EFFECTIVENESS AND MECHANISM OF ACTION OF VANADYL SULFATE IN INCREASING ß CELL PROLIFERATION OF DM MICE DUE TO STREPTOZOTOCIN INDUCTION

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EFFECTIVENESS AND MECHANISM OF ACTION OF VANADYL SULFATE IN INCREASING β CELL PROLIFERATION OF DM MICE DUE TO STREPTOZOTOCIN INDUCTION

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia as a result of damage to insulin secretion, insulin action, or both. Vanadyl sulfate is one of the form of vanadium which has begun to be used to treat diabetes in humans. In this study, investigation on the effectiveness of vanadyl sulfate in increasing pancreatic β cell proliferation of diabetes mice due to streptozotocin induction. There were 30 healthy mice were subjected to this experimental study. Findings from this experiment proved that administration of vanadyl sulfate at various doses can significantly increase the amount of pancreatic β cell proliferation and eventually reduced blood glucose levels in a meaningless manner. However, those mice that administrated of vanadyl sulfate at a dose of 30 mg/kgBW had a higher amount of Langerhans Islet than those in the vanadyl sulfate group at a dose of 5 mg/kgBW and 100 mg/kgBW. Similar observations were obtained for the Ki-67 expression. The highest Ki-67 expression was obtained at a dose of 30 mg/kgBW but it decreased with a dose of 100 mg/kgBW of vanadyl sulfate.

Keywords: Vanadyl sulphate, proliferation, β cell, Islet of Langerhans, Ki-67

1. Introduction

Over the past two decades, the development of new therapeutic approach for DM has been directed towards efforts to induce the regeneration of pancreatic β cell and shows that inducing pancreatic β cell as a promising strategy as a curative strategy for DM (1,2). Taken together, further studies on how to restore pancreatic β cell in DM conditions are still needed. Vanadium, one of important trace elements for human, has been reported to mimic the work of insulin. A number of *in vitro* and *in vivo* studies showed that vanadium stimulates glucose uptake, glycogen synthesis, and glucose oxidation in adipose cell and hepatocyte (1,3–5). A study by Missauoui *et al*. (6) showed that vanadyl sulfate may contribute to the proliferation

of pancreatic β cell but there are minimal marker for proliferation. The proliferation marker needs to be identified to find out whether the proliferation process is normal or exaggerated that will lead to malignancy. It is also necessary because of the use of vanadyl sulphate has been up to phase 1 clinical trials in human (7) .

Ki-67 has been the common marker for the proliferations of both pancreatic endocrine and exocrine. Ki-67, a core protein during interphase, relocates on the chromosome surface where it can be detected during the whole mitosis phases (8). Ki-67 expresses in minimum amount at the end of G1 phase and at the beginning of S phase. Ki-67 increases along with cell growth and reaches maximum amount at the end of S and G2 phase. Thus, Ki-67 also known as a marker of this phase (9). Ki67 is also a good proliferation marker, but an excessive increase of Ki-67 markers also has the potential to be a marker of malignancy (10–12).

Proliferation of pancreatic beta cells can lead to increased insulin production that may be useful for lowering blood glucose levels and may be a therapy for diabetics as it aims to increase the production of insulin that is expected to be definitive therapy for diabetes mellitus. A number of studies showed the latest therapy application and studied also manipulated cell proliferation mechanism (13). The increase of β cells proliferation is expected to increase β cell number that will increase the insulin production and ameliorate the progression of DM. The present study was expected to study the effect of vanadyl sulfate in the improvement and proliferation on the number of Islet of Langerhans, the number of β cells and to study the effect on Ki-67.

2. Materials and method

2.1 Materials

The material used in the present study; vanadyl sulfate (Sigma-Aldrich, St. Louis, USA), Streptozotocin (Sigma-Aldrich), citrate buffer pH 4.5 (Sigma-Aldrich)., CMC Na (Sigma-Aldrich), Neutral buffered formalin pH 6.8 (Sigma-Aldrich), *Hematoxylin-Eosin, Aldehyde Fuchsine,* Antibody Ki-67 (Biocare Medical, California, USA), *On Call Plus Blood Glucose Monitoring System®* (ACON Labs, San Diego, USA), Microscope (Olympus BX41, Tokyo, Japan).

