The Role of Cdk5 and TRPV1 in Meloxicam Resistance Signal Transduction in Rat Experiencing Chronic Pain

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Abstract

Chronic pain is a common case and become a serious problem. Chronic pain management are inconclusive. One of the common treatment option is NSAID. Treatment Failed are around 34-79% of total case. These treatment failed suspected as NSAID resistance. Treatment failed can be caused by a some molecule that make NSAID loss of efficacy. Cdk5 is one of the molecule that active in chronic pain condition. Cdk5 can increase transmembrane insertion and activate TRPV1. The aim of this research is analyzed the role of cdk5 and TRPV1 in NSAID resistance of chronic pain rat. This research used 42 rats as a subject and divided into 6 groups with random allocation method and factorial design. Meloxicam treatment was given orally every day for 7 days after rats have a chronic pain (28 days). Chronic pain induction used a CFA injection. Results : cdk5 and TRPV1 expression at the dorsal root ganglia of chronic pain groups are increase, no significant different of pain threshold and inflammation sign between treatment and no treatment groups after chronic pain occurred. Conclusion : chronic pain can induce cdk5 and TRPV1 expression, and induced by meloxicam treatment. Cdk5 and TRPV1 have a positive correlation with meloxicam resistance.

Keywords *: Chronic pain, cdk5, TRPV1, resistance, meloxicam*

Introduction

Chronicpain is a common case in the worldwide and still become a serious problem. These serious problem arising from chronic pain complication and consequences. The prevalence of chronic pain in Netherland is about 10-30% of 12,5 million population, and 14,6-64% of population in US[1,2].The common etiology of chronic pain is degenerative joint disease[3]. That's why the one of common treatment in chronic pain management is nonsteroid analgesic and anti-inflammatory drug (NSAID). Meloxicam is one of the NSAID that be prescribed very often. The failed on treating chronic pain using a NSAID is about 34% to 79% of total cases[4]. This treatment failed is suspected because of a resistances mechanism. Drug resistances are common fenomen in antibiotic or chemotherapeutic use. Drug resistances mean : the loss of drug efficacy after the drug reach a maximum plasma concentration.

Cdk5 (cyclin dependent kinase 5) and TRPV1 (transient receptor potential for vanilloid type 1) are molecule that have a key role in pain transmission. These molecule could be have a role in meloxicam resistance. Cdk5 activated TRPV1 by phosphorylation. Cdk5 is also stimulated TRPV1 translocation from golgi apparatus into nerve membrane[5,6].TRPV1 is a calcium ion channel that have a role in pain induction. This ion channel activation and translocation will increase pain transmission. Cdk5 is also elevated pain transmission by NMDA activation. Cdk5 activated NMDA receptor by phosphorylation process. This receptor activation can increasepost synaptic pain transmission[5]. All of these process can maintain a pain perception even without any significant noxious stimulation on pain origin. Cdk5 can

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be activated by MAPK pathway or ERK1/2 pathway. MAPK is a molecule that active on chronic tissue damage or chronic inflammation. ERK1/2 pathway can be activated by leukotriene elevation[7].Meloxicam inhibit COX pathway and increase LOX activity on the other hand. This condition will elevated metabolism of arachidonic acid into leukotriene[8]. These MAPK – cdk5 pathway and leukotriene-ERK1/2-cdk5 pathway could be a basic mechanism of the meloxicam resistance in chronic pain.The aim of this research is analyzing the role of cdk5 and TRPV1 expression on the basic mechanism of meloxicam resistance in chronic pain rats.

Material and Methods

Ethical Clearance : The experiment protocols have been approved by Animal Care and Use Committee of Veterinary Faculty of Airlangga University (Approved Reference Number : 2.KE.145.08.2018)

Table1. Subject grouping by factorial desain. H group determinant is chronic pain induction by CFA injection. T group determinant is meloxicam treatment

Group	H1 (no CFA injection)	H2 (CFA injection)
T1 (without meloxicam)	T1H1	T1H2
T2 (meloxicam dose 1)	T2H1	T2H2
T3 (meloxicam dose 2)	T3H1	T3H2

Chronic Pain Induction

Complete Freud's adjuvant (CFA) (Sigma Aldrich) 100 µl was injected to the left hind paw of the rats to promote inflammation and pain. This procedure was repeated weekly to maintain inflammation for 28 days. CFA injection using 1ml syringe and 27G needle (Therumo).

