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ANTINOCICEPTIVE SYNERGY OF IMIPRAMINE AND ASPIRIN IN A NEUROPATHIC PAIN MODEL IN THE RATS

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ABSTRACT

Neuropathic pain (NP) is a damage of the somatosensory nervous system and a globally accepted clinical burden. Pyridoxine (vitamin B6) is an essential cofactor used in the metabolism of proteins, fats, and carbohydrates but its higher doses produce NP. So, in the present study individual response of imipramine (IMP) and aspirin (ASA) as well its co-administration was investigated by keeping the dose of ASA constant against pyridoxine (PYR) induced NP. In Sprague Dawley rats, PYR was administered with the individual treatment of IMP, ASA, and their co-administration for 14 days. Behavioral and motor parameters were investigated on days 3, 6, 9, 12, and 14. Further, the level of oxidative biomarkers and histopathology were also recorded at the end of the study. The result of the study indicates that individual dose of IMPH (10mg/kg) & IMPL (20mg/kg) work more efficiently than ASA (100mg/kg), while its co-administration at IMPL+ASA (10+100mg/kg) and IMPH+ASA (20+100mg/kg) have better activity from 9th day of treatment. The level of oxidative biomarkers was also protected in sciatic and muscular tissues. This attenuation and improvement in therapeutic effect were confirmed by histopathological studies. In the presence of aspirin, the activity of IMPL and IMPH was improved.

Keywords: Pyridoxine; neuropathic pain; imipramine; aspirin

INTRODUCTION

Pyridoxine (PYR; vitamin B6) is a water-soluble and often nontoxic compound. Studies on humans and animals suggest that its consumption at its mega dose (800 mg/kg) induces peripheral neuropathy which causes the damage of sensory nerve fibers of dorsal root ganglion (DRG) followed by the destruction of long-myelinated fibers which end up with cell death [1-3]. Neuropathic pain (NP) is unbearable torture and is a progressive type of pain affecting the somatosensory nervous system [4-7]. It is a combination of paraesthesia as well as difficulty in locomotion [8]. It becomes a global burden due to its multifactorial causes which include diseases (diabetes, cancer, HIV, etc.), chemicals (alcohol), drugs (chemotherapeutic agents, vitamin B 6, etc.) [9-12]. NP causes the damage of the myelinated A-fibers (Aβ and Aδ fibers) and C fibers. Hence it affects work, sleep, mind, the psychological and recreational capacity of the individual [4]. It is recognized by the state of allodynia, hyperalgesia, and burning kind of pain [8, 13-15]. 26-45% of cases of NP were reported in Malaysia, the USA, and the UK. It mostly affects women & middle-aged people [16, 17]. In India, about 30% of the population is suffering from NP due to diabetes, and its number increases due to impairment of the immune system [18]. There are various treatments available so far for NP, like antidepressants, NSAIDs, opioids, anticonvulsants, etc. But all these medications bring about partial and insufficient pain relief [4]. Among all the models of induction of NP in an animal, the use of chemical agents for induction is one of the most acceptable models as it mimics NP similar to human without unnecessary torture to the animals [1, 19, 20]. As per previous scientific evidence, the dorsal horn of spinal cord, injured sciatic nerve lessens, and the expression of GABA with its receptors [21] are mostly affected segments in NP. It is also reported that fix dose of aspirin (ASA) with imipramine (IMP) significantly increases reaction time and potentiating analgesic effect [22]. So, a broader range of severe/chronic pain can be relieved by combining or co-administrating different classes of analgesics. These combinations must provide a reduction in doses as well as side effects of conventional drugs. Analgesic impingement using this combination can provide effective pain management with improvement in recovery from NP. Therefore, the present study was designed to investigate the therapeutic potential of IMP and ASA alone as well as in co- administration against PYR-induced NP in rats. The present research also evaluates the possible potentiating of the analgesic effect by keeping the dose of ASA constant with the low and high doses of IMP in rats.

