Chapter 4

Mesenchymal Stem Cells Secretome for Diabetic Wound

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A. Diabetes and Its Complications

Diabetic complications can be generalized into two categories, microvascular and macrovascular complications. Microvascular complications are related to complications affecting microvessels such as retinopathy, neuropathy, and nephropathy. On the other hand, macrovascular complications are related to those affecting macrovessels such as coronary artery disease, peripheral artery disease, and stroke (Mauricio et al., 2020; Ohiagu et al., 2021). The macrovascular complications, such as coronary artery disease, had a high prevalence among people suffering from diabetes. Besides that, one of the most common complications of diabetes is peripheral artery disease, which is associated with other diseases, particularly diabetic

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peripheral neuropathy. Both these disorders could lead to diabetic foot which usually leads to nonhealing foot ulcers (Mauricio et al., 2020; Ohiagu et al., 2021). There are still many diabetic complications other than those mentioned above.

B. Underlying Mechanism of Diabetic Complications

According to Shi et al. (2018), several studies have shown that hyperglycemic conditions may affect microvascular and macrovascular disruptions. This disease is a major contributor to the development of vascular problems and can mediate the negative effects through many mechanisms (Shi et al., 2018). The mechanisms include increased oxidative stress, mitochondrial dysfunction, excessive cellular apoptosis, and abnormal cellular autophagy, which is caused by long-term diabetic condition and have a potential effect on diabetes complications, whether it is directly or indirectly (Shi et al., 2018). In brief, this excessive production of ROS will increase the synthesis of strong oxidative peroxynitrite, induced damage to DNA, and increased the risks of diabetic microvascular complications throughout several mechanisms (Shi et al., 2017, 2018). The next mechanism is related to cellular and tissue injury in diabetes is cellular apoptosis. Apoptosis is a mechanism for genetically programmed cell death, and it has a major role in the survival of an organisms (Hamzawy et al., 2017). Cell apoptosis related to diabetes is mediated by two substantial forms: stress on endoplasmic reticulum and damage to mitochondria. There are two key pathways that take part in this mechanism, the extrinsic death receptor pathway and intrinsic mitochondrial pathway (Huang et al., 2017; Shi et al., 2018). Another major mechanism involved is autophagy which is another significant way to maintain environmental homeostasis in intracellular (Hamzawy et al., 2017). Being in a hyperglycemic condition for a prolonged period of time, abnormal cellular autophagy could occur. This mechanism is activated when the cell condition is in various stress situations such as lack of essential nutrients and high glucose environment. Autophagy is necessary for cellular functions, the deficiency of this mechanism could lead

to cellular degeneration and disruption of intracellular homeostasis (Hamzawy et al., 2017; Shi et al., 2018). Apart from the mechanisms mentioned above, there are some that believe that growth factors play a role in the development of abnormal growth and impaired regeneration in diabetics (Shi et al., 2018). Thus, it is important to understand the underlying mechanism that is impaired due to this condition.

C. Diabetic Ulcer as One of Serious Concern Complications of Diabetes

Diabetic ulcers are arising as a serious concern due to the complications from amputation cases. The incidence rate of minor and major amputations related to diabetes was 139.97 and 94.82 events respectively, per 100,000 diabetic patients per year. In the case of lower extremity amputations related to diabetes, it is more than two-fold higher in patients with type 1 DM than those with type 2 DM (Ezzatvar & García-Hermoso, 2022). This condition is a result of disruption in all phases of wound healing as a result of diabetes itself (Okonkwo et al., 2020). There are numerous factors that are responsible for the impaired wound healing process in diabetic patients, including impaired growth factor production, angiogenic response, and collagen formation (Fui et al., 2019). Consequently, even slight skin abrasions or scratches tended to develop into chronic wounds in diabetic patients (Avishai et al., 2017).

1. Impaired Wound Healing Process in Diabetes

Normally the wound healing process can be divided into four phases, specifically hemostasis, inflammation, proliferation, and finally the remodeling process (Fui et al., 2019). In brief, hemostasis is the first phase of the process in which the constriction of blood vessels occurs and platelet cells aggregate to clot the wound. The next phase is the inflammatory stage, when inflammatory cells migrate to the wound and blood circulation is increased (4 to 6 days). Then, proliferative

stage, which is when the wound bed is filled with granulation tissue during this phase, followed by angiogenesis which is when the new blood vessels are formed (4 to 21 days). Epidermal cells such as fibroblasts and keratinocytes are proliferated and migrated across the wound. After that, the collagen accumulation happens, then finally the wound edges are contracted. The final phase is remodeling phase which occurs 21 days to 2 years post injury. The tensile strength is increased by collagen crosslinking and the scar is matured (Fui et al., 2019). If there are one or more of these phases impaired, consequently the wound healing process becomes delayed and it will lead to chronic ulcer development.