2.2 Method.

2.2.1 Animal and Care

Male 8-10 week-old BALB/c mice were used. All mice were cared with equal treatment, standard chow diet *ad libitum*, with 12 hours light-dark cycle. All experiment were perform in accordance with The Guiding Principles for the Care and Use of Animal Research of Airlangga University No. 602-KE. All efforts were made to minimize animal suffering and to reduce the number of animal used. Animals were used only once.

2.2.2. The DM model

DM animals were made by intra peritoneal injection of 100 mg/kg streptozotocin (STZ), followed by 50 mg/kg STZ 14 days after the first dose. STZ was prepared in citric buffer pH 4.5. Blood glucose evaluation was conducted on day 0, 7, 14 and 21 (14). Mice with a glucose level exceeding 300 mg/dL were considered diabetic mice. Blood samples were taken from the tail vein. The blood glucose level was measured using *On Call Plus Blood Glucose Monitoring System®* (ACONLAB).

2.2.3 Procedure:

30 mice were divided into 5 groups. The first group consisted of normal mice (control group). The second group was DM animal group. These groups were treated with CMC Na 0.6%. The treatment groups consisted of 3 groups which were treated with 3 different doses of vanadyl sulfate 5, 30, and 100 mg/kg orally for 7 days. After 7 days, all animals were sacrificed. Tissues were immediately removed and fixed with formalin buffer pH 6.8. Histological analysis and immunohistochemistry were conducted to examine proliferation effect of vanadyl sulfate on pancreatic β cells. Histological analysis was conducted by counting the number of Islet of Langerhans after staining with *Hematoxylin-Eosin* (15). Pancreatic β cell calculation was conducted using *Aldehyde Fuchsine* staining (16). Calculation on the islet number was conducted according to Abdel Rahim. 2013 (17).

2.2.4 Statistical analysis

All data are presented as the mean ± standard error. Data were statistically analyzed using one-way Anova with Tukey's HSD test using Prism software (GraphPad Software, San Diego, CA). Differences were considered significant at the level of $p < 0.05$.

3.1 Result

3.1.1 STZ induced diabetic mice model

The morphological change of Islet of Langerhans between normal and DM mice was shown on Figure 1. The data of blood glucose levels of DM animals showed that blood glucose levels were significantly increased after STZ induction from 126,04 ± 8,55 mg/dL to 434,96 ± 45,37 mg/dL (*p<0,001). In the normal group, cross section of pancreatic tissue shows normal structure of Islet of Langerhans (figure 1A). In the DM group, showed degenerative and necrotic change of Islet of Langerhans also marked with cell shrinkage as the result of apoptosis (figure 1B).

3.1.2 The effect of vanadyl sulfate on blood glucose level

The result showed that blood glucose level on DM mice are significantly higher than normal mice group. Vanadyl sulfate 5 mg/Kg and 30 mg/Kg are slightly are slightly decrease blood glucose level compare to the DM groups. Vanadyl sulfate 100 mg/kg significantly decrease blood glucose level compared to DM mice groups. Blood glucose levels of treatment group after vanadyl sulfate treatment 5 mg/kg, 30 mg/kg, and 100 mg/kg respectively are 369.17 ± 48.47 mg/dL., 297.67± 73.68 mg/dL and 255.75 ± 229.64 mg/dL (Table 1).

3.1.3 The effect of vanadyl sulfate on morphology and calculation of pancreatic tissue with *hematoxylin eosin* staining on normal, DM, and treatment animal groups

The result showed the picture of normal structure of Islet of Langerhans (figure 2A) compared to degenerative and necrotic due to streptozotocin administration that cause destruction on Islet of Langerhans (figure 2B). The DM mice's pancreatic tissues administered with vanadyl sulfate experienced morphological improvement of Islet of Langerhans. Vanadyl sulfate 5 mg/kg showed marked improvement Islet of Langerhans compared to DM group, the picture showed irregular cells, blurred color cell nucleus yet sharper than that in the DM animal group (figure 2C). Vanadyl sulfate 30 mg/kg showed more intact shape of Islet of Langerhans shows, cell nucleus is clearly visible, clear nucleus boundary, more regular cell size compared to those in 5 mg/kg dose (figure 2D). Vanadyl sulfate 100 mg/kg showed size reduction of pancreatic islet, irregular cell order, widening cell nucleus distance (figure 2E). However, cellular degeneration was less marked on dose 30 mg/kg group compared to other doses that result nearing normal pancreatic condition.

The normal animal group without streptozotocin induction shows significantly higher Islet of Langerhans mean compared to the DM animal group ($p=0.0263$). The result of Islet of

Langerhans number shows no change in the number of Islet of Langerhans due to the administration of vanadyl sulfate of various doses in comparison to the DM animal group (table 2).