Meloxicam Treatment

Meloxicam treatment being started after rats showing a chronic inflammatory sign (28 days). Meloxicam given orally every day for 7 consecutive days. The dose of meloxicam calculated by conversion formula. The doses are converted from daily human dose preparation (7.5mg and 15mg).

HED (mg/Kg) = *Animal doses* (mg/Kg) x (*animal* Km */ human* Km)

HED : Human equivalent doses

Animal Km : Correction factor of animal (rat 200 gr = 6; rat 250 gr = 7)

Human Km : Correction factor of human (Human weight 60 kg =37)

Based on that formula, than the meloxicam doses for rats are;

Dose 1 (T2) : 0,77 mg/KgBWdaily for 200 gr rat

0.66 mg/KgBW daily for 250gr rat

Dose 2 (T3) : 1,54 mg/KgBWdaily for 200gr rat

1.32 g/KgBWdaily for 250gr rat

Pain Threshold Examination

This experiment used a hotplate (UgoBasile-Italy) to evaluate pain threshold. Temperature of hotplate was 51^0C. Pain threshold level of the rat determined by the time tolerance (second) of the rat on the hotplate. This test was stop when the rat showing one of the three signs : jump out, wagging the hind paw, lick the hind paw. The longer the mouse is able to survive on the hotplate means

the pain threshold increases. This procedure is done three time : a week before pain induction, 2 weeks after pain induction, and a week after meloxicam treatment.

Inflammation Sign Measurement

The sign of inflammation determined by the left hind paw volume (ml) using a pletismometer (UgoBasile-Italy). All research subject following this procedure. This procedure done three times : a day after CFA injection, after rats have a chronic inflammation (28 days), after meloxicam treatment.

Cdk5 and TRPV1 Expression

Cdk5 and TRPV1 expression on the dorsal root ganglia were seen by immunohistochemistry. Dorsal root ganglia was fixated by formaldehyde 40%, aquadestilata, NaH_2PO_4, and $Na2HPO_{4-}$. Dedihradration of this organ using etanol, clearing with xylol and that embedding with paraffin. After all, the dorsal root ganglia being cut with microtome for 4-8 micron of thickness. Haematoxylin eosin coloration following the Harris formula. This specimen than put on the Polylysine Coating Object Glass. Deparaffinisation and rehidration using alcohol. After this process, the specimen was incubated for 15 minutes. Wash the specimen twice with PBS for 2 minutes than give an antiretrieval (citrate plus). Wash again four time with PBS for 2 minutes than give a superblock. Wash again with PBS than give either CDK5 polyclonal antibody or TRPV1/ VR1 polyclonal antibody (Bioss antibodies). Cdk5 and TRPV1 expression determined by *Immunoreactive score of Remmele and Stegner* (IRS) classification[9].

Result

Cdk5 Expression in Dorsal Ganglion of Rat Experiencing Chronic Pain and Given Meloxicam Treatment

