

# THE EFFECTS OF CARBON TETRACHLORIDE (CCl<sub>4</sub>) IN INDUCING OSTEOPOROSIS TO WHITE RATS (*RATTUS NORVEGICUS*) JUDGING FROM THE MUSCOSKELETAL TISSUE DAMAGE

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## Abstract

Osteoporosis is one of the degenerative diseases which is a disease due to a decrease in the structure and function of the tissue or organ. The cause of osteoporosis is a disturbance in bone metabolism by various factors including environmental factors like the presence of xenobiotic toxic agents. Carbon tetrachloride (CCl<sub>4</sub>) is one of the toxic agents and can cause oxidative stress condition, pass through the cell membrane and distributed to all organs cause damage to cell death (apoptosis). This study aims to prove that the CCl<sub>4</sub> induced osteoporosis seen from musculoskeletal tissue damage. The study was carried out experimentally in the laboratory with a completely randomized design (CRD) factorial pattern using white rats (*Rattus norvegicus*) Wistar male aged 2-3 months weighing 180-200 totaled 35. The mice were randomized into six treatment groups with 5 replicates, K (control) of olive oil dose of 1.4 ml/g body weight (BW) rats; P1 (CCl<sub>4</sub> dose of 0.8 ml/g BW rats); P2 (CCl<sub>4</sub> dose of 1.1 ml/g BW rats); P3 (CCl<sub>4</sub> dose of 1.4 ml/g BW rats); P4 (CCl<sub>4</sub> dose of 1.7 ml/g BW rats) and P5 (CCl<sub>4</sub> dose of 2 ml/g BW rats). The treatments given every other day for one week via intramuscular injections. Data were analyzed using ANOVA and Duncan test. Test results showed that the administration of P3 (CCl<sub>4</sub> dose of 1.4 ml / gr BW rats) very significantly ( $P > 99\%$ ) cause atrophy of muscle tissue, muscle fibers decrease in diameter (43.08  $\mu\text{m}$ ), a decrease in diameter cartilage (17.78  $\mu\text{m}$ ) and bone tissue collagen (2.77  $\mu\text{m}$ ). The conclusion CCl<sub>4</sub> is prove to induce osteoporosis seen from musculoskeletal tissue damage rats (*R. norvegicus*) Wistar male.

**Key words:** CCl<sub>4</sub>, osteoporosis, white rats (*Rattus norvegicus*), musculoskeletal tissue

## INTRODUCTION

Osteoporosis is a chronic autoimmune disease that causes high morbidity and mortality worldwide. Osteoporosis is induced by a variety of different pathophysiological conditions and the prevalence is increasing due to exposure to toxins/high level of environmental toxic agent [1,2]. Although the pathogenesis of the musculoskeletal tissue is not fully understood, the reactive oxygen species (ROS) have important functions in the network pathophysiological changes. Cell membrane is very vulnerable to the effects of

ROS. Peroxidation of unsaturated fatty acids in the cell membrane will cause a decrease in fluidity, loss of function, impaired integrity and eventual cell death [3].

Carbon tetrachloride (CCl<sub>4</sub>) is a toxic xenobiotic agent widely used in industry as an organic solvent which can pass through the cell membrane and is distributed to all organs. Research states that mice given CCl<sub>4</sub> dose of 1 m/kg body weight (BW) mice showed hepatotoxic and pancreatic-toxic [4,5,6]. It is characterized by the accumulation of liver and pancreatic cells polymorfonuclear, hemorrhagic, loss of cell boundaries, hydropic degeneration and fat to apoptosis and necrosis as a whole. Although the relationship between oxidative stress and cell damage has not been fully clarified, the

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nuclear factor (NF) -  $\kappa$ B is believed to hold functions and important role. The increase in these factors has led to several diseases including osteoporosis. NF- $\kappa$ B signaling induces cell inflammatory response that causes progressive damage to the extracellular matrix (ECM) and the cell. The next critical stage is lipositol cell activation. Exposure to ROS activates NF- $\kappa$ B cell, so that it cause an inflammatory reaction. Exposure to ROS also promotes apoptosis of cells that leads to necrosis tissue damage. CCl<sub>4</sub> acute poisoning causes depression and gastrointestinal and neurological effects such as nausea, vomiting, diarrhea, headache, incoordination and speech defect [7,8].

