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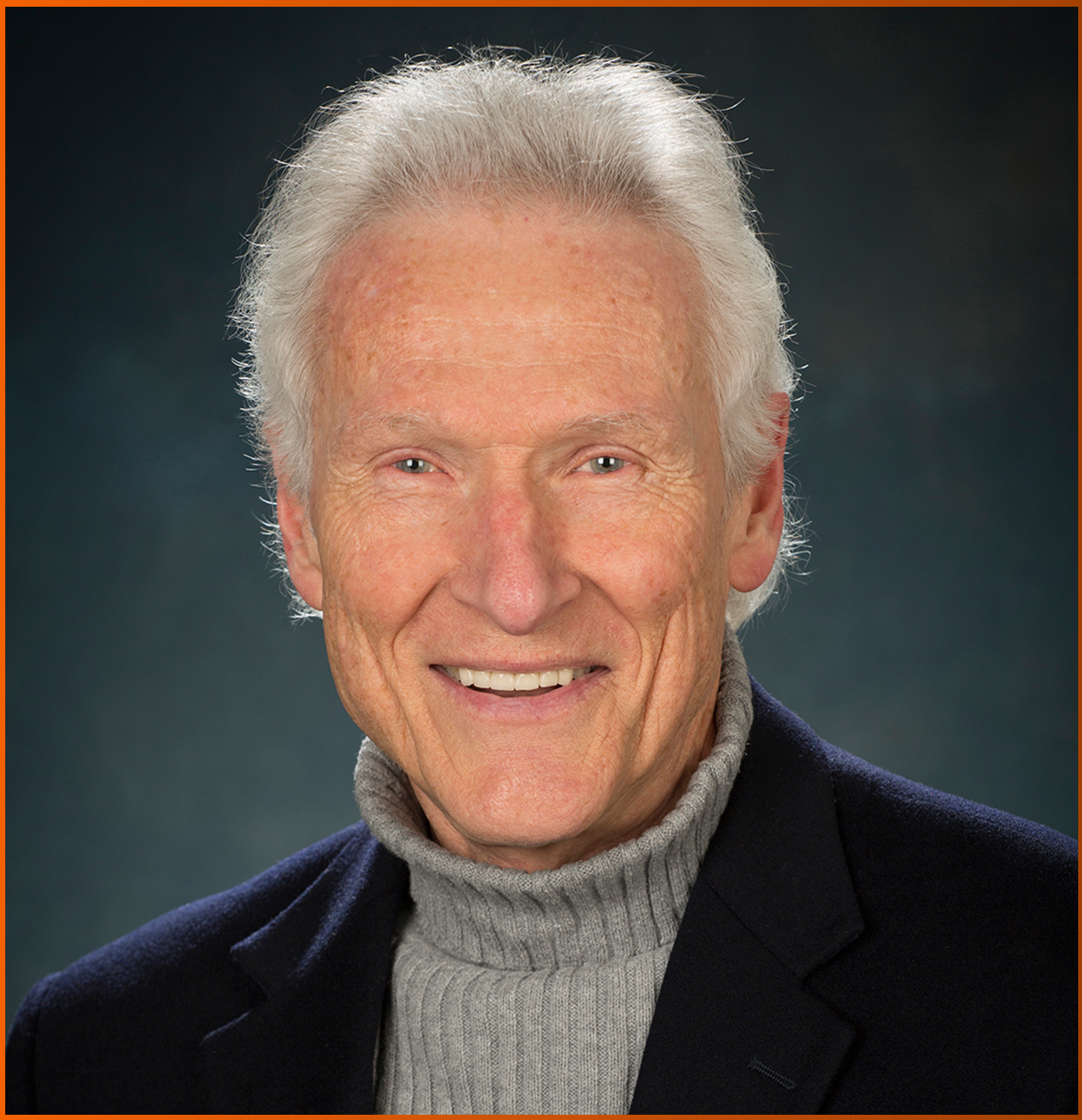

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MINIREVIEWS

Current progress and emerging technologies for generating extrapancreatic functional insulin-producing cells

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Abstract

Diabetes has been one of the major concerns in recent years, due to the increasing rate of morbidity and mortality worldwide. The available treatment strategies for uncontrolled diabetes mellitus (DM) are pancreas or islet transplantation. However, these strategies are limited due to unavailability of quality pancreas/islet donors, life-long need of immunosuppression, and associated complications. Cell therapy has emerged as a promising alternative options to achieve the clinical benefits in the management of uncontrolled DM. Since the last few years, various sources of cells have been used to convert into insulin-producing β-like cells. These extrapancreatic sources of cells may play a significant role in β-cell turnover and insulin secretion in response to environmental stimuli. Stem/progenitor cells from liver have been proposed as an alternative choice that respond well to glucose stimuli under strong transcriptional control. The liver is one of the largest organs in the human body and has a common endodermal origin with pancreatic lineages. Hence, liver has been proposed as a source of a large number of insulin-producing cells. The merging of nanotechnology and 3D tissue bioengineering has opened a new direction for producing islet-like cells suitable for *in vivo* transplantation in a cordial microenvironment. This review summarizes extrapancreatic sources for insulin-secreting cells with reference to emerging technologies to fulfill the future clinical need.

Key Words: Diabetes mellitus; Cell-based therapy; Insulin producing cells; Extrapancreatic sources; Biomaterials; Tissue engineering

Core tip: The currently accepted available treatment strategies for uncontrolled diabetes mellitus (DM) are pancreas and islet transplantation. However, these strategies are limited due to unavailability of quality pancreas/islet donors, life-long need of immunosuppression, and associated complications. Exogenous insulin administration is one of the clinically established options for patients with type 1 DM. Liver could be an appropriate extrapancreatic source to isolate insulin-producing cells due to their similar embryonic developmental origin. The need to develop more effective diabetes cell-based therapies lies in the advancements of robust sensitive micro- and nanodevices for exogenous insulin delivery.

