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Temporal Recovery of Pancreatic β-Cells in Type 2 Diabetes Mellitus Induced by Mesenchymal Stem Cell-Conditioned Medium

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Abstract
Conditioned medium (CM) derived from human umbilical cord mesenchymal stem cell culture has been studied in Type 1 Diabetes Mellitus (T1DM) and showed a good prospect as an alternative treatment. However, effects of CM in Type 2 Diabetes Mellitus (T2DM) have not been studied. The purpose of this study was to investigate the ability of human umbilical cord mesenchymal stem cell-derived conditioned medium (CM) for the recovery of pancreatic β-cells in T2DM-induced Wistar Rats (*Rattus norvegicus*). The T2DM-induced rats were prepared by applying combination injection of nicotinamide (NA) and streptozotocin (STZ). The T2DM-induced rats were treated with 0.2 ml CM fourth times periodically, with a week interval. One week after each CM treatment, the pancreas glands were collect, fixed in Bouin's solution, processed by paraffin method and carried out immunohistochemistry staining for insulin detection. The blood samples were collected for glucose concentration evaluation. Microscopic observation indicated the decrease of immunoreactive cell number and its intensity towards insulin after the combination injection of NA and STZ. One week after first and second CM treatment, the presence of numerous and hight intensity of insulin- immunoreactive (IR) cells in the pancreatic islets could be observed. However, the number of insulin-IR cells and its intensity decreased dramatically 1 week after the third CM treatment and almost disappeared in 1 week after the fourth CM treatment in all of the pancreatic islets. The histological features changes of insulin-IR cells are in agreement with the profile dynamics of glucose concentration in the blood. This study showed that conditioned medium (CM) derived from human umbilical cord mesenchymal stem cell culture has ability to recover the insulin production from pancreatic β-cells in T2DM-induced Wistar rats (*Rattus norvegicus*) temporary.
Keywords: conditioned medium, type 2 diabetes mellitus, pancreatic β-cells, temporal recovery, nicotinamide-streptozotocin

1. Introduction

Type 2 Diabetes Mellitus (T2DM) occurs due to insufficient insulin production from β-cells within the setting of insulin resistance (David & Dolres 2011). Insulin resistance is the inability of cells to respond adequately to normal levels of insulin (Tfayli & Arslanian 2009). In the liver, insulin normally suppresses glucose release. However, in the setting of insulin resistance, the liver inappropriately releases glucose into the blood (Szkudelski *et al*. 1998). The proportion of insulin resistance and β-cell dysfunction differs among individuals, with some having primarily insulin resistance and only a minor defect in insulin secretion and others with slight insulin resistance and primarily a lack of insulin secretion (David & Dolres 2011).

The number of people affected by T2DM is approximately 90% compared with 10% of Type 1 Diabetes Mellitus (T1DM) (Chabot 2002). International Diabetic Federation declared that there were 387 million diabetics worldwide and Indonesia was in seventh position. Type 2 Diabetes Mellitus (T2DM) often can treated by losing weight and exercising more, as these increases the body's sensitivity to insulin. Metformin drug, that works by helping the fat and muscle cells of the body listen to the signal from insulin to take up sugar from the blood, often given to diabetic. Insulin treatment may either, be added to oral medication or used alone (Ripsin *et al*. 2009). Latest study reported that conditioned medium derived from human umbilical cord mesenchymal stem cell can regenerate the pancreatic β-cells damage in T1DM-induced Wistar rats (*Rattus norvegicus*), both in structure and function (Kitada *et al*. 2003).

Stem cells have ability to supply trophic factors which may regenerate damaged tissues (Yang *et al*. 2013). Moreover, some studies on stem cell-derived secreted factors indicated that the secreted factors alone without the stem cell will repair tissue in varied conditions involving damaged tissue or organ (Timmers *et al*. 2011; Mishra

& Banarjee 2012; Hynes *et al*. 2013). The secreted factors often detected exceedingly in the medium where the stem cells are cultured, therefore referred to as conditioned medium or CM (Kim & Choi 2013). Various study reported that conditioned medium contains varied growth factors, interleukin and others regenerative agents (White *et al*. 2008; Sze *et al*. 2007).

There is advantage on the utilization of CM compared to the stem cells. Since there is no cell in CM, the rejection problem by recipient to the donor does not exist. Therefore, the conditioned medium features a promising prospect as alternative treatment in regenerative disease (Pawitan 2014). The evidence of potency CM as regenerative agent in T2DM is necessary. This study were carried out to investigate the ability of human umbilical cord-mesenchymal stem cell-derived conditioned medium for the recovery of pancreatic β-cells in T2DM induced Wistar Rats (*Rattus norvegicus*).