During the whole past 10 years the pathophysiology of osteoporosis has been described through linkages between the various processes at the tissue level, cellular, and molecular regulate the activity of osteoblasts and osteoclasts balance during the formation and dismantling (remodeling) bone. Bones are constantly absorbed and reshaped (remodeling) in a very dynamic process that leads to metabolic imbalance of bone diseases, such as osteoporosis. Cellular communication osteoblasts and osteoclasts in bone tissue plays an important role in the process of bone remodeling. Osteoblasts produce two ligands that RANKL and OPG regulating bone resorption. RANKL is a member of the tumor necrosis factor (TNF) superfamily of proteins synthesized by osteoblasts as transmembrane proteins. RANKL expression allows differentiation and activation of osteoclasts. Osteoclast activation triggers osteoclastogenesis (bone resorption). On the other hand, CCl<sub>4</sub> is trans membrane protein so that it can bind with the cell membrane, activating the cell producing more toxic metabolite CCl<sub>3</sub> (free radicals). CCl<sub>4</sub> can bind to cellular molecules (nucleic acid, protein, fat), affect DNA synthesis by causing apoptosis, fibrosis and malignancy. Osteoprotegerin (OPG) members of the TNF receptor superfamily acts as a receptor for RANKL negative feedback thus preventing differentiation and activation of osteoclasts even promoting osteoclast apoptosis. Therefore, the balance between RANKL and OPG determine bone resorption [9,10,11].

## MATERIAL AND METHOD

The materials used include CCl<sub>4</sub>, distilled, xilol, alcohol, paraffin, dye hematoxylin, eosin and white rats (*R. norvegicus*) Wistar male obtained from the Animal Structure and Development Laboratory, Department of Biology, Faculty of Math and Natural Sciences, Universitas Padjadjaran.

The tools used include glass tools, staining jar, rotary microtome HM 310 and light microscope Olympus CH20.

Experimental descriptive with a completely randomized design factorial pattern, using 35 laboratory animals white male rats 2-3 months of age and body weight of 180-200 grams with diversity coefficient < 10 %. Mice were randomized grouped into six treatments with five replicates, namely:

K : negative control (1.4 ml Olive oil/g body weight rats )

P1 : 0.8 ml CCl<sub>4</sub>/g body weight rats

P2 : 1.1 ml CCl<sub>4</sub>/g body weight rats

P3 : 1.4 ml CCl<sub>4</sub>/g body weight rats

P4 : 1.7 ml CCl<sub>4</sub>/g body weight rats

P5 : 2.0 ml CCl<sub>4</sub>/g body weight rats

The treatments gave via intramuscular injections every other day for one week. Furthermore, mice were killed by cervical dislocation; and muscle tissue and femur is collected for making the preparations of histologist hematoxylin eosin by staining of paraffin method. Data were analyzed with ANOVA and Duncan test (12).

Mice are kept in the animal enclosures in the Laboratory of Structure and Development Animal, Department of Biology with lighting arrangements 12 hours of light and 12 hours of dark. Feed (pellets CP 551) and drinking (tap water) provided *ad libitum*. Each two days, the rice husk as rat cage pads is replaced and the rats body weight were weighed.

CCl<sub>4</sub> dose given to the mice, has been converted, one mL / kg body weight as a dose for pangreatotoxic and hepatotoxic in mice, is becomes 1.4 ml/g body weight. Then the treatments was developed into five treatments: 0.8; 1.1; 1.4; 1.7 and 2.0 ml/g body weight.

The histological preparations using methods of paraffin and hematoxylin eosin staining. Femur bone and muscle tissue of mice were fixed in Bouin solution for  $\pm$  24 hours, then decalcified in 5% formic acid, processed for paraffin embedding, sliced using a rotary microtome with a thickness of 7  $\mu$ m

and then stained with hematoxylin eosin for examination under a light microscope.

## RESULTS AND DISCUSSIONS

### The Effect CCl<sub>4</sub> to Histopathologic Muscles Tissue Femur Male White Rats (*R. norvegicus*)

The Figure 1. is a diameter femoral muscle male white rats (*R. norvegicus*) influence CCl<sub>4</sub>. The measurement results using 400 x light microscope magnification, showed that P3 has lowest muscle fibers (34.08 μm) diameter, compared to the other treatments.