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INTRODUCTION

Currently diabetes mellitus (DM) and associated complications have been a major concern due to the continuous increasing rate of morbidity and mortality worldwide. DM is caused by insufficient insulin production due to autoimmune destruction of functional β-cells [insulin-dependent type 1 DM (T1DM)] and insulin resistance [non-insulin-dependent type 2 DM (T2DM)] and subsequent β-cell apoptosis within the pancreas[1]. The pancreas is an acinar gland and a critical controller of blood glucose levels. It has both exocrine and endocrine functions. The endocrine part of the pancreas consists of small islands of cells, called the islets of Langerhans, that encompass α-cells (involved in secretion of glucagon), β-cells (involved in insulin secretion), δ-cells (involved in somatostatin secretion), ε-cells (involved in release of ghrelin), and PP cells (release of pancreatic polypeptide). These hormones upregulate or downregulate depending upon the blood glucose levels and inadequate secretion of these hormones may result in uncontrolled glycemic regulation in our circulation causing T1DM or T2DM[2].

The currently available treatment approaches for uncontrolled DM are pancreas and islet transplantation[3,4]. However, these strategies are limited due to unavailability of quality pancreas or islet donors, life-long need of immunosuppression, and associated complications. Exogenous insulin administration is one of the clinically established approaches for patients with T1DM[5]; however, this strategy does not completely control blood glucose level and impaired insulin release, resulting in continuous abnormal functioning of β-cells. Hence, there is an urgent need to develop clinically relevant and scalable strategies to replenish the loss of β-cell function to provide long-term recovery. Cell therapy has emerged as one of the promising alternatives to achieve the required clinical benefits in both T1DM and T2DM.

Since the last decade, various sources other than pancreas-derived cells have been used to generate insulin-producing β-like cells[6-8]. These extrapancreatic sources of cells may play a significant role in β-cell turnover and insulin secretion in response to environmental stimuli. However, the most critical limitation with such extrapancreatic source of cells is that they do not respond to glucose stimuli, and do not mimic clinical conditions. In addition, the production of insulin-producing cells (InPCs) *in vitro* as well as *in vivo* requires complicated protocols which should be glucose responsive. Among various extrapancreatic sources, the liver has been considered as one of the most appropriate options for isolating large numbers of cells with the potential to dedifferentiate into InPCs due to their similar embryonic developmental origin from the endoderm. The stem/progenitor cells from the liver respond well to glucose stimuli *in vitro* and *in vivo*. Hence, the liver could be one of the most suitable sources of large numbers of functional InPCs. This review summarizes the few crucial extrapancreatic sources that are currently proposed to generate InPCs, along with other advancements in this field that may fulfill the current clinical needs for the welfare of DM patients.

EXTRAPANCREATIC SOURCES OF β–CELLS

Hepatic stem/progenitor cells

Studies have demonstrated that liver cells can be converted into InPCs under the influence of several growth factors and a high-glucose environment[9-11]. However, several questions remain to be addressed regarding which cell type undergoes differentiation and the minimum cascade of genes essential to activate or inactivate to generate fully functional β-cells. An initial study by Zalzman *et al* [12] in 2003 reported successful reversal of hyperglycemia in mice using fetal liver progenitor cells that were converted into InPCs. This study gave a boost for utilization of human fetal liver progenitors to obtain significant numbers of InPCs and develop relevant protocols to be applied in clinical settings. In

support of the above study, Cao *et al*[13] in 2004 reported the importance of liver cells in reversal of hyperglycemia in diabetic mice, using a rat liver cell line cultured in high-glucose medium. However, cells failed to produce enough insulin when injected *in vivo*, which is comparatively very less to desired insulin content (< 1%) obtained from the native β–cells. Hence, it was assumed that primary cells are of utmost important for further *in vivo* studies.

In our preliminary study, we have demonstrated the successful production of InPCs from human fetal liver-derived EpCAM$^+$ stem/progenitor cells in response to glucose stimuli *in vitro*[14]. Following to this study, we also reported a cascade of transcription factors that are required to convert EpCAM$^+$ human fetal liver-derived stem/progenitor cells into InPCs *in vitro* under a high-glucose microenvironment (Figure 1). This study revealed that activation of master regulator Pdx-1 with β-cell-specific transcription factor Nkx-6.1 in combination with Ngn-3, Pax-4, Pax-6 and Isl-1 is required to transdifferentiate hepatic stem/progenitor cells into InPCs under hyperglycemic challenge without the need of any genetic manipulation, which is crucial for its potential clinical relevance. The study also showed that the amount of insulin production was higher compared to that with other developed protocols. This particular strategy has the additional advantage for its clinical potential of scalability and lack of genetic manipulation.

Embryonic stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts. ESCs are pluripotent and can produce cells present in all three germ layers, including InPCs, when placed in appropriate *in vitro* or *in vivo* conditions. Due to self-renewal and differentiation potential, ESCs can be used to produce large numbers of desired cell types for downstream applications. Different derivatives of ESCs have shown restoration of functional and structural benefits in injured organs/tissues[15]. Hence ESCs are considered a good source for the generation of large numbers of InPCs. D'Amour *et al*[16] in 2006 demonstrated a stepwise protocol involving five stages for the conversion of ESCs into InPCs in response to a number of secretagogues *in vitro*. Recently, Vegas *et al*[17] in 2016 also developed a multistep protocol for converting ESCs into β-cells and achieved long-term glycemic control in immunocompetent mice using an encapsulation approach. However, these strategies are not clinically relevant due to their non-responsive nature to glucose stimuli, and ethical issues. Zhang *et al*[18] in 2009 developed a four-step protocol for converting ESCs into insulin/C-peptide-producing cells in response to glucose stimuli. Based on the clinical applicability of this protocol, Hua *et al*[19] in 2014 developed a four-step protocol to convert human ESCs into pancreatic InPCs and successfully corrected the hyperglycemia in immunodeficient mice. These studies have boosted our knowledge for the experimental conversion of ESCs into functional β-cells; however, the clinical applicability of these approaches is still questionable due to ethical concerns and complicated protocols, and importantly, these experiments were conducted in immunocompetent mice that do not mimic clinical conditions. Hence, there is still a need to discover alternative sources of human β-cells that can mimic clinical conditions and can be applied in clinical settings without the need for immunosuppression. More studies are desired to obtain homogeneous populations of functional human InPCs from ESCs and further to answer whether an inductive or selective mechanism of differentiation can be clinically relevant or not.