2. Materials and Methods

This study was approved by Ethical Clearence Committee from Universitas Gadjah Mada. Fifty four male Wistar rats (*Rattus norvegicus*), weight 150-250 gram, were used in this study. The rats were divided into 3 groups: group I (normal control group), group II (diabetic group), and group III (CM-treated diabetic group). Type 2 Diabetes Mellitus in rats were achieved by single injection nicotinamide (NA) dose 230 mg/BW and streptozotocin (STZ) dose 65 mg/BW intraperitoneally (Ghasemi *et al*. 2014).

Conditioned Medium (CM) was obtained from the culture medium of human umbilical cord mesenchymal stem cells at the third passage. Group II and III were injected with single dose NA and STZ at same time. In the fifth days post injection of NA and STZ, group III were treated with 0.2 ml CM by intramuscular injection. The CM treatment was repeated 4 times with interval a week. Group I as control normal group were not received any treatment. One day post adaptation, five days after NA-STZ injection, and one week after each CM treatment, the blood samples were collected from all groups for evaluation. Glucose concentration was measured using GlucoDr® (All Medicus Co., Ltd). At the same time, four rats sample were euthanized, pancreas glands were collected, and fixed in Bouin's solution for 24 hours. The pancreatic tissues were processed with paraffin method and cut serially in 5 µm thickness. One slide of pancreatic tissue in every samples were used to detect the immunolocalization of insulin in the pancreatic islets using N-Histofine®*simple stain rat* MAX PO Kit (Cosmo Bio, Ltd).

3. Results

Photomicrograph showed reduction on the number and intensity of insulin-IR cells (Fig.1B) in the pancreatic islets after single dose injection of streptozotocin and nicotinamide compared with the number and intensity of insulin-IR cells in the pancreatic islets of normal rats (Fig. 1A). One week after first CM treatment, the number and intensity of insulin-IR cells were increased excessively (Fig. 1C). Insulin-IR cells still could be detected constantly one week after second CM treatment (Fig. 1D). In contrast, the number and intensity of insulin-IR cells dramatically decreased in one week after third CM treatment (Fig. 1E) and almost could not be detected after fourth CM treatment (Fig. 1F) in all pancreatic islets.

The histological changes (Fig. 1) on the number and intensity of insulin-IR cells in pancreatic islets were in agreement with dynamics of glucose concentration (Table 1). Five days post NA-STZ injection, diabetes mellitus has been achieved as indicated by high level of glucose concentration, 319.50 mmol/L in group III and 270.50 mmol/L in group II compared to 113.21 mmol/L in normal control group (group I). The glucose concentration of diabetic rat was in high level continuously and tendency to increase (428.67 mmol/L) in the end of treatment. In CM-treated diabetic group, the glucose concentration was decrease one week post first CM treatment from 319.5 to 132 mmol/L. There were dynamics of glucose concentration level in CM-treated diabetic group, although it still lower almost a half than glucose concentration level of diabetic group, but still higher than glucose concentration level of normal control group.

Table 1. Average glucose concentration (mmol/l) in group I, group II, and group III.

Groups	Days of treatment					
	1 day Post adaptation	5 days post NA-STZ treatment	1 week post treatment 1	1 week post treatment 2	1 week post treatment 3	1 week post treatment 4
Group I	91,60	113,21	85,1	113,25	113,17	120,33
Group II	77,00	270,50	240,27	271,78	335,17	428,67
Group III	90,80	319,50	132	140,9	120	152,00

*NA= nicotinamid, STZ= streptozotocyn, Group I= normal control group, Group II= diabetic group, Group III= CM-treated diabetic group.

4. Discussion

Insulin-IR cells are identified as β-cell in pancreatic islets (Budipitojo *et al*. 2016). The present studies showed decreasing of the insulin-IR cells number in the pancreatic islets after single dose injection of nicotinamide (NA) and streptozotocin (STZ) intraperitoneally, although some pancreatic β-cells were still detected with very light insulin immunoreactivity. Insufficient of insulin production from pancreatic β-cell will make glucose can not be used effectively by the other cells (David & Dolores 2011). Such condition refers to diabetes mellitus, as indicated by high glucose concentration in blood stream, same as our finding in this research. Injections of nicotinamide and streptozotocin have been used widely to generate Type 2 Diabetes Mellitus (T2DM) in other species, such as rabbit, dog, monkey, and cat (Goldner & Gomori 1944). Single dose injection of NA-STZ will lead to fast pancreatic β-cell death (Saini 2010).

