Effects of Curcumin Against *Matrix Metalloproteinase-2* (MMP-2) and *Tissue Inhibitor Metalloproteinase-2* (TIMP-2) Serum Level on Rat Model of Liver Fibrosis Resolution Process

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ABSTRACT

Background: *Liver fibrosis is an effect from continuous fibrogenesis and fibrolysis process. During fibrogenesis, MMP-2 and TIMP-2 that produced by hepatic stellate cell (HSC) have a role to regulate extracellular matrix (ECM) homeostastic. Otherwise, curcumin inhibits both MMP-2 and TIMP-2 expression and enhances HSC apoptosis, thus inhibit fibrogenesis. Role of curcumin, MMP-2, and TIMP-2 in a fibrolysis process has not been widely studied. This study aimed to determine the correlation between curcumin administration and the decline of MMP-2 and TIMP-2 on rat model of liver fibrosis.*

Method: *This is an experimental study done in male Wistar rats. There are 8 groups consist of 4 rats each. Both control and intervention group were exposed to CCl_4 1 cc/kgBW intraperitoneally 2 times per week for 9 consecutive weeks to form F3 fibrosis. Negative control group was injected with normal saline. After CCl_4 injection, control group was given curcumin solvent as placebo while intervention groups were given curcumin 200 mg/kgBW for 2, 5, and 9 weeks. Statistical analysis then conducted in the end of study.*

Results: *MMP-2 and TIMP-2 were remarkably increased in positive control group, but found decreased in control group 5 and 9. There are remarkable decrease of MMP-2 and TIMP-2 serum level in intervention group 2, 5, and 9, but MMP-2 and TIMP-2 level was significantly lower in intervention group 2 compared to the control group.*

Conclusion: *MMP-2 and TIMP-2 serum level were decreased after giving of curcumin for 2 weeks. The duration of curcumin administration correlated with decrease of TIMP-2 serum level but not correlated with MMP-2 serum level in rat model of liver fibrosis.*

Keywords: *Carbon tetrachloride, curcumin, MMP-2, TIMP-2, rat model of liver fibrosis*

ABSTRAK

Latar belakang: *Fibrosis hati merupakan hasil dari proses fibrogenesis dan fibrolisis. Pada proses fibrogenesis, MMP-2 dan TIMP-2 yang diproduksi sel stellata hati (HSC), mengatur homeostasis matriks ekstraseluler (MES). Kurkumin menghambat ekspresi MMP-2 dan TIMP-2, serta mendorong apoptosis HSC sehingga menghambat fibrogenesis. Peran kurkumin, MMP-2 dan TIMP-2 dalam proses fibrolisis belum banyak diteliti. Penelitian ini bertujuan mengetahui hubungan antara pemberian kurkumin terhadap penurunan MMP-2 dan TIMP-2 pada tikus model fibrosis hati.*

Metode: *Penelitian eksperimental pada tikus jantan strain wistar. Terdapat 8 kelompok, masing-masing terdiri dari 4 tikus. Kelompok KP dipapar CCl4 1cc/kgbb 2x perminggu intraperitoneal selama 9 minggu untuk membentuk fibrosis F3. Kelompok KN diinjeksi Normal Salin. Setelah diinjeksi CCl4, Kelompok Kontrol (KK2, KK5, KK9) diberikan plasebo dan Kelompok Perlakuan (KP2, KP5 dan KP9) diberikan kurkumin 200mg/kgbb 2,5 dan 9 minggu. Dilakukan analisis kadar serum MMP-2 dan TIMP-2 dengan menggunakan uji T independen, uji pengaruh pemberian dan lama pemberian kurkumin dengan ANOVA.*

Hasil: *Terdapat peningkatan signifikan kadar serum MMP-2 dan TIMP-2 pada kelompok KP, dan penurunan signifikan kadar serum MMP-2 dan TIMP-2 pada kelompok KK5 dan KK9. Pada kelompok kurkumin, terdapat penurunan signifikan kadar serum MMP-2 dan TIMP-2 pada KP2, KP5, dan KP9, dan pada perbandingan kelompok plasebo dengan kelompok yang diberikan kurkumin, terdapat penurunan signifikan kadar MMP-2 dan TIMP-2 pada KP2.*