Bone-marrow-derived stem cells

Bone-marrow-derived stem cells (BMSCs) are considered one of the potential sources for generating functional pancreatic β-cells[20,21]. Several studies have demonstrated experimental conversion of BMSCs into InPCs[22-25]. However, the amount of insulin/C-peptide secreted by these cells was less compared to that from isolated pancreatic islets and was not enough to reverse hyperglycemia in diabetic rats. Several other studies have reported similar problems with BMSCs[26,27]. Some of the results of these studies were nonreproducible, which warrants their further investigation for clinical applicability.

Umbilical cord blood cells

Human umbilical cord blood (UCB) is known to contain stem/progenitor cell populations that can be converted into different types of organ-specific cells including InPCs. UCB is considered one of the most appropriate sources of stem/progenitor cells because of fewer ethical concerns and biological waste materials. Human UCB can also be readily available in sufficient amounts with low risk of graft rejection[28,29]. A few studies have successfully reported the conversion of human UCB-derived stem cells into functional InPCs by activating several crucial pancreatic transcription factors (Pdx-1, Isl-1, Pax-4 and Ngn-3), which is capable of correcting hyperglycemia in diabetic mice[30-33]. However, for their clinical applicability, more appropriate protocols need to be developed with suitable transplantation strategies to support long-term cell survival and function post-transplantation *in vivo*.

Fibroblast cells

A recent study by Zhu *et al*[34] in 2016 demonstrated that fibroblasts of adults or neonates can generate precursors of endodermal lineages following the cell activation and signaling directed transdifferentiation paradigm. They developed conditions for expansion of glucose responsive β-like transdifferen-

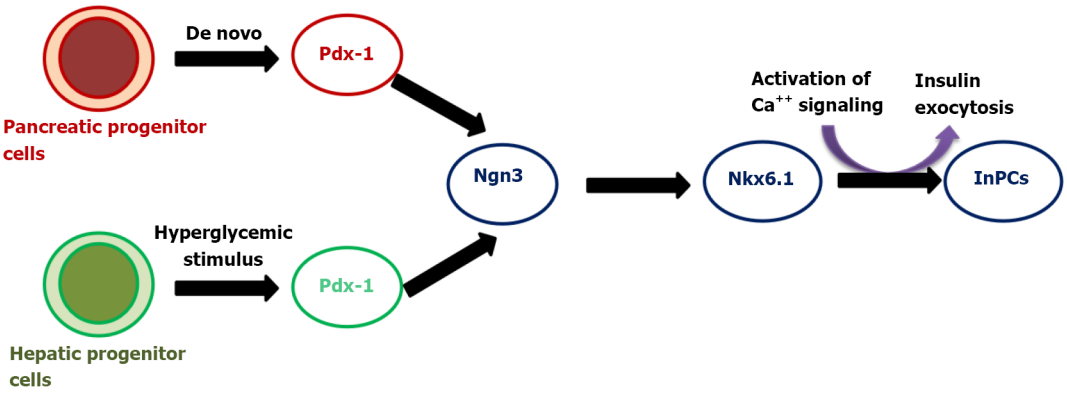

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Figure 1 Schematic representation showing activation of pancreatic transcription factors in human hepatic progenitor cells under hyperglycemic conditions to generate insulin producing cells similar to de novo pathway. InPCs: Insulin producing cells.

tiated pancreatic endodermal cells into the progenitor stage. These transdifferentiated cells can control hyperglycemia in mice and provide a new approach for the production of patient-specific InPCs for studying unresolved questions in pancreatic biology, disease modeling and drug testing strategies.

Induced pluripotent stem cells

Patient-specific cell lines can be generated using induced pluripotent stem cells (iPSCs), which can then be converted into cells of interest for disease models or cell replacement therapy. Current advances in iPSCs research have evolved several robust protocols for the production of patient specific functional β-cells. These differentiation protocols resemble with the same developmental stages ultimately targeting the final differentiated β-cells[35]. To trigger the pathways required for differentiation phases, a mixture of signaling molecules and growth factors are often utilized, with the aim of mimicking embryonic stages of developmental. For converting somatic cells towards iPSCs, the Yamanaka factors (OCT4, KLF4, SOX2 and c-MYC) are commonly used[36].

Several studies have examined the role of iPSCs-derived β-cells in the context of T1DM[37,38]. Maehr *et al*[39] in 2009, for the first time demonstrated conversion of iPSCs derived from T1DM patients into glucose-responsive functional InPCs[39]. Millman *et al*[40] in 2016 compared differentiation potential of iPSCs derived from diabetic and nondiabetic individuals and concluded that both the sources of iPSCs encompass similar expression patterns of the specific surface markers, and capacity of insulin secretion both *in vitro* and *in vivo*[40]. Despite progress towards potential applicability of iPSC-derived β-cells, there are technical difficulties and financial concerns related to iPSC therapy in clinical settings[41]. To alleviate the financial concern, iPSC biobanks with ability to match the majority of HLA types and occasional blood types are continuously being explored. However, the genomic instability involving chromosomal aberrations or mutations, as well as tumor formation remain obstacles for bench to bedside clinical translation of iPSCs.

Other sources

In addition to the above extrapancreatic sources, several other source of tissues have also been investigated that contain progenitor cell populations which can be converted into InPCs. Among these sources, spleen[42], adipose tissues[43], blood[44], amniotic membrane, and central nervous system[45] have showed β-cell-specific markers during *in vitro* or *in vivo* transdifferentiation (Table 1). In particular, Kodama *et al*[42] in 2003 demonstrated that injected splenocytes can be converted into InPCs and minimize the onset of autoimmunity. Transplantation of these cells combined with Freund's adjuvant improves diabetes in nonobese diabetic (NOD) mice. However, subsequent reports[46,47] found no such evidence which questions their clinical applicability.