3.1.4 The effect of vanadyl sulfate on pancreatic β cells calculation and morphological using Aldehyde Fuchsine on normal, DM, and treatment animal groups.

Histopathological image of pancreatic β cells in normal animal group shows spherical shape on pancreatic β cell and dense β cell granulation compared to the DM animal group (figure 3A, figure 3B), while the group treated with vanadyl sulfate 5 mg/kg showed loose β cell granulation (figure 3C). Vanadyl sulfate 30 mg/kg showed improvement on the Islet of Langerhans boundaries and denser β cell granulation than those with 5 mg/kg (figure 3D). Vanadyl sulfate 100 mg/kg shows more spherical boundaries of Islet of Langerhans and dense β cell granulation nearing those in the normal animal group (figure 3E). Mean score of pancreatic β cells count showed that DM group significantly lower than normal group ($p=0.0001$). Vanadyl sulfate 5 mg/kg, 30 mg/kg, and 100 mg/kg showed significant difference on the number of pancreatic β cells compared to the DM animal ($p\leq0.005$)(Table 3).

3.1.5 The effect of vanadyl sulfate on Ki-67 expression with immunohistochemically method on normal, DM, and treatment animal groups.

The Ki-67 expression in the Islet of Langerhans marked with black arrow and pancreatic β cells marked with white arrow. The normal animal groups showed the immunoreactivity β cells absorbing brown color with medium to high intensity. The DM animal group shows some fairly drastic change with the β cells absorbed lighter color (figure 4A and 4B). Vanadyl sulfate 5 mg/kg showed Ki-67 expression marked with brown color with low intensity. While on 30 mg/kg showed increase color intensity of Ki-67 expression marked with medium to

high intensity (figure 4C). However, the group of 100 mg/kg vanadyl sulfate showed the number of positive cells is lower than those on 30mg/kg (figure 4E). The group induced with vanadyl sulfate 5 mg/kg, 30 mg/kg, and 100 mg/kg showed no change compared to those in DM group (table 4).

3.2 Discussion

The administration of vanadyl sulfate on treatment group in comparison to the DM animal group is capable of decreasing mice's blood glucose level as shown on table 3.1. Vanadyl sulfate is capable of increasing glucose transport and metabolism in skeleton muscles, hepatic and fat tissues, inhibiting *phosphotyrosine phosphatase* and PTP-1β both of which are enzymes responsible for insulin receptor dephosphorization that causes insulin resistance. Vanadyl sulfate also activates PKB/Akt kinase that increases glucose uptake by GLUT4 transporter. PKB/Akt activation also stimulates *glycogen synthase kinase-3* (GSK3) phosphorylation that causes glycogen synthesis stimulation (18). Akt modulates β cell proliferation through its various downstream targets including GSK3, FoxO1, and *tuberous sclerosis proteins* (TSC)/*mammalian target of rapamycin* (mTOR). Recent studies relating *cyclin/cyclin-dependent kinase*-4 (CDK4) complexes with Akt on β cell proliferation (19).

To determine the effect of pancreatic β cell proliferation from vanadyl sulfate, histochemical and immunohistochemically method were conducted. The result of this study shows no significant difference of Islet of Langerhans number on administration of vanadyl sulfate of varying doses in comparison to the DM animal group. There are increases sum of Islet of Langerhans on the doses of 30 and 100 mg/kg, but shows no increase on 5 mg/kg. Which means that the administration of vanadyl sulfate of 5 mg/kg is incapable of increasing the number of Islet of Langerhans after STZ destruction. The administration of vanadyl sulfate of 30 mg/kg and 100 mg/kg was capable of increasing the sum of Islet of Langerhans

compared to the number of islet on DM animals but without any statistical significance. Although table 2 shows the highest number of Islet of Langerhans occurs on the group with vanadyl sulfate 30 mg/kg compare to than that in the 100 mg/kg but not statically different. It is also should be emphasized that as a whole the administration of vanadyl sulfate does not increase the number of Islet of Langerhans to exceed the number of Islet of Langerhans in normal animal group which means that indicating no occurrence of excessive effect of proliferation.

