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Therapeutic potential of certain drug combinations on paclitaxel-induced peripheral neuropathy in rats

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Abstract

Background and Aims: Experimental research and clinical data support the potential combination therapy for the treatment of neuropathic pain. We aimed to investigate the analgesic effect of the following associations: gabapentin + etifoxine; tramadol + etifoxine; gabapentin + tramadol, in an experimental model of peripheral neuropathy induced by paclitaxel. *Materials and Methods*: Neuropathy was induced in male Wistar rats by the daily administration of 2 mg/kg body weight (bw) paclitaxel intraperitoneally, four days in a row. Analgesics were given concomitantly with paclitaxel, in the following doses: tramadol 15 mg/kg bw, etifoxine 100 mg/kg bw, gabapentin 300 mg/kg bw. Tactile allodynia and mechanical hyperalgesia were assessed using the Dynamic Plantar Aesthesiometer apparatus (Ugo Basile). After 18 days of treatment, the brain and liver tissue susceptibility to lipid peroxidation was evaluated and the sciatic nerve histological examination of the effect on myelin fibers was performed. *Results and Conclusions*: Experimental data have shown a strong analgesic effect of these three tested combinations expressed mainly by the statistically significant increased maximum response time, both in the assessment of allodynia and hyperalgesia. The gabapentin + tramadol combination lead to the maximum analgesic effect, immediately after the discontinuation of paclitaxel (44.94%, $p<0.0001$) and throughout the study. The treatment associated with tramadol caused a reduction in lipid peroxidation in the brain as compared to paclitaxel group. Combination therapy showed reduced damage to myelinated fiber density in the sciatic nerve. The drug combinations used in the experiment showed therapeutic potential in the fight against neuropathic pain induced by the administration of taxanes.

Keywords: gabapentin, tramadol, etifoxine, paclitaxel, neuropathic pain.

Introduction

The incidence of chemotherapy-induced peripheral neuropathy (CIPN) in the population of cancer patients is estimated at 1–12% [1]. Moreover, it is estimated that cytostatic monotherapy generates CIPN with a frequency of 3–7%, while polytherapy can affect as much as 38% of the patients [2]. Taxane (paclitaxel) therapy is routinely used in breast, ovarian and lung cancer, as well as Kaposi's sarcoma. Paclitaxel favors the development of abnormal microtubule bundles during the cell cycle and multiple asters during the interphase and mitosis leading to cell cycle arrest and apoptosis [3]. Clinical evaluations have revealed that paclitaxel therapy produces neuropathic pain in up to 64% of the treated patients (of which 4% manifest severe symptoms) [4, 5]. Signs of neuropathy may appear after a single treatment regimen, while intensity may increase with subsequent doses.

The mechanisms responsible for generating neuropathic pain are diverse and imply peripheral as well as central pathophysiological phenomena. The available options for the management of neuropathic pain imply multiple lines of therapy. A wide selection of drugs has been proposed, including tricyclic antidepressants (TCAs), anticonvulsants (gabapentin), *N*-methyl-D-aspartate receptor antagonists, gamma-aminobutyric acid (GABA) B receptor agonists and topical capsaicin. However, sufficient pain relief is reported only by as much as half the treated patients [6]. The wide array of pharmacodynamic properties of these drugs stands testament to the fact that there does not appear to be one single, ideal treatment [7–9]. Combination therapy (gabapentin combined with opioids or TCAs) is also recommended [10, 11] for patients who show partial response to single drugs, but more large-scale trials are needed for confirmation [12].

Using an experimental model of paclitaxel-induced painful peripheral neuropathy in rats, we sought out to evaluate changes in mechanical sensitivity and to identify potential analgesic effects of certain drug combinations. As such, in the attempt to cover a broad array of

mechanisms, we used combinations of gabapentin (which binds to the $\alpha 2\delta$ site of voltage gated calcium channels) with etifoxine (a non-benzodiazepinic drug that binds to the GABAA receptor $\beta 2$ and $\beta 3$ subunits [13]) or tramadol (a mixed mechanism analgesic drug used in the acute phase of neuropathic pain [14, 15]), and etifoxine with tramadol.

Oxidative stress may occur due to imbalances between reactive oxygen species (ROS) production and native neutralizing or repair capacity. This has been shown to be correlated to the presence of pain [16, 17]. Measuring ROS directly is arduous due to their brief half-life; hence, we focused our efforts on evaluating their effects by quantifying malonyldialdehide (MDA), a product of lipid peroxidation, which retains the position of one of the most frequently used indicators of oxidative stress [18].

Clinical and neurophysiological data conclude that paclitaxel induces a distal axonal neuropathy [19]. Examination of the sciatic nerve of paclitaxel-treated rats revealed broadened myelin layers, leading to inconsistent thickness throughout the length of the myelin sheet, and axonal diameter shrinkage [20].

In this regard, we aimed to evaluate the paclitaxel-induced peripheral neuropathy by the immunohistochemical analysis of the myelin sheet corresponding to the sciatic nerves of treated rats.

Materials and Methods

Animals

Adult male Wistar rats (235.9±27.33 g, *N*=47), were supplied by the rodent farm of the "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania, and housed in groups of nine or 10 on sawdust bedding in Plexiglas cages, having free access to water and food. Experiments were carried out between 8:00 a.m. and 2:00 p.m. All animals were habituated to the testing environment. The temperature was maintained between 20^0C and 24^0C and the relative humidity was generally maintained at 45–60%. All procedures were carried out in accordance with the *European Directive* 2010/63/UE/ 22.09.2010 regarding the protection of animals used for experimental and other scientific purposes. The experimental protocol was approved by the Ethics Committee of the Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy.

Rat model of paclitaxel-induced peripheral neuropathy

Paclitaxel 2 mg/kg body weight (bw) was injected intraperitoneally (i.p.), once daily, for four days, following the method described by Zbârcea *et al.* (2011) [21].

Mechanical sensitivity

This sensitivity was assessed using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). Animals were placed on an elevated wire mesh bottomed cage (22×16.5×14 cm) and left for habituation for 15 minutes. The unit raises a metal rod (0.5 mm diameter) until it touches the plantar surface of the hind paws and begins to exert an upwards force until the paw is withdrawn or the preset cut-off is reached. Tactile allodynia was measured by assessing hind paw withdrawal thresholds in response to a mechanical stimulus exerting a linearly increasing force (2 g/s; cut-off force: 20 g). For the mechanical hyperalgesia, we measured the response to mechanical stimulation (force-increasing rate: 4 g/s, cut-off force: 40 g). Three readings were taken on each paw at each time point with a 3–5 minute interval between trials. The individual data are presented as the mean of the six readings. The force and the time required to elicit a withdrawal responses are measured, respectively, in grams and seconds.

Tissue preparation and immunohistochemical analysis

Tissue sample collection

The animals were beheaded under ether anesthesia. Livers and brains were rapidly excised and frozen. In addition, sciatic nerves and glabrous as well as pilose skin samples were taken and submerged in 4% formaldehyde [22].