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MATERIAL AND METHODS

2.1. Drugs and chemicals

Aspirin was procured from Central Drug House (CDH) Pvt. Ltd. Mumbai, India. Pyridoxine and imipramine were procured from Yarrow biochem. Pvt. Ltd., India. Protein kit was procured from Erba Mannheim, Trichloroacetic acid, HCL, EDTA were procured from Loba Chemie, and Thiobarbituric acid was purchased from Himedia Labs.

2.2. Procurement of animals

Sprague-Dawley (SD) rats of either sex (200-250 g each) were procured from the National Institute of Pharmaceutical Education and Research (NIPER, Hyderabad). The animals were given 12 h light and 12 h dark cycles and were kept at ambient temperature and humidity conditions. The animal study was approved by the Institutional Animal Ethics Committee (IAEC).

2.3. Induction of NP

PYR solution was prepared by diluting in 0.9% (w/v) sodium chloride sterile solution & administered to the rats intraperitoneally (i.p.) twice a day. The induction of neuropathy takes place at a dose of (400 mg/kg) to the rats. It is important to note that the fresh solution of pyridoxine solution was required to prepare immediately before each injection [23].

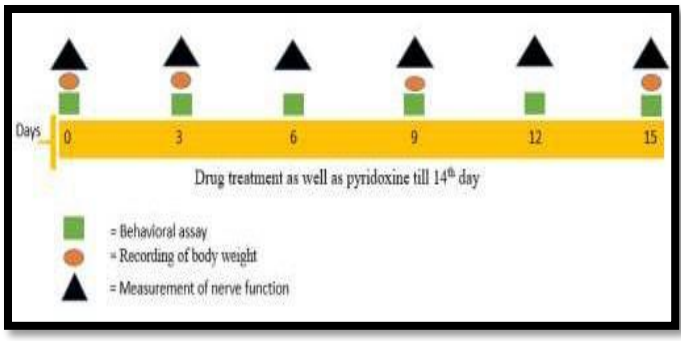

Fig: 1. Experimental design

Table.1. Experiment protocol for in vivo study

Groups	Treatment	Doses (mg/kg); daily, p.o.
Animal without pyridoxine intoxication		
I	**Normal control (N)**	
Animal intoxicated with pyridoxine (400 mg/kg, i.p., twice daily		
II	Pyridoxine (PYR)	400
III	Imipramine (IMPL)	10
IV	Imipramine (IMPH)	20
V	Aspirin	100
VI	Imipramine +Aspirin (IMPL+ ASP)	10+100
VII	Imipramine + Aspirin (IMPH + ASP)	20+100

2.4. Treatment schedule

Rats (200-250 g) were divided into 7 groups (n=6). Rats of the control group (NC; Group I) received the vehicle and all the other groups of rat's i.e II-VII were subjected to pyridoxine (i.p.) twice a day at a dose of 400 mg/kg till 14 days (Fig.1.). Table.1. indicates the group of rats receiving their respective treatment. Imipramine (IMP) at 10 and 20 mg/kg was given to rats of group III (IMPL) and IV (IMPH) respectively while rats of group V received aspirin (ASP) at 100 mg/kg dose. Now in group VI & VII by keeping the dose of ASP constant animals were received IMPL + ASP (10+100 mg/kg) and IMPH + ASP (20+100 mg/kg). Three hours before each treatment pyridoxine was administered to the rats. All neurobehavioral evaluation of their appearance was conducted on 0, 3, 6, 9, 12, & 15th day of the experimental period. On the final day of the study, all the animals were sacrificed for biochemical as well as histomorphological study.

2.5. Effect on general appearance

2.5.1. Body weight

Assessment of body weight was performed on 0, 3, 9, and 15th days before the administration of drugs [24].

2.5.2. Effect on neurobehavioral assay

2.5.2.1. Motor test

The ability to walk & maintenance balance was assessed by placing the rats at the center of the 1-meter wooden rod and the score of motor characteristics was assigned as mentioned given in the table.2 [24].