In diabetic patients with a constant state of hyperglycemia, the endothelial cells lose their integrity and eventually become susceptible to apoptosis and detachment, resulting in impairment of the wound healing process (Okonkwo & Dipietro, 2017; Velnar & Gradisnik, 2018). The study conducted by Okonkwo et al. (2020) also found that there was a decreased amount of functional endothelial cells present in the diabetic skin. There was also impairment in the pruning and refinement process of the capillary bed (Bodnar et al., 2016; Caporali et al., 2017; Okonkwo et al., 2020). Therefore, it leads to the conclusion that skin wounds on diabetic patients are significantly reduced due to neovascularization and pro-angiogenic factor expression after injury (Okonkwo et al., 2020). Moreover, the overproduction of ROS in diabetics will result in cellular damage of endothelial cells (Fui et al., 2019).

Although many comprehensive treatments are currently available, the number of complications related to diabetic wounds, especially diabetic foot ulcers, is still alarming, with the data reported by the IDF showing that 9.1–26.1 million people will develop diabetic foot ulcers (Armstrong et al., 2017; Everett & Mathioudakis, 2018). Hence, advanced treatments for diabetic wounds have been studied intensively in the last decade. Growth factors are known to be involved in every phase of wound healing through their inhibitory or stimulatory effect (Burgess et al., 2021; Fui et al., 2019). Although there is some belief

that in the diabetic condition, growth factors play a major role in repairing tissue, nevertheless, it is still one of the promising targets for diabetic wound healing therapy.

2. Growth Factors That Related to Wound Healing Process

There are some major growth factors that play an important role in the wound healing process. Platelet-derived growth factor (PDGF) is released by platelets as a key factor that functions to increase the infiltration of immune cells, activating macrophages, promoting fibroblast proliferation, and accumulation of the extracellular matrix (ECM). It is involved in the inflammatory, proliferative, and remodeling phases of wound healing (Gardner et al., 2016; Patel et al., 2019). Epidermal growth factor (EGF), which is involved in the proliferation phase, is also released by platelets. It has the function of enhancing cell motility, migration, and cell proliferation (Bai et al., 2016; Fui et al., 2019; Patel et al., 2019; Shin et al., 2022). Transforming growth factor beta 1 (TGF-β1), involved in inflammatory and proliferation phase, it is produced by several cells such as macrophages, fibroblasts, keratinocytes, and platelets. The functions of TGF-β1 are increasing leukocytes and fibroblast migration, promoting angiogenesis, and also stimulating the production of ECM components (Bai et al., 2016; Fui et al., 2019; Mori et al., 2016). Vascular endothelial growth factor (VEGF) is the most known growth factor for its potent angiogenic properties. It increases capillary density and improves the blood metabolism in wounded tissue. It also mediates migration and proliferation of endothelial cells, angiogenesis and tissue granulation during inflammatory and proliferation phases (Fui et al., 2019; Gardner et al., 2016; Patel et al., 2019). Finally, basic fibroblast growth factor (bFGF) is usually highly expressed during late inflammatory stage. This growth factor has the functions to enhance the proliferation of fibroblast, promotes angiogenesis and collagen maturation during the proliferation and remodeling phase of the wound healing (Bai et al., 2016; Fui et al., 2019). Most of these growth factors are secreted by

mesenchymal stem cells, which is why it has become the major focus for advanced therapy in diabetes.

D. Stem Cell-Based Therapy for Diabetes

Recently, stem cells-based therapy has arisen as one of promising alternative treatment to many diseases, including diabetes. Interestingly, mesenchymal stem cells (MSCs) are well known for their regenerative ability and immunomodulatory attributes. MSCs can be found in many perivascular tissues such as bone marrow, adipose tissue, teeth, placenta, umbilical cord, amniotic fluid, and cord blood (Shin et al., 2021). The following are some studies, both pre-clinical and clinical trials, that used mesenchymal stem cells from varied sources to treat diabetic condition such as insulin resistance and control the hyperglycemia condition.

