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RESEARCH ARTICLE

Effect of Andrographolide and Resveratrol on OX1R and Prepro-orexin mRNA expression in CIPN-induced hypothalamus of mice with oxaliplatin

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ABSTRACT:

Chemotherapy-induced peripheral neuropathy (CIPN) is a one of side effect in cancer patients that receive antineoplastic agent, like oxaliplatin. Orexinergic system in the hypothalamus is the one of system that modulate nociceptive and neuropathy. Because there is flavonoids such as resveratrol and andrographolide that may prevent chemotherapy induced peripheral neuropathy, this study analyzed effects of andrographolide and resveratrol treatment on PPOrx and OX1R mRNA expression in hypothalamic oxaliplatin-induced mice. **Materials and Methods** This study was conducted for 22 days in mice. Mice injected with oxaliplatin followed by andrographolide or resveratrol. Chemotherapy induced peripheral neuropathic pain was assessed based on withdrawal threshold, mRNA PPOrx expression, and mRNA OX1R expression. **Results** The results showed that intraperitoneal injection of 100mg/kg resveratrol and 20mg/kg andrographolide increased the withdrawal threshold after oxaliplatin induction. Resveratrol administration also increased the relative expression of PPOrx mRNA significantly, but not the OX1R mRNA relative expression. On the other hand, administration of andrographolide did not cause a change in the expression of PPOrx and OX1R in the hypothalamus. **Conclusions** Intraperitoneal injection of andrographolide and resveratrol reduces the mechanical allodynia response in oxaliplatin-induced mice significantly. The mechanism of andrographolide increases the withdrawal threshold does not via the orexinergic system, but the mechanism of resveratrol via the orexinergic system.

KEYWORDS: Antioxidant, Cancer, CIPN, Hypothalamus, Orexin.

INTRODUCTION:

Neuropathic pain is pain that caused by a lesion or disorder in the somatosensory nervous system[1]. It occur in the central nervous system (CNS) or peripheral nervous system (PNS) depending on the location of the lesion[2]. Common conditions associated with neuropathic pain include diabetic, HIV infection, leprosy, amputation, and stroke. Peripheral neuropathic pain occurs due to aging and the consequence of chemotherapy on cancer survivor, which affect all sensory fibres (Aβ, Aδ, and C fibres)[3]. Oxaliplatin is a third-generation platinum chemotherapeutic agent used in the first-line treatment of colon cancer. The use of oxaliplatin may cause acute and chronic side effects of peripheral neuropathy with the highest incidence.

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Oxaliplatin is rapid nonenzymatic transformation to reactive platinum and leaving-group oxalate. Reactive platinum binds to mitochondrial DNA to form platinum–mDNA complexes, which causes neurotoxicity in dorsal root ganglion (DRG) and responsible for the initiation of chronic peripheral neuropathy. On other hand, oxalates are known to be Ca^{2+} and Mg^{2+} chelators, may lower the mechanical threshold, and are thought to be responsible for acute neuropathy[4]. Oxaliplatin administration activates microglia and astrocytes which have a significant effect on the inflammatory process[5]. This activation causes the release of pro-inflammatory cytokines such as interleukins and chemokines that cause peripheral nerve hyperexcitability leading to neuroinflammation[4].

Treatment or prevention of these adverse effects using various available approaches is inadequate. Therefore, the search for neuroprotective agents that may prevent

or reduce the side effect is imperative need. Natural compounds like Andrographolide and Resveratrol can be candidates to be adjuvant treatment to treat chemotherapy-induced peripheral neuropathy. Andrographolide is one of the secondary metabolites that are widely found in the plant *Andrographis paniculata* Ness. Andrographolide act as an anti-inflammatory, analgesic, and antioxidant agent[6,7]. Resveratrol (3,5,4'-trihydroxystilbene) is a naturally stilbene-derived polyphenolic compound found in several plants such as grape skins[9]. Studies revealed that resveratrol has neuroprotective and antiallodynic effects in different experimental models of nervous system injury and or persistent pain[8]. Resveratrol is identified as a potent activator of SIRT1 and activates Nrf2, which plays a role in upregulation of antioxidant genes and reduce oxidative stress[11,12]. Administration of resveratrol downregulates the M1 form of microglia that secretes pro-inflammatory cytokines and upregulates the M2 form of microglia that secretes anti-inflammatory cytokines[10].