Table. 2. Score for motor activity and muscle power test

Characteristics	Score
Motor activity	
Normal	+4
Mild abnormal (ataxia and wide based gait)	+3
Moderate abnormal (ataxia and unstable gait)	+2
Inability to walk	+1
Muscle power test	
At 180° angle stay longer than 5s	+4
At 180° angle stay shorter 5s but on 120° degree longer than 10s	+3
At 120° angle stay shorter 10 s but 90° degree longer than 15s	+2
At 90° degree shorter than 15s	+1

2.5.2.2. Muscle power test

Rats were trained one week before the conduct of the experiment and the ability of muscle strength was recorded by moving wired screen at an angle of 90°, 120°, and 180° [24]. The ability of muscle strength was expressed as per scores represented in the table. 2.

2.5.2.3. Walking test

Rats are allowed to move on the rod of 5.5 cm diameter and 100 cm in length. The rod was placed horizontally 40 cm above a table. Movement of rats was measured from one end of the rod to distance traveled by them up to 1 m & each trail took 60 s maximum [25].

2.5.2.4. Test for hypersensitivity

2.5.2.4.1. Heat hyperalgesia test

Rats were allowed to place on an eddy's hotplate at 52±3ºc and the reaction time was recorded in the form of paw licking or jumping response [25].

2.5.2.5. Measurement of nerve function

2.5.2.5.1. Thermal tail-flick test

Restrained rats were allowed to leave their tail hanging freely and immersed in preheated water (50°C). Response time was recorded as the time of tail withdrawal [24].

2.5.3. Biochemical parameters

After the completion of all behavioral studies, on the last day of treatment animals were sacrificed for biochemical studies using cervical dislocation. Immediately, the sciatic nerve was isolated and the sample was kept free from moisture in a humidity chamber at 37 °C with 85% RH. The sciatic nerve homogenate (10%, w/v) was prepared with 0.1 M Tris-HCl buffer (pH 7.4) and deionized water for total protein [26, 27]. Further, surrounding muscular tissue was homogenated with phosphate buffer (pH 7.4) and employed for myeloperoxidase (MPO) estimation.

2.5.3.1. Estimation of total protein content

Protein concentration was estimated according to the method of Lowry 1951 [28], using bovine serum albumin (BSA) as a standard. The absorbance was determined spectrophotometric ally at 750 nm [9, 26, 27].

2.5.3.2. Estimation of superoxide anion generation

It is recorded in terms of the amount of reduced nitroblue tetrazolium (NBT) according to Wang et al. [29]. Briefly, sciatic nerve homogenate reacts with NBT to form formazan under the specific chemical condition and is determined spectrophotometrically at 540 nm [26, 27].

2.5.3.3. Estimation of myeloperoxidase (MPO) activity

It was recorded as described by Grisham et al. [30]. The absorbance for MPO was measured at 460 nm spectrophotometrically at pH 7.0 and 25°C. The results of MPO activity were expressed as units per milligram of protein at one minute [26, 27, 31].

2.5.3.4. Estimation of reduced glutathione (GSH)

The tissue homogenate of the sciatic nerve was centrifuged at 3000g for 10 min. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5,5′-dithio, bis (2-nitrobenzoic acid), and 0.4 ml double distilled water were added. The mixture was vortexed and the absorbance was recorded at 412 nm within 15 min of the completion of the reaction. The concentration of reduced GSH was expressed as µg/mg of protein [9, 32].

2.5.4. Histopathological evaluation

In the end, sciatic nerves were stored in 10% formalin. Staining was done by using hematoxylin and eosin [26, 33] and analyzed under a light microscope for axonal degeneration.

2.5.5. Statistical analysis

The data from the behavioral results were statistically analyzed by one-way analysis of variance followed by Bonferonni's posthoc-test and the results of the biochemical study were statistically analyzed using one-way ANOVA followed by Tukey's multiple range tests using GraphPad Prism Version-5.0 software. The p-value < 0.05 was considered to be statistically significant.