Conditioned Medium derived from human umbilical cord mesenchymal stem cell has been tried in Type 1 Diabetes Mellitus (T1DM)-induced rat. The result showed a good prospect as alternative therapy for diabetic. Conditioned Medium treatment in T1DM-induced rat not only regenerate pancreatic β cell but also maintain their function to produce insulin (Nugroho *et al*. 2016). Latest result showed CM treatment in T2DM-induced rat has different effect. Treatment could regenerate pancreatic β-cell after first and second administration but it progressively decrease again in third and fourth administration. However, insulin production still could maintenance glucose concentration lower than diabetic rat and almost same with normal rat. The dose and how long CM treatment should be given in T2DM patient still need to investigate furthermore.

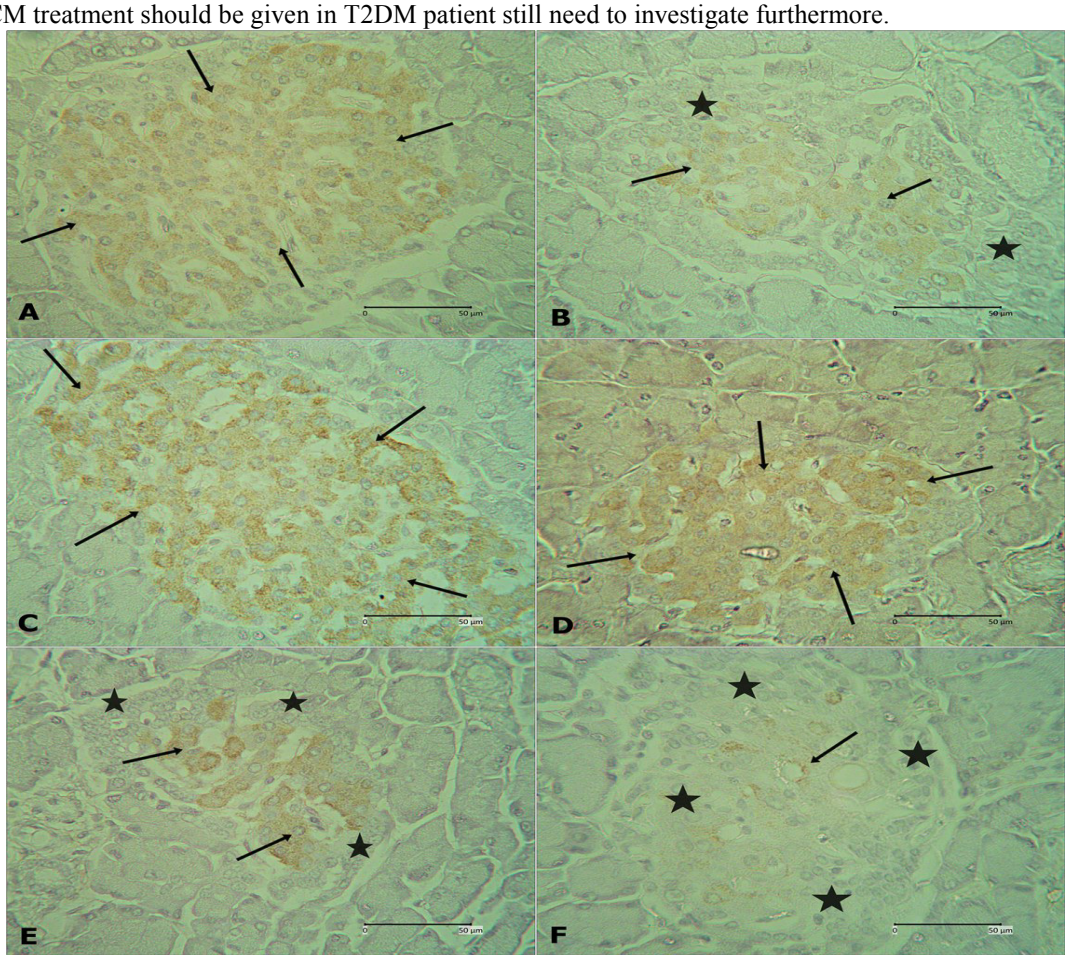

Figure 1. Immonolocalization profile of insulin immunoreactive cells in pancreatic islets. Numerous and stronger intensity of insulin-immunoreactive (IR) cells were detected in the pancreatic islets of normal control group (Fig. 1A) compared with diabetic group (Fig. 1B) as indicated by arrows. The presence of abundance insulin-IR cells with strong intensity were detected one week after the first CM treatment in the pancreatic islets (Fig. 1C). The number and intensity of insulin immunoreactive cells were detected constantly until one week after second CM treatment (Fig. 1D). The number of insulin-IR cells and the intensity decreased dramatically one week after third CM treatment (Fig. 1E) and almost dissapeared after the fourth CM treatment (Fig. 1E), in all the pancreatic islets. Stars indicated non β cells or β cells with no insulin immunoreactivities in the pancreatic islets

Some proteomic study reported that various growth factor and cytokine presented in the CM (Pawitan 2014). In present study, CM could regenerate pancreatic β-cell and restore their function to produce insulin in early CM treatment. Type 2 diabetes mellitus (T2DM) were characterized with pancreatic β-cell dysfunction or insulin insensitivity on target cells (Saiedullah 2016). Inflammatory cytokines may play a role in pancreatic β-cell dysfunction and insulin resistance in type 2 diabetes mellitus. Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine found in CM (Pawitan 2014) and naturally secreted by macrophages, lymphocytes and also adipocytes. TNF-α could be a promising treatment of T2DM before progression of pancreatic β-cell dysfunction. There were correlation between TNF-α with percentage of pancreatic β-cell function and insulin. It may indicate pancreatic β-cell compensated to insulin resistance produced by TNF-α in peripheral tissue (Swaroop *et al*. 2012).