There is a part of the brain that plays a role in pain regulation, namely the hypothalamus. Hypothalamus linked the sympathetic and parasympathetic nervous systems to the preganglionic nerves. Changes in the hypothalamus may cause disturbances in the body's homeostatic functions. In the study of Crowell et al. (2016), there was a change in the MMP-2 marker in the hypothalamus after mice induced spinal cord injury (SCI) and caused neuropathic pain[13]. In the hypothalamus, the orexinergic system plays a role in neuropathic pain regulation[14].

Orexins (Orx) are endogenous neuropeptides produced from a prepro-orexin (PPOrx) molecule and cleaved into two isoforms, orexin-A and orexin-B. Orexinergic system also have two G-protein coupled receptors (GPCR), orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R)[15]. The orexinergic system influences energy metabolism and pain regulation. Ablation of orexin nerves causes mechanical allodynia and thermal hyperalgesia in mice[16]. Based on the research of Duffy et al. (2015), OX1R that bound to orexin-A prevents the activation of M1 microglia and prevent the production of proinflammatory cytokines such as TNF-, IL-6, and iNOS in microglia[17]. In addition, orexin-A also increases the activation of M2-type microglia and increase the production of anti-inflammatory cytokines[17]. However, there is no information regarding the involvement of OX1R and prepro-orexin in the neuroprotection mechanism of andrographolide and resveratrol in oxaliplatin-induced peripheral neuropathy models. Therefore, the present study was designed to evaluate the influence of andrographolide and resveratrol on the hypothalamus' orexinergic systems in an experimental model of oxaliplatin-induced neuropathy.

MATERIALS AND METHODS:

Animals:

Experiments were performed in male Bals/c mice weighing 22-26g. Animals were housed in cages lined with sawdust. The mice are maintained under a 12 h light/dark cycle and provided free access to water and rodent chow ad libitum. Animals underwent an acclimatization period of at least 7 days before use in our study.

Murine Models of Chemotherapy Induced Peripheral Neuropathy:

Forty-eight male BALB/c mice were randomly divided into 8 groups (n=6), normal group, oxaliplatin group, oxa+andrographolide 1mg/kgBW group, oxa+andrographolide 10mg/kgBW group, oxa+andrographolide 20mg/kgBW group, oxa+resveratrol 10mg/kgBW group, oxa+resveratrol 50 mg/kgBW group, and oxa+resveratrol 100mg/kgBW group. In brief, the study was conducted for 22 days, where mice in the oxaliplatin group were injected with oxaliplatin (3mg/kgBW; i.p) on the first week, then followed by giving solvent on the second week, the mice in andro/resv group were injected with oxaliplatin (3 mg/kgBW;i.p) on the first week, then followed by giving andrographolide or resveratrol (i.p) on the second week.

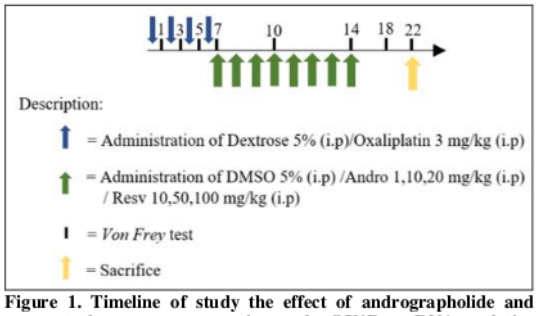


Figure 1. Timeline of study the effect of andrographolide and resveratrol on prepro-orexin and OX1R mRNA relative expression in oxaliplatin induced peripheral neuropathy mice

Pain Behavioral Study: Evaluation of Pain Response Using von Frey Test:

The Von Frey test was performed for the assessment of mechanical allodynia using a series of von Frey filaments. An individual acrylic box with a wire mesh floor was placed 50cm above the bench and utilized for the mice placements. Evaluation is carried out in a quiet room and mice were allowed to habituate to the behavioral testing environment for 15minutes. Mice were evaluated before initiating oxaliplatin administration in order to obtain normal baseline values. The filaments were applied to the mid-plantar surface of hind paw in a series 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, and 2.0g force. Each filament was delivered to the central plantar area of each hind paw following the "up-

and-down" method, initiated with the 0.6g filament, and tested five times. Withdrawal of mice hind-paw was noted as a positive response. Whenever a positive response occurred, the next weaker von Frey filament was applied. On other hand, a negative response occurred, the next stronger filament was applied. The mean value of five measurements was determined as the withdrawal threshold.

