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IN VIVO BIODISTRIBUTION OF ^{99m}Tc-MDP FOR EARLY OSTEOPOROSIS MONITORING IN OVARIECTOMIZED BALB/C MICE

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ABSTRACT

IN VIVO BIODISTRIBUTION OF ^{99m}Tc-MDP FOR EARLY OSTEOPOROSIS MONITORING IN OVARIECTOMIZED BALB/C MICE. Technetium-99m Methylene Diphosphonate (^{99m}Tc-MDP) has been utilized in a variety of clinical situations to identify bone areas due to the strong affinity of hydroxyapatite crystals in the mineral phase of the bone with the diphosphonate compounds. Osteoporosis is a disease characterized by decreased bone mass and increased fracture risk and represents a significant population health issue. It has been observed that ^{99m}Tc-MDP can be used for bone scintigraphy especially in case of bone cancer, but biodistribution study of ^{99m}Tc-MDP on ovariectomized mice for early monitoring of osteoporosis model remains unclear. Therefore, we aimed to investigate the biodistribution of ^{99m}Tc-MDP both in normal and ovariectomized mice. The experiment was performed on BALB/c mice weighing approximately 30 g. Mice were divided into a normal and ovariectomized group. After the first, second and third hours, mice were euthanized using the accepted protocol and the tissue of interest was collected. All tissue and blood were weighed using an analytical scale and counted for radioactivity using Automatic Gamma Counter with NaI(Tl) detector. Administration of ^{99m}Tc-MDP showed in normal mice compared with an animal model of osteoporosis, there are significant differences at 1 hour post-injection from (20.32±1.38) %ID/g decreased to (7.42±2.61) %ID/g, 2 hours from (13.75±0.01) %ID/g to (5.25±0.25) %ID/g and 3 hours from (12.18±1.44)%ID/g to (4.86±1.34) %ID/g uptake in the bones with (p<0.05). This study can be a consideration for the clinical application of ^{99m}Tc-MDP for early detection of osteoporosis conditions by looking at bone uptake and become a concern in the application for bone scintigraphy if the patient is indicated osteoporosis because it will affect visualization of the organ.

Keywords : Radiopharmaceutical, ^{99m}Tc-MDP, Osteoporosis, Ovariectomized, Biodistribution.

ABSTRAK

BIODISTRIBUSI IN VIVO ^{99m}Tc-MDP PADA MENCIT BALB/C YANG DIOVARIEKTOMI UNTUK DETEKSI DINI OSTEOPOROSIS. Technetium-99m Methylene Diphosphonate (^{99m}Tc-MDP) telah digunakan dalam berbagai aplikasi klinis untuk mengidentifikasi area tulang karena afinitas senyawa difosfonat yang kuat pada kristal hidroksiapatit. Osteoporosis adalah penyakit yang ditandai dengan penurunan massa tulang dan peningkatan risiko patah tulang dan merupakan masalah kesehatan yang mengancam populasi secara signifikan. Penelitian sebelumnya telah diamati bahwa ^{99m}Tc-MDP dapat digunakan untuk skintigrafi tulang terutama dalam kasus kanker tulang, tetapi studi biodistribusi ^{99m}Tc-MDP pada mencit yang diovariektomi untuk pemantauan dini osteoporosis masih belum dilakukan. Oleh karena itu, studi ini bertujuan untuk menyelidiki biodistribusi ^{99m}Tc-MDP pada mencit normal dan model ovariektomi. Percobaan dilakukan pada mencit BALB/c dengan berat sekitar 30 g. Mencit dibagi menjadi kelompok normal dan kelompok yang diovariektomi. Kemudian setelah 1, 2, dan 3 jam di-

