LAPORAN

HIBAH KOMPETITIF PENELITIAN UNTUK PUBLIKASI INTERNASIONAL BATCH 1

TAHUN ANGGARAN 2009

KKA
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PROTECTIVE EFFECT OF VANADYL SULPHATE ON THE MORPHOLOGICAL CHANGE OF PANCREAS, MUSCLE AND ADIPOSE TISSUES IN STREPTOZOTOCIN-INDUCED DIABETIC MICE

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HALAMAN PENGESAHAN LAPORAN
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1\. Judul : Protective effect of vanadyl sulphate on the morphological change of pancreas, muscle and adipose tissues in streptozotocin-induced diabetic mice.

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j. Pendanaan dan Jangka Waktu Penelitian :

Jangka Waktu penelitian : 1 tahun
Biaya total yang diusulkan : Rp. 200.000.000,-
Biaya yang disetujui tahun 2009 : Rp. 40.000.000,-

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1. Judul : Protective effect of vanadyl sulphate on the morphological change of pancreas, muscle and adipose tissues in streptozotocin-induced diabetic mice

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I. PENDAHULUAN

Telah dilakukan penelitian tambahan untuk hibah penelitian publikasi internasional sebagai data pelengkap dalam submission manuscript ke Journal Internasional. Penelitian berupa pemeriksaan sel beta pancreas dengan pewarnaan yang lebih spesifik secara histopatologi.

II. MATERIAL DAN METODOLOGI PENELITIAN

2.1 Bahan

- Jaringan pancreas
- Obyek glass
- Aldehid Fuchsin

2.2 Instrumen

- Mikrotom
- Mikroskop Konfokal dan inverted

2.3 Desain Penelitian

Kelompok mencit diinduksi diabetes mellitus dengan injeksi intraperitonial streptozotocin 100 mg/kg bb dan diulangi pada hari ke 14 pada dosis 50 mg/kg bb. Pada hari ke 21 diberikan treatment vanadil sulfat dengan konsentrasi sehari sekali selama 7 hari. Pada hari ke 28 mencit dikorbankan dan diambil jaringan pancreas. Selanjutnya dilakukan slicing dan pewarnaan dengan menggunakan aldehid fuchsin. Irisan diamati dibawah mikroskop konfokal dan inverted.

III. HASIL PENELITIAN DAN KEMAJUAN PUBLKIKASI

Data tambahan hasil penelitian digabung dengan data dari Hibah Bersaing Tahun 2008 menjadi manuscript terlampir. Manuscript sudah dievaluasi oleh expert baik dari kajian ilmiahnya (Prof. Dr. Tsutomu Suzuki dari Hoshi University, Tokyo, Japan) maupun linguistiknya (Dr. Dyah Ariani Arimbi dari Fakultas Ilmu Budaya, Universitas Airlangga). Manuscript siap disubmitt secara on line ke Journal of Pharmacology and Experimental Therapy.

Pada tanggal 6 September 2009 telah dilakukan submission di Journal of Neuroscience (IF = 16). Tetapi setelah direview pada bulan September-Oktober 2009 telah diterima hasil bahwa ada revisi major yaitu penambahan data. Selanjutnya penulis membuat keputusan untuk disubmit pada journal yang mempunyai IF yang lebih rendah. Manuscript telah disubmit pada journal Journal Pharmacology and Experimental Therapeutic (IF = 3) pada 30 Oktober 2009

IV. PENUTUP

Demikian draft laporan, atas perhatian dan masukkannya kami sampaikan terima kasih.

PROTECTIVE EFFECT OF VANADYL SULPHATE ON THE MORPHOLOGICAL CHANGE OF PANCREAS, MUSCLE AND ADIPOSE TISSUES IN STREPTOZOTOCIN-INDUCED DIABETIC MICE

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ABSTRACT

This present study is designed to investigate the protective effect of vanadyl sulphate on the morphological change of pancreas, muscle and adipose tissues in streptozotocin-induced diabetic mice. The diabetic mice model was induced twice by intraperitoneally administration of streptozotocin at 100 mg/kg body weight followed by 50 mg/kg at 14 days after the first injection. To observe the development of diabetes mellitus, blood glucose level was measured on day 0, 7, 14 and 21 with portable glucometer. On day 21, vanadyl sulphate suspension at 5, 30, or 100 mg/kg was given orally once a day for 7 consecutive days. The mice were sacrificed on the day 28. Then, pancreas, muscle, and adipose tissues were dissected for histopathological approaches. We found that the islet langerhans and number of beta pancreatic cells were significantly decreased in the diabetic group. In addition, the diameter of hind paw muscle and glycogen were drastically reduced. Likewise, there was a structural change of adipose tissue and a decrease in fatty drop. The repeated treatments of vanadyl sulphate 5, 30 or 100 mg/kg once a day for 7 consecutive were days significantly decreased the blood glucose level from 228.3 ± 5.7; 228.3 ± 10.0 and 266.3 ± 34.3 mg/dL to 151.0 ± 22.3; 140.7 ± 19.9 and 113.3 ± 26.7 mg/dL, respectively ($F_{(4,43)} = 8.0004$, $p = 0.001$). The repeated treatments of vanadyl sulphate could restore the diameter size of islet langerhans which was previously atrophied in diabetic state. At 5 mg/kg dose, there was an initiation of beta pancreatic cell repairs, apparent activity of the nucleus cell, and a decrease in apoptosis. This condition was closer to normal state with higher dose. Moreover, treatment with 100 mg/kg showed proliferation of beta pancreatic cells. At hind paw muscles, the diameter size and glycogen had increased. At adipose tissue, vanadyl sulphate administration caused an increase of fatty drop in adipose cell and more homogenous cell without pseudopodia. Taken together, repeated treatments of vanadyl sulphate were effective to protect the morphological change of beta pancreatic cell, muscle and adipose tissue in the streptozotocin-induced diabetic mice.

Key word: Adipose tissue, beta pancreatic cell, diabetes mellitus, glycogen, islet langerhaens, muscle, streptozotocin.

1. Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia associated with abnormal metabolism of carbohydrate, fat and protein. Based on its etiology, diabetes mellitus is classified into two major groups i.e. diabetes mellitus type 1 and 2 (Harris, 2000; Setter et al, 2000; Oki & Isley, 2002; American Diabetes Association, 2008). Diabetes type 1 is caused by total destruction of beta pancreatic cell mediated by immune system or viral infection while type 2 is caused by combination of decreased insulin secretion and action, from major insulin resistance with relatively insulin deficiency to major insulin secretion with insulin resistance (Harris, 2000; Nolte & Karam, 2001). Diabetes type 2 makes up of 90% diabetic cases and are generally caused by insulin resistance and relative insulin deficiency (Schwinghammer, 2003).