Degenerative changes of the myelin fibers experimentally induced by paclitaxel

After fixation for 24 hours in 4% formaldehyde, the skin samples and the sciatic nerves were dehydrated in alcohol solutions. Then, the samples were submerged in paraffin. Afterwards, tissue microarrays were manufactured using arrays provided by HistoBest Diagnostics SRL, Bucharest. Using a Leica 2035 microtome, the skin samples were sliced and treated with aminosilan [23].

Immunohistochemical assay

The slices were cleaned of paraffin and rehydrated in distilled water [24]. After being repeatedly washed in 0.05% Tween 20 supplemented phosphate buffer (pH 7.4), the slices were incubated with a non-seric universal agent (DakoCytomation, Denmark, A/S). Following the removal of the blocking agent (without washing), the slices were incubated with anti-major basic myelin (mouse IgG1, clone 7H11, Leica Biosystems Newcastle Ltd., UK). Endogenous peroxidase activity was blocked afterwards by means of 2% H_2O_2 incubation for 30 minutes. The slices were incubated with EnVision Flex anti-mouse (Dako) and with 3,3'-diaminobenzidine (DAB) (Dako) [DAB was used as chromogen for WNT10A staining (brown color)]. Nuclear counterstaining was performed with methyl green (1% in acetate buffer, pH 4). The prepared samples were viewed with a Nikon Eclipse 50i microscope and images were captured with a Nikon Digital Sight DS-Fi1 camera.

Evaluation of lipid peroxidation, and susceptibility to oxidative stress in rat tissues

Determination of tissue lipid peroxidation

Lipid peroxidation was measured as thiobarbituric acid reactive substance (TBARS). The amount of tissue TBARS was measured by the thiobarbituric acid assay as previously described [25]. The extent of the peroxidative reactions was determined by measuring MDA. MDA extinction coefficient (0.156 $\mu M^{-1}cm^{-1}$) was used for calculation of TBARS content. TBARS were expressed as nmoles MDA/mg tissular proteins:

$$E_{535}/(\varepsilon \times l) \times f \times (1/c)$$

where E_{535} – optical density of the sample compare to control at a wavelength of 535 nm; $\varepsilon \times l$ – molar extinction coefficient for MDA of 0.156 cm/mol; f – dilution factor of 55; $1/c$ – protein concentration of the tissue homogenates.

Protein measurement was performed using the Folin–Ciocâlteu reagent, modified by Lowry *et al.* (1951) [26].

Experimental design

Assessment of tactile allodynia and mechanical hyperalgesia was performed right before and at 4, 10 and 18 days after the first administration of paclitaxel by measuring mechanical-induced sensitivity to pain with Dynamic Plantar Aesthesiometer (Ugo Basile, Italy).

The five animal groups received daily, for 18 days, the following:

- Group C: Control group (N=9) – normal saline 0.1 mL/100 g bw, i.p., four days and distilled water, 1 mL/100 g bw, i.p., 18 days;
- Group P: Paclitaxel group (N=9) – paclitaxel 2 mg/kg bw, i.p., four days and distilled water, 1 mL/100 g bw, i.p., 18 days;
- Group GE: Gabapentin and etifoxine group (N=10) – paclitaxel 2 mg/kg bw, i.p., four days, gabapentin 300 mg/kg bw and etifoxine 100 mg/kg bw, *p.o.*, 18 days;
- Group TE: Tramadol and etifoxine group (N=10) – paclitaxel 2 mg/kg bw, i.p., four days, tramadol 15 mg/kg bw and etifoxine 100 mg/kg bw, *p.o.*, 18 days;
- Group GT: Gabapentin and tramadol group (N=9) – paclitaxel 2 mg/kg bw, i.p., four days, gabapentin 300 mg/kg bw and etifoxine 100 mg/kg bw, *p.o.*, 18 days.

After the last measurement of tactile sensitivity on day 18 of the treatment, animals were sacrificed and tissue samples were collected. Sciatic nerve biopsies (2–3 samples/group), glabrous skin (plantar area) and pilose skin (ear) were harvested after euthanasia. In sections from these tissues, we observed myelinated fibers.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM) of nine or 10 animals per group. Results were processed using GraphPad Prism 5 software (San Diego, California, USA, www.graphpad.com). We established distribution normality in the groups using the D'Agostino & Pearson's test. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's (when compared to the baseline response) or Bonferroni's test (when compared to control group or paclitaxel group). If the results of the group are abnormally distributed, we used the Kruskal–Wallis test followed by Dunn's Multiple Comparison Test (when compared to the baseline response, control group or paclitaxel group).

Results

Mechanical sensitivity

The mechanical response was assessed by two parameters: time to response (s) and applied force (g). Paclitaxel treatment established and maintained neuropathic states characterized by hypersensitivities to mechanical stimulation (allodynia – 2 g/s and hyperalgesia – 4 g/s).

Treatment with paclitaxel significantly reduced the withdrawal thresholds to mechanical allodynia (reduces both the time to response and also the force applied for paw retraction) on day 18 after the first drug administration (9.17±1.24 s and 15.30±0.68 g) compared to the control mechanical thresholds (15.83±1.48 s and 18.54± 0.70 g) (p<0.05; Table 1).

Table 1 – ***Modifications in time to response and applied force during allodynia (2 g/s) following paclitaxel and each of the co-administered association: gabapentin/etifoxine, tramadol/etifoxine, gabapentin/tramadol***

Group		Behavioral tactile allodynia in response to stimulus 2 g/s							
		Basal		*4 days*		*10 days*		*18 days*	
		Time [s]	*Force [g]*	*Time [s]*	*Force [g]*	*Time [s]*	*Force [g]*	*Time [s]*	*Force [g]*
Control (C)	Mean/group ± SEM	16.54 ±2.63	18.05 ±0.507	15.40 ±0.10	18.94 ±0.34	15.45 ±1.31	18.51 ±0.37	15.83 ±1.48	18.54 ±0.70
	Percentage change *vs.* baseline			-6.89	4.93	-6.59	2.55	-4.29	2.71
	Kruskal–Wallis test followed by Dunn's *post-hoc* test *vs.* baseline (*p*)			ns	ns	ns	ns	ns	ns
Paclitaxel (P)	Mean/group ± SEM	16.55 ±1.87	17.23 ±0.72	12.09 ±1.74	15.6 ±1.23	12.25 ±2.54	15.78 ±1.16	9.17 ±1.24	15.30 ±0.68
	Percentage change *vs.* baseline			-26.95	-9.46	-25.98	-8.42	-44.59	-11.20
	ANOVA test followed by Dunnett's *post-hoc* test *vs.* baseline (*p*)			ns	ns	ns	ns	ns	ns
	Percentage change *vs.* control group			-20.06	-14.39	-19.39	-10.97	-40.29	-13.91
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* control group (*p*)			ns	*	ns	ns	*	*
Gabapentin / etifoxine (GE)	Mean/group ± SEM	17.15 ±2.59	18.32 ±0.48	24.91 ±3.30	19.14 ±0.42	23.96 ±4.06	18.87 ±0.45	23.54 ±4.64	19.07 ±0.45
	Percentage change *vs.* baseline			45.25	4.48	39.71	3.00	37.26	4.09
	Kruskal–Wallis test followed by Dunn's *post-hoc* test *vs.* baseline (*p*)			ns	ns	ns	ns	ns	ns
	Percentage change *vs.* control group			52.14	-0.45	46.30	0.45	41.55	1.38
	Kruskal–Wallis test followed by Dunn's *post-hoc* test *vs.* control group (*p*)			ns	ns	ns	ns	ns	ns
	Percentage change *vs.* paclitaxel group			72.20	13.94	65.69	11.42	81.85	15.30
	Kruskal–Wallis test followed by Dunn's *post-hoc* test *vs.* paclitaxel group (*p*)			*	*	*	ns	**	*