RESULTS

3.1. Induction of NP

Successful induction of NP using was a mega-dose of PYR observed from 1st week only. It causes an inability to walk and stand with the animals. Animals found tried to move their body by dragging but no signs of toxicity were recorded. It indicates decreased function and strengthening of the hind paw due to deprivation of motor as well as sensory coordination.

3.2. Effect of treatment on general appearance:

3.2.1. Effect on body weight of rats

The intoxication of PYR resulted in a significant alteration in body weight whether they were subjected to the drug treatment or not. Here the progressive reduction in body weight was observed in the group of rats who received PYR (Fig.2.). On days 3, the decrease in body weight was a bit less but as the day progresses its severity increases. Treatment with ASA didn't alter much more alteration in a reduction in body weight but co-administration of IMP+ASA exhibited better improvement in fall in body weight concerning PYR treated group of rats. Here IMP at any doses whether they were administrated alone or co-administrated with ASA didn't bring a positive result. Potentiating effect ASA for IMP at both the doses resulted in improvement in the reduction in body weight. Overall in each group fall in body weight was observed.

3.3. Effect on neurobehavioral assay

3.3.1. Effect on body motor coordination

In the motor co-ordination test, IMPL+ASA and IMPH+ASA showed a pattern of treatment starts from day 9 and it was recorded as 3.43±0.16 and 3.63±0.10 respectively and at the end of the study, it reaches 3.58±0.17 and 3.72±0.18 for IMPL+ASA and IMPH+ASA respectively. Group of rats received IMPL and IMPH the improvement was observed from day 12th only i.e 2.74±0.05 and 2.89±0.11 respectively while ASA treated group showed a delay in the treatment. Hence, this study indicates that ASA and IMPL in a co-administrated form able to treat disease in an early state only (Fig.3 a) and decrease the severity of abnormal motor coordination. A group of rats treated with PYR alone appears to show abnormal co-ordination and these effects appeared from day 3.

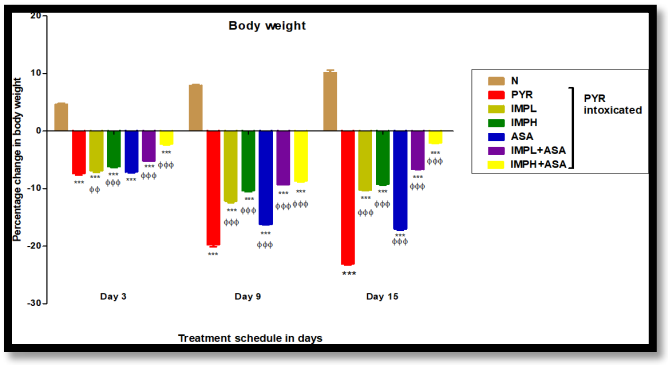

Fig-2. Effect on body weight

Each data represent mean±SD. * p<0.05, ** p<0.01 and ***p<0.001 when compared with control while ϕ p<0.05, ϕ ϕ p<0.01 and ϕ ϕ ϕ p<0.001 with respect to PYR

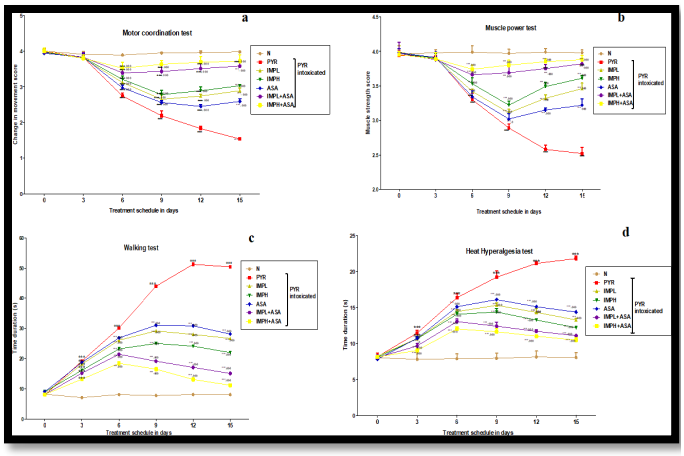

Fig-3. Effect on neurobehavioral assay a. Motor co-ordination test, b. Muscle power test, c. Walking test, d. Heat hyperalgesia test