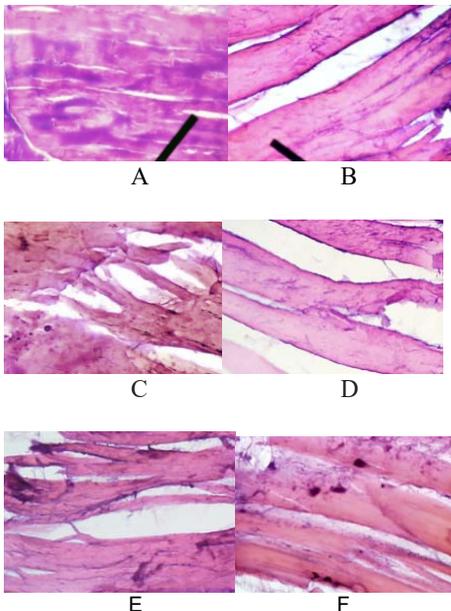
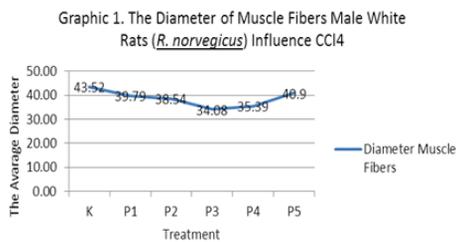


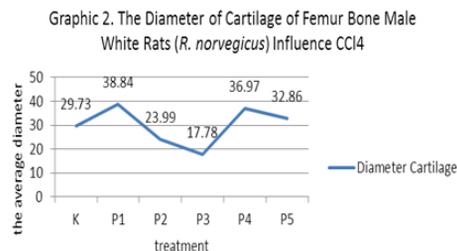
Figure 1. The Muscles Tissue Femur Male White Rats (*R. norvegicus*) after a given treatment  
**Note:** A. Control (K) 1.4 ml olive oil/g BW; B. P1 (0.8 ml CCl<sub>4</sub>/g BW); C. P2 (1.1 ml CCl<sub>4</sub> /g BW); D. P3 (1.4 ml CCl<sub>4</sub> /gr BW rats); E. P4 (1.7 ml CCl<sub>4</sub>/gr BW); F. P5 (2.0 mL CCl<sub>4</sub> / g BW).  
 In light microscope 400 x magnification, Hematoxylin Eosin Stained

It appears that under the microscope, when the dose of CCl<sub>4</sub> increase, the muscle fibers becomes irregular and the death cells of muscle increases. In addition, the muscle fibers endomyceum was degenerates and lysis, then the space between muscle fibers was longer. The ANOVA test shows that the treatment significantly different at 5% level. The Duncan test shows that the P3 treatment is the most toxic dose, was significantly different damaging the muscle tissue, characterized by the lowest muscle fibers diameters (34.08 μm), compared to other treatments. The P4 treatment shows a significantly (muscle fibers diameter 35.40 μm) different from the K, P1 and P5 treatments; although P3 and P2 are not significantly different. The P2 treatment show a significantly different (muscle fibers diameter 38.54 μm) with K although the P1 and P5 treatments are not significantly different. The P1 treatment (muscle fibers diameter 39.78 μm) is not significantly different from the P5 treatment (muscle fibers diameter 40.88 μm). The muscle fibers of control diameter (43.52 μm) shows the highest significantly different.

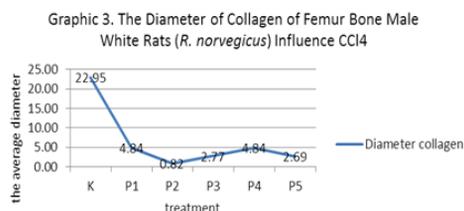
CCl<sub>4</sub> is a membrane trans protein, so it can bind to the cell membrane, and activate the cell; resulting the most toxic metabolite CCl<sub>3</sub> (free radicals). CCl<sub>4</sub> can bind cellular molecules (nucleic acids, proteins, fats), influencing DNA synthesis so that it causes apoptosis, fibrosis and malignancy [13].

### Histopathologic Tissue of Femur Bone of Male White Rats (*R. norvegicus*) Influenced by CCl<sub>4</sub>

In the Graphic 2, the cartilage diameter of histopathologic observation of white rat (*R. norvegicus*) femur bone includes the measuring of cartilage and collagen diameter. The result of measuring using light microscope 100 x magnification shows, that P3 has the lowest cartilage diameter (17.78 μm) compared to other treatments.



From the Graphic 3, the collagen diameter as the results of measurement using light microscope 100 x magnification, shows that P2 has the lowest collagen diameter (0.82 µm) compared to other treatments.



Based on observations under microscope, the results of all treatments can be seen in Figure 2.