Wharton's jelly derived MSCs and hematopoietic stem cells derived from umbilical cord blood have been analyzed for treatment of type 1 and type 2 diabetes (Bani Hamad et al., 2021). A clinical trial in China used Wharton's jelly derived MSCs on a recently diagnosed type 1 DM patients. This clinical trial was designed as randomized controlled study, and the stem cells were administered via intravenous injection and were combined with insulin administration prior and throughout the follow up period. The dose of implanted MSCs was not disclosed by the authors. The result showed improvement in hemoglobin A1c (HbA1c) levels on the stem cells therapy group, the dosage of insulin administration was significantly decreased, and interestingly, a fifth of patients in the therapy group become insulin-independent, for almost 1.5 years. Moreover, there were no adverse events reported during the study (Bani Hamad et al., 2021; Hu et al., 2013). Other clinical trial was done on type 2 DM patients in China. Wharton's jelly derived MSCs is used and administered twice. First, it was delivered via intravenous injection, then for the second dose it was directly injected through splenic artery using catheter. The first and second dosage was given five days apart. The result showed

there was a decrease in HbA1c and blood glucose levels, moreover the dosage of insulin and other anti-diabetic medication is reduced. Although there are no control group to compare the result in this trial, this result showed that administration of Wharton's jelly derived MSCs can improve the regulation of metabolic pathway and β cell function in type 2 DM patients (Bani Hamad et al., 2021; Liu et al., 2014). The limitation for both studies is the small sample size. Overall, it proved that stem cell therapy was much safer compared to islet and organ transplantation (Bani Hamad et al., 2021). Umbilical cord (UC) derived MSCs are considered a better choice for clinical applications due to its high paracrine potential and it has low immunogenicity (Wang et al., 2018; Xiang et al., 2020). MSC have the ability to repair the cell damage through paracrine mechanisms from several factors such as immunomodulation factors, angiogenic factors, antiapoptotic factors, antioxidative factors, and also cell migration, and targeting and stimulation, although their fundamental and detailed biological mechanism still required further elucidation (Gnecchi et al., 2016; Liang et al., 2014).

Despite its promising results, there are some limitations of this stem cells therapy. These are related to the administration of stem cells, which is via infusion route in most of the studies or via clinical trials regarding type 1 or type 2 DM (Cho et al., 2018). One of the significant challenges is the low survival rate of the engrafted cells. Many transplanted cells eventually will die within hours or days post transplantation (Mitrousis et al., 2018; Sortwell et al., 2000). Numerous efforts have been attempted by researchers to overcome this problem. Pre-conditioning, genetic modification, and mimicking extracellular matrix such as hydrogel have been used to improve the survival of the cells (Li et al., 2016; Zhao et al., 2019). Nonetheless, further strategies and research are needed, including various cells condition and environment, the delivery system, and dosages that must be consider.

E. Secretome Based Therapy for Diabetes

This last decade, researchers were racing to develop cell free therapy derived from stem cells. As described above, stem cells, such as MSCs, have been used in clinical trials to treat diabetes and there are several trials that have proven their positive effects. However, due to some challenges to have the optimal effect from the usage of MSCs, cell-free therapy such as secretome or CM is more preferrable. MSCs from various sources have been known to release numerous paracrine factors that classified as bioactive molecules (Hsiao et al., 2011). These bioactive molecules which secreted into the extracellular space are known as secretome and is secreted by MSCs as a response to specific microenvironment conditions. According to González-González et al. (2020), secretome contains two different components. The first component is a soluble part, mostly comprised of cytokines, chemokines, immunomodulatory molecules, and broad spectrum of growth factors (Madrigal et al., 2014). The second component is a vesicular fragment, consisted of variety type of extracellular vesicles (EVs) (González-González et al., 2020; Teixeira & Salgado, 2020) such as microvesicles (Bruno et al., 2009), microparticles (Kim et al., 2012) and exosomes (Lai et al., 2010, 2015). Various studies on these secreted factors showed that even without the cells, it still has the regenerative ability to repair tissue or organ damage (Pawitan, 2014). This secreted factor can be found in the media where the cells were cultured, therefore the media is called conditioned medium (CM) (Kim et al., 2013).