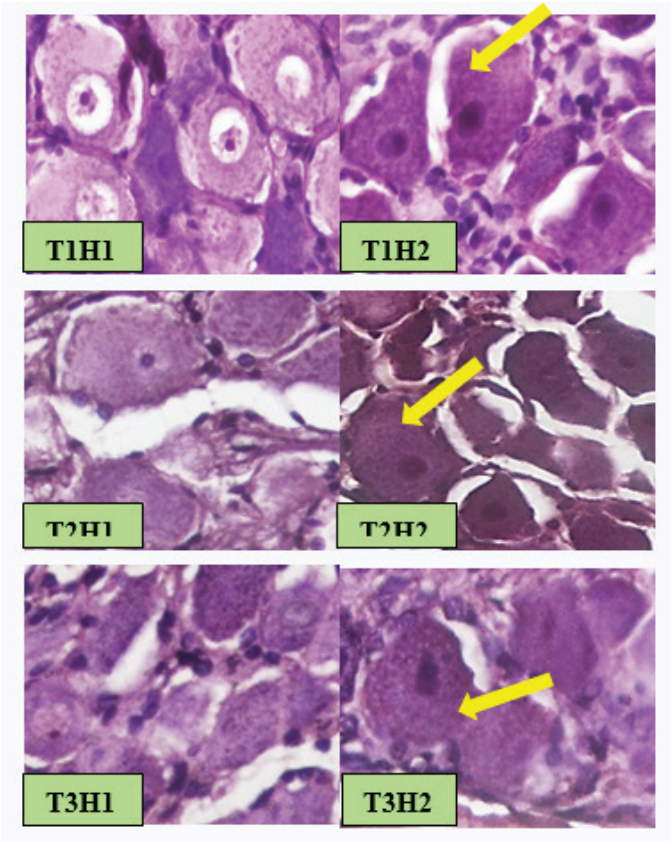

Figure 1. Expression of cdk5 in the dorsal ganglion of rat experiencing chronic pain and given meloxicam. A. Group T1H1, B. Group T2H1, C. Group T2H1, D. Group T2H2, E. Group T3H1, and F. Group T3H2. Cells that show expression cdk5 appear brownish (yellow arrows are indicated). Most expressions in the T2H2 (D) group.

Table 2 Average CDK5 expression in the dorsal ganglion of rat experiencing chronic pain and those who did not experience chronic pain and given meloxicam

Group	Cdk5 expression				Kruskal Wallis (p)
	X	SD	Min	Max	
T1H1	1.2	0.2	1	2	0.01
T1H2	2.6	0.6	1	4	
T2H1	1.6	0.25	1	2	
T2H2	9a	0.95	6	12	
T3H1	1.4	0.25	1	2	
T3H2	3.6	0.4	2	4	

The mean of cdk5 expression in the group experiencing chronic pain was greater than the group that did not experience chronic pain. The results of statistical tests using the Kruskal Wallis show the value of $p = 0.01$ ($p < 0.05$). The results of the Mann-Whitney test showed that those who had a significant difference to the mean value of CDK5 expression were between the T1H2 and T2H2 groups and between the T2H2 group and the T3H2 group.