Reverse Transcription Polymerase Chain Reaction

Mice from control group, oxa group, oxa+andrographolide 20mg/kg and oxa+resveratrol 100 mg/kg were sacrificed on day 22 and the hypothalamus tissue extracted. The total RNA was extracted using Life Technology reagents following the manufacturer's instructions. The RNA was reverse-transcribed into cDNA according to the Promega instructions. The primer sequence of the target genes (PPOrx and OX1R) and the β-actin gene were designed using the Primar Blast software (Table 1). Then, the amplification products were analyzed by 1.5% agarose gel electrophoresis, a gel imaging, and an analysis system.

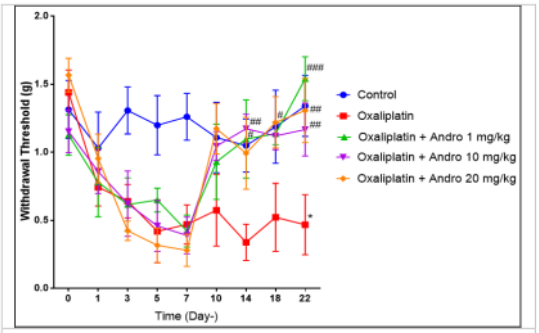
Table 1. The primers used in RT–PCR

mRNA	Sequence	PCR product (bp)
PPOrx F	'5 – CAG CCT CTG CCC GAC TGC TGT- 3'	270
PPOrx R	'5 – TAA AGC GGT GGT AGT TAC GGT CGG AC- 3'	
OX1R F	'5 – GCT TTT TCA TTG TCA CCT ACC- 3'	284
OX1R R	'5 – CAG ATA ACA GAG TGC AAA AAC C- 3'	
β-actin F	'5 - TGT TAC CAA CTG GGA CGA CA - 3'	573
β-actin R	'5 - AAG GAA GGC TGG AAA AGA GC - 3'	

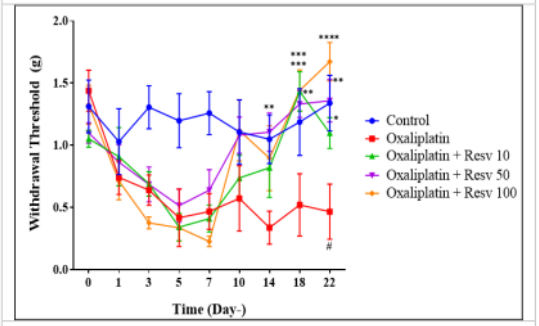
RESULT:

Effect of Andrographolide and Resveratrol on Peripheral neuropathic Response Using von Frey Test:

The results of the behavioral test before the induction of oxaliplatin as a baseline showed no significant difference in all groups. Then, in oxa group (mice receiving oxaliplatin 3mg/kg/day, i.p), was detect a gradual decrease in hindpaw mechanical withdrawal threshold, compared to the control group until the end of the study. The decrease in oxa group mainly occurred on the last day (Fig. 1a and 1b, $p < 0.05$ vs control group). As it is shown in Figure 1, andrographolide at doses 1, 10, and 20mg/kg/day (Fig. 1a) and resveratrol at doses 10, 50, 100mg/kg/day (Fig. 1b) for 8 days after oxaliplatin injection showed and increase in the withdrawal threshold significantly (Two-way ANOVA, treatment, $F_{4,25} = 3,593$ (Fig. 1a) $F_{4,25} = 4,233$ (Fig. 1b), $p < 0.01$).



a.

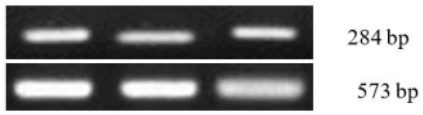


b.