The result of pancreatic β cell count shows that administration of vanadyl sulfate is capable of increasing pancreatic β cell proliferation marked with increase in the number of pancreatic β cells along with the increase of vanadyl sulfate dose. It can be concluded that pancreatic β cell proliferation process as a result of vanadyl sulfate may occur due to hyperplasia or increase in pancreatic β cell number. The result of scoring on the number of pancreatic β cell number as shown in Table 3 shows that administration of vanadyl sulfate at various doses indicates a dose-dependent manner but without significant differences.

The increasing number of pancreatic β cell may be due to proliferation (hyperplasia), increase of cell volume in individual cell (hypertrophy), or neogenesis islet due to ductal cell differentiation (20, 21). Also, a study conducted by Pirmoradi (22) shows that vanadyl sulfate administration on diabetic mice can prevent islet atrophy, increase density volume, and pancreatic β cell number. The result of our previous unpublished work also shows that there also occurs an increasing in the Islet of Langerhans diameter of group which treated with vanadyl sulfate. Like any other cells, a pancreatic β cell in normal condition is actively regulated within its lifetime. Cellular and molecular mechanism of the β cell regulation has not yet been fully known but involves three main processes: 1. β cell replication, 2. β cell differentiation and neogenesis from precursor cell, 3. β cell apoptosis inhibition (23).

The result of Ki-67 expression with Allred method is presented in Table 3.4. Scoring result with Allred method shows that administration of vanadyl sulfate on the dose of 5 mg/kg, 30 mg/kg, or 100 mg/kg show an increase in the Ki-67 expression compared to that in the DM group. The highest Allred scoring occurs with the dose of 30 mg/kg, which shows increase in the proliferation of β cells when compared to the DM group with vanadyl sulfate doses of 5 mg/kg or 100 mg/kg. No significant difference in Ki-67 expression may be caused by activation of Ki-67 in DNA damage and DNA restoration conditions (24). The DNA damage and restoration process naturally occurs as the effort of pancreatic β cells for functional defense due to streptozotocin damage. Pancreatic β cells proliferation occurs from the remains and surviving pancreatic β cells from damage caused by streptozotocin induction which is the source of pancreatic β cell regeneration (21).

Other factors are due to proliferation process involves cell cycle stream on complex eukaryotic. Pancreatic β cell proliferation is influenced by various factors, both through receptor tyrosine kinase (RTKs), *G-protein coupled receptors* (GPCRs), JAK *binding receptors*, and other signaling pathways (20). Some other paths predicted to play some role in β cell proliferation both on animals and humans include Akt/PKB, PI3K, LKB1, *glucokinase*, mTOR, AMPK, *Calcineurin*, EGF, PDGF, *β-catenin*, leptin, estrogen and progesterone (25). These factors affect Ki-67 expression which is the downstream of pancreatic β cell proliferation. This shows possibility that proliferation process may not only occur through Ki-67 increase but also other unknown mechanisms. It should be noted that when DM occurred, β cell damage process continues when the blood sugar is uncontrolled on the other hand the capacity of pancreatic β cells to proliferate is limited. This may also conclude that vanadyl sulfate mechanism tends to inhibit apoptosis higher than induced proliferation but need a further studies. More studies are need to be done to investigate the regulation of proliferation and apoptosis with other markers. The main thing to consider from the results of this study is

the Ki-67 score for all groups increased compared to the DM animal group but the score did not exceed the negative control group. This indicates that there is no excessive proliferation process leads to malignancy.

4. Conclusion

The administration of vanadyl sulfate on various doses significantly increase pancreatic β cell proliferation but not the number of Islet of Langerhans. Vanadyl sulfate administration of various doses can increase Ki-67 expression insignificantly. Vanadyl sulfate may improve pancreatic β cells, Islet of Langerhans and Ki-67 expression. The proliferation process on pancreatic β cell may occur due to hyperplasia. The is no excessive proliferation leading to malignancy on the use of vanadyl sulfate but the exact mechanism still need a further study.

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7. Conflict of interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Table 1 Characteristics of blood glucose levels on normal animals, DM animals and animals with DM + VS 5 mg/kg, DM + VS 30 mg/kg, and DM + VS 100 mg/kg for 7 days.