Group		Behavioral tactile allodynia in response to stimulus 2 g/s							
		Basal		***4 days***		***10 days***		***18 days***	
		Time [s]	*Force [g]*	*Time [s]*	*Force [g]*	*Time [s]*	*Force [g]*	*Time [s]*	*Force [g]*
Tramadol / etifoxine (TE)	Mean/group ± SEM	16.45 ±1.46	19.29 ±0.29	40.02 ±5.91	19.48 ±0.19	42.59 ±8.05	18.96 ±0.51	32.44 ±4.93	19.6 ±0.18
	Percentage change *vs.* baseline			143.28	0.98	158.91	-1.71	97.20	1.61
	ANOVA test followed by Dunnett's *post-hoc* test *vs.* baseline (*p*)			*	ns	**	ns	ns	ns
	Percentage change *vs.* control group			150.18	-3.95	165.50	-4.26	101.50	-1.10
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* control group (*p*)			**	ns	***	ns	ns	ns
	Percentage change *vs.* paclitaxel group			170.23	10.44	184.89	6.70	141.79	12.81
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* paclitaxel group (*p*)			***	**	***	*	**	***
Gabapentin / tramadol (GT)	Mean/group ± SEM	16.01 ±2.21	17.47 ±0.98	44.06 ±4.49	19.96 ±0.04	37.72 ±3.98	19.59 ±0.17	30.38 ±3.46	19.87 ±0.09
	Procentual change *vs.* baseline			175.20	14.25	135.60	12.14	89.76	13.74
	Kruskal–Wallis test followed by Dunn's *post-hoc* test *vs.* baseline (*p*)			***	***	***	ns	*	**
	Percentage change *vs.* control group			182.10	9.32	142.19	9.59	94.05	11.03
	Kruskal–Wallis test followed by Dunn's *post-hoc* test *vs.* control group (*p*)			***	*	***	ns	**	ns
	Percentage change *vs.* paclitaxel group			202.15	23.71	161.58	20.55	134.34	24.94
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* paclitaxel group (*p*)			***	***	***	ns	***	***

SEM: The standard error of the mean; ns: Not significant; ANOVA: Analysis of variance; *p<0.05; **p<0.01; ***p<0.001.

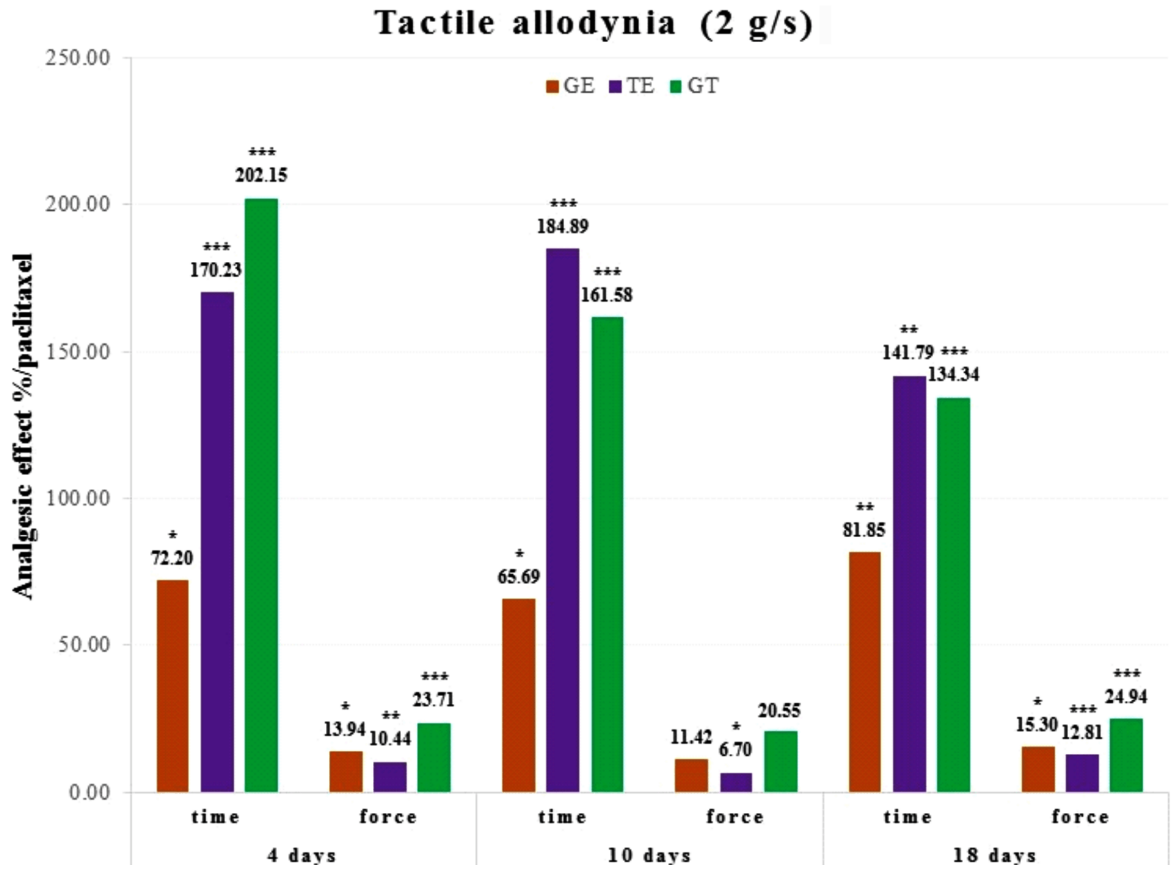

Figure 1 – ***Analgesic effect (%) of each co-treatment (GE: gabapentin 300 mg/kg bw + etifoxine 100 mg/kg bw; TE: tramadol 15 mg/kg bw + etifoxine 100 mg/kg bw; GT: gabapentin 300 mg/kg bw + tramadol l5 mg/kg bw) in tactile allodynia induced with paclitaxel group. Group comparisons were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test or Kruskal–Wallis test followed by Dunn's post-hoc test, in comparison to paclitaxel. *p<0.05; **p<0.01; ***p<0.001.***

Oral administration of the association GE (gabapentin 300 mg/kg bw + etifoxine 100 mg/kg bw) produced a significant inhibition of mechanical allodynia induced by paclitaxel (increases both the time to response and also the force applied for paw retraction) on days 4, 10 and 18 after the first drug administration compared to paclitaxel treated animals (p<0.05; Table 1).