Growth factors and cytokines level released in CM depend on cell type, cell number, medium condition and culture process. Tumor necrosis factor-α (TNF-α) level in umbilical cord mesenchymal stem cell CM have not been known yet, so it has to investigate furthermore. Administration of anti TNF-α in T1DM and T2DM mice might recover pancreatic β-cell and insulin-IR cells (Koulmanda *et al*. 2012). Furthermore, production conditioned medium should be adjusted as a new candidate for DM treatment. Cell type, cell number, medium condition and culture process for DM treatment should be treated as needed to reduce inflammatory cytokines released in CM. In this present study, insulin IR-cells on pancreatic islet were decrease progressively after third and fourth CM administration. Inflammatory cytokines (Donath *et al.* 2003) and oxidative stress (Kaneto *et al*. 2006) associated with pancreatic β-cell apoptosis and reduce their ability to produce insulin (Rhodes 2005; Lin &Sun 2010). High level of cytokines, such as TNF-α, interlukin-6 (IL-6) and IL-10 (Pawitan 2014) have a role in pancreatic β-cell apoptosis in the late CM treatment. Beside, high serum TNF-α level associated with insulin insensitivity in T2DM progression. High serum TNF-α were secreted by macrophages and lymphocytes, indicated T2DM represent a chronic inflammation (Miyazaki *et al*., 2003). Inflammation could increased reactive oxygen species (ROS). Several study reported that this oxidative stress markers supported insulin insensitivity in T2DM (Lin *et al*. 2005). However, temporal insulin production induce by CM could maintain blood glucose concentration in T2DM.

Nowadays, some cell types, such as stem cell, endocrine progenitor, others mature cells in the pancreas gland, and pancreatic β-cell itself have been developed to generate the pancreatic β cell (Borowiak & Melton 2009). In addition, there are possibilities of generate pancreatic β-cell from duct-lining and acinar cells (Bonner-Weir & Weir 2005), or hepatocyte (Porat & Dor 2007). However, it remains unclear which cell type can generate to pancreatic β-cell successfully in clinical administration. It needs more effort to develop an applicable diabetic therapy from cell line, since CM treatment have a good prospect and more easily administered. Diabetic therapies in the future may combine the role of microenvironment factor that suitable for pancreatic β-cell development and cell line (Alismail & Jin 2014). Conditioned Medium derived from human umbilical cord mesenchymal stem cell may contain signal molecules and biomaterials that suitable for pancreatic β-cell development.

5. Conclusion

The results of present study showed very strong evidence that conditioned medium derived from human umbilical cord mesenchymal stem cell could recover the insulin production from pancreatic β-cells in early administrated. However, after long treatment the insulin-immunoreactive cells dramatically decreased and almost could not be detected in pancreatic islets.

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