Insulin plays an important role in the metabolism of carbohydrate, fat and protein. While there is an overproduction of carbohydrate and fat, it initiates the process of storing carbohydrate into glycogen in hepar and muscle cell, and fat into adipose tissue. Meanwhile, an overproduction of protein triggers insulin to directly stimulate amino acid uptake by cell and then transform the amino acid into protein. In addition, insulin prevents breakdown of protein in the cell (Guyton & Hall, 1996). Molecular studies show that insulin can interact with its receptor on cell membrane with high specifity and affinity. This receptor consists of 2 sub unit (alpha and beta) with different functions. Sub unit alpha is located in the extracellular and has a binding site of insulin, while sub unit beta is transmembrane protein with tyrosine protein kinase activity. The binding of the insulin to its receptor triggers tyrosine kinase activity by tyrosine phosphorylation. Then, the phosphorylated tyrosine stimulates some intracellular molecules which cause an increase of glycogenesis and a decrease of glycogenolysis and gluconeogenesis (Nair & Karki, 1992; Davis & Granner, 2001; Khan & Saltiel, 2001; Lizkano & Alessi, 2002; Galic, 2004). This activation causes phosphorylation of 2 residual tyrosine in sub unit beta which is more known as bis-phosphorylation. As a result, this increases kinase activity in sub unit beta. A quick phosphorylation of the third residual tyrosine takes place following bis-phosphorylation to tris-phosphorylation. Then, glucose transporter 4 moves from

intracellular to membrane cell causing glucose to enter the cell. On one hand, glucose is then transformed into glycogen by glycogen synthetase enzyme. On the other hand, tris-phosphoryation of insulin receptor becomes a substrate for protein tyrosine phosphatase, inhibits kinase activity in the insulin signaling (Goldstein, 2000; Galic, 2004; Syahriel, 2007).

In vitro study shows that purified insulin receptor maintains autophosphorylation state and does not dephosphorylate itself eventhough insulin has been cleared up from binding site. *In situ* study using intact cell has revealed that the dissociation of insulin receptor is followed by dephosphorylation of sub unit beta and deactivation of kinase receptor together. This condition is caused by protein tyrosine phosphatase (PTPase) which regulates insulin by dephosphorylation and inactivation of insulin receptor causing end of insulin signaling pathway. In insulin resistance, the activity of PTPase increase causes premature insulin signaling and prevents metabolic effect to glucose that then develops to diabetes mellitus (Goldstein, 2000; Galic, 2004).

Insulin and oral antidiabetes drugs have shown to work effectively, however, the use of insulin and its analog in the long term can cause insulin resistance and side effects such as hypoglycemia and weight gain. Sulphonylurea and non-sulphonylurea increase the risk of cardiovascular disease and hypoglycemia. Biguanide can lower the absorption of vitamin B_{12}, thiazolidinedione causes oedema and anemia. Alpha-glucosidase inhibitor has side effects in gastrointestinal tract, decreases Ca serum dan vitamin B_6. The use of chronic oral antidiabetes can cause a resistance to insulin receptor (Nolte & Karam, 2001).

Therefore, it will need a method to manage diabetes mellitus with more effective and specific strategy such as specific inhibition in tyrosine phosphatase pathway. Several reports have shown that the administration of vanadium could lower blood glucose level in diabetic mice induced by streptozotocin. Moreover, the chronic vanadium ingestion increases basal hexosa transport to muscle and liver (Verma *et al*, 1998, Goldstein, 2000). Vanadium is a trace element and can be found in lower concentration in mammalia tissue (Tsiani *et al*, 1998). The mechanism of action of vanadium salt is by a specific inhibition of protein tyrosine phosphatase. The most common vanadium salt to use is vanadyl sulphate

(Cadene *et al*, 1996; PDR Health, 1999; Dey *et al*, 2002; Devandra, 2004, Hasianami, 2007). Inhibition of autophosphorylation of tyrosine phosphatase enzyme using vanadium therefore can prevent insulin receptor desensitization. Hence, vanadium can help glucose to enter from blood to tissue and to lower blood glucose level (Asante-Appiah & Kennedy, 2003).

Vanadium with its mechanism of action, hopefully, could lower the blood glucose level and could be a choice in the more specific management of diabetes mellitus type 2. Therefore, this study will be carried out to evaluate the effectivity of vanadyl sulphate in a range of doses and then compared with glibenclamide, a sulphonylurea oral antidiabetic drug.

2. Methods

2.1 Instruments

This study used convocal microscope (Olympus) in the Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University.

2.2 Materials

In this study, vanadyl sulphate was obtained from Fluka while streptozotocin, citric acid, sodium citric, haemoxylin-eosin dye, fuchsin aldehyde and PAS were purchased from Sigma.

2.3 Animals

For this experiment, 8-week-Balb-C-male mice were used from Animal Laboratory of Airlangga University.

2.4 Research Protocol

Mice were grouped into 8 groups in standard treatment maintained at a room temperature of 27 ± 1°C. A light was set to 12 hours day and night (a day cycle started from 6.00 to 18.00). Their food and drink were added ad libitum. After a week of adaptation, mice were first injected with 100 mg/kg streptozocin in citrate buffer intraperitonially to induce diabetes mellitus. On day 14, second intraperitonial injection of 50 mg/kg streptozotocin was given. Blood glucose

level was measured on day 0, 7, 14, and 21. On day 21, vanadyl sulphate suspension was given orally to 3 groups of treatment with dose of 5, 30, dan 100 mg/kg once a day for 7 consecutive days. A decrease of blood glucose level was measured on day 23, 25, and 28. Blood sample was taken by cutting mice's tail. Blood glucose level was measured with *On Call Now Blood Glucose Monitoring System*.

2.5 Sample preparation and staining of pancreas, muscle and adipose tissues

A surgery procedure was applied to each mouse with ether anaesthesia to take its tissue. Pancreas, muscle and adipose tissues were fixed in neutral buffered formaldehyde 10%. Formaldehyde was well absorbed by tissue in relatively long time i.e. 1 – 4 hours. Formaldehyde buffer acts by preventing acidosis that could induce autolysis and pigmen precipitation in the tissue. After the tissue was washed with xylene, it was embeded in parafin and cut with microtom for 4 – 5μm in thickness. Tissue slice had to be directly placed in glass object to be reacted with dye staining.

2.6 Haematoxylin-Eosin and Aldehid Fuchsin staining

The first step of pancreas tissue staining with hematoxylin-eosin was deparafination. This process was conducted by flowing aquadest to tissue slice and then soaked into hematoxylin for 5 minutes and washed with flowing water. The tissue slice was then stained with blue color by soaking in carbonate lythium and washed with flowing water. Next, the slice was put into acid alcohol 1% for couple of seconds before rewashing it with flowing water and soaking it in eosin for 5 minutes. After washing it with flowing water, dehydrated, and cleaned, sample preparation was ready to be observed. In general, haematoxylin is oxidized into hematein and makes a complex with mordant, metal ion like alumunium. The positive charge of the complex is bound with negative charge of the phosphate ion from nucleic acid to make blue purple color, a specific color for haematoxylin. The result of this staining is blue black for nucleus, pink for sitoplasm, and purple or dark red for red blood cell (Lowe, 1997).