Simpulan: *Terdapat penurunan signifikan kadar serum MMP-2 dan TIMP-2 pada pemberian kurkumin selama 2 minggu. Lama pemberian kurkumin berkorelasi dengan penurunan kadar serum TIMP-2 tetapi tidak signifikan pengaruhnya pada kadar serum MMP-2 tikus model fibrosis hati.*

Kata kunci: *karbon tetraklorida, kurkumin, MMP-2, TIMP-2, tikus model fibrosis hati*

INTRODUCTION

Liver fibrosis is an effect from continuous fibrogenesis and fibrolysis process. At the beginning of fibrosis, several hepatotoxic factors insult hepatocytes, induce inflammatory response, and activate quiescent hepatic stellate cell (HSC) to activated HSC (aHSC). Later, aHSC would produce huge amount of extracellular matrix (ECM) protein, mainly collagen type I and III, that increase the stiffness of ECM.[1] Liver fibrosis is a dynamic process which involve deposition and degradation of ECM. A balance between matrix metalloproteinase (MMP), an enzyme to degrade ECM, and its specific inhibitor known as tissue inhibitor matrix metalloproteinase (TIMP) is important to regulate homeostasis of liver connective tissue.[2] Previous study on rat by Iredale et al and Issa et al showed that improvement in fibrolysis process towards normal liver histology takes about 4-6 weeks after its fibrosing etiology removed. This showed that liver fibrosis is a reversible process and further known as spontaneous resolution.[3]

MMPs that largely produced by HSC is MMP-2 which its act on ECM degradation was regulated by TIMP-2. MMP-2 and TIMP-2 level in blood was corelate to its concentration in liver and also corelate to fibrosis degree.[4] To induce liver fibrosis in experimental animal, carbon tetrachloride (CCl_4) as a hepatotoxic agent is widely used. CCl_4 is metabolized by cytochrome P450 2EI to form two radicals: reactive trichloromethyl (CCl_3^-) and trichloromethyl peroxyl (CCl_3O_2). Those radical will bond to DNA, fat, protein, and carbohydrate to cause lipid peroxidation, cell necrosis and apoptosis, and collagen deposition in liver. Kupffer cell which activated by those free radicals will produce several proinflammatory mediator that induce inflammation cascade.[5]

Curcumin was previously known to induce HSC apoptosis by inhibit transforming growth factor (TGF)- β1 and platelet derived growth factor (PDGF).[6] Recent study by Banerji, et al showed a significant inhibitory effect of curcumin on MMP-2 activation. Curcumin inhibitor MMP-2 activity by down regulate expression of MT1-MMP, an enzyme which together with TIMP-2, activate pro-MMP-2 to MMP-2. Lower MMP-2 activity will lead reduce liver basal membrane degradation during liver fibrogenesis.[7]

METHOD

This is an in vivo experimental study in Wistar strain rat with post test control group design and randomization. Intervention consist of CCl_4 induction and curcumin administration. Sample analysis of MMP-2 and TIMP-2 level was done in physiology laboratory Faculty of Medicine, Brawijaya University, Malang. This study was conducted in June to November 2016.

We divide subject into 8 groups with 4 rats in each group. Negative control group was given normal saline injection twice a week for 9 weeks, while positive control group was given CCl_4 injection 1 cc/kgBW twice a week intraperitoneally for 9 weeks to form a F3 fibrosis, then continued by placebo injection of curcumin solvent CMC Na 1% 1 cc/kgBW daily for two weeks (CG2), five weeks (CG5), and nine weeks (CG9). Other intervention groups were given CCl_4 injection for 9 weeks than continued by 200 mg/kgBW/day of curcumin for two weeks (IG2), five weeks

(IG5), and nine weeks (IG9). Rats were terminated after the last 72 hours injection. This study was ethically approved by Ethical Committee of Faculty of Medicine, Brawijaya University.

Inclusion criteria is male rat, aged ± 3 months, weight of 150-250 grams, and physically active. Drop out criteria is rat that refuse to eat, sick, and died during intervention period. Independent variable were CCl_4 exposure and curcumin administration and duration, while dependent variable were MMP-2 and TIMP-2 serum level measured using Rat MMP-2/ TIMP-2 Platinum ELISA BMS635/BMS635TEN kit from eBioscience.

Statistical analysis was done using IBM SPSS statistics, version 22.0 for windows. We use 95% of confidence interval (α = 0,05). A normal distribution and homogenous variation data will be analyzed using parametric analysis Two Way ANOVA and Post Hoc Tukey. If not, non-parametric analysis using Kruskal Wallis and Mann Whitney is used. We also done a Pearson correlation analysis and independent t-test.