Figure 2. Effect of (a) andrographolide and (b) resveratrol treatment on peripheral neuropathic pain by oxaliplatin induction using a von Frey test. Data are expressed as mean ± SEM, n = 6. The control group was injected with 5% dextrose solution + 10% DMSO solution ip, the oxa group was injected with oxaliplatin in 5% dextrose solution at a dose of 3 mg/kg/day + 10% tween solution, the oxa + andro group 1, 10, 20 was injected with oxaliplatin in 5% dextrose solution at a dose of 3 mg/kg/day + andrographolide in 5% DMSO solution at a dose of 1, 10, 20 mg/kgBW/day, and the oxa + resv group 10, 50, 100 was injected with oxaliplatin in 5% dextrose solution at a dose of 3 mg/kg/day + resveratrol in 10% tween solution at a dose of 10, 50, 100 mg/kgBW/day. On Figure a, *p<0.05 vs control and #p < 0.05, ##p < 0.005, ###p < 0.001 vs oxa. #p < 0.05 vs control. On Figure b, *p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001 vs oxa.

Effect of Andrographolide on the Level of OX1R and PPOrx mRNA relative expression in hypothalamus:

The relative expression of OX1R and PPOrx mRNA in the oxaliplatin group decreased compared to the control group. Meanwhile, the relative mRNA expression of OX1R and PPOrx in the andrographolide 20 mg/kg BW group tended to remain unchanged when compared to the oxaliplatin group (Fig. 2b, *Oneway ANOVA;* $F(2.15) = 0.3036$, n = 6; Fig. 2d, *Oneway ANOVA;* $F(2.15) = 1.406$, n = 6).



(a)

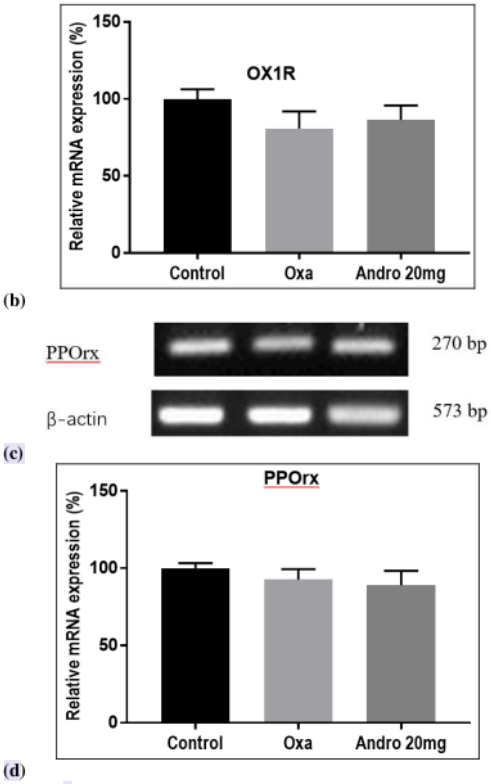


Figure 3. Effect of andrographolide on OX1R and PPOrx mRNA expression in hypothalamus of peripheral neuropathic mice by oxaliplatin induction. (a) representative RT-PCR for the control group (control), oxaliplatin (oxa), and oxaliplatin + andrographolide 20 mg/kg/day (oxa + andro) of OX1R. (b) OX1R semi-quantitative intensity of each band. (c) representative RT-PCR for the control group (control), oxaliplatin (oxa), and oxaliplatin + andrographolide 20 mg/kg/day (oxa + andro) of PPOrx. (d) PPOrx semi-quantitative intensity of each band. Histograms are shown as mean ± SEM, n = 6.

Effect of Resveratrol on the Level of OX1R and PPOrx mRNA relative expression in hypothalamus:

A significant impact of treatment on both, OX1R (One-way ANOVA, treatment, $F_{2,15} = 10.42$, $p < 0.01$) and PPOrx (One-way ANOVA, treatment, $F_{2,15} = 6.059$, $p < 0.05$) mRNA expression in hypothalamus was observed. The result showed a decrease OX1R/β-actin mRNA relative expression in the oxa group compared to the control group (Fig. 3b, $p < 0.01$ vs control group). No statistically significant differences were observed between oxa group compared to the oxa + resv group. Therefore, there was a significant decrease in the relative expression of OX1R/β-actin mRNA in the oxa + resv group compared to the control group (Fig. 3b, $p < 0.01$ vs control group). Different result were shown for the PPOrx mRNA relative expression. There was a tendency to decrease the relative expression of PPOrx/ β-actin mRNA in the oxa group compared to the control group. Resveratrol treatment at dose 100 mg/kg/day, oxa + resv group, was significantly increase the relative expression of of PPOrx/ β-actin mRNA compared to the oxa group (Fig. 3d, $p < 0.05$ vs oxa group).