Each data represent mean±SD. * p<0.05, ** p<0.01 and ***p<0.001 when compared with control while ϕ p<0.05, ϕ ϕ p<0.01 and ϕ ϕ ϕ p<0.001 with respect to PYR

3.3.2. Effect on muscle strength of rats

IMPL+ASA and IMPH+ASA showed an improvement in muscle strength from day 9th only as 3.69±0.09 and 3.80±0.07 respectively which reaches 3.82±0.05 and 3.88±0.06 on day 15th. It indicates that ASA and IMPL co- administration able to treat disease in the early days concerning a drug given in individual forms (Fig.3 b). Muscle score was improved in a group of rats who received IMPL and IMPH from day 12th i.e. 3.32±0.05 and 3.49±0.06 respectively and at the end of the study it reached 3.46±0.08 and 3.61±0.04 for IMPL and IMPH respectively. Severity in muscle weakness was observed more intensified in a group of rats treated with PYR alone from day 3rd. Rats intoxicated with PYR received a treatment of ASA, showed the least treatment in abnormal muscle strength score.

3.3.3. Effect on the walking ability of rats

In this parameter again co-administration of IMPL+ASA and IMPH+ASA showed a similar pattern of treatment that starts from day 9th i.e. 19.16±0.16s and 16.56±0.10s respectively while it reached 15.09±0.17s and 11.19±0.18s on day 15th. Hence it can be assumed that ASA and IMPL giving more prominent effects when co- administrated as they can begin treatment in the early state. The ability of walking was improved in a group of rats received IMPL and IMPH from day 12th i.e. 28.02±0.05 and 24.15±0.11 respectively and at the end of the study, it reached 26.70±0.08 and 22.01±0.04 for IMPL and IMPH respectively. Rats intoxicated with PYR showed irregular walking and took a longer duration of time to travel 1m of length (Fig.3 c).

3.3.4. Effect on Sensitivity test: heat hyperalgesia

In the case of heat hyperalgesia test, a group of rats received PYR intoxication exhibited a delay in the reaction time i.e. delay of paw withdrawal (Fig.3 d) while significant improvement was recorded in the group of rats treated with IMPL+ASA and IMPH+ASA on the 9th day i.e. 12.41±0.56s and 11.63±0.48s respectively while on day 15th it becomes 11.09±0.06s and 10.47±0.36s respectively. It reflects the potentiating activity in the presence of ASA using IMP (at both doses). In ASA treated groups very minute kinds of changes were observed but lesser than both the doses of IMP. Low and high doses of IMP attenuate the symptoms of NP from day 12th.

3.3.5. Thermal tail-flick test

Latency in thermal response is represented in fig.4. From the 9th day onwards improvement in thermal latency was recorded in a group of rats treated with IMPL+ASA and IMPH+ASA while IMPL and IMPH exhibited treatment from the 12th day onwards. Rats treated with PYR alone showed less sensitivity and reacted to heat slower than the other groups (Fig.4.).

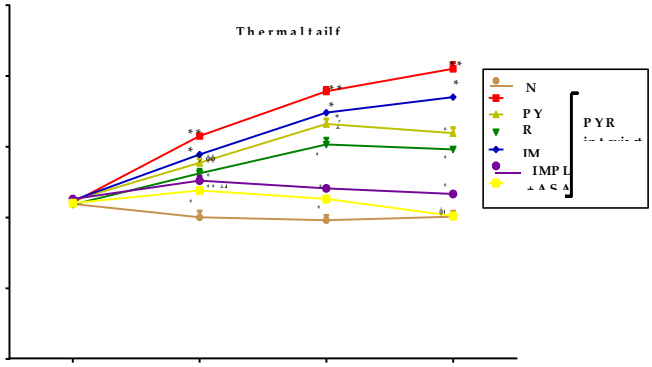

Fig. 4: Effect of treatment on nerve function of rats.