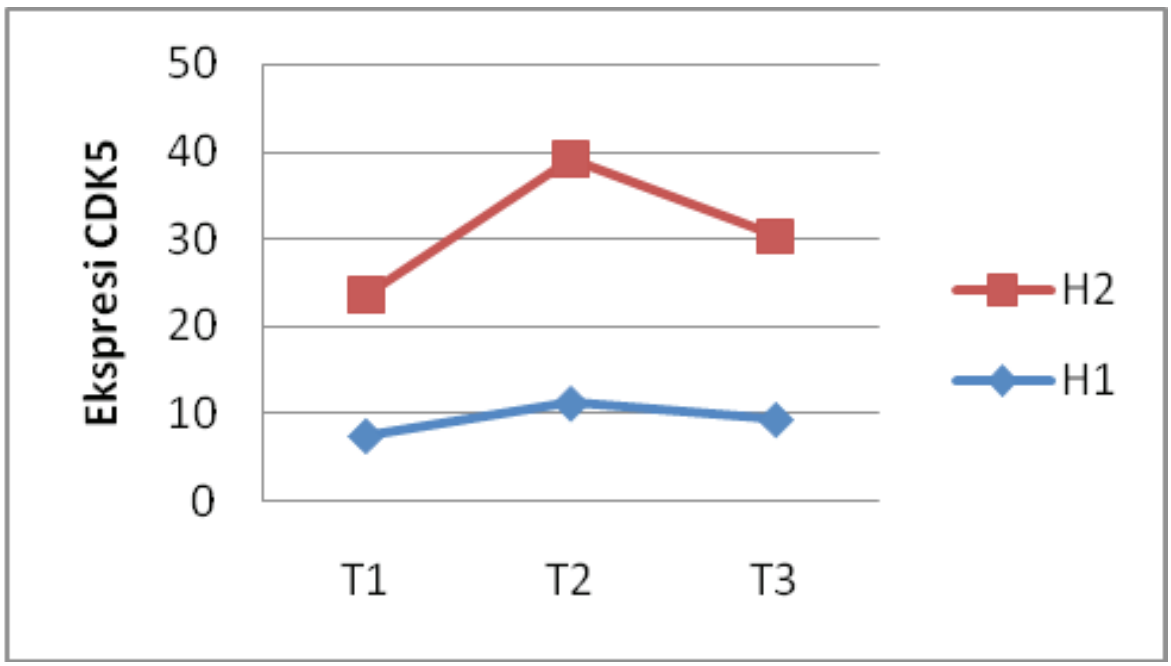

Figure 2. Expression of cdk5 in the dorsal ganglion of rat. The group that experienced chronic pain (red line) had a higher average expression than the group that did not experience chronic pain (blue line). Giving meloxicam causes an increase in cdk5 expression (T2 and T3).

Expression of TRPV1 in Dorsal Ganglion of Rat Experiencing Chronic Pain and Given Meloxicam

Table 3 Expression of TRPV1 in the dorsal ganglion of rat experiencing chronic pain and those who did not experience chronic pain and given meloxicam

Group	TRPV1 Expression				Kruskal Wallis (p)
	X	SD	Min	Max	
T1H1	1.6	0.4	1	3	0.00
T1H2	3.8	0.8	1	6	
T2H1	1.6	0.25	1	2	
T2H2	9a	0.0	9	9	
T3H1	1.8	0.2	1	2	
T3H2	5.2	0.49	4	6	

TRPV1 expression in the group that experienced chronic pain was greater than the group that did not experience chronic pain. The results of these statistical tests show the value of $p < 0.05$ so that it can be concluded that the expression of TRPV1 is significantly different.

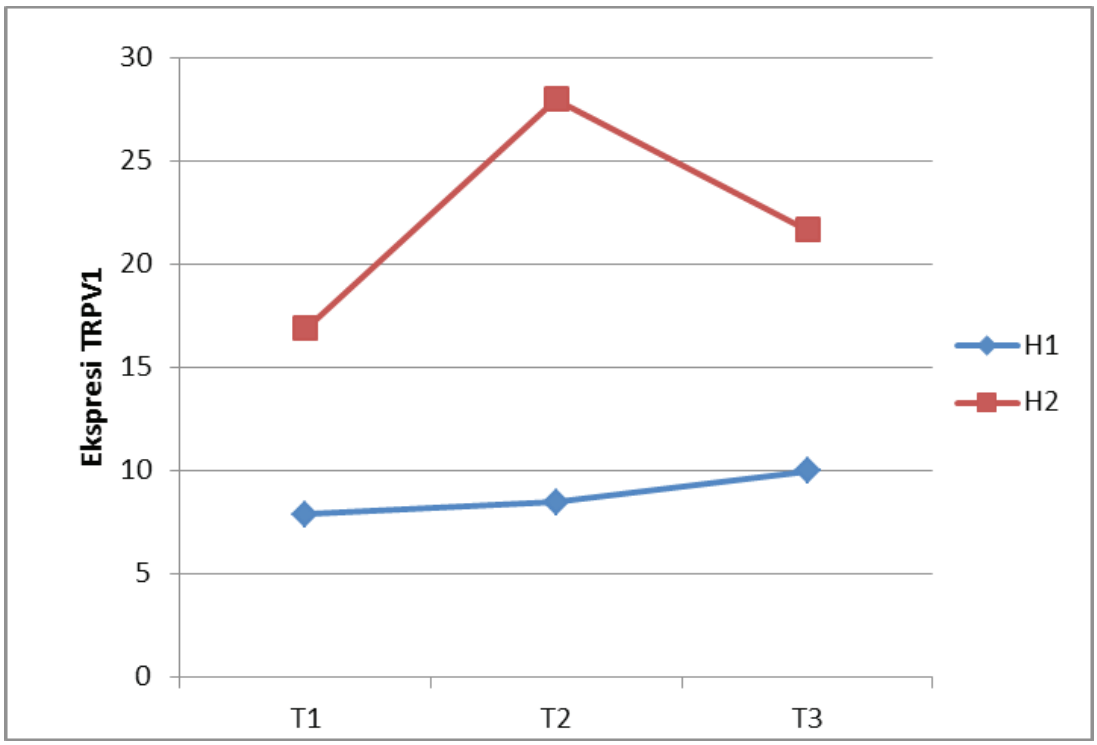

Figure 4 TRPV1 expression in the dorsal ganglion of rat. The group that experienced chronic pain (red line) had a higher average expression than the group that did not experience chronic pain (blue line). The administration of meloxicam causes an increase in the expression of TRPV1 (T2 and T3) in groups experiencing chronic pain.