There are some theories stating that the origin of the MSCs may have a difference in the protein expression. A study conducted by Shin et al. (2021) has done the comparative analysis of the secretome from different sources which are adipose and bone marrow (adult stem cells) and placenta and Wharton's jelly (fetal stem cells). There were plenty of proteins that involved in cellular migration and apoptosis reduction in the secretome derived from adipose, placenta, and Wharton's jelly, but not from bone marrow, though the level is varied between the sources. On adult stem cells, protein secreted by adipose MSCs is associated with the organization such as the development of

cytoplasm, while protein secreted by bone marrow MSCs is related to cellular development, and epithelial-mesenchymal transition. Protein that associated to cell migration and survival were detected similarly on both sources (Shin et al., 2021). Nonetheless, secretome secreted by fetal MSC group was expected to have higher potential than the adult stem cells due to the higher quantity of protein and more diverse proteins they have (Shin et al., 2021).

Secretome or CM has been used in several pre-clinical research particularly for wound healing in diabetes case. In brief, these are some of the studies related to that case. First is an in vitro study using secretome isolated from human adipose tissue-derived MSC has showed its ability to accelerate cutaneous wound healing. The results showed that the epidermal and dermal thickness, vascularized granulation tissue, and dermal collagen layers were increased on the wound treated by the stem cell secretome. The secretome stimulates collagen synthesis and migration of dermal fibroblasts through upregulating the transcription of collagen type I and III. It also may promote wound healing by increasing re-epithelization of the dermal tissue (Park et al., 2018). MSC and MSC-CM accelerated epithelialization, increasing granulation tissue formation. In response to MSC and MSC-CM, dermal fibroblast secrete increased the amounts of collagen type I and alter gene expression (Gnecchi et al., 2016).

We also have done an in vivo study using conditioned medium isolated from human umbilical cord-derived MSCs in diabetes induced rats to observe the wound healing potential. All animal experiments in this study were approved by Institutional Animal Care and Use Committees (IACUC) of the Faculty of Medicine, Tarumanagara University, approval number 001.KEPH/UPPM/FK/IV/2019. The cells were processed at Stem Cell and Cancer Institute Laboratory, Jakarta, Indonesia. The MSCs were cultured under hypoxic condition then the CM was collected. The result showed that this pre-conditioning hypoxic condition could stimulate MSCs to produce higher growth factors such as VEGF, bFGF, and pro-collagen 1 and promote better wound closure in rats. Intriguingly, VEGF was not secreted in CM collected

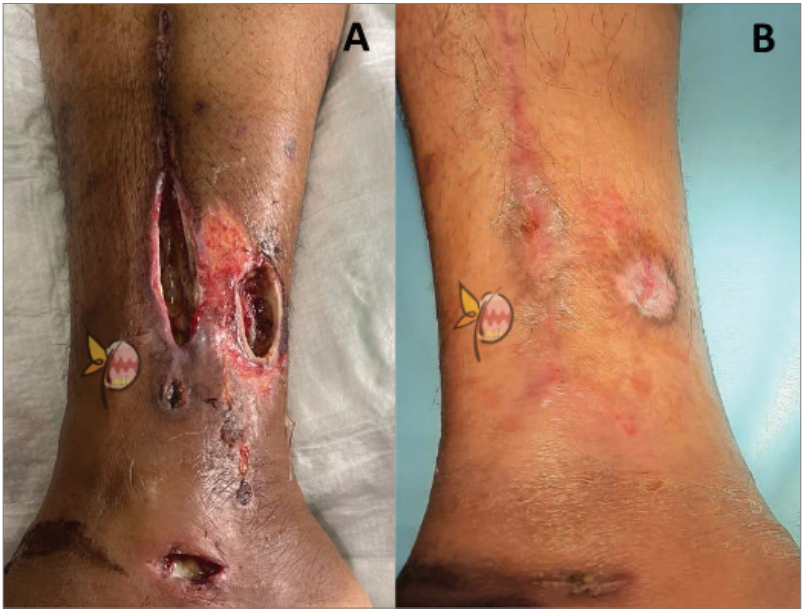
from umbilical cord MSCs that was cultured in normoxic condition. It has been proven that pre-conditioning certain factors such as hypoxia could enhance growth factors secretion. The histopathological analysis on the wound site showed that there is an increase in re-epithelization and also has the largest collagen deposition in the group treated using hypoxic umbilical cord-CM compared to the other group. Therefore, we concluded that the CM collected from umbilical cord MSCs cultured in hypoxic condition has positive effects towards wound healing process based on the result of re-epithelization and collagen formation on the wound site (Hendrawan et al., 2021).