eutanasia menggunakan protokol yang sudah disetujui dan jaringan yang dibutuhkan dikumpulkan. Semua jaringan dan darah ditimbang menggunakan timbangan analitik dan radioaktivitasnya dihitung menggunakan *Automatic Gamma Counter* dengan detektor NaI(Tl). Pemberian ^{99m}Tc-MDP menunjukkan pada hewan normal dibandingkan dengan model osteoporosis, terdapat perbedaan signifikan pada 1 jam pasca penyuntikan dari (20,32 ± 1,38) %ID/g menurun menjadi (7,42 ± 2,61) %ID/g, 2 jam dari (13,75 ± 0,01) %ID/g menurun hingga (5,25 ± 0,25) %ID/g dan 3 jam dari (12,18 ± 1,44) %ID/g menurun menjadi (4,86 ± 1,34) %ID/g serapan dalam tulang dengan (p<0,05). Penelitian ini dapat menjadi pertimbangan untuk aplikasi klinis ^{99m}Tc-MDP untuk deteksi dini kondisi osteoporosis dengan mengamati akumulasi pada tulang. Aplikasi skintigrafi tulang dengan ^{99m}Tc-MDP juga harus diperhatikan jika pasien diindikasikan osteoporosis karena akan mempengaruhi visualisasi pada organ.

Kata Kunci : Radiofarmasi, ^{99m}Tc-MDP, Osteoporosis, Ovariektomi, Biodistribusi

1. INTRODUCTION

Osteoporosis is a disease characterized by decreased bone mass and increased fracture risk. It becomes a significant population health issue because of its negative effects on mortality, quality of life, and other health outcomes (1). A study of the prevalence of osteoporosis cases in the US in 2013-2014 for adults above 50 years revealed 6% cases at the femur neck, 8% at the lumbar spine, and 11% at other bone locations (2). The prevalence of cases in China also shows an increase in the last 12 years in which cases of osteoporosis affect 1 in 3 people 50 years or older (3).

Diagnosis of osteoporosis is based on Bone Mineral Density (BMD) and Dual X-Ray Absorptiometry (DXA) and is assisted with a minimal number threshold (4). The use of BMD and DXA is widely accepted for screening and fracture risk assessment. However, this method has poor accuracy due to several circumstances, such as vertebral disease, fracture, bone calcification and obesity (5)(6).

In studies related to the condition of osteoporosis, the main choice is to use rodents as animal models with an ovariectomy procedure(7). Hormonal changes especially estrogen, on the ovariectomized rodent model are permanent compared to other estrogen deficiency models. Osteoporosis animal models with ovariectomy can mimic the condition of accelerated bone loss in postmenopausal women (8).

The use of radionuclides for bone scan imaging using ^{99m}Tc-MDP is still the first choice for clinicians in hospitals to detect bone disease and its metabolism. Research in Indonesia was observed uptake of ^{99m}Tc-MDP in prostate cancer patients with bone metastatic cases at 20 prostate cancer patients, and the uptake was increased in metastatic cases (9). Research conducted by Moore *et al* showed that there is a visual change from ^{99m}Tc-MDP when giving teriparatide drug therapy in osteoporosis patients (10). The mechanism of ^{99m}Tc-MDP accumulation is attached to areas of calcification or active bone metabolism especially the surface of hydroxyapatite from bones and crystalline structure of hydroxyapatite (11). Another mechanism was found that the accumulation of ^{99m}Tc-MDP

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was related to its binding with immature collagen (12).

It has been observed that ^{99m}Tc-MDP can be used for bone scintigraphy especially in case of bone cancer and some interaction with osteoporotic drugs in osteoporosis patient, but biodistribution data of ^{99m}Tc-MDP on ovariectomized mice for early monitoring of osteoporosis model remains unclear. This study aimed to examine the biodistribution of ^{99m}Tc-MDP both in normal and ovariectomized mice to identify whether ^{99m}Tc-MDP can be used for early detection of osteoporosis.

2. METHODOLOGY

Animal Models

Eighteen female BALB/c mice with 30 g body weight from PT Biofarma, Tbk Bandung, Indonesia were housed in polypropylene cages (38x30 cm) at constant temperature (26±1^{o}C) and humidity (60±5%) with 12 hours dark cycle. The mice were given dry feed and water ad libitum and divided into normal group and ovariectomized groups. All protocol was approved by the Ethics Committee for the Care and Use of Laboratory Animal (KEPPHP BATAN) under protocol number 003/KEPPHP-BATAN/IX/2019.