The results of different tests between groups using the Mann-Whitney test showed significant differences between the T1H1 and T1H2 groups (p = 0.049), T1H2 with T2H2 (p = 0.005), the T3H2 group did not differ significantly from T1H2. Based on the results of these tests it appears that the most TRPV1 expression in the dorsal ganglion is in the T2H2 group and has a significant difference with all groups.

Correlation of CDK5 Expression with TRPV1 Expression in Dorsal Ganglion of Rat Experiencing Chronic Pain and Given Meloxicam

The correlation of CDK5 expression with TRPV1 expression in dorsal diganglion of experimental animals was tested using the Spearman correlation test. The test results show p value 0.867. the results show that CDK5 has a strong positive correlation (close to 1). The correlation of the CDK5 expression with the expression TRPV1 can be seen in the graph

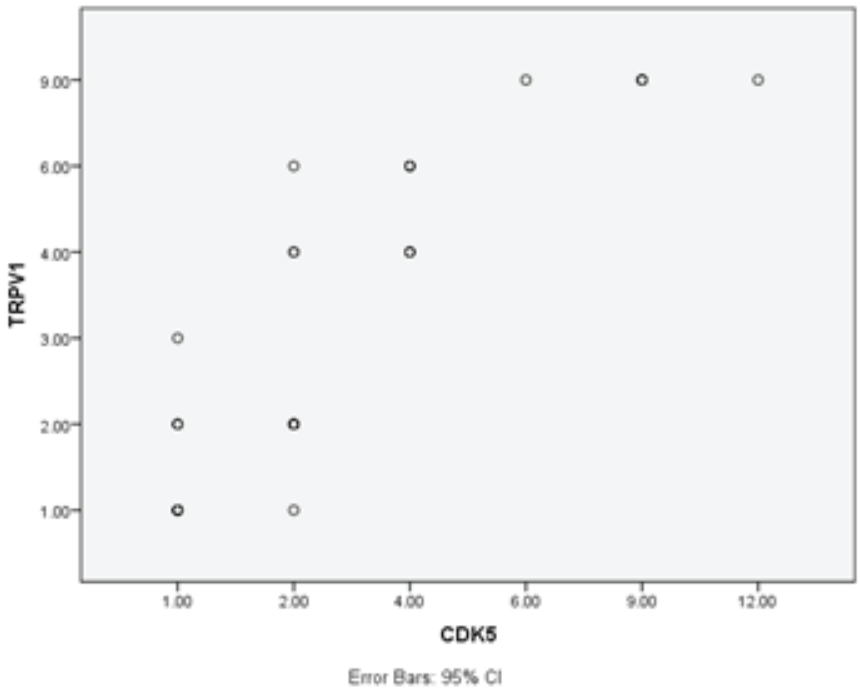

Figure 5 The relationship of CDK5 expression with TRPV1 expression shows that the more expression of CDK5, the greater the expression of TRPV1

Pain Threshold of the Rat Experiencing Chronic Pain and Given Meloxicam

The mean value of the pain threshold I assessed one week before the CFA injection obtained a normal distributed value and did not differ significantly. These results show that the characteristics of the pain threshold of experimental animals before being treated are not different.The pain threshold value II assessed one week after the CFA injection was normally distributed and did not differ significantly between the inflammatory group and those without inflammation.

Table 4 Pain threshold values III of rats experiencing chronic pain assessed one week after being given meloxicam medication every day (7 days)

Group	Pain Threshold Value III				One- way Anova (p)
	X	SD	Min	Maks	
T1H1	14.64	2.29	6.5	19.9	0.288
T1H2	13.88	1.77	8.2	19.3	
T2H1	11.02	1.46	6.5	14.8	
T2H2	16.68	3.6	9.2	27.3	
T3H1	11.2	1.59	6.6	15.7	
T3H2	10.44	1.32	7.7	14.2	

The pain threshold value III is normally distributed and does not show a significant difference between the group who received the drug meloxicam and the group that received meloxicam at one and two doses.