The association TE (tramadol 15 mg/kg bw + etifoxine

100 mg/kg bw) reduced mechanical allodynia induced by paclitaxel (increases both the time to response and also the force applied for paw retraction) at all time measurements (Table 1).

Rats treated with the association GT (gabapentin 300 mg/kg bw + tramadol 15 mg/kg bw) experienced a significant reduction of mechanical allodynia especially on days 4 and 10 (Table 1).

In this study, repeated administration of all three associations inhibited the development of paclitaxel-induced mechanical allodynia. Remarkable effects were recorded in the two combinations containing tramadol, in particular for the parameter time to response (Figure 1).

Paclitaxel produced a significant reduction in response withdrawal thresholds to mechanical stimulus of 4 g/s (mechanical hyperalgesia), on day 18 after first drug administration compared to the baseline and control group (Table 2).

Paclitaxel-induced mechanical hyperalgesia in rats was decreased by administration of the tested associations. The parameter time to response was changed significantly at all moments of determinations. The strongest analgesic effect was registered for the combination GT (gabapentin 300 mg/kg bw + tramadol 15 mg/kg bw), which increased the time to response after 18 days with 70.6% compared to paclitaxel group (p<0.001) (Figure 2).

Table 2 – ***Modifications in time to response and applied force during hyperalgesia (4 g/s) following paclitaxel and each of the co-administered association: gabapentin/etifoxine, tramadol/etifoxine, gabapentin /tramadol***

Group		Behavioral tactile hyperalgesia in response to stimulus 4 g/s							
		Basal		*4 days*		*10 days*		*18 days*	
		Time [s]	*Force [g]*	*Time [s]*	*Force [g]*	*Time [s]*	*Force [g]*	*Time [s]*	*Force [g]*
Control (C)	Mean/group ± SEM	6.83 ±0.39	27.32 ±1.51	6.75 ±0.34	26.95 ±1.29	6.70 ±0.33	26.64 ±1.13	6.79 ±0.57	26.47 ±1.99
	Percentage change *vs.* baseline			-1.14	-1.35	-1.89	-2.49	2.71	-3.11
	ANOVA test followed by Dunnett's *post-hoc* test *vs.* baseline (p)			ns	ns	ns	ns	ns	ns
Paclitaxel (P)	Mean/group ± SEM	7.08 ±0.47	27.28 ±1.54	5.90 ±0.71	23.33 ±2.59	5.85 ±0.53	23.54 ±2.1	5.22 ±0.51	21.06 ±2.00
	Percentage change *vs.* baseline			-16.66	-14.48	-17.37	-13.71	-26.28	-22.80
	ANOVA test followed by Dunnett's *post-hoc* test *vs.* baseline (p)			ns	ns	ns	ns	**	*
	Percentage change *vs.* control group			-15.52	-13.13	-15.48	-11.22	-29.00	-19.69
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* control group (p)			ns	ns	ns	ns	*	ns
Gabapentin / Etifoxine (GE)	Mean/group ± SEM	7.14 ±0.46	27.77 ±1.71	7.86 ±0.46	29.75 ±1.24	8.07 ±0.314	31.19 ±0.9	8.32 ±0.48	32.10 ±1.55
	Percentage change *vs.* baseline			10.18	7.13	13.10	12.32	16.59	15.59
	ANOVA test followed by Dunnett's *post-hoc* test *vs.* baseline (p)			ns	ns	ns	ns	ns	ns
	Percentage change *vs.* control group			11.32	8.48	14.99	14.80	13.88	18.70
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* control group (p)			ns	ns	ns	*	*	**
	Percentage change *vs.* paclitaxel group			26.83	21.61	30.47	26.03	42.88	38.39
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* paclitaxel group (p)			*	*	**	*	***	***
Tramadol / etifoxine (TE)	Mean/group ± SEM	6.81 ±0.44	27.00 ±1.60	8.36 ±0.59	31.80 ±1.74	7.92 ±0.74	29.60 ±2.40	8.27 ±0.37	31.96 ±1.11
	Percentage change *vs.* baseline			22.77	17.78	16.26	9.63	21.31	18.37
	ANOVA test followed by Dunnett's *post-hoc* test *vs.* baseline (p)			ns	ns	ns	ns	ns	ns
	Percentage change *vs.* control group			23.91	19.13	18.15	12.12	18.60	21.48
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* control group (p)			ns	ns	ns	ns	ns	ns
	Percentage change *vs.* paclitaxel group			39.42	32.26	33.63	23.34	47.59	41.17
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* paclitaxel group (p)			*	*	ns	*	**	**
Gabapentin / tramadol (GT)	Mean/group ± SEM	6.87 ±0.48	27.41 ±1.80	11.12 ±0.72	35.76 ±0.96	9.92 ±0.64	35.85 ±0.85	9.91 ±0.61	35.97 ±0.87
	Percentage change *vs.* baseline			61.89	30.46	44.42	30.79	44.32	31.23
	ANOVA test followed by Dunnett's *post-hoc* test *vs.* baseline (p)			***	***	*	***	*	***
	Percentage change *vs.* control group			63.03	31.82	46.31	33.28	41.60	34.34
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* control group (p)			***	***	**	***	**	***
	Percentage change *vs.* paclitaxel group			78.55	44.94	61.78	44.50	70.60	54.03
	ANOVA test followed by Bonferroni's *post-hoc* test *vs.* paclitaxel group (p)			***	***	**	***	**	***

SEM: The standard error of the mean; ns: Not significant; ANOVA: Analysis of variance; *p<0.05; **p<0.01; ***p<0.001.