RESULTS

After CCl_4 administration for 9 weeks, F3 fibrosis (septal fibrosis) was expected which also followed by increase of MMP-2 and TIMP-2 serum level in intervention group compared to normal group. Here was the different of MMP-2 and TIMP-2 increasement level between intervention and normal group:

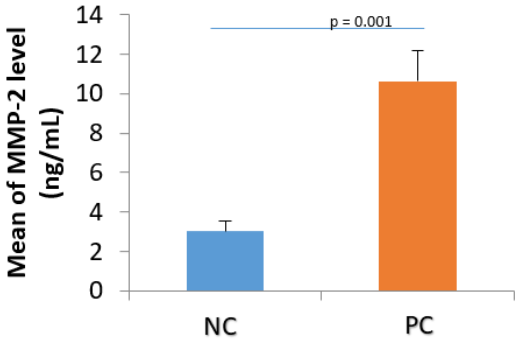

Figure 1. Histogram of mean MMP-2 level in positive and negative control group (NC: negative control; PC: positive control)

From Figure 1, it showed that a significant increase in MMP-2 level in fibrosis was occurred in rat model that induced by CCl_4 for 9 weeks.

In Figure 2, we compare positive control group to control group that administered curcumin solvent. There were no significant difference in mean MMP-2 level between positive control group and IG2. Otherwise, comparison between positive control group to IG5 and IG 9 showed a significant lower MMP-2 level with $p < 0,05$. It can be concluded that MMP-2 level was significantly lower in week 5 and 9 of intervention.

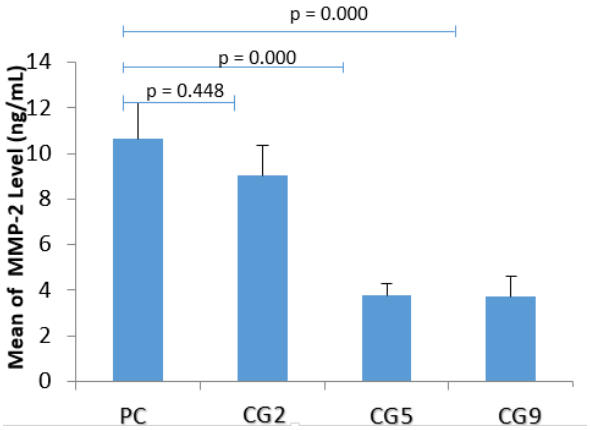

Figure 2. Comparison of MMP-2 level between positive control (PC) group and control group (CG)

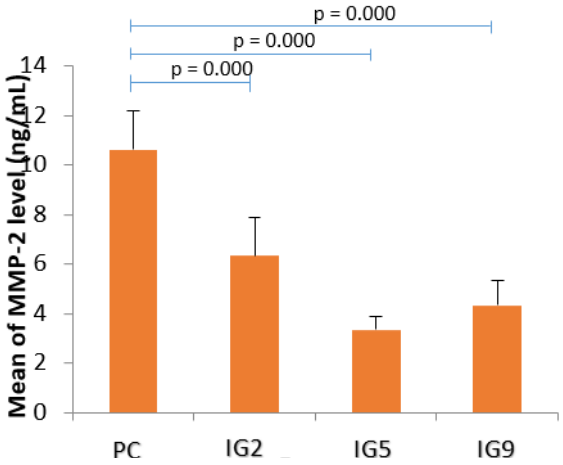

Figure 3. Comparison of MMP-2 level between positive control (PC) group and intervention group (IG)

In all group that administered curcumin, MMP-2 level was found to be significantly lower ($p < 0,05$). This result showed that curcumin administration for 2, 5, and 9 weeks have been proven to significantly reduce MMP-2 level.

Table 1. Test of interaction between curcumin administration and its duration to MMP-2 serum level

Intervention	Mean ± SD	P
CG2	9.03 ± 1.32	
IG2	6.34 ± 1.53	
CG5	3.75 ± 0.54	0.015
IG5	3.34 ± 0.56	
CG9	3.71 ± 0.89	
IG9	4.34 ± 1.01	

Note: if mean ± SD column contains 2 different alphabet, it means that there is a significant difference ($p < 0.05$). But if it contains same alphabet, it means that there is no significant different ($p > 0.005$)

We than conduct a statistical analysis using Two Way ANOVA followed by Post Hoc Tukey analysis

to investigate the interaction between curcumin administration and its duration after the test of normality and homogeneity (Table 1).