EMERGING TECHNOLOGIES FOR CELL TRANSPLANTATION IN DIABETES

Microencapsulation

Cell encapsulation technology is based on the concept of immunoisolation. Because islet cells can be effectively harvested and transplanted, encapsulation technology is promising for future clinical transplantation. Therefore, during the last three decades, various encapsulation strategies have been demonstrated in different animals such as mice[48], rats[49], dogs[50] and monkeys[51]. Encapsulation technology also offers enhanced cell survival post-transplantation without the use of immunosup-

Table 1 Summary of target cells and the degree of β-cell similarity

Characteristics	Target cell				
	Liver	Pituitary	Muscle	Kidney	Bone marrow
Endodermal origin	+	-	-	-	+
Possession of β-cell transcription factors	-	-	-	-	+
Glucose sensing system	+	-	-	+	-
Processing enzymes	-	+	-	+	-
Glucose-regulatable promoter	+	-	-	+	-
Exocytosis system	-	+	-	+	-
Autologous use	+	+	+	+	+
Allogenic use	-	-	-	-	+

pressive drugs. This technology involves an artificial compartment of semipermeable membrane as a capsule that contains cells and allows oxygen and nutrient supply. The capsule protects cells from injuries against antibodies, proteins, and potent immune cells. Hence, these capsules are also referred to as an immunoisolation device. Diffusion of insulin, glucose, nutrients and oxygen across the capsule has additional advantages that allows efficient glucose homeostasis. Moreover, additional intravascular devices have been designed that contain a small planar or tubular diffusion chamber which is directly connected with the host vascular system and also referred to as an encapsulation device[52]. This device does not require anastomosis after implantation, and encompasses better clinical application compared to the intravascular device.

In a recent study of Vegas *et al*[17] in 2016, long-term glycemic control was achieved in immunocompetent hyperglycemic mice using polymer-encapsulated human stem-cell–derived β-cells. This study highlighted the decreased obscurity in successful immunoprotection against xenogenic human cell implants in diabetic mice. This report provided groundwork for future studies in autoimmune animal models using xenogenic cells transplantation with the goal of achieving long-term glycemic control and cell survival to offer insulin independence in patients with DM. However, this study had the limitation of generating β-cells using complex long duration protocols and was conducted in immunodeficient mice. Hence, further investigations are required to prove that this technology is clinically acceptable using a wild-type diabetic model without the need of immunosuppression.

In a similar direction, our group has also been working to generate functional insulin-producing β-like cells *in vitro* through transdifferentiation of human fetal liver-derived $EpCAM^+$ enriched progenitor cells in response to high glucose concentration (Figure 2). This protocol has been simplified and encapsulated functional β-like cells in alginate beads (with modified protocol) have been transplanted into C57BL6 hyperglycemic wild-type mice without the need of immunosuppression. The mice were able to restore the blood glucose level within 30 d after transplantation of encapsulated cells and maintained for up to 90 d (unpublished data). In our view, this is the only study where xenogenic human functional β-like cells have been transplanted into wild-type mice without immunosuppression. The encouraging results of this study have shown a path to further investigate their potential in clinical applications.

Hydrogel-based cell transplantation

Suspending viable cells in an aqueous medium of hydrogel precursors, infusing the cocktail to target areas, and stimulating crosslinking (gelation) to generate 3D gel matrices *in situ* are the three primary phases in cell transplantation utilizing hydrogels. Cell transplantation using a hydrogel-based strategy has several crucial advantages. Hydrogels are injectable, and can be used to construct cell-encapsulated gels that have a uniform distribution of cells in the transplanted space. They have water content that can be used to construct different shapes and sizes. Hydrogels can be used for cell therapy when they are constructed with limited pore size that can facilitate diffusion and metabolic wastes but prevent leakage in the cells[53]. Hydrogel scaffolds can be coated with encapsulated InPCs and placed at the site of transplantation[54].

Despite these advances, using hydrogels to control the fate of transplanted therapeutic cells still poses significant obstacles including unsatisfactory cell survival rate due to their death in the new microenvironment post-transplantation. Cells that survive in the hydrogels have uncontrolled interactions, proliferation and differentiation. The overall therapeutic efficacy was unsatisfactory using this method. The nonautologous cells were rejected by the host immune system and the cells were strictly confined. The encapsulated cells were not able to proliferate freely, the cell viability varied for different cell types and in many cases it decreased to nearly 50% when assessed after 10 wk. Hence, the methodologies were modified to improve the drug delivery and integration with the target tissue for successful delivery of