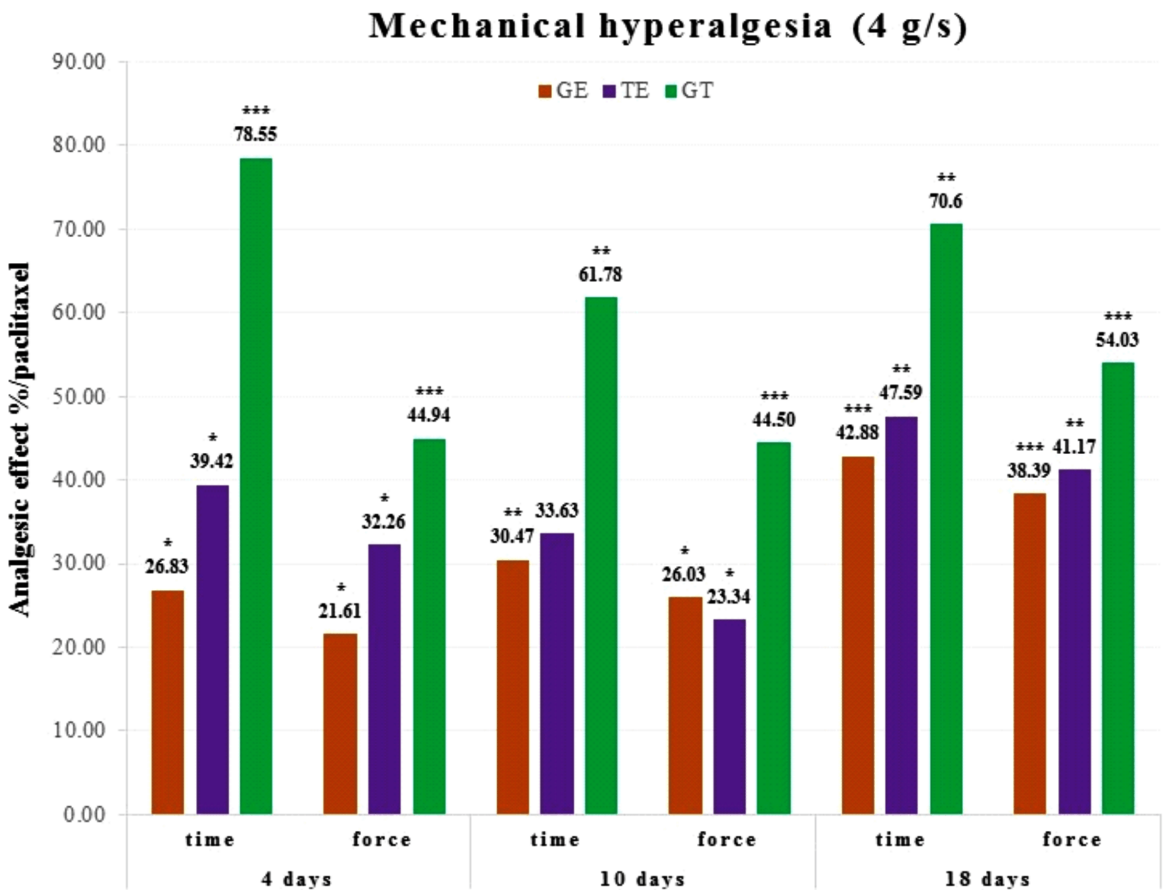

Figure 2 – ***Analgesic effect (%) of each co-treatment (GE: gabapentin 300 mg/kg bw +etifoxine 100 mg/kg bw; TE: tramadol 15 mg/kg bw + etifoxine 100 mg/kg bw; GT: gabapentin 300 mg/kg bw + tramadol 15 mg/kg bw) in the mechanical hyperalgesia induced with paclitaxel. Group comparisons were performed using the one-way analysis of variance (ANOVA) followed by Bonferroni's test, in comparison to paclitaxel group. *p<0.05; **p<0.01; ***p<0.001.***

Evaluation of lipid peroxidation, and susceptibility to oxidative stress in rat tissues

Susceptibility to lipid peroxidation, expressed as nmol/mg MDA protein in the brain and liver tissue homogenate, and the statistical significance of the results is presented in Table 3.

Table 3 – ***Susceptibility to lipid peroxidation values, expressed as nmol/mg MDA protein in the brain and liver tissue homogenate, for control (C), paclitaxel (P), gabapentin/etifoxine (GE), tramadol/etifoxine (TE) and gabapentin/tramadol (GT) groups. Percentage change vs. control group or paclitaxel group and the statistical significance of the results***

	nmoles MDA/mg tissular proteins from brain homogenate				
	Group				
	C	*P*	*GE*	*TE*	*GT*
Mean/group ± SEM	13.17 ±0.678	20.64 ±1.028	11.03 ±0.865	8.78 ±1.178	7.35 ±1.077
Percentage change *vs.* control group		56.76	-16.20	-33.29	-44.15
ANOVA test followed by Bonferroni's *post-hoc* test *vs.* control group (*p*)		***	ns	*	***
Percentage change *vs.* paclitaxel group			-46.54	-57.44	-64.37
ANOVA test followed by Bonferroni's *post-hoc* test *vs.* paclitaxel group (*p*)			***	***	***

	nmoles MDA/mg tisular proteins from liver homogenate				
	Group				
	C	*P*	*GE*	*TE*	*GT*
Mean/group ± SEM	13.3 ±2.005	12.57 ±2.099	11.77 ±1.74	9.88 ±1.454	12.91 ±2.162
Percentage change *vs.* control group		-5.48	-11.54	-25.74	-2.90
ANOVA test followed by Bonferroni's *post-hoc* test *vs.* control group (*p*)		ns	ns	ns	ns
Percentage change *vs.* paclitaxel group			-6.41	-21.43	2.73
ANOVA test followed by Bonferroni's *post-hoc* test *vs.* paclitaxel group (*p*)			ns	ns	ns

MDA: Malonyldialdehide; SEM: The standard error of the mean; ns: Not significant; ANOVA: Analysis of variance; *p<0.05; **p<0.01; ***p<0.001.

For groups treated with paclitaxel 2 mg/kg bw, i.p., four consecutive doses (P, GT, TE, GT), the liver MDA values have not changed significantly comparing to the control group (Table 3).

In the brain, there was an enhanced susceptibility to lipid peroxidation for the group that received just paclitaxel. Treatment with tramadol/etifoxine (-33.29%; p<0.05) and gabapentin/tramadol (-44.15%, p<0.001) reduced the malondialdehyde formation in the brain (Figure 3).

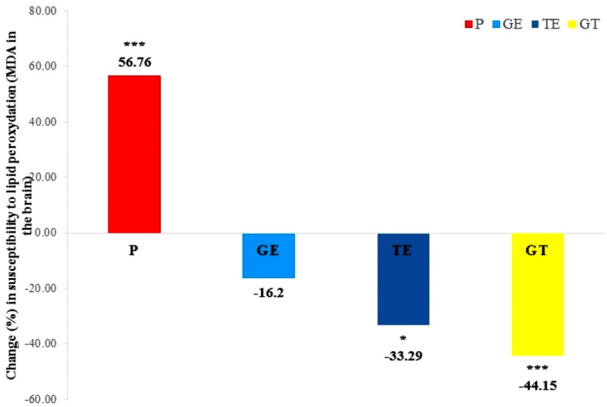

Figure 3 – ***Change (%) in susceptibility to lipid peroxidation (MDA in the brain) to groups: paclitaxel (P), gabapentin/etifoxine (GE); tramadol/etifoxine (TE); gabapentin/tramadol (GT). Group comparisons were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test, in comparison to control group. MDA: Malonyldialdehide; *p<0.05; ***p<0.001.***

Immunohistochemistry on formalin-fixed paraffin-embedded tissue slices. Observations on myelinated fibers

The nerves showed areas of demyelination and clear signs of axonal degeneration highlighted by reduction in the density of myelinated fibers (Figure 4). Following the morphological analysis under the optical microscope, it was concluded that C group exhibited sciatic nerves rich in myelinated fibers, while P group showed a marked decline in myelinated fiber density.

However, the GE group showed only a moderate reduction of myelinated nerve fiber density (moderate axonal degeneration) with the prevalence of thick fibers. The best outcome was observed in the TE group, which exhibited only a slight reduction in myelinated nerve fiber density (minimal axonal degeneration) with numerous myelinated thick fibers. The GT group displayed the most severe reduction in myelinated nerve fiber density (severe axonal degeneration). In Figure 4H, the sciatic nerve is rich in myelinated fibers.