Each data represent mean±SD. * p<0.05, ** p<0.01 and ***p<0.001 when compared with control while ϕ p<0.05, ϕ ϕ p<0.01 and ϕ ϕ ϕ p<0.001 with respect to PYR

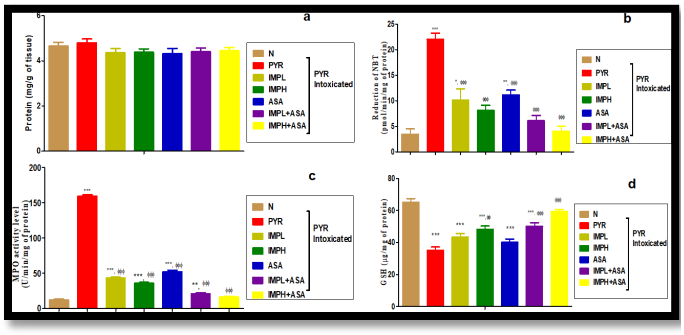

Fig-5: Effect on oxidative biomarkers a. Level of protein, b. Level of SOD, c. Level of MPO d. Level of GSH

Each data represent mean±SEM. * p<0.05, ** p<0.01 and ***p<0.001 when compared with control while ϕ p<0.05, ϕ ϕ p<0.01 and ϕ ϕ ϕ p<0.001 with respect to PYR

3.4. Estimations of oxidative biomarkers

No significant changes in the level of protein were observed in any group (Fig.5a). Induction of PYR causes significant enhancement of SOD in the sciatic nerve with respect to the control group. Significant attenuation was recorded in all the groups of rats with respect to group II i.e. PYR treated groups. But most effective attenuation was observed in a group of rats receive IMPL+ASA and IMPH+ASA i.e. 6.11±1.03 and 4.07±0.93 respectively (fig.5b). The level of MPO was also higher due to the induction of PYR are but its effect also protected in the same pattern as observed in SOD. The level of attenuation was observed better in IMPL+ASA and IMPH+ASA treated group of rats i.e. 21.06±1.19 and 16.23±1.17 respectively (fig.5c). It is well known that GSH causes direct chemical neutralization of reactive oxygen species like singlet oxygen, hydroxyl radicals, an superoxide radicals. Sometimes it acts as a cofactor for antioxidant enzymes (fig.5d). It is responsible to cause the regeneration of certain vitamins like C and E [34]. The decrease in the level of GSH was observed due to the administration of PYR in the sciatic nerve. But attenuation was investigated as 43.36±2.20, 48.13±2.10, 40.14±2.06, 50.16±2.11, and 59.34±1.17 in the group of rats treated with PYR, IMPL, IMPH, ASA, IMPL+ASA, and IMPH+ASA respectively.

3.5. Histopathology

A histopathological study was performed on the sciatic nerve. At the end of the study, animals were sacrificed to collect the sciatic nerve. In the control group, the normal architecture of neurons was observed but the group that received pyridoxine only showed loss of myelination, swelling of the axon, an increased number of Schwann as well as satellite cells. Protection in seen damage was observed more in the IMPH group while the IMPL groups of rats have less protective. It was also observed that a group of rats that receive ASA showed lesser protection than IMPL. This study indicated that co-administration of IMPL+ASA and IMPH+ASA gave a greater level of protection against pyridoxine. IMPH+ASA exhibited architecture closer to the normal group.

DISCUSSION

In the present investigation, it was observed that a high dose of PYR produces neuropathy in rats without any systemic morbidity and mortality. The intoxication of PYR causes a significant reduction in weight loss, abnormality in motor coordination, decreased muscle strength, delayed response to heat, and finally reduction in latency in the thermal tail-flick test. PYR intoxication leads to necrosis of dorsal route ganglia and damage of peripheral as well as sensory fibers of the central nervous system causes neuropathy.