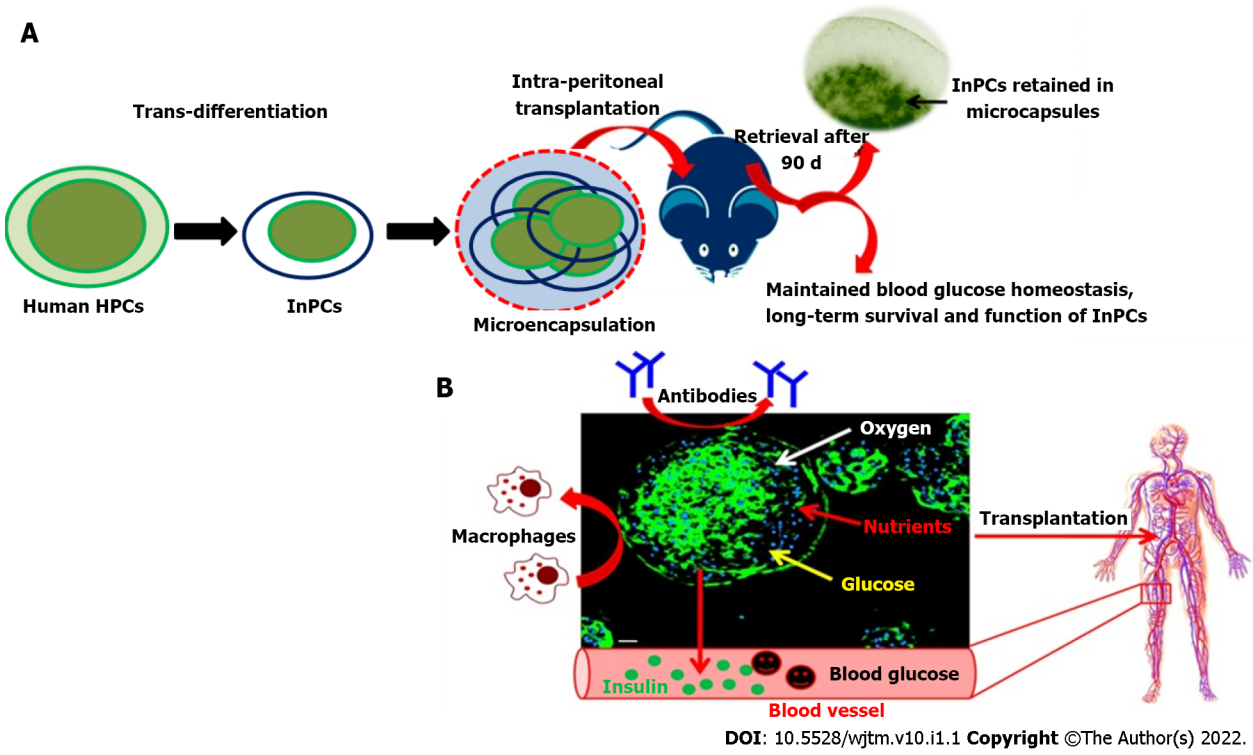

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Figure 2 Representation of encapsulation technology for glycemic control in diabetes. A: Schematic representation showing the applicability of microencapsulation of intermediate, progenitor cells derived from human hepatic progenitor cells under hyperglycemic microenvironment post intraperitoenal transplantation into diabetic mice. The strategy demonstrated the long-term maintenance of blood glucose homeostasis in diabetic mice; B: The microencapsulation strategy overcomes on immunological barriers and allows efficient supply of oxygen and nutrients to encapsulated cells in 3D-environment which can control the hyperglycemia in human from ectopic site. InPCs: Insulin producing cells; HPC: Hepatic progenitor cells.

the cells.

To improve the functional response and stability of the implanted cells, dual-layer hydrogels as well as silk hydrogels are being tested for efficient transfer of islet cells to the required site. Silk hydrogels are formed using extracellular matrix (ECM) proteins such as collagen IV and laminin. These hydrogels have been tested for cell signaling and survival by immunostaining and oscillatory theometry and found to be stable. The silk hydrogels encapsulated with islet cells in laminin had high insulin response compared with nonencapsulated cells when stimulated over a 7-d period. Silk hydrogels allow coencapsulation of other ECM proteins and cells that can ultimately be used as a transplant device for the treatment of T1DM[55].

Gene therapy

The generation of functional β-cells by genetic engineering necessitates a thorough knowledge of pancreas development and a good understanding of the organ. The production of a cascade of transcriptional regulators, as well as their involvement in regulating endocrine cell fate and maturation, are important considerations for creating artificial β-cells. Furthermore, the type of vector used for gene delivery and the selection of suitable cell type candidates for differentiation into functional β-cells are key challenges.

Selecting an ideal cell type: The ultimate aim of gene therapy is to produce cells with the ability to produce insulin and maintain blood glucose level *in vivo*. Different types of cells have been investigated over the last few years, in particular, pituitary cells that contain both the proinsulin processing enzymes and the secretory granules. However, these cells were non-glucose responsive and later became glucose responsive after transfection with glucose transporter 2 and glucokinase genes. However, *in vivo* production of adenocorticotropic hormone inhibits insulin function, which limits its clinical efficacy [56]. Muscle cells, liver cells, mesenchymal stem cells from UCB and bone marrow cells have also been investigated by gene transfer technology and demonstrated the reversal of hyperglycemia in immunodeficient diabetic mice. More recently, a combination of gene and cell therapy was used to produce glucose-responsive InPCs after retroviral transfection with Pdx-1. Transplantation of this combination reversed hyperglycemia in immunodeficient diabetic mice[57].

Choice of vector for gene delivery: The ideal method of gene delivery in cells relies on integrating viral vectors for sustained gene transfer into the daughter cells to provide sustained therapeutic benefits

throughout the life of the patient. Four main different kinds of viral vectors have been used in *in vitro* and *in vivo* models for gene delivery purposes: (1) Retroviral vectors[58]; (2) Adenoviral vectors[59]; (3) Adeno-associated vectors[60]; and (4) Lentiviral vectors[61]. Among these, lentiviral vectors have become a popular choice for gene delivery in animal models of diabetes. However, the clinical applicability of these strategies is limited due to virus-mediated complications and resultant pancreatic transdifferentiation.

β-cell transcription factors: Transcription factors play a significant role in determining the phenotype of β-cells. Homeobox factor Pdx-1 is considered to be the master regulator and has a crucial role in early development of the pancreas[62]. Hes-1 and Neurogen-3 are present in pancreatic progenitor cells and direct the respective compartmental fates through Notch signaling[63]. However, for subsequent differentiation into respective pancreatic cell lineages, a cascade of transcription factors are required. All endocrine cells express Neurogen-3, which activates NeuroD1, and maintains the endocrine cell differentiation program[64]. As soon as the endocrine cell differentiation program is activated, Pax-4 and Pax-6 direct the differentiation into different kinds of endocrine cells[62]. In these two transcription factors, Pax-4 is responsible for the fate of β- and γ-cells, and Pax-6 is essential for α-cell fate. More importantly, NK homeobox factors Nkx2.2 and Nkx6.1 are responsible for driving the β-cell fate. Both these transcription factors are imperative in β-cell differentiation. Nkx6.1 is also responsible for preservation of β-cell function during its interaction with Pdx-1, the master regulator, and the other transcription factor NeuroD1 that modulates insulin transcription. Alteration in the expression of these transcription factors and their subcellular localization alters the cellular processes related to β-cell differentiation, and cell cycle modulation and function.