Correlation of Cdk5 Expression and Pain Threshold

The results of the Spearman statistical test showed that cdk5 levels had a very weak negative correlation with the pain threshold (r = - 0.045 for pain threshold II; r = - 0.047 for pain threshold III).

Inflammation sign (Hind Paw Volume)

The volume of rat hind paw is used as a parameter of swelling of the feet due to an inflammatory process. The results of the foot volume examination using a pletismometer showed that in the T1H1, T2H1, and T3H1 groups (groups without treatment of meloxicam or CFA injection) there was no swelling of the legs from the beginning to the end of the study period.

Table 5: Hind paw volume III of rats measured after being given meloxicam for 7 days

Group	Hind paw volume III (ml)				Kruskal Wallis (p)
	X	SD	Min	Max	
T1H1	3.86	0.15	6.09	9.5	0.000
T1H2	7.66	0.76	6.09	9.5	
T2H1	3.97	0.14	3.64	4.32	
T2H2	6.76	0.33	5.97	7.68	
T3H1	4.33	0.14	3.93	4.7	
T3H2	6.35	0.57	5.61	8.6	

Correlation of Cdk5 and TRPV1 Expressions with Hind Paw Volume

The results of the statistical test with the Spearman test showed a positive correlation between cdk5 expression and volume of foot III (r = 0.625). The results of the statistical test with the Spearman test showed a positive correlation between TRPV1 expression and hind paw volume III (r = 0.731)

Discussion

Expression of Cdk5 and TRPV1 of the Rat Experiencing Chronic Pain and Given Meloxicam

The expression of cdk5 in the dorsal ganglion of rat that experienced chronic pain had a greater average than the group that did not experience chronic pain. This shows that in chronic pain conditions changes in biomolecular signals can cause changes in treatment outcomes. Repeated footprint using CFA to make models of chronic pain in animals in this study shows the same results as previous studies that have shown that recurrent injury can increase expression of cdk5 through TNFα and MAPK pathways[10].

The administration of meloxicam group with chronic pain caused cdk5 expression to increase, especially the meloxicam group dose 0.66 mg / KgBW ($p < 0.05$). The expression of cdk5 in the dose group was 1.32 mg / KgBW higher than the condition without meloxicam ($p > 0.05$) but the expression of cdk5 in this group was lower than the group receiving meloxicam dose of 0.66 mg / KgBW. These results indicate that administration of meloxicam in chronic pain can increase the expression of cdk5.

In accordance with the results of previous studies on the relationship of dose and analgesic effect of meloxicam. Various results of the study stated that the greater the dose of meloxicam, the greater the analgesic effect obtained, while in this study the results were different. This can be seen from the results of statistical tests and Figure 5.2 which show lower expression of CKD5 with the addition of meloxicam doses.

The results of this study have not been able to explain whether the greater cox enzyme barriers will reduce cdk5 expression because in this study only two doses were used. The reason for choosing two doses in this study is based on preparations that are routinely used in the practice of daily medicine, namely 7.5 mg and 15 mg. In this study also did not examine the level or expression of leucotriene, as well as other biomarkers (eg interleukin) that are associated with suspected new mechanism of action of meloxicam[8], so that a path analysis cannot be performed which has a strong relationship with expression cdk5. Based on the results of this study it can be seen that cdk5 undergoes very large changes in chronic pain conditions and has increased expression after administration of meloxicam, but the pathways that affect these expression changes cannot be explained.

The expression of TRPV1 in the dorsal ganglion of rat that experienced chronic pain increased when compared to groups that did not experience chronic pain. The description of TRPV1 expression obtained in this study has the same pattern as the description of cdk5 expression. The results of the statistical test also show that the expression cdk5 and TRPV1 have a very strong positive correlation (r = 0.867). This very strong positive relationship illustrates that CDK5 has a very important role in TRPV1 expression.