Upon analysis of the skin samples from the ear, all treated groups presented small myelinated nerve fiber endings (Figure 5).

Discussion

CIPN affects up to 80% of patients during chemotherapy and it is a severe adverse effect that can limit dose and choice of chemotherapy. Mechanisms involved in CIPN include disruption of axonal transport, altered ion channel and receptor activity, neuronal injury and inflammation, oxidative stress, and mitochondrial damage [27].

The aim of our study was to investigate the effect of three combinations of active substances on the peripheral neuropathy induced by paclitaxel in rats. Using the three drugs in combinations was supported by their different mechanisms of action in order to achieve a therapeutic effect potentiation. Data from preclinical literature shows a wide scale use of gabapentin doses (300–900 mg/kg) and in the case of these associations, we used the minimum therapeutic dose, especially for the safety profile of the active substance. For etifoxine, the used dose was equal to 1/10 of the LD_{50} determined in rats.

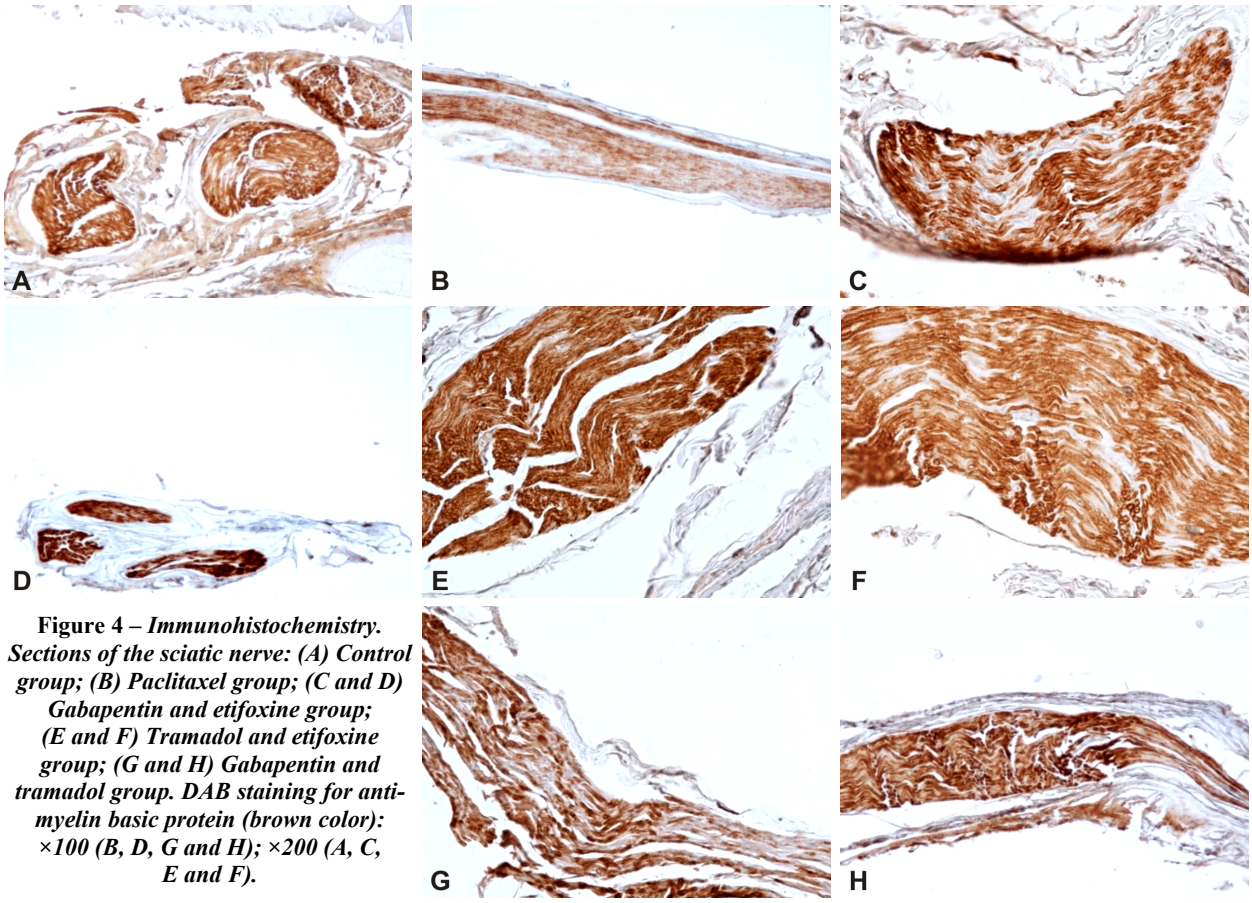

Figure 4 – ***Immunohistochemistry. Sections of the sciatic nerve: (A) Control group; (B) Paclitaxel group; (C and D) Gabapentin and etifoxine group; (E and F) Tramadol and etifoxine group; (G and H) Gabapentin and tramadol group. DAB staining for anti-myelin basic protein (brown color): ×100 (B, D, G and H); ×200 (A, C, E and F).***