Another in vivo study was done by Saheli et al. (2020) which evaluated the impact of CM collected from human bone marrow derived MSCs for diabetic wound healing in rats. The result showed the healing progress on the diabetic wound treated by CM was improved and comparable to the progress on non-diabetic group. They also found that the inflammation was significantly reduced on day 4 in the group treated with the CM compared to the diabetic control group. Higher expression of EGF and bFGF was also observed on the diabetic wound treated by the CM. Additionally, the collagen density was also increased, the inflammation was repressed, number of fibroblasts and microvessels was significantly elevated on the CM-treated group when compared to the diabetic wound. Therefore, this study also demonstrated that administration of MSC-CM has the potential to effectively improve the quality of healed wounds in chronic diabetes condition, which mainly through the modulation of fibroblast behaviors (Saheli et al., 2020).

Even though numerous researches were done using secretome or CM in relation to wound healing, especially diabetic wound, however, there are still only a few pre-clinical trials using CM or secretome systemically to treat hyperglycemic condition and other diabetic complications. We have done a pilot study to see the effect using CM from hypoxic human umbilical cord MSCs via intravenous injection on diabetic induced rats. The pilot study was approved by IACUC of PT. Bimana Indomedical, Bogor, ethical approval number R.02-21-IR.

The CM was injected intravenously through rat tail vein and the blood glucose concentration was monitored for 1 month. The results showed that the insulin concentration was decreased in CM group and was comparable to the normal rats. Based on this result, it suggested that the administration of CM could reduce the hypersensitivity of β cells. There were no side effects observed during the study (unpublished data) (Hendrawan et al., 2021; Tan et al., 2021).

We also performed a clinical study (number NCT04134676), in which we evaluated the therapeutic potential of CM on chronic ulcer wounds especially on diabetic patients. The ethical clearance for this study was obtained from Human Research Ethics Committee, Institute of Research and Community Engagement of Tarumanagara University, number 1007-Int-KLPPM/Untar/VI/2020. Umbilical



Notes: (A) Before treatment and (B) After treatment

Photo: Sukmawati Tansil Tan (2021)

Figure 4.1 Representative Image of Diabetic Chronic Ulcer Wound Treated with MSC CM

cord was obtained with the parental consent and was processed at Tarumanagara Human Cell Technology Laboratory, Jakarta, Indonesia. The CM was collected from MSC cultured under hypoxic condition (Figure 4.1a). The CM and other active ingredients were mixed in the form of 10% gel for topical use. An ample amount of the gel was applied to the wound. The results showed that the width and length of the wound decreased. Moreover, the bed of wounds is improved after 2 weeks post treatment (Figure 4.1B). The wound was treated for one month. There are no adverse effects observed in this study. Overall, the study showed that the topical administration of 10% gel CM can effectively enhance wound healing, in particular diabetic chronic ulcers (Tan et al., 2023).

F. Future Prospect and Challenge Against Secretome Wide Clinical Application

Stem cell-based therapy has emerged as a prominent alternative therapy to various degenerative diseases (Park et al., 2018). However, there are numerous challenges for clinical application of this therapy. The process of manufacture, sources, cell culture protocols, level of expansion and status of the cells can influence the therapeutic effectiveness of MSCs. Until now, allogenic or autologous MSCs have been used in clinical trials, while xenografts of MSCs are only applied in pre-clinical studies (Shin et al., 2021). The concerns are delivery route and dosage of the MSCs. Viable cells usually were delivered to the body via injection or catheter. However, the data showed that injection of live cells through a syringe needle can reduce the cell viability below 32% and it could cause irreparable damage to the cell membrane. It could also lead to a raise of an immune response that can be harmful for healing process (Ahangar et al., 2020). Moreover, there are side effects that are associated to MSC administration such as transient fever, constipation, and fatigue. Neither serious adverse events nor mortality were discovered across the clinical studies (Wang et al., 2021).

On the contrary, secretome provides an option for cell-free therapy with lower immunogenicity reaction. Secretome has the advantage that it can be prepared ahead in larger quantities and immediately become available for application (Xia et al., 2019). Despite the fact that it is easier to produce, handle, and store compared to the viable cells, it also has several drawbacks and challenges to bring it to bedside application (González-González et al., 2020).