2.7 PAS staining

The first step of muscle staining with periodic acid schiff was deparafination by flowing aquadest into tissue's slice. Next, the slice was soaked into periodic acid for 5 minutes and washed with aquadest. A schiff reagent was added at room temperature, allowing it for 30 minutes, heating with high power microwave for 45 – 60 seconds until its colour turned into dark magenta. Then, it was washed with flowing water for 5 minutes, adding hematoxylin as counterstain for 3 minutes, washed with water, blue hematoxylin and aquadest. The final step was to dehydrate the tissue slice with alcohol, cleaned and covered with glass cover. The mice's tissue slice was ready for analysis. Periodic acid schiff staining is a hystochemistry reaction with periodic acid oxydize carbon-carbon bond (C – C) to form aldehyde which then reacts with sulphate-fuchsin acid to form magenta color. The color of sample preparation is magenta which indicates glycogen while blue indicates nucleus (Crookham & Dapson, 1991)

2.8 Statistical analysis

The effectivity of lowering blood glucose level by vanadyl sulphate/sodium orthovanadate for each dose on day 21 dan 28 will be compared with positive control and analyzed with Anova. The significant difference will then analyzed with LSD. A descriptive qualitative analysis for hystologycal examination will be carried out.

3. Results

This study aims to evaluate the vanadium to decrease a blood glucose level and its effect toward morphology changes of pancreatic, muscle and adipose tissue of streptozocin induced diabetic mice (*Mus musculus*). 80 male, 8 weeks mice were grouped into 8 groups i.e. 1 negative control group, 1 streptozotocin induced diabetic mice, and 3 treatment groups which received vanadyl sulphate with doses of 5, 30 and 100 mg/kg. Diabetic mice were developed by injecting intraperitonially 100 mg/kg streptozotocin in citrate buffer pH 4-5 followed by 50 mg/kg on day 14.

Table 1 shows the changes in blood glucose level in mice injected by intraperitonial streptozotosin 100 mg/kg and followed by 50 mg/kg. There was no increase of blood glucose level in the control group which received intraperitonial citrate buffer pH 4.5 while groups received intraperitonial streptozotocin showed random increase of blood glucose level from average of 151.5 ± 4.4 mg/dL to 224.6 ± 9.9 mg/dL. Statistical analysis using Anova showed that the injection of streptozotosin significantly increased blood glucose level compared with negative control ($F_{(1,37)} = 5.332$; $p = 0.023$).

A surgery procedure was carried out to evaluate the morphological changes in adipose, muscle and pancreatic tissues in control and diabetic groups by a hystochemical preparation with appropriate staining procedure such as aldehyde fuchsin for staining beta pancreatic cell, PAS for glycogen and hematoxylin-eosin for general staining procedure. Muscle tissue was taken from hind paw and adipose tissue was taken from the abdominal skin.

Figure 1 and 2 show a decrease in the size of islet langerhan and the number of beta pancreatic cells due to injection of streptozotosin. Figure 1A shows islet langerhans's position among glandula acini whose colour is paler than other surrounding glandula, consisting of a group of granules. The morphology of islet langerhans can be seen with 400 times microscope (Figure 1C). Cross section of naive pancrease mice shows that the islet langerhans is intact, well organized cells, showing apparent border among cells and chromatin inside of the cells. Figure 1B shows the diabetic mice's pancreatic tissue. Morphological change of islet langerhans due to diabetes mellitus can be seen with 400 times microscope (Figure 1D). The destruction of islet langerhans has started as smaller sizes are shown compared to those of normal group, having irregular cell structure, smaller nucleus size, the sparse of distance among nucleus. Some nucleus increase their sizes but it remains unclear which ones show degenerative and apoptosis cell. Apoptosis cell is characterized with the decrease of nucleus size due to fragmentation while sitoplasm is intact. Using 400 times microscope, apoptosis cell can be seen with red yellow sitoplasm and surrounded by smaller size of nucleus compared with others. Using aldehyde fuchsin staining (Figure 3), it can be seen that there is a decrease in the number of beta pancreatic cell in diabetes

mellitus induced by streptozotocin. It indicates that injection of streptozotocin is effective to induce diabetes mellitus by destructing beta pancreatic cells.

Figure 3. shows the morphological change of diabetic mice muscle. Longitudinal section of the diabetic mice muscle shows more fibroblast, decreasing number of nucleus in the muscle cell compared with control (3b). Cross section also shows fibroblast and decreasing diameter size of mucle cell (3d). Figure 4 shows glycogen drops spreaded in sarcoplasmic of muscle cell. With PAS staining, glycogen drops are magenta in color. In that figure, the muscle nucleus is long, oval and has dark color. Figure 4 shows a change in muscle tissues due to streptozotocin induced diabetes mellitus. With 400 times microscope magnification, there is a decrease in the number of glycogen drops due to glycogenolysis. Also, it shows that muscle fibres are not compact, thin, pale and swollen nucleus as a result of the decrease in metabolic activity and in the number of cromatin fibres.

Figure 5 shows more of detailed changes of muscle cells due to diabetes mellitus. Longitudinal section of the tissue shows that the major change is in the nucleus cell. In normal condition (a) nucleus muscle looks thin and compact, while in diabetes mellitus shows swollen nucleus cell (b) and some nucleus fused in sitoplasm (c) indicating necrosis of the muscle cell. The cross section shows smaller diameter muscle cell in diabetic mice compared with control, indicating atrophic state or a decrease in the volume of the muscle cell.

Figure 6. shows the influence of diabetes mellitus into mice's adipose tissues. With 100 times magnification, large vacuola without nucleus is seen among other fat cells in diabetic mice (b), while in control group, compact fat cells in adipose tissue are seen. The diameter of the fat cells is smaller compared to the normal condition. With 400 times magnification, the vacuola without nucleus comes from lysis of the citoplasm fat cel (d) indicating degenerative cell.

After the diabetic mice with streptozotocin injection had been developed for 21 days, 3 groups of mice were received oral vanadyl sulphate in CMC Na 0.5% with the doses of 5, 30 and 100 mg/kg once a day for 7 consecutive days. The control positive group received only CMC Na. Any changes in blood glucose

level were monitored on day 28. Table 2 shows the blood glucose level measurement.

Vanadyl sulphate is effective to lower blood glucose level in the diabetic mice. The blood glucose level measurement on day 28 showed a significant differentce between all diabetic groups received vanadyl sulphate at various doses and the diabetic mice group received only CMC Na ($F_{(4,43)} = 8.004$; $p < 0.001$). Subsequently, histopathological study following vanadyl sulphate treatment at various doses was carried out by surgery and preparation of mice's pancreatic, muscle and adipose tissue.

Figure 7 and Figure 8 show any changes in pancreatic tissue before and after pretreatment with vanadyl sulphate at the dose of 5, 30 dan 100 mg/kg BB once a day for 7 consecutive days in the diabetic mice group. Pancreatic tissue in the mice received treatment with vanadyl sulphate showed morphological repair of islet langerhans. At the dose of 5 mg/kg, islet langerhans starts to be re-intact, having irregular cell organization, showing wide distance between cell, and nucleus color still is not apparent but sharper compared to the diabetic mice and many more apoptosis cells. At the dose 30 mg/kg, islet langerhans looks better than that previous dose with apparent nucleus cell and its border, demonstrating uniformity in cell size (7A-B), The apoptosis cells are rarely apparent and the process of proliferative cell starts to develop. Proliferative process is characterized by nucleus division. At the dose 100 mg/kg, well characterized cell organization looks like the normal group (7C-D), displaying very apparent nucleus and its border, with uniformity in cell size, having almost no apoptosis. Compared with other doses, at the dose of 100 mg/kg vanadyl sulphate, the morphology of pancreatic tissue in diabetic mice is closely similar to a normal pancreatic tissue in normal mice. It suggests that there is a repair mechanism toward the islet langerhans destruction in diabetes mellitus.