Result from ANOVA test showed a p value of 0,015 so it can be concluded that there were significant effect from curcumin administration and its duration to MMP-2 serum level. In other words, a significant lower MMP-2 level was correlate to curcumin administration and its duration.

Based on Tukey test 5% in Table 1, in comparison between CG2 and IG2, a significant lower MMP-2 level was found after curcumin administration in fibrosis rat model. A comparison between both CG5 to IG5 and GC9 to IG9 did not showed a significant reduction in MMP-2 level. This result explained that curcumin administration for 5 and 9 weeks were not significant to reduce MMP-2 level in liver fibrosis. Mean MMP-2 levels are shown in following histogram:

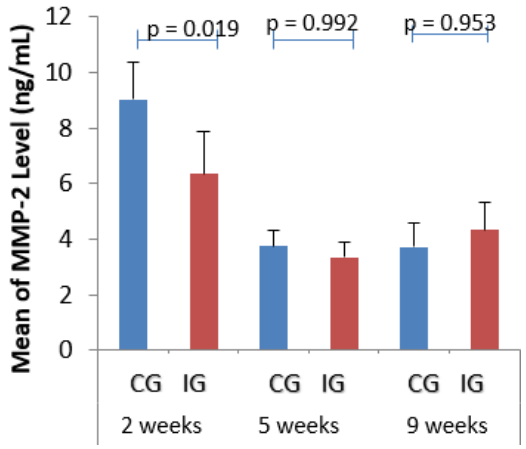

Figure 4. Histogram of mean MMP-2 level in all intervention group

To investigate TIMP-2 level in fibrosis rat model, an independent t-test was conducted. As shown in Figure 5, we found a significant increase of serum TIMP-2 level after CCl_4 induction for 9 weeks.

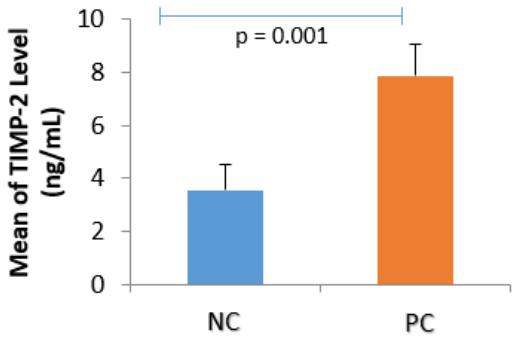

Figure 5. Histogram of mean TIMP-2 level in negative control (NC) and positive control (PC) group

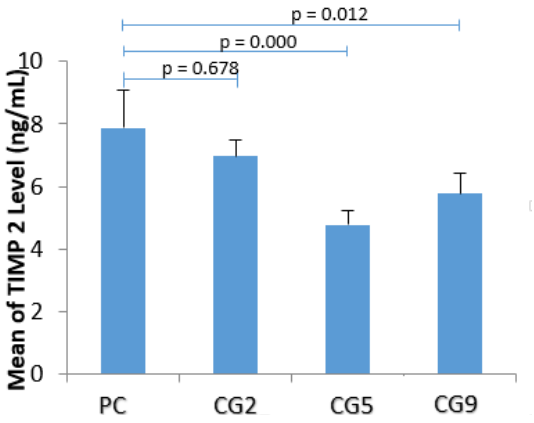

Figure 6. Comparison of TIMP-2 level between positive control group and control group that have been administered curcumin solvent (PC: positive control; CG: control group)

To know whether spontaneous resolution from TIMP-2 level improvement, we compare the result from positive control group to group that administered with curcumin solvent (CG) as shown in Figure 6. There was lower mean TIMP-2 level in CG5 and CG9 ($p = 0,000$ and $p = 0,012$, respectively). This result showed a significant reduction in TIMP-2 level after CCl_4 administration was stopped for 5 and 9 weeks.

Curcumin administration effect in lowering TIMP-2 level could be analyze by comparing positive control group to intervention group (IG). From Figure 7, a significant reduction of TIMP-2 level ($p < 0,05$) was found between positive control group and IG2, IG5, and IG9. This prove that curcumin administration for 2, 5, and 9 weeks can reduce TIMP-2 level significantly.