When the dose of CCl<sub>4</sub> increased, the growth of cartilage cells (chondrocytes) in the epiphyseal regions decreased, bone cells (osteocytes) in trabecular also diminishing, and the appearance of space in the epiphyseal and trabecular area; and also the thinner diameter of the collagen. The interesting thing is, reinforcement appears or growth of cartilage cells (chondrocytes) in the trabecular area in the treatment P5 (2.0 mL CCl<sub>4</sub>/gr BW rats). This conditions show that exposure high doses of CCl<sub>4</sub> cause the growth of abnormal cells.

The ANOVA results the cartilage diameter showed that the treatment very significantly different ( $P < 0.01$ ). The Duncan test shows that the P3 result the lowest cartilage diameter (17.78µm) very significantly different ( $P < 0.01$ ) compared to other treatments. The next critical condition followed by P2, K, P5, P4 and P1 treatments which showed very significantly different ( $P < 0.01$ ), and the P1 treatment shows the best results cartilage diameter is 38.84 µm.

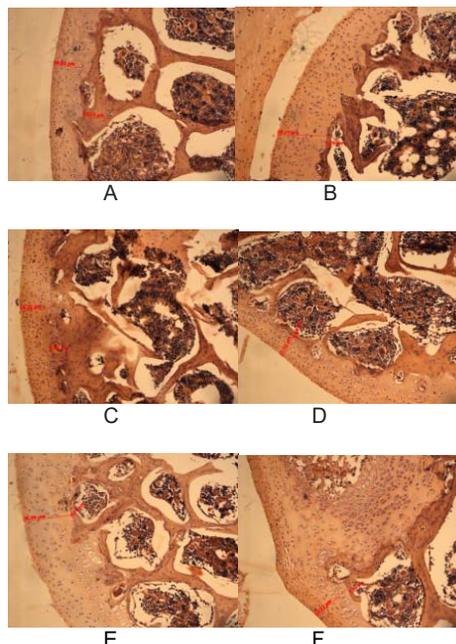


Figure 2 The Tissue of Femur Bone Male White Rats (*R. norvegicus*) after treatments

Note: A. Control (K) 1.4 mLolive oil / gr BW; B . P1 (0.8 mL CCl<sub>4</sub> / gr BW) ; C . P2 (1.1 mLCCl<sub>4</sub> / gr BW) ; D . P3 (1.4 mLCCl<sub>4</sub> / gr BW rats) ; E . P4 (1.7 mLCCl<sub>4</sub> / gr BW rats) ; F . P5 (2.0 mL CCl<sub>4</sub> / gr BW rats). Light microscope magnification of 400x, Hematoxylin Eosin Stained.

The ANOVA test result at the collagen diameter showed that the treatment very significantly different ( $P < 0.01$ ). Duncan test shows that the P2 treatments, is the lowest collagen diameter (0.82µm) very significantly different compared to other treatments, then P5 shows a very significantly different (collagen diameter 2.69 µm) from the K, P1, P3 and P4 treatments. The P3 treatment shows the collagen diameter (2.77 µm) very significant different compared to K, P1 and P4 treatments, even though the P1 treatment (4.84 µm) and P4 (4.84 µm) was not very significantly different. The K treatment shows the highest collagen diameter (22.95 µm).

Based on the test results, the P3 treatment (1.4 ml CCl<sub>4</sub>/g BW ) is a toxic dose to male white rats (*R. norvegicus*), causing musculoskeletal tissue damage characterized by muscle fibers diameter, collagen and cartilage of femur bone diameter decrease, supported by the data of mice body weight treated with CCl<sub>4</sub>,the heavier body weight

than the control treatment (olive oil). This shows decreased mobility of mice exposed with CCl<sub>4</sub> due to pain and musculoskeletal tissue damage.

The CCl<sub>4</sub> also induces an inflammatory response cells, causing progressive damage to the extracellular matrix (ECM) and the cell. This is appears from the reduction in the diameter of the femur bone collagen. The exposure CCl<sub>4</sub> promote cell apoptosis that leads to tissue damage necrosis.

Cellular communication between osteoblasts and osteoclasts in bone tissue plays an important role in the process of bone remodeling. Two ligand in osteoblasts that RANKL and OPG regulate bone resorption. Excessive RANKL expression allows differentiation and activation of osteoclasts. Osteoclast activation trigger osteoclastogenesis (bone resorption). The result is a cell apoptosis. Therefore, the balance between RANKL and OPG determine bone resorption.

## CONCLUSIONS

The CCl<sub>4</sub>as toxic agent shown to induce osteoporosis seen from musculoskeletal tissue damage in male white rat (*R. norvegicus*) Wistar.

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