Group	Blood glucose level (mg/dL)			
	Post STZ ± SE	Day 2 ± SE	Day 4 ± SE	Day 8 ± SE
Normal animals	135.17 ± 4.01	133.5 ± 9.02	145.33 ± 4.04	147.33 ± 7.97
DM animals	413.00 ± 50.97	552.83 ± 30.55	493.83 ± 40.71	471.5 ± 49.05
DM + VS 5 mg/kg	430.00 ± 47.08	414.17 ± 81.28	431.5 ± 50.10	369.17 ± 48.47
DM + VS 30 mg/kg	444.17 ± 40.00	514.5 ± 29.09	363.33 ± 75.9	294.67 ± 73.68
DM + VS 100 mg/kg	452.67 ± 106.36	343.67 ± 177.41	269.2 ± 210.07 (n=5)	255.75 ± 229.64 (n=4)

Table 2 Means of Islet of Langerhans number of each group (n=6) after vanadyl sulfate treatment of 5 mg/kg, 30 mg/kg, or 100 mg/kg doses for 7 days.

Group	No.of Islet of Langerhans (unit)
	Mean ± SE
Normal animals	8.00 ± 1.1*
DM animals	3.78 ± 0.58
DM+VS 5mg/kg	3.08 ± 0.44
DM+VS 30mg/kg	6.11 ± 0.89
DM+VS 100mg/kg	4.44 ± 0.61

* Significant difference $p<0,05$ compared to DM animal group

Table 3 Means of the number of pancreatic β cells on each group (n=6) after 7 day treatment with vanadyl sulfate of 5 mg/kg, 30 mg/kg, or 100 mg/kg doses.

Group	Number of pancreatic β cells (unit)
	Mean ± SE
Normal animals	5.03 ± 0.26
DM animals	1.87 ± 0.43
DM+VS 5mg/kg	2.67 ± 0.24*
DM+VS 30mg/kg	4.07 ± 0.18**
DM+VS 100mg/kg	4.27 ± 0.27***

*/**/*** Significant difference $p<0,05$ compared to DM animal group

Table 4 Result of mean scoring Allread method on Ki-67 expression on Islet of Langerhans on each of the treatment animal groups (n=6)

Group	Allred Scoring (unit)
	Mean ± SE
Normal animals	4.06 ± 0.29
DM animals	3.28 ± 0.18
DM+VS 5mg/kg	3.67 ± 0.12
DM+VS 30mg/kg	3.89 ± 0.25
DM+VS 100mg/kg	3.33 ± 0.19

Figure 1

Pancreatic β cell nucleus appears as a darker sphere

Islet of Langerhans appears as a group of pancreatic β cells gathering and forming regular sphere with clear boundaries with the surrounding cells

Transversal section of pancreatic tissue with *hematoxyllin-eosin* coloring on the normal animal group (a); DM animal groups (b). The picture was taken with 400x enlargement. The thick white arrow shows the pancreatic β cell nucleus appearing in darker sphere on normal and treatment animals. The black arrow shows Islet of Langerhans on both normal and DM conditions.

Figure 2

Pancreatic β cell nucleus appears as darker sphere

Islet of Langerhans appears as a group of pancreatic β cells gathering and forming regular sphere with boundaries by the surrounding cells

Transversal section of pancreatic tissue with *hematoxyllin-eosin* colouring on the normal animal group (a); DM animal group (b); DM + vanadyl sulfate 5 mg/kg (c); DM + vanadyl sulfate 30 mg/kg (d); DM + vanadyl sulfate 100 mg/kg (e);. The picture was taken with 400x enlargement. The thick white arrow shows the β cell nucleus and the black arrow shows Islet of Langerhans on both normal and DM conditions.

Figure 3

Pancreatic β cell on Islet of Langerhans appears as a cell absorbing blue color on both normal, DM and treatment animal groups.

Pancreatic β cell with *Aldehyde fuchsin* colouring and 400x enlargement. Normal animal group (a); DM animal group (b); DM + vanadiyl sulfate 5 mg/kg; DM + vanadyl sulfate 30

mg/kg (c); DM + vanadyl sulfate 100 mg/kg. The pancreatic β cell is marked in the figure with thick white arrow and appears as the cell absorbing blue color.

Figure 4

⟶ Islet of Langerhans appears as a group of β cells with distinct boundaries compared to surrounding cells colored in blue and brown.

⟹ Ki-67 expression appears as a pancreatic β cell in Islet of Langerhans colored in light and dark brown depending on the intensity or capacity to absorb antibody Ki-67.

Ki-67 expression on an Islet of Langerhans: Normal animal group (a); DM animal group (b DM + vanadyl sulfate 5 mg/kg; DM + vanadyl sulfate 30 mg/kg (c); DM + vanadyl sulfate 100 mg/kg. The picture was taken with 400x enlargement. The thick white arrow shows Islet Lengerhans, while the thin black arrow shows Ki-67 expression.

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