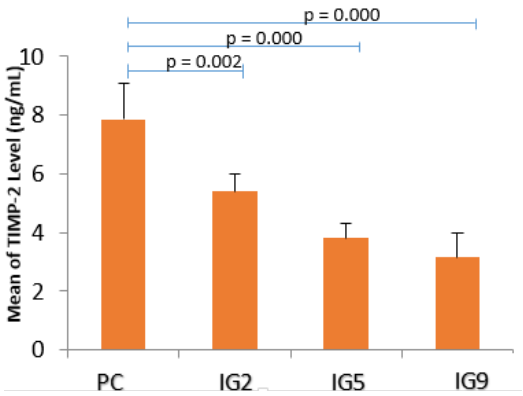

Figure 7. A Comparison of TIMP-2 level between positive control (PC) group and curcumin intervention group (IG)

Table 2. A test of interaction between curcumin administration and its duration to serum TIMP-2 level

Intervention	Mean ± SD	p value
CG2	6.96 ± 0.55	
IG2	5.4 ± 0.58	
CG5	4.79 ± 0.44	0.015
IG5	3.81 ± 0.51	
CG9	5.78 ± 0.62	
IG9	3.15 ± 0.82	

Note: if mean ± SD column contains 2 different alphabet, it means that there is a significant difference ($p < 0.05$). But if it contains same alphabet, it means that there is no significant different ($p > 0.005$)

We then conduct Two Way ANOVA and Post Hoc Tukey test as shown in Table 2. From ANOVA test, a p value of 0,039 showed a significant improvement effect of curcumin administration and its duration to TIMP-2 level. In Tukey analysis, a significant reduction in TIMP-2 level was found a comparison between CG2 to IG2 and CG9 to IG9 (Figure 8). This showed that curcumin administration for 2 and 9 weeks lowered fibrosis rat model TIMP-2 level.

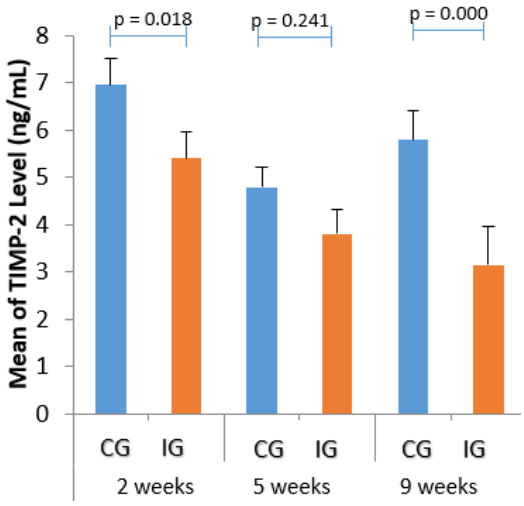

Figure 8. Mean TIMP-2 level in all intervention group (IG)

Table 3. Correlation between curcumin administration duration and MMP-2 and TIMP-2 level

Correlation	Coeff. Correlation	p
Curcumin administration duration and MMP-2 level	-0.468	0.125
Curcumin administration duration and TIMP-2 level	-0.815	0.001

A correlation study using Pearson correlation test was done (Table 3). There was significant correlation between curcumin administration and TIMP-2 level (r = -0,815; p = 0,001), but no significant correlation to MMP-2 level (p > 0,005)

DISCUSSION

This study was using CCl_4 as fibrosis inducer in rat liver tissue. CCl_4 administration for 9 weeks was aimed to induce septal fibrosis (F3). CCl_4 is metabolized by cytochrome P450 2E1 to form a reactive trichloromethyl (CCl_3^-) and trichloromethyl peroxyl (CCl_3O_2) radicals. Both radicals would further bond to DNA, lipid, protein, and carbohydrate to cause lipid peroxidation, cell necrosis, and collagen deposition in liver. Kupffer cell was later activated by those free radicals and further produce proinflammatory mediators that trigger inflammatory cascade.[9]

From previous study, CCl_4 intraperitoneal injection for 9 weeks showed a formation of F3 fibrosis in positive control group with only one subject forming F2 fibrosis, while negative control group that given saline injection form F0 fibrosis in three rats and F1 fibrosis in one rats, using histologic Hematoxylin-Eosin examination.[10]