Ovariectomy was performed using double dorsolateral incisions in eight week-old mice under anesthesia. Anesthesia was performed using ketamine and xylazine then the flank area between the last rib and above the pelvis was shaved. The skin was disinfected with chlorhexidine solution and incision was made in the skin on the right side. Musculature was separated with curved tip scissors. The ovarian was carefully pulled and fat pad out of the incision. The sterile thread was used to make two knots to delimitate the area. The ovary was removed from both sides. Analgesia was injected to control pain and mice were placed on a heating pad until it is recovered. Maintenance of pain was performed by adding 1.4 mL acetaminophen solution to 300 mL water (final concentration, 0.47 mg/mL) and maintain ad libitum for 3 days. The animal was observed for any inflammatory signs that suggest pain (13).

Histologic evaluation

The femur from normal mice and ovariectomized mice were isolated and fixed with 10% formalin for 24 hours, decalcified in EDTA. The bone sample was embedded into the paraffin and then cut into 4 mm slices along the sagittal plane through the longitudinal axis. The histopathology analysis stained with Hematoxylin Eosin (HE) and examined under a light microscope with 20X magnification.

Preparation of ^{99m}Tc-MDP

The ^{99m}Tc-MDP preparation was conducted aseptically, natrium pertechnetate ($Na^{99m}TcO_4$) (111 MBq/3 mL) was added into a vial containing MDP kit from PSTNT BATAN. The solution was shaken and incubated for 30 minutes at room temperature, resulting in ^{99m}Tc-MDP radiopharmaceutical. The radiochemical purity of ^{99m}Tc-MDP was determined using two paper chromatography systems. In the first step, The Whatmann 3MM

chromatography paper was used as a stationary phase and 100% acetone as mobile phase for separating reduced ^{99m}Tc ($^{99m}TcO_2$) and ^{99m}Tc-MDP from pertechnetate ion ($^{99m}TcO_4^-$). Then, used Whatmann 3MM chromatography paper as stationary phase and 0.9% NaCl as a mobile phase for separating $^{99m}TcO_2$ from ^{99m}Tc-MDP and $^{99m}TcO_4^-$ (14).

Biodistribution Studies

The experiment was performed on BALB/c mice weighing approximately 30 g. Mice were divided into normal and ovariectomized groups with n=3 for every time point, referring to International Atomic Energy Agency (IAEA) technical report series No.466 for ^{99m}Tc radiopharmaceutical biodistribution (15). The ^{99m}Tc-MDP was injected into the tail vein (100 µl) with 1.85 MBq radioactivity. At the time point of 1, 2, and 3 hours mice was euthanized using accepted protocol and the tissue of interest was collected. All tissue and blood were weighed using an analytical scale and counted for radioactivity using Automatic Gamma Counter with NaI(Tl) detector. The percentage of radioactivity per gram of tissue weight (%ID/g) was determined using the following formula :

$$\%ID/g = \frac{count\ per gram\ organ}{count\ dose\ given} x\ 100\%$$

Data Analysis

Numerical data that conformed to a normal distribution were expressed as mean ± SD. The biodistribution for a normal group and ovariectomized group for every time interval using One Way ANOVA. Data comparison between normal and ovariectomized groups were compared using independent t-test with significance level $p<0.05$.

3. RESULT AND DISCUSSION

Based on Figure 1, ovariectomy procedures affect the bone structure in the femur of BALB/c mice. Samples showed that quite clear the femoral head and middle of the diaphysis bone loss at figure 1 (B), compared with normal mice figure 1 (A). Estrogen deficiency caused by the procedure causes bone loss is the stimulation of osteoclast differentiation (16).