Figure 9 and Figure 10 show any changes in diabetic mice before and after the treatment with vanadyl sulphate at the doses of 5, 30 and 100 mg/kg for 7 consecutive days. The mice receiving vanadyl sulphate treatment showed an increasing cell volume which can be seen from an increase in diameter of muscle

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cell compared to the diabetic group without treatment (a). This indicates repair of the atrophic state in the muscle cell following diabetes mellitus repairing glucose transport from blood to tissue. The nucleus cell of the treatment groups (Figure 3b; 400 times magnification) does not show any sign of necrotic muscle cell compared to the diabetic mice without treatment. An increasing volume of muscle corresponds to an increasing dose of vanadyl sulphate from 5 mg/kg BB to 100 mg/kg BB. At the highest dose, the muscle mass is compact and closely similar to normal muscle in normal mice. Apparent nucleus cell is seen and no sign of necrotic muscle cell. Also, there is no fibroblast among muscle fibres compared to the diabetic mice (400 times magnification).

Figure 11. and Figure 12. show any changes in adipose tissue as a result of vanadyl sulphate treatment in diabetic mice. An adipose tissue contains fat cells with more than one nucleus which indicate regeneration process. This process is frequently found in the mice receiving vanadyl sulphate dose 100 mg/kg (d). At 400 times magnification, it can be seen that treatment group with vanadyl sulphate has a compact and bigger size of fat cell as well as no lysis of citoplasm compared to the diabetic mice without treatment. At the dose of 5 mg/kg BB vanadyl sulphate, it shows that a large, round fat cell and apparent border among fat cells are found. In addition, there is a recovery process of the fat tissue to a normal condition with a lot of endothelial vascular and an increase of fat cell. At the dose of 30 mg/kg BB, the border among fat cells is more apparent; big, round and homogenous fat cell, excentric nucleus cell with apparent color and no more citoplasm lysis or pseudopodia. At the dose of 100 mg/kg BB fat cell is almost similar to the normal condition in naive mice. The cross section of the adipose tissue shows that the fat cells have more than one nucleus which indicates regeneration process in mice's adipose tissue.

4. Discussion

The study of the effectiveness of vanadium to lower blood glucose level and its effect toward morphological changes in diabetic mice's (*Mus musculus*) pancreatic, muscle and adipose tissue had been carried out. The influence of streptozotocin toward the development of *diabetes mellitus* can be shown in Table

1. On day 1, the diabetic mice group was injected with streptozotocin in citrate buffer pH 6,8 with a single dose of 100 mg/kg body weight intraperitonially, but this dose could not induce diabetes mellitus to all mice so that second injection of 50 mg/kg body weight was given on day 14. On day 21, diabetes mellitus had been developed. From 70 mice injected with streptozotocin, 50 mice developed diabetes mellitus (71.42 %). The usual dose of streptozotocin to induce type 2 diabetes mellitus is 40 – 100 mg/kg (Szkudelski, 2001), but in this study, a single induction did not develop diabetes mellitus. It might be due to the mice's genetic and laboratory condition. Genetic resistance toward streptozotocin had been studied before in which injection with streptozotocin 40 mg/kg for 6 consecutive days in Balb/c cannot develop severe diabetes mellitus (Leiter, 1982). Negative control only received citrate buffer injection on day 1 and day 14 intraperitonially. Blood glucose level measurement was conducted by cutting the mice's tail to take blood sample and was measured using glucometer. Groups of mice receiving streptozotocin showed a significant increase of blood glucose level compared to control group receiving only citrate buffer on day 21 after streptozotocin injection ($F_{(1,37)}$ = 5,332; p = 0,023). The average random blood glucose level in the diabetic mice was 224.6 ± 9.9 mg/dL.

After measuring the body weight and blood glucose level of the mice, on day 28 a surgery procedure was carried out to take pancreatic, muscle and adipose tissue from control and diabetic groups. Morphological changes in those tissue were detected using hematoxylin-eosin, PAS and aldehyde fuchsin staining. Hematoxylin-eosin is not specific to differentiate beta pancreatic cell so that all cells in islet langerhans (alpha, beta, delta and PP cells) look similar (Fawcett, 2002). Therefore, additional staining such as aldehyde fuchsin is needed. Hystologically, morphological changes in islet langerhans in diabetes mellitus are vary in size and number of islet langerhans, alpha : beta cell ratio, and degranulation beta cell. In type 2 diabetes mellitus, pancreatic beta cell is in normal structure until a decrease in the number of islet langerhans in which degranulation occurs to pancreatic beta cell. Histopathological analysis with haematoxylin-eosin staining toward rat's pancreatic tissue induced with streptozotocin intraperitonially showed a decrease in the number of islet

langerhans as well as pancreatic beta cell; irregular structure or defect of islet langerhans, relatively small and atrophied as well as having degranulation in pancreatic beta cell (Leiter *et al*, 2002; Conrad *et al*, 2001; Yazdanparast *et al*, 2005).

Using aldehyde fuchsin, a decreasing number of beta pancreatic cell in diabetes mellitus due to injection of streptozotocin can be shown. Several studies report that more than 60% of beta pancreatic cells are due to an increase of beta pancreatic cell apoptosis (Butler *et al*, 2002). Morphology of the cell in apoptosis state includes cell shrinkage, sitoplasm and chromatin condensation as well as chromatin aggregation. Apoptosis involves one or group of cell that with hematoxyllin-eosin staining looks round or oval mass with large sitoplasm. Compact nucleus chromatin may aggregate in peripheral as well as fragmented DNA into pieces of nucleosom (Loo & Rillema, 1998; Kumar *et al*, 2007). An increase of apoptosis and a decrease of beta pancreatic cell proliferation cause hyperglycemia (Donath & Halban, 2004).

Changes in pancreatic tissue between the diabetic and the control groups can be shown in Figure 2 and Figure 3. More detail changes can be seen in figure with 400 times magnification. Horizontal view of normal mice (Figure 2.B) shows that islet langerhans structure is intact, a well organized cell with apparent border among cells, and clear chromatin. In reverse, pancreatic tissue in the diabetic mice (Figure 3.B), the islet langerhans structure is defect, in smaller size due to atrophy compared to a normal (Figure 2.B), irregular cell organization with smaller nucleus cell (Figure 2.B) or shrinkage nucleus cell, demonstrating a widen distance among nucleus. Furthermore, unclear big nucleus is found thus indicating degenerative cell as well as apoptosis. Apoptosis cell is characterized with shrinkage nucleus due to fragmentation but its sitoplasm is still intact. In Figure 3.B, apoptosis cell is shown with big red yellowish sitoplasm surrounding smaller nucleus compared to others. Changes in pancreatic tissue are due to specific destruction by streptozotocin. Streptozotocin causes diabetes mellitus by destructing beta pancreatic cell DNA via NO production process, hydrogen peroxide radical, hidroxyl radical. Streptozotocin increases production of H_2O_2 i.e. molecule which responsible for DNA fragmentation and thus apoptosis of beta

pancreatic cell (Le May, 2006; Li, 2001). DNA destruction stimulates ribosilation poly ADP which induces NAD^+ and ATP depletion in cell. As a result, disruption in insulin production occurs thus decreasing insulin secretion. Destruction in beta pancreatic cell depends on time exposure and dose of streptozotocin (Szkudelski, 2001; Szkudelski, 2006).