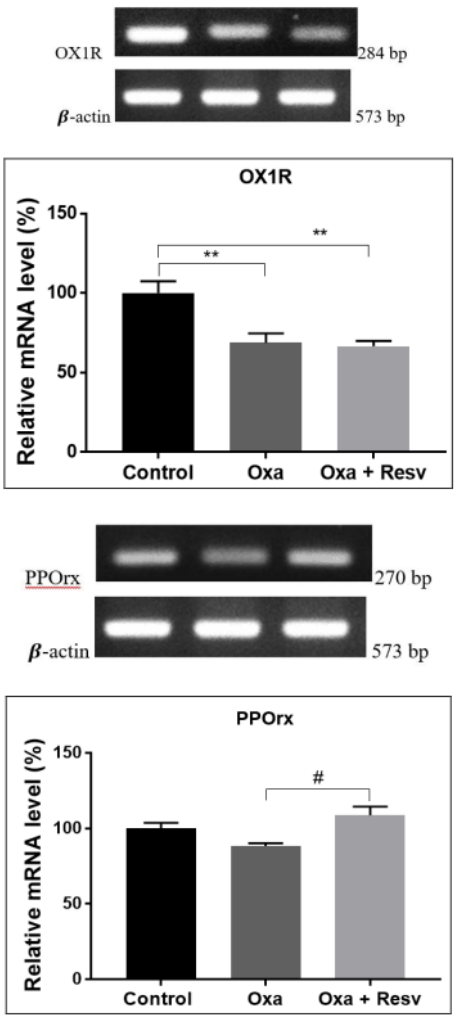


Figure 4. Effect of resveratrol on OX1R and PPOrx mRNA expression in hypothalamus of peripheral neuropathic mice by oxaliplatin induction. Oxaliplatin in 5% dextrose solution (3 mg/kg/day, i.p.) was injected on days 0, 2, 4, and 6. Resveratrol in 10% tween solution (100 mg/kg/day, i.p.) was injected from day to day -7 to the 14. Brain extraction was performed on day 22. (a) representative RT-PCR for the control group (control), oxaliplatin (oxa), and oxaliplatin + resveratrol 100 mg/kg/day (oxa + resv) of OX1R. (b) OX1R semi-quantitative intensity of each band. (c) representative RT-PCR for the control group (control), oxaliplatin (oxa), and oxaliplatin + resveratrol 100 mg/kg/day (oxa + resv) of PPOrx. (d) PPOrx semi-quantitative intensity of each band. Histograms are shown as mean ± SEM, n = 6. **p < 0.01 vs control, #p < 0.05 vs oxa.

DISCUSSION:

The purpose of this study was to examine the effect of resveratrol and andrographolide treatment on PPOrx and OX1R mRNA relative expression in the hypothalamus of mice that induced by oxaliplatin. Oxaliplatin induction was given in a dose of 3 mg/kg/day four times with a cumulative dose of 12mg/kg, showing that the withdrawal threshold of the oxa group decreased from the first day after induction until the 22nd day. This result showed that the peripheral neuropathy model is successful and causes mechanical allodynia in mice. These results are consistent with other studies showing that oxaliplatin injection in certain doses and duration induces chronic peripheral neuropathy in a CIPN model[18,19]. Thus, the oxaliplatin dose and duration used in the present study is the chronic model of oxaliplatin-induced peripheral neuropathy.