Radiochemical Purity

In vivo application of radiopharmaceutical has a quality requirement that is clarity, sterility, non-pyrogenicity, pH, and radiochemical purity. In this study, radiochemical purity testing was performed using two ascending chromatography system. Figure 2A showed a radiochromatogram of ^{99m}Tc-MDP, where acetone used as a mobile phase, ^{99m}Tc-MDP and $^{99m}TcO_2$ stayed at origin give Rf = 0 while $^{99m}TcO_4^-$ moved with solvent to give Rf = 1. Figure 2B showed the radiochromatogram result of ^{99m}Tc-MDP where saline solution used as a mobile phase. $^{99m}TcO_2$ stayed at origin give Rf = 0 while ^{99m}Tc-MDP and $^{99m}TcO_4^-$ moved with the solvent to give Rf = 1. Two ascending chromatography tests showed that ^{99m}Tc-MDP had 99,98% radiochemical purity with n=3. The result has been confirmed to United States of Pharmacopoeia that the radiochemical purity should be greater than 95% for in vivo application.

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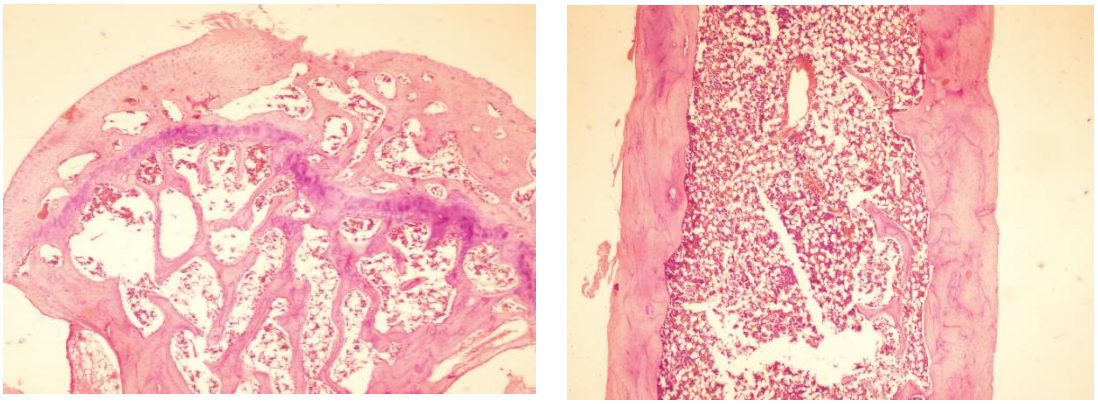
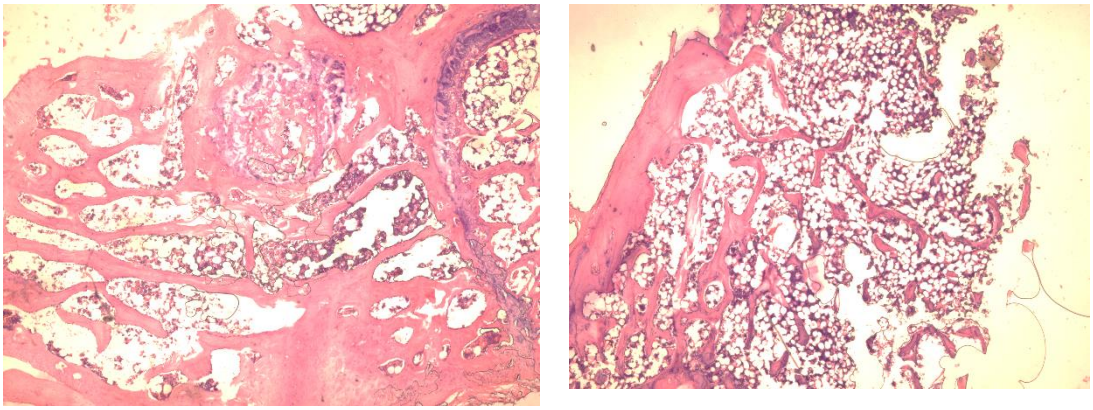
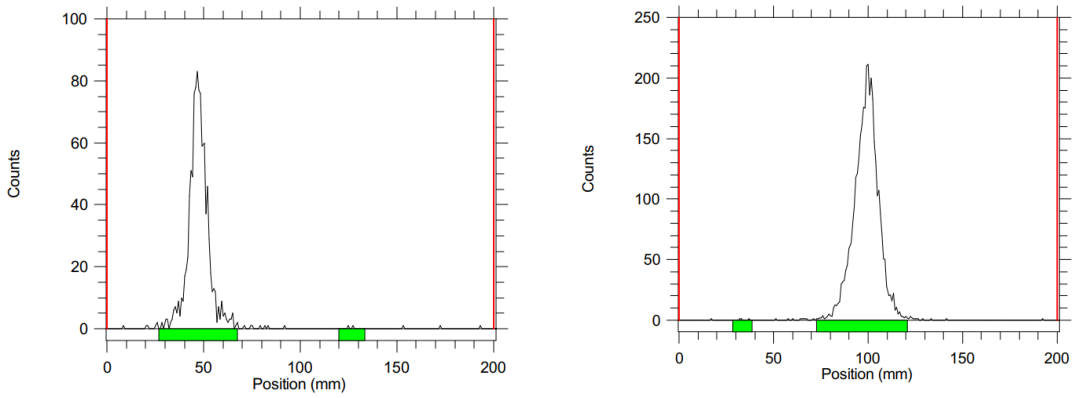