Direct gene transfer: In relation to β-cell replacement therapy, direct gene delivery represents one of the potential techniques to obtain β-like cell phenotype in autologous tissues[65]. Although in past decades a lot of work was focused on direct transcription factor/gene transfer in hepatocytes, the emergence of stem cells has changed the way to look into the cells and needs further investigation due to their clinical suitability. However, before developing a suitable strategy, one needs to consider the appropriate type of transcription factor for direct delivery and true conversion into functional β-cells without causing future complications.

Nano-bioengineering

Nano-bioengineering is an emerging field that has the potential to revolutionize DM treatment. Merging of nanotechnology with medical biology has given a new direction to create smart delivery systems that can regulate blood glucose levels within the body and could produce the desired amount of insulin. Recent advances have been made in this field and novel blood glucose measurement and insulin delivery methods are being developed to improve the quality of life of DM patients (Figure 3).

Nano-bioengineering has been used to develop a novel smart insulin patch that can deliver glucose-responsive insulin with the help of a painless microneedle-array patch. This device is based on the glucose-responsive enzymatic mechanism that can regulate the blood glucose level in T1DM faster than the commonly used pH-sensitive formulations. In addition, it can also avoid the risk of developing hypoglycemia. The study by Lee *et al*[66] in 2016 showed the diversified application of a graphene-based electrochemical device to monitor DM and efficient transcutaneous delivery of drugs to reduce blood glucose levels in hyperglycemic mice.

In our recent study, we have demonstrated the applicability of an unique strategy that enables effective transdifferentiation of human hepatic progenitor cells (hHPCs) into InPCs on 3D-nanostructured TiO_2 substrate developed on conducting surfaces[67]. This 3D-TiO_2 cellularized chip was able to reverse hyperglycemia in wild-type mice (C57BL6) when transplanted into the peritoneal cavity. We also observed enhanced cell survival and insulin production, and long-term glycemic control in hyperglycemic animals where it does not elicit significant immunological response after ectopic transplantation. Another advantage of this approach includes a sufficient amount of insulin production within a short time post-transplantation in hyperglycemic animals. Due to rapid insulin production in the bloodstream, this approach is more successful in reducing the need for exogenous insulin in T1DM. Ongoing investigation has shown that by expanding the surface area of microchips, this strategy can be used to scale up the procedure for incorporating a sufficient number of InPCs. However, more specialized 3D packaging of InPCs, as well as pancreatic exocrine and ductal cells using TiO_2 nanostructures supported by conducting substrates, would be required to construct a full pancreatic organotypic system to evolve a more effective approach for regulating hyperglycemia. Therefore, further exploration of this approach is required to achieve its real therapeutic possibility for the clinical management of hyperglycemia.

Neo-organ bioengineering

Neo-organ bioengineering is one of the most promising approaches, which includes decellularization and repopulation of whole organs harvested from human or xenogenic sources. This approach generates a whole functional neo-organ construct for future clinical applications as a bridge therapy for

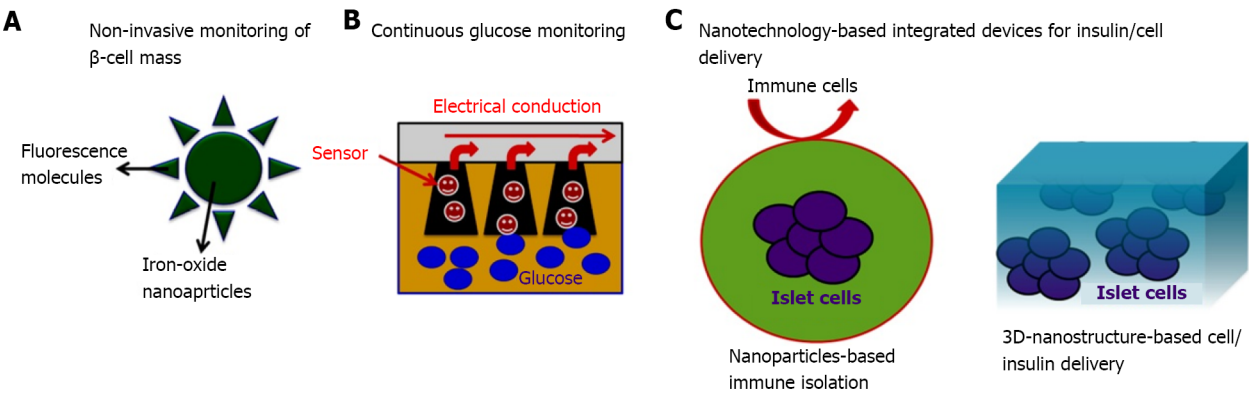

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Figure 3 Nanotechnology-based emerging strategies to address the challenges in diagnosis and management of diabetes. A: Iron-oxide nanoparticles-based approach (such as enhanced contrast imaging) for non-invasive monitoring of progressive β-cell loss which could be used to address the patient needs at the various stages of disease progression; B: Nanoparticle-based glucose sensors can assist improved accuracy and patient comfort for regular monitoring of blood glucose levels; C: Nanotechnology-based devices may provide immunological protection of transplanted pancreatic cells which could help to maintain normoglycaemic conditions in patients with diabetes.

organ transplantation. It offers a unique strategy of using natural organ platforms to produce natural organ systems using different sources of InPCs. Therefore, this particular technology can overcome the above-mentioned critical concerns and has attracted a lot of attention to generate different organs, including the pancreas[68].

A few recent studies have reported the use of decellularized pancreas to produce functional neo-organs for *ex vivo* insulin secretion[69-71]. However, a critical stumbling block is the lack of sufficient pancreata for decellularization and repopulation. Furthermore, the mechanical integrity of the neopancreas has not been reported. The developing concept of using heterografts to create viable humanized neo-organ systems has opened a new avenue for meeting transplant demand[72,73]. Nevertheless, due to the small amount of research conducted, some important questions remained unanswered. To date, no effective decellularization and repopulation of xenogeneic spleen sources with endocrine cells has been documented. As a result, it is still unknown if decellularized spleen can be repopulated with human InPCs and perform similarly to islets or the entire pancreas.