In type 2 diabetes mellitus, insulin resistance occurs because there is insulin action failure to its target such as muscle, adipose and liver. Consequently, a disruption to carbohydrate, fat, and protein metabolism occurs at those tissue target (Belfiore & Iannello, 2000; Guyton & Hall, 1997). Inflamation and necrosis occur in muscle fibre in diabetes mellitus. A decrease in insulin secretion causes direct impact on muscle contractile protein synthesis which initiates muscle atrophy. Because of this, a decrease in diameter muscle fibre occurs and this is shown in horizontal and vertical view in histological sample. The most common early change in necrotic muscle cell is waxy degeneration which then is followed with fibres protein dissolution and cross section of muscle. Progressively, in line with nucleus degeneration, there appears an aggregation that after the removal of the sarcoplasm, what is left is only empty sarcolema (Thomas *et al*, 1984).

Insulin resistance increases triglyceride lipolysis (TG) and prevents free fatty acid esterification in adipose tissue (Adams *et al*, 2005). Lipolysis causes fat tissue to release its fat storage. In fat tissue, lipodystrophy occurs in which there is a decrease in fat tissue mass. Furthermore, in diabetic state, it is often found a magnification of fatty cell because of the incapability of fatty tissue to develop and receive high energy influx (Ronti *et al*, 2006). An increase of free fatty acid in serum will be deposited in liver. In type 2 diabetes mellitus, dislipidemia is a common case which is characterized with an increase of plasma triglyceride, a lower HDL; and an increase of VLDL which is also often seen in *nonalcoholic fatty liver disease* (NAFLD) (Cassader *et al*, 2001). In *fatty liver* disease, big drop triglyceride deposited in hepatocyte is common (Belfiore & Iannello, 2000).

Figure 3 to Figure 6 show muscle and adipose morphological change in the diabetic mice group and control group. The diabetic mice group shows atrophy in its diameter of the muscle fibre; small, thin, unclear color of nucleus muscle. Figure 6D shows that the diabetic mice group has irregular organization of fatty

cells whereas the control group shows compact and well organized fatty cells, the diameter of its fatty cells (Figure 4B) is bigger than diabetic group.

With PAS staining, glycogen drops show bright red in color and are distributed over muscle sarcoplasm. In addition, horizontal view of the normal liver shows groups of glycogen drops in sitoplasm of the liver parenchyme while in the diabetic mice there is an increasing activity of glycogenolysis. As a result, the number of glycogen in liver and muscle is decreasing (Belfiore & Iannello, 2000).

Following the diabetic state due to streptozotocin injection for 21 days, oral vanadyl sulphate or sodium ortho vanadat in CMC Na 0.6 % was given to 3 groups of treatment with the doses of 5, 30 dan 100 mg/kg body weight once a day for 7 consecutive days and only CMC Na 0.6 % to negative and positive control. Blood glucose level was measured on day 28. Table 2 dan Figure 9 show the blood glucose level measurement. The optimum dose to lower blood glucose level is achieved at 30 mg/kg body weight of vanadyl sulphate (Hasmono dkk , 2005; Arijanto, 2006), but in this study at the dose of 5 mg/kg body weight vanadyl sulphate, the blood glucose level was shown to be significantly reduced compared to the diabetic mice group ($p = 0,013$). A lower blood glucose level was seen in group that received vanadyl sulphate 30 mg/kg body weight ($p = 0,001$). The lowest blood glucose level was seen in mice that received vanadyl sulphate 100 mg/kg body weight at day 4 (day 25) but resulted in the death of some mice. Higher dose of vanadyl sulphate (100 mg/kg body weight) may lower drastically the blood glucose level and cause severe hypoglycemia. Previous study has shown that the administration of vanadyl sulphate dose 100-200 mg/kg body weight may lower blood glucose level dramatically in diabetic mice (Arijanto, 2006). In addition, vanadyl sulphate side effect i.e. diarhoea may contribute to this condition. Almost all mortality in mice was due to diarhoea. Vanadyl sulphate contains vanadium which has osmotic action and draws water into gastrointestinal tract so that it increases stool water content. Consequently, dehydration occurs in mice and causes death. In general, vanadyl sulphate is effective to lower blood glucose level in the diabetic mice. Nevertheless, no effect has been found in the introduction of sodium ortho vanadate to mice.

Vanadyl sulphate in the modest dose can protect beta pancreatic cell by preventing hypersecretion insulin response and maintaining insulin storage due to induction by streptozotocin, preventing further destruction of beta cell (Cam *et al*, 1999). Also, it can regenerate beta pancreatic cell in diabetic rat due to streptozotocin injection (Bolkent *et al*, 2005). An increasing cell proliferation or a decreasing apoptosis may regenerate cell (Butler *et al*, 2002, Kumar *et al*, 2007). Thus, to evaluate any changes in pancreatic tissue after vanadyl sulphate *pretreatment* in some doses, a surgery procedure and sample preparation was carried out to the control and diabetic group.

The morphological change in pancreatic tissue in the diabetic mice after vanadyl sulphate *pretreatment* with the doses of 5, 30 dan 100 mg/kg body weight once a day for 7 consecutive days can be seen in Figure 7 and Figure 8. The vanadyl sulphate pretreatment resulted in the morphological repair of islet langerhans. With dose 5 mg/kg body weight,the islet langerhans started to re-intact, forming irregular cell organization, widening distance among cells, showing unclear color of the nucleus, and having plenty of apoptosis cells (Figure 7.B). At the dose of 30 mg/kg body weight, the islet langerhans looks better with apparent nucleus cell and its border, shaping uniformity in cell size. Compared to the mice receiving 5 mg/kg body weight of vanadyl sulphate (Figure 7.C), the 30mg/kg body weight group shows 1-2 apoptosis cell yet proliferative cell process starts to develop. Proliferative process is characterized by nucleus division (Figure 7.C). Proliferative cell indicates cell regeneration in which islet langerhans cells contain 75% of pancreatic beta cells (Nolte & Karam, 2001). At the dose of 100 mg/kg, well organized cell organization that look like the normal group is indicated (Figure 1.B), demonstrating very apparent nucleus and its border, uniformity in cell size with almost no apoptosis. Compared to other doses, at the dose of 100 mg/kg vanadyl sulphate, the morphology of pancreatic tissue in the diabetic mice is closely similar to a normal pancreatic tissue in naive mice. It is suggested that there is a repair mechanism toward the islet langerhans destruction in diabetes mellitus.