(A) Normal Mice



(B) Osteoporosis Mice

Figure 1. Normal mice (A) and Osteoporosis mice (B) photomicrographs showing a significant bone loss in the femur at the femoral head and middle of the diaphysis (hematoxylin and eosin stain; original magnifications, 20X)



(A) (B)

Figure 2. (A) Radiochromatogram of ^{99m}Tc-MDP using aceton as mobile phase (B) Radiochromatogram of ^{99m}TC-MDP using saline solution as mobile phase

Biodistribution Study

Biodistribution of ^{99m}Tc-MDP both in normal and ovariectomized mice group was studied 1, 2, and 3 hours post-injection to evaluate the optimum time of radiopharmaceutical accumulation for every time interval.

Data from Table 1 shows for the first three hours post-injection higher physiological uptake was observed in bone, liver, and kidney both in normal and ovariectomized mice. Data analysis conducted in the normal group showed a decrease in the radioactivity accumulation in each organ at different time intervals. On the other hand, the ovariectomy group showed retention of radioactive accumulation in kidney and liver organs related to increased bone resorption and reduced of bone formation in osteoporosis conditions, the exact mechanism still remains unknown (17). Statistical analysis using independent t-test proves that for normal mice physiological uptake was significantly different in the bone as target organ with $p<0.05$.

Table 1. (A) Biodistribution Data of ^{99m}Tc-MDP in normal and ovariectomized mice (Ovx) expressed as %Injected dose/g tissue (ID/g) (mean value ± SD, n = 3).

Organ	Normal			Sig (p<0.05)	Ovx			Sig (p<0.05)
	1	2	3		1	2	3	
Muscle	0.08±0.02	0.06±0.01	0.05±0.02	0.08	0.08±0.15	0.06±0.01	0.05±0.02	0.08
Bone	20.32±1.38	13.75±0.01	12.18±1.44	0.00	7.42±2.61	5.25±0.25	4.86±1.34	0.00
Blood	0.44±0.01	0.29±0.10	0.14±0.01	0.00	0.50±0.09	0.30±0.11	0.22±0.06	0.00
Intestine	0.19±0.01	0.15±0.04	0.14±0.05	0.08	0.21±0.03	0.22±0.12	0.13±0.03	0.08
Liver	4.04±0.41	3.64±0.60	2.96±0.39	0.07	2.73±0.21	3.13±0.10	4.07±0.59	0.67
Kidney	1.56±0.39	1.20±0.21	1.02±0.20	0.11	2.35±0.11	1.45±0.39	1.68±0.59	0.11
Hearth	0.34±0.07	0.31±0.08	0.19±0.01	0.03	0.22±0.05	0.15±0.02	0.11±0.03	0.03
Lung	0.69±0.19	0.36±0.02	0.33±0.02	0.00	0.54±0.15	0.37±0.01	0.32±0.14	0.00
Stomach	0.31±0.05	0.20±0.02	0.16±0.04	0.01	0.31±0.05	0.19±0.02	0.16±0.04	0.01