Our recent study has demonstrated a heterograft approach of using whole decellularized xenogenic scaffold of spleen to generate functional constructs that are capable of producing the desired amount of insulin in response to hyperglycemic stimulus[74,75]. In our preliminary study, we standardized a unique strategy for activating pancreatic transcription factors of $EpCAM^{+}$-enriched human hepatic progenitor cells repopulated within acellular spleen harvested from rats[74]. This indicates that 3D, intact acellular splenic scaffolds can provide a superior microenvironment for long-term survival of cells, activation of crucial transcription factors, and transdifferentiation of hHPCs into functional InPCs. Our subsequent study demonstrated that the heterograft approach developed in our previous study generates secondary neo-organoids during ectopic transplantation in diabetic rats and is capable of transporting insulin into the bloodstream, which is essential to manage uncontrolled blood glucose levels[75]. Moreover, this study provides the first proof-of-concept for creating bio/immunocompatible, humanized insulin-producing neo-organoids, which could evolve into more acceptable functional biological implantable devices for long-term diabetes management.

IMPORTANCE OF TRANSPLANTATION SITES

The ideal choice for cell transplantation offers optimum engraftment and long-term cell function. The appropriate site for cell transplantation should include: (1) Membrane drainage for the permeabilization of blood glucose and to avoid systemic hyperinsulinemia; (2) Rich arterial supply; (3) Minimal invasive infusion; (4) Access for morphological and functional follow-up of the transplant; (5) Microenvironment with maximum cell survival; and (6) Immunological tolerance. Such types of transplantation sites need to be defined. Over the last few years, various sites have been attempted for islet cell transplantation[76] to manage DM in different animal models. Among different proposed sites/routes, the portal vein has been the site of choice for clinical transplantation (Table 2). However, inflammatory reactions and low oxygen tension lead to cell loss and varied responses among patients. Peritoneal transplantation has the advantage of being an immunologically privileged site, and offers enough space for housing the cells. Hence, the peritoneum overcomes the limitations of other identified transplantation sites and could be an ideal choice for ectopic transplantation. In our recent study, we have demonstrated the usefulness of

Table 2 The pros and cons of different transplantation sites used for cell delivery in diabetic condition

Transplantation site	Pros	Cons	Ref.
Liver	Ease of transplant, Portal insulin delivery	Portal hypertension, bleeding, portal vein thrombosis, inflammation, portal circulation	Kim *et al*[77], 2010
Kidney subcapsule	Graft retrieval, immunological tolerance	Not ideal site, invasive procedure, graft tissue oxygen tension	Reece-Smith *et al*[78], 1981
Spleen	Good vascular supply, portal vein insulin delivery	Rich in lymphocytes, risk of bleeding	Gray[79], 1990; Alderson *et al*[80], 1984
Pancreas	Home of islets, ideal oxygen tension	Autoimmune destruction, invasive procedure	Stagner *et al*[81], 2008
Omental pouch	High vascular density, neoangiogenesis, good blood supply, impure islets can be implanted	Large graft unstable, islet cells estimation is empirical	Gustavson *et al*[82], 2005
Gastro intestinal wall	Ease of accessibility, portal vein delivery of insulin	Thickening of gastric walls after islet cells transplantation	Wszola *et al*[83], 2009
Bone marrow	Ease of accessibility, no graft rejection	Conserved differentiation into α and β cells	Salazar-Bañuelos *et al*[84], 2008; Cantarelli *et al*[85], 2009

the peritoneal site in diabetic animal models and have reported that this site increases animal survival and faster recovery of normoglycemia within 30 d post-transplantation without the need for immunosuppression (unpublished data).

CLINICAL CHALLENGES

The major challenges in DM cell therapy are: (1) Identifying clinically acceptable sources of cells that can be used to produce homogeneous populations and therapeutic doses of insulin-secreting cells; (2) Prevention of immunological rejection post-transplantation; (3) *in vivo* glucose responsiveness of transplanted cells; (4) Long-term cell survival and function post-transplantation; and (5) No need for immunosuppression. Other clinical challenges include: Safety of the transplantation procedure; determining the cell delivery and engraftment efficiency using live clinical imaging systems; cell delivery at the targeted site within a clinically relevant time; identification of ways to promote regeneration of resident β-cells; ease of source tissue collection and clinical grade cell isolation; and cost-effectiveness of procedures. These key considerations and challenges need to be resolved to successfully translate the stem-cell-based therapeutic possibilities for timely management of T1DM into clinical practice. Given the current debate on such issues, clinical applicability of stem-cell-based therapies for the treatment of DM is still a future goal.

CONCLUSION

In summary, in the next decade, we expect stem-cell-based therapeutic strategies in combination with nanotechnology and other potential areas of science for improved management of DM. Recent developments in neo-organ bioengineering and United States Food and Drug Administration-approved nanotechnology-based formulations with the success of insulin-delivery are encouraging and provide newer opportunities for DM treatment. In our view, the need to develop more effective microencapsulation, neo-organ bioengineering, and nanotechnology-based diabetes therapies lies in the development of robust sensitive micro- and nanodevices for insulin delivery, using clinically acceptable platforms.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Cell-free mitochondrial DNA quantification in ischemic stroke patients for non-invasive and real-time monitoring of disease status

Nusrath Fathima, Sandhya Manorenj, Sandeep Kumar Vishwakarma, Aleem Ahmed Khan

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Abstract

BACKGROUND

Acute ischemic stroke (AIS) is one of the major causes of the continuous increasing rate of global mortality due to the lack of timely diagnosis, prognosis, and management. This study provides a primitive platform for non-invasive and cost-effective diagnosis and prognosis of patients with AIS using circulating cell-free mitochondrial DNA (cf-mtDNA) quantification and validation.

AIM

To evaluate the role of cf-mtDNA as s non-invasive, and affordable tool for real-time monitoring and prognosticating AIS patients at disease onset and during treatment.