The morphological changes were also evaluated by calculating the diameter of islet langerhans. Semi quantitative method was used with 400 times

magnification. On the average, the diameter of islet langerhans in a diabetic state is smaller (1800.00 μm) than normal (2365.72 μm). This is because of the destruction of nucleus DNA by streptozotocin, an increasing cell apoptosis, degranulation of nucleus so that the structure of the islet langerhans is defect (Yazdanparast *et al*,2005). The destruction of and the decreasing number of pancreatic beta cell result in the shrinkage of the diameter of islet langerhans (Ahmadi *et al*, 2003). Pretreatment with vanadyl sulphate can increase the diameter size of the islet langerhans. Increasing the dose of vanadyl sulphate will increase the diameter size of the islet langerhans so that at the dose of 100 mg/kg body weight, the diameter size almost returns to normal (2398.28 μm). This is because vanadyl sulphate can regenerate pancreatic beta cell (Bolkent *et al*, 2005) so that the islet langerhans mass returns to normal and its diameter increases.

The morphological changes also occur in muscle, adipose and liver tissue of the diabetic mice following treatment with vanadyl sulphate. Figure 12 to 16 describe the above changes. In mice's muscle tissue, atrophy of the muscle has been repaired with the increasing volume of the muscle cell which is shown with a bigger diameter of the muscle cell compared to the diabetic mice without treatment. At the dose of 30 mg/kg body weight, a more compact in which its structure is closer to normal muscle cell can be seen. Apparent and clear nucleus mass and no sign necrosis of the muscle cells show. Figure 11 and Figure 12 show compact and well organized fatty cells. At the dose of 5 mg/kg body weight vanadyl sulphate (15B), it shows big and round fatty cells with clear border among them. At the dose of 30 mg/kg body weight vanadyl sulphate (12C), it shows clearer border of fatty cells, round and big as well as homogenous fatty cells, excentric shape with sharp color and no destruction of sitoplasm or pseudopodia. At the dose of 100 mg/kg body weight (12D) fatty cells structure almost returns to normal.

Overall, vanadyl sulphate is effective toward repair of the morphology of pancreatic beta cells, liver, muscle and adipose tissue in *diabetes mellitus*. The repair mechanism of vanadyl sulphate acts by decreasing apoptosis frequency and increasing cell proliferation so that pancreatic beta cell can be regenerated. Therefore, a further research should be carried out to evaluate the influence of

vanadyl sulphate toward decreasing apoptosis of pancreatic beta cell quantitatively with specific staining method to detect apoptosis cell such as TUNEL (*Tdt-mediated dUTP Nick-End Labeling*).

5. Conclusions and Suggestions

Based on this study, it can be concluded that:

1. Vanadyl sulphate is effective to lower blood glucose level in diabetes mellitus.
2. Vanadyl sulphate can repair morphological destruction of beta pancreatic cell in diabetes mellitus.
3. Vanadyl sulphate can repair atrophy and necrosis of the muscle tissue in diabetes mellitus as well as stimulate glycogen synthesis.
4. Vanadyl sulphate can take lipid storage in diabetes mellitus and increase fatty cells regeneration on adipose tissue.

6. Acknowledgements

This study was funded by Hibah Bersaing XI Direktorat Jenderal Pendidikan Tinggi Indonesia. We would like to express gratitude to Elisa, Elvina and Anton Rahman for their technical assistance in this project.

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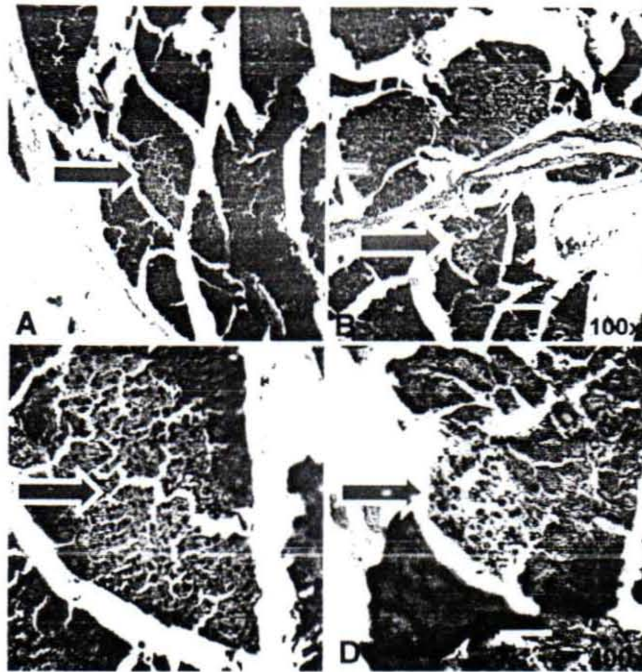

Figure 1. Cross section of pancreatic tissue with hematoxylin-eosin staining. Red arrow indicates islet langerhans. (A and C) mice pancreatic tissue which receive citrate buffer (control) and (B and D) mice pancreatic tissue suffering diabetes mellitus induced by streptozotocin. (A-B) 100 times magnification, (C-D) 400 times magnification

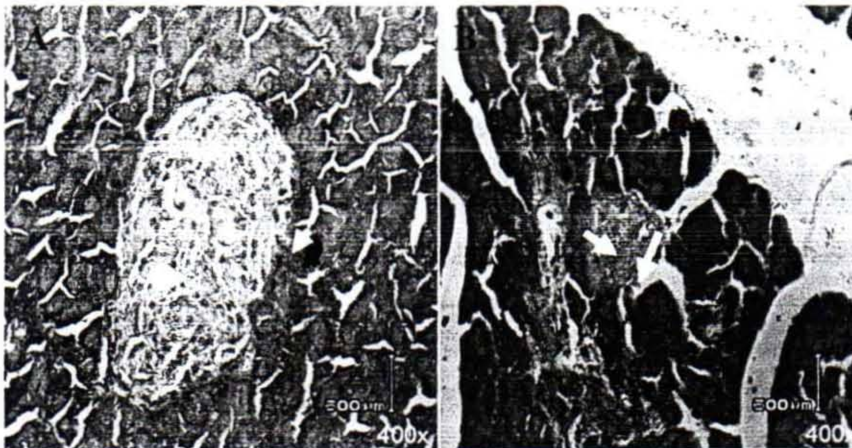

Figure 2. Cross section of pancreatic tissue with aldehyde fuchsin staining. (A) Mice pancreatic tissue which receive citrate buffer (normal) and (B) mice pancreatic tissue suffering *diabetes mellitus* induced streptozotocin. (A-B) 400 times magnification