The results of the behavioral test showed that the group of mice that received andrographolide treatment at a doses of 1, 10, 20mg/kg/day and resveratrol treatment at doses of 10, 50, and 100mg/kg/day was able to increase the withdrawal threshold and inhibit the effect of oxaliplatin. In the oxaliplatin+andrographolide group, there was an increase in the withdrawal threshold since day 10 for all doses. The withdrawal threshold value also increased until day-22. This result indicates that andrographolide with various doses administration improve the mechanical allodynia and reduce neuropathic pain symptoms. This result was following the study of Sulaiman et al. (2010), andrographolide 10 mg/kg, 25mg/kg, and 50mg/kg can reduce pain in the hot-plate test[22]. This result may occur through several mechanisms, namely a decrease in an nf-κB activity wherein the study of Chan et al. (2010), the administration of andrographolide at a dose of 1 mg/kg can reduce the effect of Nf-κB activation[23]. Decreased Nf-κB activation can decrease microglial activation[21]. Another mechanism of andrographolide in reducing pain is through reduced production of ROS, TNF- and NO. Decreased ROS production may occur because andrographolide can induce HO-1 protein expression through the activity on the Nrf2/ARE pathway[20]. Increased HO-1 expression and Nrf2 activation can result in neuroprotection against oxidative damage and cell death[24,25]. Based on the results of these behavioral tests, it is indicated that andrographolide reduces oxaliplatin-induced neuropathic pain.

In the oxaliplatin+resveratrol group, on the 10th day, the withdrawal threshold began to increase in all groups oxa + resv. As a whole, the administration of resveratrol 10, 50, and 100mg/kg/day was able to improve mechanical allodynia in mice. The behavioral test showed that the resveratrol administration significantly increase the withdrawal threshold until the end of the study. These results show that resveratrol not only has an analgesic effect but also showed an effect that was able to improve the condition of mechanical allodynia due to oxaliplatin induction. These results are supported by other studies which state that resveratrol is the potential to reduce allodynia and has an effect for several days after the last administration[9,26]. However, in this study, it was shown that the higher the dose of resveratrol didn't show a consistent increase in the withdrawal threshold in each test so that increasing the dose did not affect the increase in the withdrawal threshold. These results are in line with the research of Zhao et al. (2014) who revealed the administration of resveratrol doses of 3, 10, and 30 mg/kg p.o. for 21 days in CCI model animals did not show a dose-dependent increase in the withdrawal threshold[27].

Administration of resveratrol doses of 10, 50, and 100 mg/kg/day i.p. for the treatment of CIPN in the present study showed a non-linear dose-response result. Resveratrol doses of 10 and 50mg/kg/day showed better results than doses of 100mg/kg/day. The administration of resveratrol 10 and 50mg/kg/day resulted in a linear increase in the withdrawal threshold from day to day. However, the administration of resveratrol 100 mg/kg/day resulted in two peaks, on the 7th and 22nd days. The profile showed that the use of a dose of 100 mg/kg/day or higher did not provide effective results, so it is not recommended for use in the next study. Some studies say that resveratrol is dose-dependent. On the other hand, there are also studies showing the results that low-dose resveratrol has more effect on clinical improvement than higher-dose resveratrol[28]. Based on the results of previous studies, it was shown that low-dose resveratrol had more pharmacological effects. Thus, low-dose resveratrol may be more effective, including for the treatment of peripheral neuropathy.

At the end of the study, mice were sacrificed and the hypothalamic was extract. Several studies have suggested that the orexinergic system contributes to nociceptive modulation[29,30].Activation of orexin neurons in the presence of inflammation may contribute to the activation of descending pain inhibitory pathways involving PAG[32]. In descending pain pathway inhibition, PAG neurons receive input from several areas of the brain, including the hypothalamus. PAG neurons project their axons on the LC and RVM. Neurons located in the LC and RVM directly project axons to the spinal dorsal horn, where nociceptive input was processed first[31]. In the spinal cord, DRG neurons are primary afferent neurons that transmit peripheral stimuli to areas that process pain. Morphological results showed the presence of orexin-A and OX1R distributed in DRG neurons. Orexin-A induces excitability and elevation of intracellular calcium concentrations in DRG neurons

isolated from mice, which is dependent on spinal OX1R activation. Based on these findings, the projection of the orexin system from the hypothalamus to the DRG allows orexin modulation pathways for the transmission of pain stimuli including peripheral neuropathic pain[33].