Figure 3 shows that the administration of ^{99m}Tc-MDP on ovariectomized mice also changed the physiological uptake in mice organs. The higher physiological uptake was found in bone, liver, and kidney. Based on statistical analysis for every time interval with ($p<0.05$), data obtained were significantly different. The highest uptake of ^{99m}Tc-MDP was found at 1 hour post-injection in bone (7.42±2.61) %ID/g. The liver has the highest physiological uptake 3 hours post-injection (4.07±0.59) %ID/g. The higher uptake of ^{99m}Tc-MDP in the liver at 3 hours as shown on Figure 4(c) may be due to changes in liver function caused by osteoporosis, although this needs further study. Research conducted by Nackhbandi showed that there is a link between liver function and osteoporosis (18).

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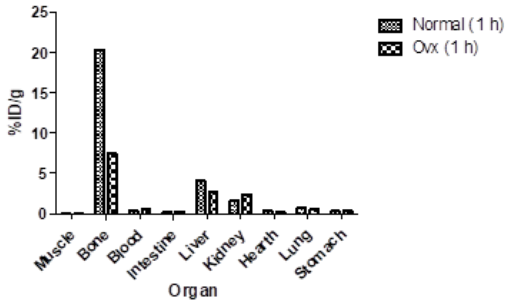
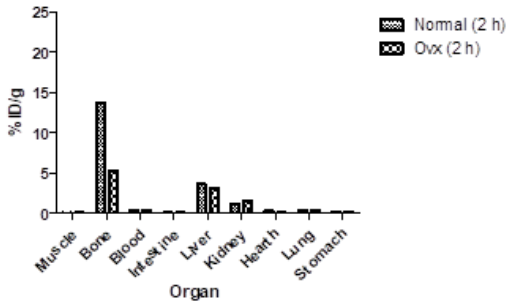
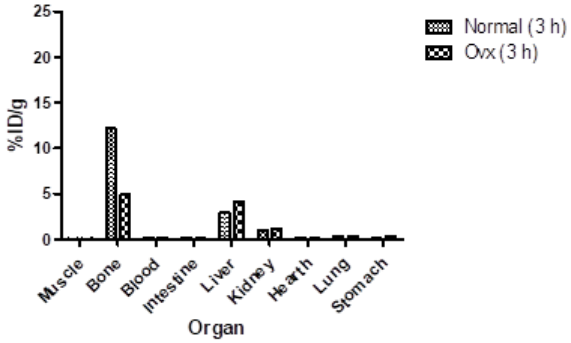


(A) 1 hours post injection



(B) 2 hours post injection



(C) 3 hours post injection

Figure 3. Biodistribution profile of ^{99m}Tc-MDP in normal mice and ovariectomized mice at 1 hour (a), 2 hours (b) , and 3 hours (c) post-injection. Data are presented as percentage Injected dose per gram organ (%ID/g; n=3)

Table 2 shows the comparison of the T/NT ratio of target organ (bone) with non target organ (muscle, liver, and kidney). The radioactivity accumulation shows optimum results based on T/NT ratio with minimum ratio 2:1 for application of gamma camera. The normal group shows T/NT ratio not significantly different for the first three hours. On the other hand ovariectomized group shows the ratio T/NT decreased at 2 hours because the ^{99m}Tc-MDP distributed in the soft tissue. The maximum value of T/NT in osteoporosis model was seen at 3 hours after administration of the tracer. The result similar according to the study of Sugiharti et al, an increase in 3 hours post-injection (14). For clinical application, it is expected that radiopharmaceuticals are localized specifically in target organs because of the activity of T/NT the structural details of the image in the target organ. Therefore, the ratio of target organs to non-target organs has a high activity ratio.