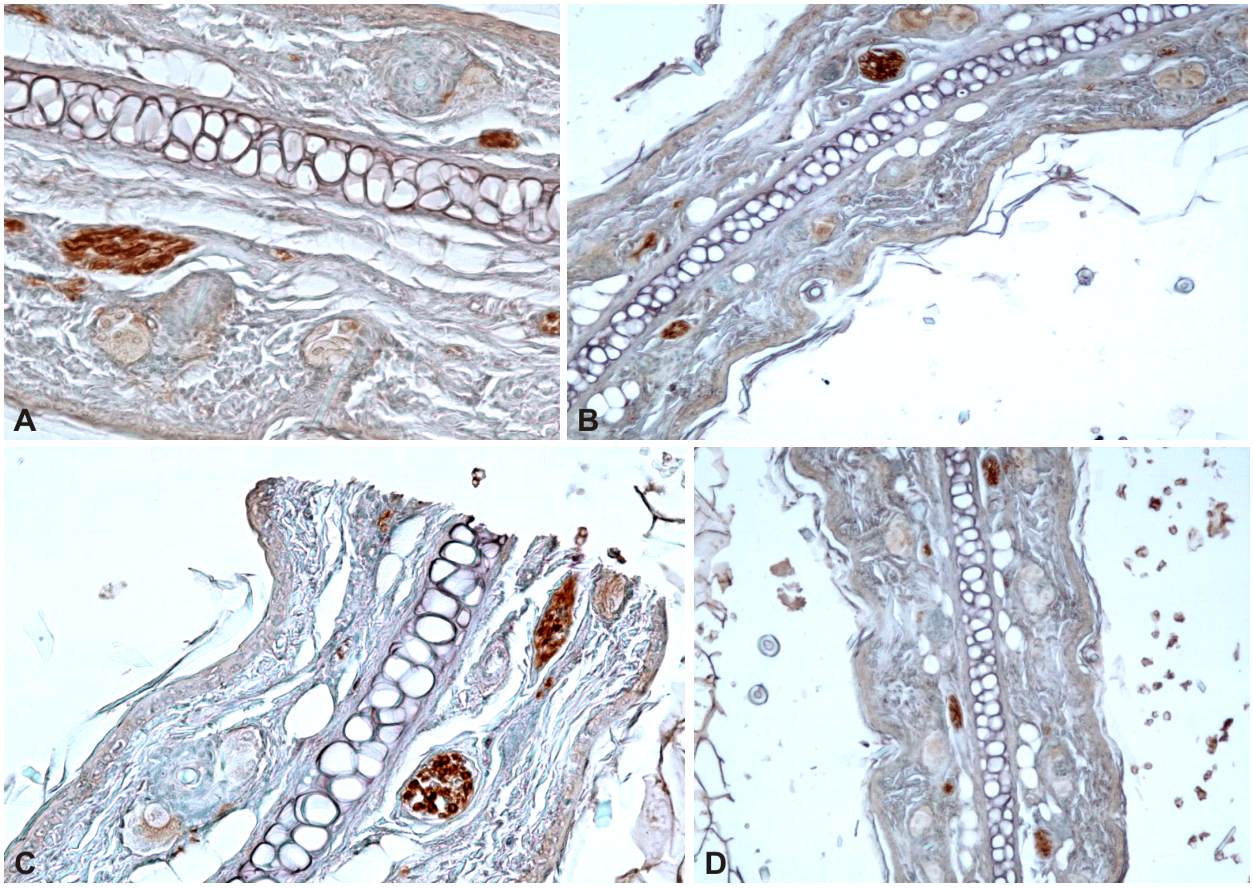

Figure 5 – ***Slices of pilose skin extracted from the ears, with observable myelinated nerve endings DAB staining for anti-myelin basic protein (brown areas): ×100 (B and D); ×200 (A and C).***

We noticed changes in tactile sensitivity, during treatment and in the end, we determined the susceptibility to oxidative stress and degenerative changes of myelin in the central and peripheral nervous system.

Allodynia and hyperalgesia following the administration of paclitaxel are highlighted by reducing the time to response and the force applied for paw retraction. The smallest tactile sensitivity threshold for the paclitaxel group is registered after 18 days, when the parameter time to response decreases *vs.* control group (allodynia -40.29%, $p<0.05$; hyperalgesia -29%, $p<0.05$) (Tables 1 and 2). These data are in line with other experimental researches [28, 29].

The experimental results have shown the analgesic effect of all three tested associations, expressed especially by significantly increasing the time to response, both in the assessment of allodynia and hyperalgesia.

Remarkable effects in reducing allodynia were recorded for the two associations containing tramadol. The combination of gabapentin 300 mg/kg bw + tramadol 15 mg/kg bw presented anti-allodynic effect *versus* paclitaxel group, most intensively after four days (202.15%; $p<0.001$) (Figure 1).

The mechanical hyperalgesia induced by paclitaxel in rats was decreased by administration of the tested combinations. The highest analgesic effect was recorded for the combination of gabapentin 300 mg/kg bw + tramadol 15 mg/kg bw, after 18 days, when the time to response increased by 70.6% relative to the paclitaxel group ($p<0.001$) (Figure 2). These data are consistent with results reported in literature that show clinical efficacy of tramadol in the treatment of acute neuropathic pain [14, 15]. Our experimental study shows that the analgesic effect of tramadol is maintained over time, when it is associated with gabapentin (Figures 1 and 2).

Mitochondrial dysfunction is a potential cause of chemotherapy-induced peripheral neuropathy [30]. Zheng *et al.* showed a link between deformation (increase in volume, "swelling") of the mitochondria in damaged nerve pathways and triggering neuropathic pain [30]. Paclitaxel is a chemotherapeutic drug, which damages mitochondrial molecular structures through abnormal apoptotic processes [31]. Neuropathic pain is favored by affecting mitochondria in nerve pathways (due to the oxidative effects of paclitaxel); reducing this phenomenon could be a possible target for the therapy.

Gabapentin shows neuroprotective effects supporting the antioxidant molecular mechanisms in neuronal mitochondria [32, 33], being recommended to be used in neurodegenerative diseases. Etifoxine is described in the literature as a potential antioxidant molecule [34]. Experimental studies present data on its beneficial anti-inflammatory effects on the nerves harmed by paclitaxel [35].

To assess oxidative stress, we determined MDA, one of the most commonly used indicators of lipid peroxidation, knowing that pain is closely related to the peroxidation of lipids [16, 17]. The experimental results showed the involvement of oxidative stress in the development and evolution of neuropathic pain. The

pharmacological therapy used by us significantly reduced the lipid peroxidation and susceptibility to oxidative stress compared to the group treated with paclitaxel. Therefore, gabapentin and etifoxine can improve peripheral neuropathy induced by paclitaxel due to their neuroprotective, anti-inflammatory, antioxidant mechanisms. These effects benefit from the addition of the analgesic effect of tramadol. Our results show neuroprotective and antioxioxidant effects especially for the combination gabapentin/tramadol, and then for the association etifoxine/tramadol.

Observations on myelinated fibers indicated towards histological changes induced by paclitaxel on the sciatic nerves and the possible protective effect of the tested substances. After examining the sciatic nerve for all those receiving paclitaxel, we observed areas of segmental demyelination, depletion and destruction of the myelin sheath as well as axonal degeneration. These observations are consistent with literature data [36]. The groups treated with combinations (etifoxine/gabapentin, tramadol/etifoxine, gabapentin/tramadol) showed reduced damage of the myelin fiber density from the sciatic nerve.

Conclusions

Our present data provide evidence that allodynia and hyperalgesia evoked by paclitaxel might be inhibited by the tested combinations (etifoxine/gabapentin, tramadol/etifoxine and gabapentin/tramadol). Importantly, the beneficial effects of the combinations used on paclitaxel-induced peripheral neuropathy might be mediated by reducing the lipid peroxidation and susceptibility to oxidative stress and by decreasing the damage on the myelin fibers. An advantage of these combinations lies in the use of lower doses than with the single active substances for the significant improvement of the profile of side effects. The gabapentin/tramadol combination might represent a strong addition to the array of therapeutic options for neuropathic pain, awaiting future high quality validating clinical trials.

Conflict of interests

The authors declare that they have no conflict of interests.

References

[1] Paice JA. Mechanisms and management of neuropathic pain in cancer. J Support Oncol, 2003, 1(2):107–120.

[2] Cavaletti G, Zanna C. Current status and future prospects for the treatment of chemotherapy-induced peripheral neurotoxicity. Eur J Cancer, 2002, 38(14):1832–1837.

[3] Park SB, Lin CS, Krishnan AV, Friedlander ML, Lewis CR, Kiernan MC. Early, progressive, and sustained dysfunction of sensory axons underlies paclitaxel-induced neuropathy. Muscle Nerve, 2011, 43(3):367–374.

[4] Cavaletti G, Bogliun G, Marzorati L, Zincone A, Marzola M, Colombo N, Tredici G. Peripheral neurotoxicity of taxol in patients previously treated with cisplatin. Cancer, 1995, 75(5):1141–1150.

