ipsilateral dorsal horn, which supports its potential role in the mechanism of maintenance and development of chronic neuropathic pain.

In a recent study conducted by Buckley et al[5] on humans suffering from chronic neuropathic back pain, they observed that the TIMP1 gene expression was upregulated as compared to healthy controls and they also deciphered a strong correlation between TIMP 1 gene and development of chronic neuropathic pain. Hence these findings also support the observations in the present study.

The expression of TIMP 1 in the present study also demonstrated similar findings as there was a significant ($p<0.05$) upregulation of TIMP1 gene in both the groups A & B at the end of 2nd and 12th week in patient with PHN. This indicates that the genetic upregulation after therapy may cause significant reduction in the pain. In comparison we found that the combination therapy was better than Pregabalin alone.

The present study is the first ever study to be done on humans which proved that ERK gene and TIMP1 play a critical contribution in pain pathways and in the chronification of pain.

Limitation of study

The study had a small sample size. The duration of disease among patients was less. Other genes of MAPK pathways were not assessed.

CONCLUSION

The drug combination of Pregabalin plus Nortriptyline holds significant superiority over Pregabalin alone in modulating the genetic expression of TIMP1 in patients with PHN while for ERK expression modulation the fixed drug combination holds lesser impact than the drug Pregabalin alone.

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