This finding further strengthens the theory which states that cdk5 can activate TRPV1 so that its expression increases with increasing expression of cdk5 and any change in cdk5 activity will be positively related to TRPV1 activity[5]. Based on the results of this study it can also be seen that changes in TRPV1 expression are not directly related to cox enzyme barriers but are more influenced by cdk5.

Rat Pain Threshold Experiencing Chronic Pain and Given Meloxicam

The mean pain threshold of experimental animals assessed before the induction of chronic pain (pain threshold I) using CFA showed results that were normally distributed and did not have a significant difference ($p = 0.893$). These results indicate that the experimental pain threshold used in this study has basic characteristics that are not much different. The threshold value of pain I really needs to be assessed because the

pain threshold value is individual, and with the results of the assessment it can be ascertained that any difference in mean pain threshold after induction is due to the induction and not due to differences in individual animal characteristics in response to pain.

The mean pain threshold assessed after induction of chronic pain still did not show a significant difference (p = 0.592). The mean pain threshold of experimental animals after obtaining CFA induction that is not significantly different cannot be interpreted as a failure of the process of induction of chronic pain because clinically there are signs of inflammation. Pain threshold values that do not differ between induced groups and those not induced by CFA can be caused by induction on one leg cannot change the character of the experimental pain threshold, or because of the weakness of the tool used to assess the pain threshold itself (hot plate) .

The results of the pain threshold assessment in experimental animals that have received meloxicam for 7 days also did not show a significant difference between the group experiencing chronic pain and those who did not experience chronic pain (p = 0.288). this result shows that administration of oral meloxicam for 7 days, both doses of 0.66 mg / KgBB or 1.32 mg / KgBB cannot reduce the pain threshold (assessed by hot plate) in both normal and chronic pain conditions. This can also be caused due to the weakness of the tool used to assess the pain threshold.

Hot plate was chosen as a tool to assess the pain threshold of animals in this study because this tool is not invasive so it does not provide additional induction. This tool also does not add chemicals that may cause drug interactions and can affect the results of the assessment. The form of stimulation from the hot plate is hot temperature (510C) and this is very suitable for stimulating TRPV1[11,12,13].

Signs of Inflammation in the Rat Hind Paw Experiencing Chronic Pain and Given Meloxicam

Inflammatory signs assessed after CFA induction showed a significant volume difference between the induced and non-induced groups (p = 0.000). This result shows that the process of making an inflammatory model is successful. This foot volume still shows a significant average difference up to 28 days (p = 0.000). These results also showed that the process of making chronic pain models with CFA was successful which was characterized by swelling of the rat legs that lasted up to 28 days.

Examination of foot volume after rats get oral meloxicam drug for 7 days with a dose according to each group found a significant volume difference (Kruskal Wallis, p = 0,000). The mean volume of the legs of rat that experienced chronic pain was greater than the group that did not experience chronic pain. based on these data it can be seen that the inflammatory process marked by swelling in the legs of rat still occurs.

Examination of foot volume after rats get oral meloxicam drug for 7 days with a dose according to each group found a significant volume difference (Kruskal Wallis, p = 0,000). The mean volume of the legs of the rat that experienced chronic pain was greater than the group that did not experience chronic pain. based on these data it can be seen that the process is marked by swelling in the legs of rat still occurs.

Inflammation in the legs of rat has a positive correlation with both cdk5 (r = 0.625) and TRPV1 (r = 0.731). The findings show that cdk5 has a very important role in inflammatory conditions and plays a role in the occurrence of resistance to the anti-inflammatory effects of meloxicam. Increased expression of CDK5 and TRPV1 above normal conditions will be able to eliminate the anti-inflammatory effects of meloxicam in both doses of 0.66 mg / KgBB or 1.32 mg / KgBW. This can be caused by the very large role of CDK5 in the activity of IL-2 and T cells in the inflammatory process.[9,14,15] Increased CDK5 activity can also cause an increase in TRPV1 activity so that influx calcium will increase and can cause hyperalgesia to allodynia[16]. This condition which can cause cox enzyme resistance by meloxicam is not effective in the treatment of chronic pain (resistance).

Conflict of Interest: The authors declare that they have no competing interest

Ethical Clearance: Committee of Veterinary Faculty of Airlangga University (Approved Reference Number : 2.KE.145.08.2018)

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