To date, there is still no clinical trials have been registered in the clinicaltrials.gov that used the mesenchymal stem cells secretome or conditioned medium, whether to treat diabetic condition such as to control the hyperglycemia or to treat the diabetic complications cases. In brief, here are some of the challenges with secretome especially secretome from MSCs as therapeutic product. First, the characterization of secretome is needed. Due to the composition of secretome, it has become highly challenging to define specific function of each components and quantify the activity (Ahangar et al., 2020; Vizoso et al., 2017). Secondly, the inconsistency during the preparation of secretome from the MSCs. It is well-known that there are many factors that could affect the quality and efficacy of the secretome. Health condition and age of the donors, also the methods for isolation and culture the MSCs, are some of the factors that must be considered. The donor should be strictly screened and free from hepatitis B virus and human immunodeficiency virus (HIV) (Ahangar et al., 2020; Lukomska et al., 2019). The source of the MSCs is also one of the hurdles, some of it possibly due to ethical issue. For example, the usage of human fetal which obtained from the abortion procedure, although it has unique properties, has ethical issue for clinical application. Conversely, human umbilical cord should be more suitable as source of MSCs because it was categorized as clinical waste, therefore, there are no ethical issue to use the umbilical cord tissue (Wang et al., 2023). The other challenges regarding inconsistency are the heterogenicity of the MSCs, number of cells, and the interval of time. The most crucial part of the challenges is to produce the secretome under pharmaceutical standard and in the Good Manufacturing Practice (GMP) certified

facility. The secretome production under good manufacturing protocols can improve the consistency from one batch to another and importantly the efficacy of the secretome can be reproducible (Ahangar et al., 2020; De Sousa et al., 2016). The other concern part of secretome application is the potential side effect of the secretome administration. Despite the fact that there are only a few reports regarding the negative effects or even adverse events of secretome, there is always a risk that potentially happens when administering foreign substance. One of the problems is the immunosuppressive properties that has been reported in some studies (Zhao et al., 2016). Therefore, there is probability that the usage of secretome could cause immunodeficiency and poses risk to an infection (Bascones-Martinez et al., 2014). Hence, the optimum dosage for secretome administration should be clearly specified to have the balance of efficacy and safety of this secretome based treatment (Ahangar et al., 2020). Another concern regarding the instability and half-life of the protein contained in the secretome could be overcome by pre-conditioning the cells to increase the paracrine activity and production of the cells (Park et al., 2018). The alteration, namely hypoxia, inflammatory stimulus, or even the usage of bioreactors on preconditioned cells, was also reported to be related to increases the therapeutic potential of secretome (Pinho et al., 2020). We have proved that pre-conditioning such as culturing the cells in a hypoxic condition will increase the growth factor production in the secretome compared to the cells culture in normal condition (Hendrawan et al., 2021). Besides the challenges, there are concerns such as the route of administration, dosage, and duration of secretome application that need to be determined and standardized. The most common route of administration is topical for wound healing treatment (Fui et al., 2019). For other therapy, there is pre-clinical trial that used injection (intramuscular, intravenous) as the delivery route. However, there is still no clinical trials for diabetes that administered secretome as its therapy. Related to dosage, it will become one of the difficult challenges to calculate the generalized

dosage and duration needed for diabetes related disease. Although there is a study that has demonstrated the repeated administration of secretome can increase the duration of secretome effects as we have described above, the available data is still very limited. Furthermore, for wider clinical application, it is necessary to apply the precautionary principle and based on scientific evidence on safety and efficacy. It is also necessary to increase the education of the general public on how to interpret and apply these new findings. In Indonesia, the support from the government is obvious and have already regulated the provision of stem cell services which has all been stated in Indonesian Minister of Health Regulation Number 32, 2018 (Permenkes No. 32, 2018). However, there are still limited GMP certified facilities that has been established in the country. Limited budget is also one of the biggest concerns, which is why there is still a long way to go to bring the secretome to bedside application widely in Indonesia. Hopefully, the government concern to decrease the number of diabetes related complications could ease the way of secretome clinical application in Indonesia. In conclusion, there are numerous treatments for diabetes; nonetheless, the complications of this disease are still happening.

There are evidences that in most of the pre-clinical and clinical trials that used stem cell-based therapy which showed positive results, especially regarding diabetic wound healing treatment. While there are many challenges, this therapy is highly potential and very promising as alternative therapy for diabetes and its complications in translational medicine. New strategies are needed for overcoming limitations of stem cells and its secretome to be applied in a broad field of disorders, such as the use of encapsulated stem cells (Freimark et al., 2010) and nanotechnology (Zaghary et al., 2021). Moreover, cooperation among all stakeholders is essential to accelerate clinical applications. Good clinical trials to prove safety and actual efficacy of stem cell therapy are required to rush application and development of commercialized products.

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