Molecular study used four groups, control group, oxa group, oxa+andro 20 group and oxa + resv 100 group. It showed that the administration of oxaliplatin was significantly reduce the expression of OX1R mRNA in the hypothalamus compared to the control group. Then the administration of andrographolide 20 mg/kg/day did not show any effect on OX1R and PPOrx mRNA relative expression. However, the administration of resveratrol at 100mg/kg/day after oxaliplatin induction did not show any effect on OX1R mRNA expression. These results are supported by several other studies which have shown that inflammation reduces OX1R mRNA expression in the central and the periphery[34,35]. Meanwhile, the administration of resveratrol therapy at 100mg/kg/day and andrographolide 20mg/kg/day was not able to increase OX1R mRNA expression in the hypothalamus, possibly because OX1R activation to antinociceptive effects was not primarily in the hypothalamus. Unaltered expression of OX1R and Prepro-orexin mRNA in the andrographolide group may occur because the dose was not large enough to affect the orexinergic system in the hypothalamus. In the study of Abu-Ghefreh et al. (2009), andrographolide can still be given up to a dose of 30mg/kg and does not cause toxic effects for experimental animals, and used as anti-inflammation on the lungs[36]. However, in this study, andrographolide 30mg/kg was not used because based on the research of Chan et al. (2010), administration of andrographolide 0.1mg/kg and 1mg/kg intraperitoneally has been able to affect the central nervous system and decrease inflammation in damaged brain tissue[23]. Therefore, it was assumed that a dose of 20mg/kg may be able to affect the central nervous system, including the brain. In addition, in this study, the higher dose of andrographolide were not used since it may cause an increased risk of side effects, such as headache, fatigue, rash, nausea, diarrhea, pruritus, and allergic reactions, when the dose increased[37].

On other hand, PPOrx mRNA showed that oxaliplatin administration tended to decrease PPOrx mRNA expression in the hypothalamus compared to the control group. Administration of resveratrol 100mg/kg/day after oxaliplatin induction increases PPOrx mRNA expression compared to the oxa group. The PPOrx mRNA expression in the oxa + resv group was not different from the PPOrx mRNA expression in the control group. This indicated that the administration of resveratrol was able to inhibit the decrease in PPOrx mRNA expression by oxaliplatin and increase to the equal as the healthy state. This result is in accordance with the behavioral test, on day 22, the oxa + resv 100group showed a significant increase in the withdrawal threshold compared to the oxa group.

The evidence showed that the hypothalamus, the central site of the orexinergic system, is associated with nociceptive processes. Administration of oxaliplatin causes a series of multifactorial mechanisms in the pathogenesis of peripheral neuropathy, where pain stimuli from the periphery are transmitted through the spinal cord central nervous system to the pain center in the brain. It is known in previous studies that the absence of orexin, both OX1R, and PPOrx, cause hyperalgesia after peripheral inflammation[32,34,35]. Different studies have shown that exogenous Orexin A administration reduces mechanical allodynia in CIPN model animals and the orexinergic system reduces pro-inflammatory cytokines in microglia. Administration of exogenous Orexin A activates OX1R in the ventrolateral PAG nerve which then activates descending pain modulation and reduces nociceptive responses[17,30]. This study showed that in chronic inflammatory conditions such as peripheral neuropathy, there was a decrease in the expression of OX1R and PPOrx mRNA in the hypothalamus of mice. Administration of andrographolide did not modulate the orexinergic system. However, the administration of resveratrol was able to modulate PPOrx. Increased expression of PPOrx mRNA causes an antinociceptive effect which is characterized by an increase in the withdrawal threshold and improvement in peripheral neuropathy. However, OX1R in the hypothalamus is not sufficiently activated so that there is no increase in OX1R mRNA expression. The mechanism of action of andrographolide in reducing pain is not through the orexinergic pathway. However, andrographolide may act via other pathways, such as Nrf2/HO-1 pathway. On the other hand, the antinociceptive effect of resveratrol is possibly by increasing the expression of PPOrx mRNA in the hypothalamus and activating descending pain modulation. The exact mechanism of these antioxidants in increasing the mechanical withdrawal threshold in CIPN-induced mice should be further verified.

CONCLUSION:

The results of this study reveal that resveratrol increased the mechanical withdrawal threshold and increased relative expression level of PPOrx mRNA. On the other hand, andrographolide increased the mechanical threshold but did not change in the relative expression of OX1R and PPOrx mRNA significantly.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation.

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