[5] Cavaletti G, Cavalletti E, Montaguti P, Oggioni N, De Negri O, Tredici G. Effect on the peripheral nervous system of the short-term intravenous administration of paclitaxel in the rat. Neurotoxicology, 1997, 18(1):137–145.

[6] Hansson P, Lacerenza M, Marchettini P. Aspects of clinical and experimental neuropathic pain: the clinical perspective. In: Hansson PT, Fields HL, Hill RG, Marchettini P (eds). Neuropathic pain: pathophysiology and treatment. Vol. 21, Progress in Pain Research and Management, IASP Press, Seattle, 2001, 1–18.

[7] Dworkin RH. Introduction: Recommendations for the diagnosis, assessment, and treatment of neuropathic pain. Am J Med, 2009, 122(10 Suppl):S1–S2.

[8] Coluzzi F, Pappagallo M; National Initiative on Pain Control. Opioid therapy for chronic noncancer pain: practice guidelines for initiation and maintenance of therapy. Minerva Anestesiol, 2005, 71(7–8):425–433.

[9] Jensen TS, Gottrup H, Sindrup SH, Bach FW. The clinical picture of neuropathic pain. Eur J Pharmacol, 2001, 429(1–3): 1–11.

[10] Amorim D. Pharmacological treatment of neuropathic pain: review of oral and topical therapy recommendations. Int J Clin Neurosci Mental Health, 2015, 2:4.

[11] Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, Gilron I, Haanpää M, Hansson P, Jensen TS, Kamerman PR, Lund K, Moore A, Raja SN, Rice AS, Rowbotham M, Sena E, Siddall P, Smith BH, Wallace M. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol, 2015, 14(2):162–173.

[12] Attal N, Cruccu G, Baron R, Haanpää M, Hansson P, Jensen TS, Nurmikko T; European Federation of Neurological Societies. EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision. Eur J Neurol, 2010, 17(9):1113–e88.

[13] Hamon A, Morel A, Hue B, Verleye M, Gillardin JM. The modulatory effects of the anxiolytic etifoxine on GABA(A) receptors are mediated by the beta subunit. Neuropharmacology, 2003, 45(3):293–303.

[14] Furlan AD, Sandoval JA, Mailis-Gagnon A, Tunks E. Opioids for chronic noncancer pain: a meta-analysis of effectiveness and side effects. CMAJ, 2006, 174(11):1589–1594.

[15] Eisenberg E, McNicol ED, Carr DB. Efficacy of mu-opioid agonists in the treatment of evoked neuropathic pain: systematic review of randomized controlled trials. Eur J Pain, 2006, 10(8):667–676.

[16] Rokyta R, Holecek V, Pekárkova I, Krejcová J, Racek J, Trefil L, Yamamotová A. Free radicals after painful stimulation are influenced by antioxidants and analgesics. Neuro Endocrinol Lett, 2003, 24(5):304–309.

[17] Smith HS. Arachidonic acid pathways in nociception. J Support Oncol, 2006, 4(6):277–287.

[18] Gaweł S, Wardas M, Niedworok E, Wardas P. [Malondialdehyde (MDA) as a lipid peroxidation marker]. Wiad Lek, 2004, 57(9–10):453–455.

[19] Pace A, Bove L, Aloe A, Nardi M, Pietrangeli A, Calabresi F, Innocenti P, Jandolo B. Paclitaxel neurotoxicity: clinical and neurophysiological study of 23 patients. Ital J Neurol Sci, 1997, 18(2):73–79.

[20] Vuorinen V, Röyttä M, Raine CS. The long-term cellular response to taxol in peripheral nerve: Schwann cell and endoneurial cell changes. J Neurocytol, 1989, 18(6):785–794.

[21] Zbârcea CE, Negreş S, Chiriţă C. Gabapentin, alone and associated with tramadol reduces peripheral paclitaxel-induced neuropathy in rats. Farmacia, 2011, 59(3):414–423.

[22] Rhodens A. Fixation of tissues. In: Suvarna SK, Layton C, Bancroft JD (eds). Bancroft's theory and practice of histological techniques. 7th edition, Churchill Livingstone–Elsevier, 2013, 69–94.

[23] Odemuyiwa SO, Ghahary A, Li Y, Puttagunta L, Lee JE, Musat-Marcu S, Ghahary A, Moqbel R. Cutting edge: Human eosinophils can regulate T-cell subset selection through indoleamine 2,3-dioxygenase. J Immunol, 2004, 107(10): 5909–5913.

[24] Renshaw S. Immunochemical staining techniques. In: Renshaw S (ed). Immunohistochemistry. Methods Express Series, Scion Publishing, Ltd., 2007, 45–95.

[25] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol, 1978, 52:302–310.

[26] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem, 1951, 193(1):265–275.

[27] Sisignano M, Baron R, Scholich K, Geisslinger G. Mechanism-based treatment for chemotherapy-induced peripheral neuropathic pain. Nat Rev Neurol, 2014, 10(12):694–707.

[28] Ciotu IC, Lupuliasa D, Zbârcea CE, Negreş S. The effect of nimodipine on a rat model of paclitaxel-induced peripheral neuropathy. Farmacia, 2016, 64(4):493–497.

[29] Ciotu IC, Lupuliasa D, Chiriţă C, Zbârcea CE, Negreş S. The antihyperalgic effect of memantine in a rat model of paclitaxel induced neuropathic pain. Farmacia, 2016, 64(5):809–812.
[30] Zheng H, Xiao WH, Bennett GJ. Functional deficits in peripheral nerve mitochondria in rats with paclitaxel- and oxaliplatin-evoked painful peripheral neuropathy. Exp Neurol, 2011, 232(2):154–161.
[31] Wang YF, Chen CY, Chung SF, Chiou YH, Lo HR. Involvement of oxidative stress and caspase activation in paclitaxel-induced apoptosis of primary effusion lymphoma cells. Cancer Chemother Pharmacol, 2004, 54(4):322–330.
[32] Davies A, Hendrich J, Van Minh AT, Wratten J, Douglas L, Dolphin AC. Functional biology of the alpha(2)delta subunits of voltage-gated calcium channels. Trends Pharmacol Sci, 2007, 28(5):220–228.
[33] Kale A, Börcek AÖ, Emmez H, Yildirim Z, Durdağ E, Lortlar N, Kurt G, Doğulu F, Kılıç N. Neuroprotective effects of gabapentin on spinal cord ischemia-reperfusion injury in rabbits. J Neurosurg Spine, 2011, 15(3):228–237.
[34] Girard P, Pansart Y, Gillardin JM. Preventive and curative effects of etifoxine in a rat model of brain oedema. Clin Exp Pharmacol Physiol, 2009, 36(7):655–661.
[35] Authier N, Balayssac D, Marchand F, Ling B, Zangarelli A, Descoeur J, Coudore F, Bourinet E, Eschalier A. Animal models of chemotherapy-evoked painful peripheral neuropathies. Neurotherapeutics, 2009, 6(4):620–629.
[36] Hashimoto K, Sakuma Y, Kotani J. Histological examination of peripheral neuropathy caused by paclitaxel. J Osaka Dent Univ, 2004, 38(1):53–59.

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