Osteoporosis condition will appear immediately after the procedure where estrogen will be replaced by 17-β-estradiol. Bone damage occurs gradually in the proximal tibial metaphysis after 14 days, in the lumbar vertebral body after 60 days, and in the femoral neck after 30 days. The reduction of cortical thickness and enlargement of the bone marrow cavity begin between 90 and 120 days after ovariectomy (7). This study observed biodistribution of

^{99m}Tc-MDP for 30 days after the ovariectomy procedure has been performed and osteoporosis condition proven by histological findings.

In ovariectomized mice, estrogen level was decreased which retards parathyroid induced calcium dissolution, resulting in imbalanced osteoblast and osteoclast activities and a high bone resorption rate. Ovariectomized also reduced BMD then the features and arrangements of collagen fibers and hydroxyapatite crystals are also expected to change. As in the ovariectomized collagen fibers are short and randomly distributed, rather than long and aligned, and hydroxyapatite crystals are loosely packed (19).

A fundamental aspect of understanding the biodistribution of ^{99m}Tc-MDP is primarily cleared by renal and osseous pathway. The study results showed that the administration of ^{99m}Tc-MDP is related to the clearance mechanism where the radioactivity accumulation of kidney and liver is high. Therefore, the degree of osseous uptake depends not only on factors relating to bone metabolism but also on renal clearance, the latter being closely approximated by the glomerular filtration rate (20).

Table 2. ^{99m}Tc-MDP radiopharmaceutical accumulation ratio Target/Non-Target (T/NT) between normal and ovariectomized mice

Target/Non- Target organ	Accumulation Ratio Target/Non-Target (T/NT)					
	1 hours		2 hours		3 hours	
	Normal	Ovx	Normal	Ovx	Normal	Ovx
Bone : Muscle	240.32	61.42	242.82	38.50	233.64	89.92
Bone : Liver	5.03	2.72	3.78	1.68	4.11	1.19
Bone : Kidney	12.99	3.15	11.50	3.29	11.96	4.11

When ^{99m}Tc-MDP is injected intravenously, its biodistribution depends on the blood flow and the uptake reflects the rate of new bone formation. This radiotracer accumulates in the inorganic hydroxyapatite crystal component of bone. It is therefore normal to see the uptake of the radiotracer throughout the entire skeleton. The radiotracer ^{99m}Tc-MDP undergoes urinary excretion and activity can be seen in the kidneys. The difference in accumulation that occurs in ^{99m}Tc-MDP is due to changes in hydroxyapatite crystals in osteoporotic bone. Bone porosity is proportional to the size of hydroxyapatite crystals, where an increase in porosity can increase the size of hydroxyapatite crystals. The normal bone will show the size of hydroxyapatite crystals that are smaller than osteoporosis because they have low porosity. Larger crystal sizes cause an increase in the number of total cavities so that the vacancy ratio also increases. The change in size and destruction of hydroxyapatite crystals by osteoclasts

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causes ^{99m}Tc-MDP radiopharmaceuticals to be unable to bind to hydroxyapatite so that the accumulation of ^{99m}Tc-MDP on osteoporosis mice becomes lower than normal mice (21).

4. CONCLUSION

The application of ^{99m}Tc-MDP as bone scintigraphy has been widely used for clinical application. Osteoporosis condition in ovariectomized mice, shown that there are differences in biodistribution patterns in organs. The clinical application of ^{99m}Tc-MDP can be used to detect osteoporosis conditions earlier by looking at bone uptake and become a concern in the application for bone scintigraphy if the patient is indicated osteoporosis because it will affect visualization of the organ. Further research is needed to investigate interactions of some osteoporotic drugs with the ^{99m}Tc-MDP application and pharmacokinetic parameters in the osteoporosis model

5. ACKNOWLEDGEMENT

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