In this study, we found an increasing MMP-2 level in fibrosis rat model that statistically significant (p < 0,005). This was consistent to study by Liang et al, which MMP-2 concentration significantly increasing in liver F2-F4 fibrosis.[11] This study also showed that a reduction in both MMP-2 and TIMP-2 level in CG5 and CG9 that means TIMP-2 level reduced after CCl_4 administration stopped for 5 weeks. A study by Issa et al revealed that aHSC was quickly undergo apoptosis in four weeks after liver injury by CCl_4 was stopped. following HSC apoptosis, MMP-2 and TIMP-2 would further reduced.[12] This is consistent with this study result in CG5 group. By removing the insult to liver injury, CCl_4 in this study, a lower MMP-2 and TIMP-2 could be found lower after 5 weeks.

Curcumin was predicted to improve liver injury and prevent liver cirrhosis. Various liver injury caused by virus, alcoholism, and other toxin increase fibrosis progressivity in which normal liver tissue replaced by collagen-rich ECM that could lead to cirrhosis. HSC played an important role in fibrosis development. After liver cell injury, HSC is activated and proliferate to produce several cytokines, chemokines, growth factors, profibrogenic cytokines, and metalloproteinase inhibitors.[13]

Latest evidence showed that liver fibrosis is a reversible process, related to HSC apoptosis. In this situation, curcumin was predicted to act as antioxidant, antiinflammation, antifibrosis, and antiproliferative agent.

Curcumin act as antifibrosis agent by reducing oxidative stress, inhibit HSC activation, and induce activated HSC apoptosis. From the last mechanism, MMP-2 expression and serum level could reduce and followed by TIMP-2. Curcumin also reduce MT1-MMP that further lower MMP-2 activation. Antiinflammation effect of Curcumin was proven by its role in reducing TGF-β1 and PDGF as activating and mitogenic factors from HSC so that it lead to HSC apoptosis.

Finally, curcumin administration was predicted to improve liver fibrognesis by lowering MMP-2 and TIMP-2 level in fibrosis rat liver model. The result of this study showed an increase in MMP-2 and TIMP-2

level in positive control group after CCl_4 administration. In contrast, a significant lower MMP-2 and TIMP-2 level was found after curcumin intervention, indicate an improvement in liver fibrosis grade.

Curcumin was safe and effective to be used as both preventive and curative agent or various disease. Yet, curcumin has a poor bioavailability when mixed with water. In a study that administer curcumin 2 g/kgBW in rat, a peak serum concentration of 1.35 ± 0.23 μg/mL was found in 0.83 hours, while similar dose in human showed nearly no curcumin detected (0.006 ± 0.005 μg/mL) in the first hour of observation.[14]

Curcumin accumulation was largely found in bowel and liver tissue, indicate that it should has effect on gastrointestinal diseases. Liver is the main organ to metabolize curcumin. After it absorbed, curcumin would then conjugate to form curcumin glucuronide or curcumin sulfate.[12] Nowadays, nanoemulsion is a substance that could increase curcumin bioavailability. Carboxymethyl cellulose (CMC) used as curcumin emulsifier because its polyphenolic component was water insoluble. [13]

From this study, a significant lower MMP-2 and TIMP-2 level were found in rats that given curcumin for 2 weeks (IG2) compared to curcumin solvent only (CG2) with $p = 0,019$ ($p < 0,05$), but insignificant in comparison of IG5 and IG9 to CG5 and CG9. It can be concluded that curcumin effect to lower MMP-2 level was optimal in two week-administration.

In intervention group of 5 and 9 weeks, insignificant results were explained as an effect of maximum aHSC apoptosis following 5 week CCl_4 injection, so that no MMP-2 reduction observed in both intervention group.

Correlation analysis between curcumin duration and MMP-2 level showed insignificant correlation. In contrast, TIMP-2 and curcumin administration was significantly corelate with correlation coefficient of $-0,815$ and $p < 0,05$. This indicate a strong correlation that the longer curcumin administration duration, the lower TIMP-2 level could be.

CONCLUSION

There were increase of MMP-2 and TIMP-2 level in liver fibrosis rat model after CCl_4 exposure for 9 weeks. CCl_4 termination then proved to reduce MMP-2 and TIMP-2 level in liver fibrosis. Curcumin administration lead to lower MMP-2 and TIMP-2 level in liver fibrosis rat model, optimally during the second week. Duration of curcumin administration only correlate to lower TIMP-2 serum level, not in MMP-2 serum level.

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