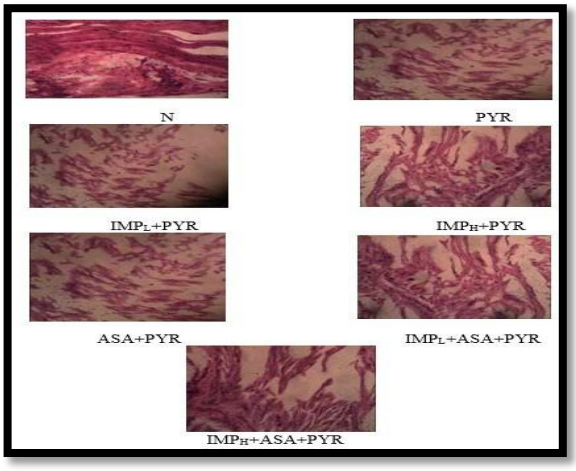

Fig. 6: Histopathology of the sciatic nerve of rats

The toxicity of PYR causes sciatic nerve axonopathy and an increase in the endoneurial area, which is probably related to axonal degeneration together with the increase of the number of small diameter fibers [1]. In our present investigation, all the abnormalities begin from day 3rd, and as time progresses its severity increases from moderate to severe. The performed parameters for general appearance, sensitivity, walking, and nerve function appeared to be sensitive and reliable to explain the painful neuropathy of rats. The aim of the study was not only to provide a new avenue of treatment but also emphasizes the co-administration of drugs at reduced doses to treat painful neuropathy in rats caused by PYR. The sensorimotor deficit in rats by this model indicates damage of large myelinated nerve fibers which was also supported by previous reports, gave a shred of evidence that this model is homology to human pathological states of neuropathy [35].

In this study, it was also established that combination therapy provides a better therapeutic effect as well as reduces the duration of treatment. It is well established that to be biologically active, PYR has to go to metabolism and converted to PLP (pyridoxal 5′-phosphate). For this conversion need phosphorylation of pyridoxine to pyridoxine 5′- phosphate, which further undergoes the process of oxidation to PLP [36]. To the best of our knowledge combination of imipramine and aspirin was never used to treat PYR-induced neuropathy. NSAIDs classes of drugs are known to possess analgesics and anti-inflammatory effects and can be used for any kind of pain. On the other hand, antidepressants are considered as the class of analgesics and can be useful in the treatment of chronic kinds of pain [22]. Combination of such two classes of analgesic provided here better pain relief than their response to reduction of pain. Potentiating kinds of responses were observed when we kept the doses of ASA constant and varying the doses of IMP. When IMP was administrated in rats at its both doses in association with ASA exhibited a dose-dependent therapeutic effect.

In our study group of rats received only PYR showed induction of pain in early days only but when treated with ASA then only slight modification in neuropathy was observed which starts from day 12 to 15th while IMP also exhibited the effect from same day only but its therapeutic effect in all parameters was better than ASA. Finally, co-administration brought major changes in NP conditions in rats as it gave potential therapeutic benefits from day 9th only till the end of experiments. In the previously reported article, it was established that a low dose of IMP in association with ASA exhibited a reduction in thermal pain [22]. Hence this study was designed to find out the activity of IMP at 10 and 20 mg/kg body in association with ASA (100mg/kg). The potentiating effect of ASA in the reduction of NP condition may due to its COX inhibition and PGs synthesis while IMP showed enhancement of descending inhibition of spinothalamic tract neurons [22]. These therapeutic benefits were supported by biochemical investigation and histopathological reports in which the level of GSH and neurons were protected. The index of such benefits is exhibited in the following manner: IMPH+ASA>IMPL+ASA>IMPH>IMPL>ASA.

CONCLUSION

It can be concluded that different pathway or mechanism to treat NP allows enhancement of reduction of pain when the drugs are used in co-administration even at lesser doses. So, co-administration increases the range of pain management, decreases dose frequency as well as side effects.

Declaration of Competing Interest

None

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