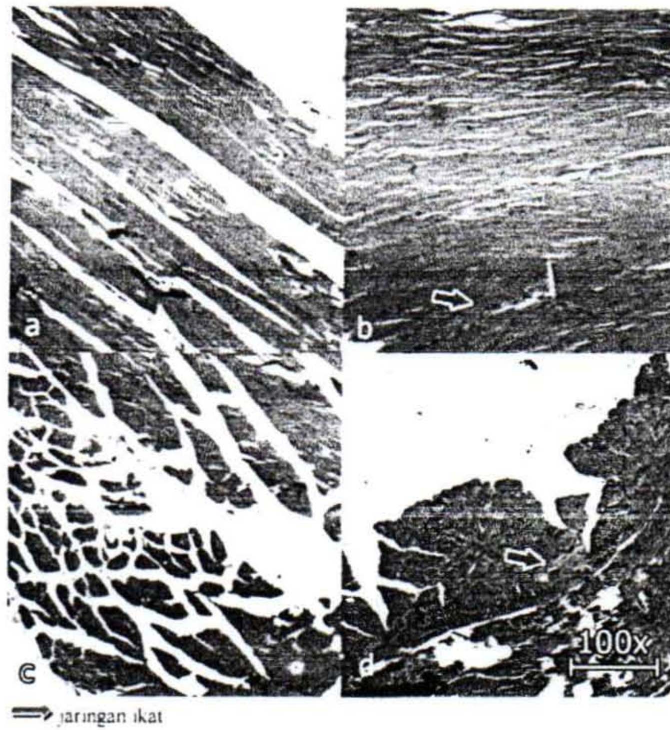

Figure 3. Hind paw mice muscle tissue using haematoxylin-eosin staining, with 100 times magnification. Longitudinal section of muscle tissue was obtain from (a-b) control and (c-d) diabetic mice.

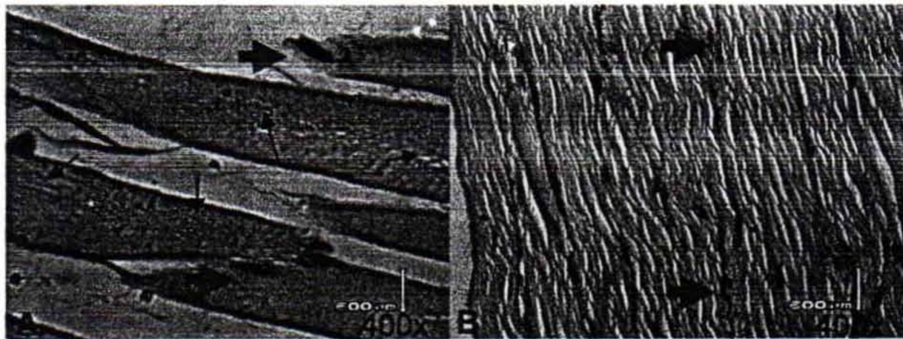

Figure 4. Hind paw mice muscle tissue using PAS staining with 400 times magnification. Longitudinal section of muscle tissue were obtained from of the control (A) and diabetic mice(B). Black arrow shows group of glycogen and red arrow shows nucleus cell.

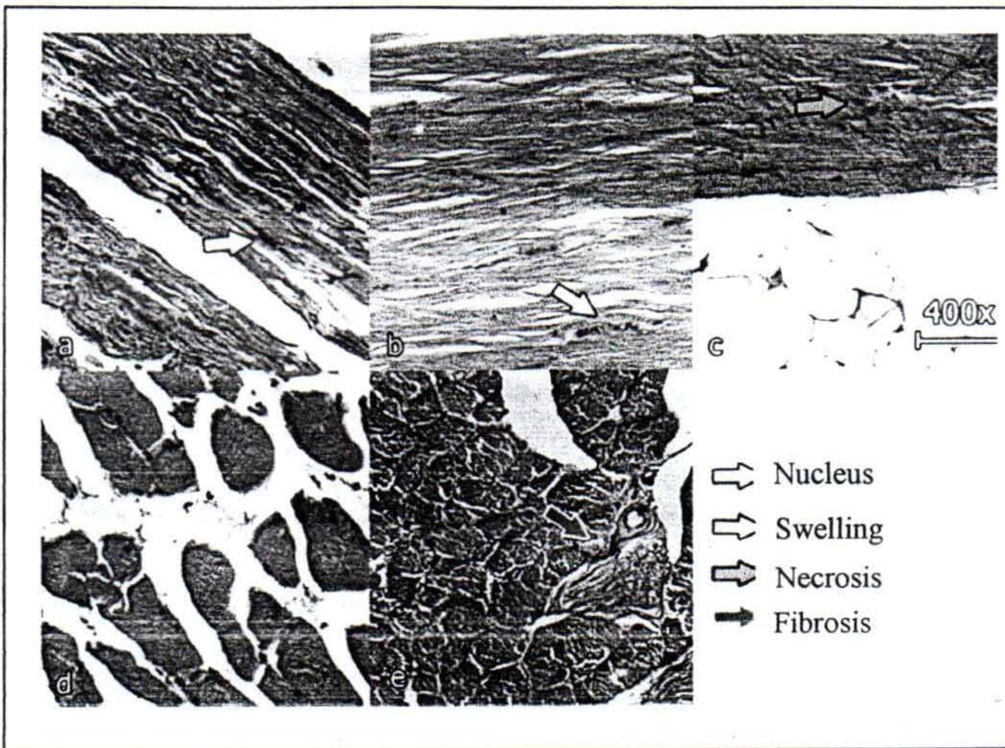

Figure 5. Hind paw mice tissue muscle using hematoxylin-eosin with 400 times magnification. Longitudinal (a-c) and cross section (d-e) section of muscle tissue were obtained from control group (a dan d) and diabetic mice (b, c, e).

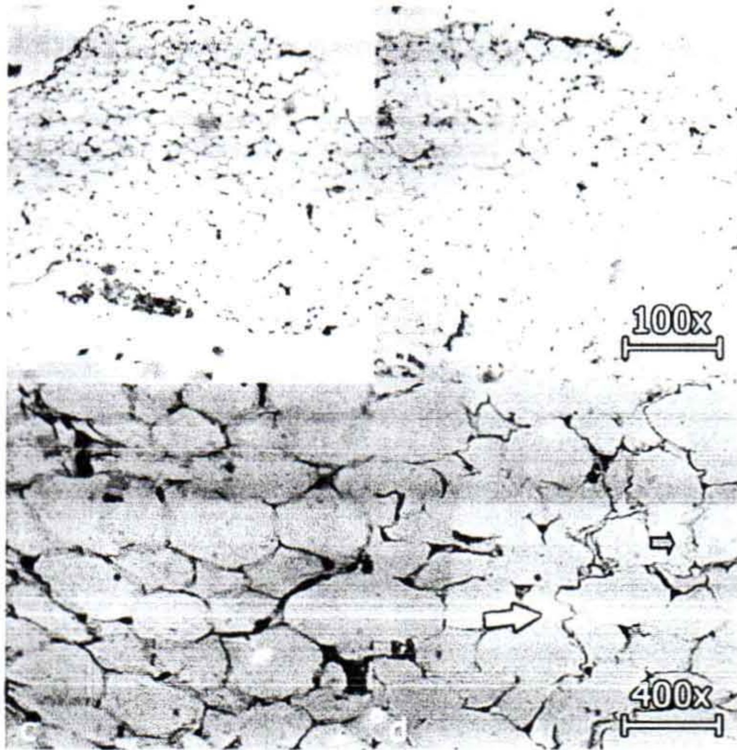

Figure 6. Cross section of the mice adipose tissue were obtained from control group (a and c) and diabetic group (b and d) using hematoxylin-eosin staining with 100 times magnification (a-b) or 400 times magnification (c-d). The white arrow is pseudopodia.

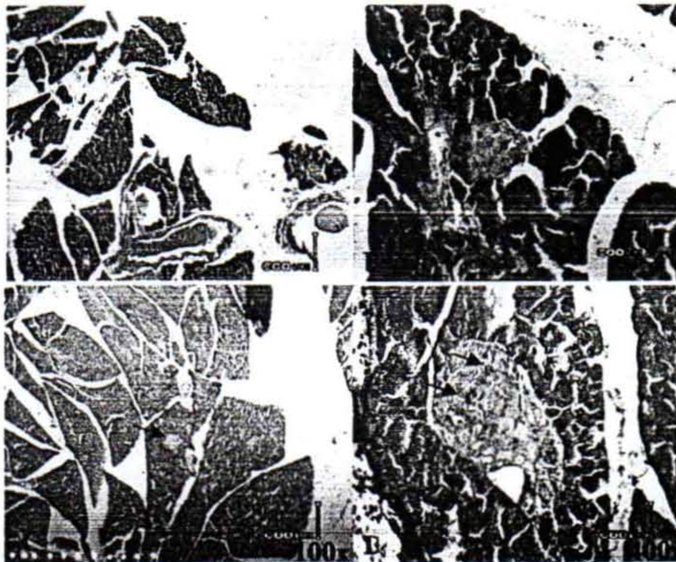

Figure 7. Cross section of pancreatic tissue in diabetic mice and receiving CMC Na (A-B) or vanadyl sulphate 5 mg/kg (C-D) using hematoxylin-eosin staining. A and C (100 times magification); B and D (400 times magification)

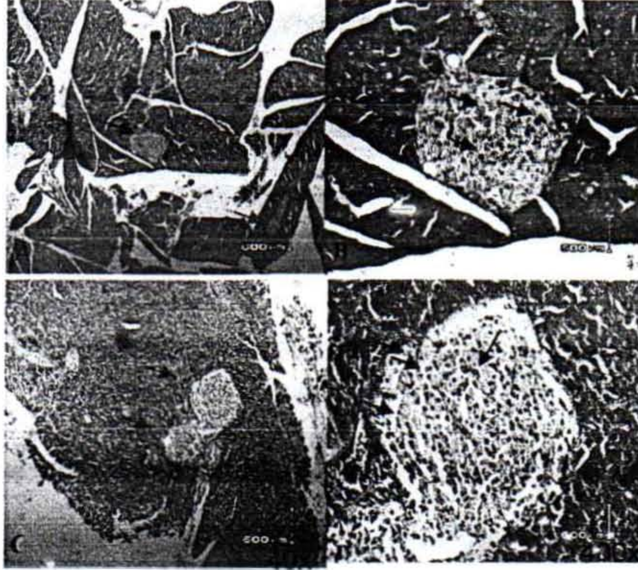

Figure 8. Cross section of pancreatic tissue in diabetic mice and receiving vanadyl sulphate 30 mg/kg (A-B) or vanadyl sulphate 100 mg/kg (C-D) using hematoxylin-eosin staining. A and C (100 times magification); B and D (400 times magification)

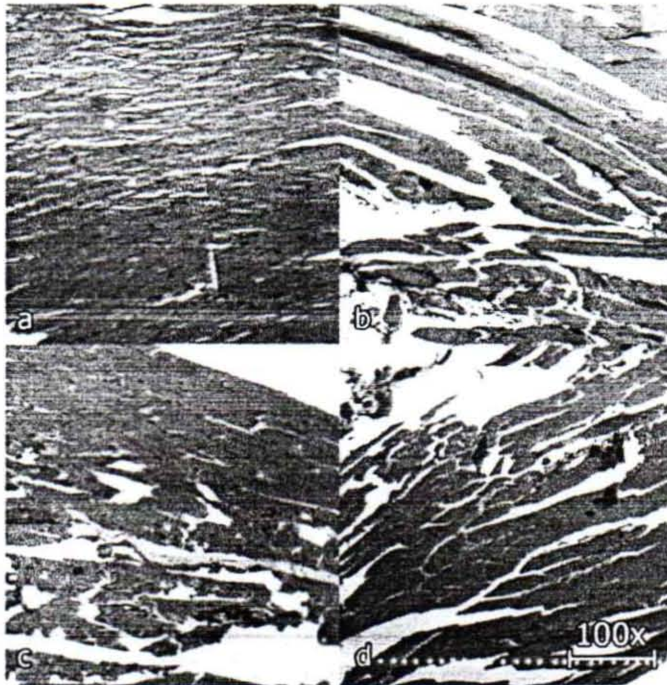

Figure 9. Longitudinal section of mice's hind paws tissue in diabetic mice and receiving CMC Na (a) or vanadyl sulphate 5 (b), 30 (c) and 100 (d) mg/kg using hematoxylin-eosin (100 times magnification).

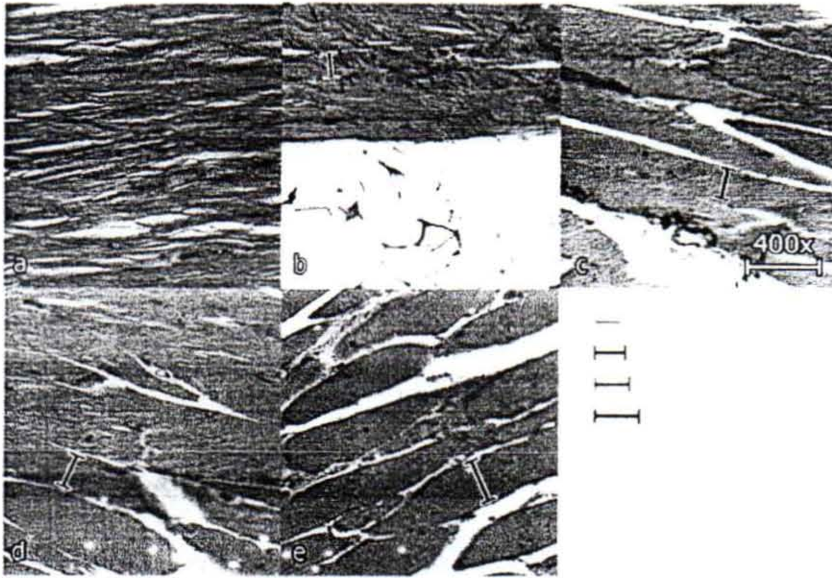

Figure 10. Longitudinal section of hind paws tissue muscle in diabetic mice and receiving CMC Na (a-b) or vanadyl sulphate 5 (c), 30 (d) and 100 (e) mg/kg using hematoxylin-eosin staining (400 times magnification)

Figure 11. Cross section of adipose tissue in diabetic mice and receiving CMC Na (a) or vanadyl sulphate 5 (b), 30 (c) and 100 (d) mg/kg using hematoxylin-eosin staining (100 times magnification)

Gambar 12. Cross section of adipose tissue in diabetic mice and receiving CMC Na (a) or vanadyl sulphate 5 (b), 30 (c) and 100 (d) mg/kg using hematoxylin-eosin staining (400 times magnification). The orange arrow indicates pseudopodia cell and white arrow show polinuclear adipose cell.