METHODS

This study enrolled 88 participants including 44 patients with AIS and 44 healthy controls with almost similar mean age group at stroke onset, and at 24 h and 72 h of treatment. Peripheral blood samples were collected from each study participant and plasma was separated using centrifugation. The cf-mtDNA concentration was quantified using nanodrop reading and validated through real-time quantitative polymerase chain reaction (RT-qPCR) of NADH-ubiquinone oxidoreductase chain 1 (ND1) relative transcript expression levels.

RESULTS

Comparative analysis of cf-mtDNA concentration in patients at disease onset showed significantly increased levels compared to control individuals for both nanodrop reading, as well as ND1 relative expression levels ($P < 0.0001$).

Intergroup analysis of cf-mtDNA concentration using nanodrop showed significantly reduced levels in patients at 72 h of treatment compared to onset ($P < 0.01$). However, RT-qPCR analysis showed a significant reduction at 24 h and 72 h of treatment compared to the disease onset ($P < 0.001$). The sensitivity and specificity were relatively higher for RT-qPCR than nanodrop-based cf-mtDNA quantification. Correlation analysis of both cf-mtDNA concentration as well as ND1 relative expression with National Institute of Health Stroke Scale score at baseline showed a positive trend.

CONCLUSION

In summary, quantitative estimation of highly pure cf-mtDNA provides a simple, highly sensitive and specific, non-invasive, and affordable approach for real-time monitoring and prognosticating AIS patients at onset and during treatment.

Key Words: Cell-free mitochondrial DNA; NADH-ubiquinone oxidoreductase chain 1; Ischemic stroke; Circulating biomarker; National Institute of Health Stroke Scale score; Stroke assessment; Severity and outcome

Core Tip: Several blood biomarkers have been studied to determine the severity and outcome of ischemic stroke with limited applications. Hence, we need to establish molecular markers which can provide more comprehensive information on the stroke pathophysiology and treatment response. Dynamic quantification of plasma cell-free DNA appears to be a valid and reliable option. Hence, we compared the real-time expression of cell-free mitochondrial DNA (NADH-ubiquinone oxidoreductase chain 1 gene) in ischemic stroke patients with healthy controls and studies its prognostic value during the treatment. This study could aid in the development of clinical values for assessing real-time, non-invasive mode of ischemic stroke status in the future.

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INTRODUCTION

Stroke is one of the leading causes of morbidity and mortality worldwide[1]. Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2017 has reported stroke as the third major cause of death and disability[2]. According to the World Health Organization, the effective prevention approaches to stroke involve decreasing the risk of hypertension, lipid levels, fasting plasma glucose levels, smoking, reduced physical activity, unhealthy diet, and high body-mass index, which is similar to the observations from GBD 2016 and GBD 2017[3,4]. The findings of GBD 2019 Stroke Collaborators indicate that the majority of the global stroke burden (86% of deaths and 89% of disability-adjusted life-years [DALYs]) persists in lower-income and lower-middle-income countries[5]. Globally, over the past three decades, the number of stroke related DALYs has increased substantially (by 33.5 million, from 91.5 million in 1990 to 125 million in 2019).

Emergency stroke treatment depends on the type of stroke, either ischemic or hemorrhagic. The hemorrhagic stroke treatment depends on controlling the amount of bleeding and reducing pressure in the brain caused by the excess fluid. While intravenous administration of tissue plasminogen activator (TPA) remains the gold standard treatment for ischemic stroke[6]. Although progress in treating acute ischemic stroke (AIS) has been slow over the last decade, the goals for treating this frequent condition have grown. The therapeutic window should be increased from 3 to 6 h to improve the therapeutic efficacy because a small proportion of patients arrive for treatment after three hours. Hence, the beneficial clinical outcome of stroke patients relies on examining the proportion of patients receiving a timely and effective diagnosis.

Recently several studies have demonstrated that blood circulating cell-free DNA (cfDNA) analysis can efficiently distinguish ischemic stroke patients from other types of strokes or healthy populations[7,8]. CfDNA is extremely fragmented and circulates freely in the blood. As a result, cfDNA can be quantified easily in the blood through standard blood DNA extraction methods[9]. Blood has been one

of the most widely used biofluids for both clinical and research purposes, mainly due to its abundance and easy access; however, both plasma and serum are considered reliable sources due to their stability after long-term storage at ultra-low temperatures (-20 °C or -80 °C). Under normal physiological conditions, small DNA fragments can cross the blood-brain barrier (BBB) and reach into plasma or serum[10]. However, pathological conditions may cause disruption of BBB which increases its permeability and allows the open flow of several molecules, cells, and DNAs between the central nervous system (CNS) and the peripheral circulation[10]. Therefore quantification of cfDNAs in circulation can reflect pathological processes that occur in the CNS[9,11,12]. Although several studies have reported the significance of cfDNA quantification in distinguishing stroke patients[13], none of the studies have evaluated its role before and after treatment with TPA or any other mode of acceptable treatment. Further, blood cfDNA has not traditionally been considered an ideal test for a condition like a stroke[14].

The above-mentioned limitations are accompanied by total DNA estimation in circulation which includes both nuclear and mitochondrial DNA. Accumulating evidences have shown that cell free-mitochondrial DNA (cf-mtDNA) is released from damaged mitochondria into circulation which acts as damage-associated molecular patterns (DAMPs) with inflammation in several pathological conditions [15,16]. Moreover, alterations in mitochondrial dynamics affect energy metabolism and post-stroke neuronal function by regulating the number, morphology, and function of mitochondria. The increased interest in mapping several crucial pathways linking stress, mitochondria, and pathophysiology is fueled by recent discoveries implicating mitochondrial signaling in cellular and physiological stress management and mental health[17].

Although cf-mtDNA haplogroups have been demonstrated for their association with stroke onset, the impact of other differential roles of cf-mtDNA on stroke outcomes remains unclear. Hence, individual quantification of cf-mtDNA in circulation may provide a more clear and sensitive tool to overcome the current dilemma of cfDNA in stroke patients[18,19]. However, an ideal clinical evaluation of cfDNA using a simple procedure with good predictive values is required which could be utilized in both prehospital and emergency departments. More importantly, for early and accurate diagnosis of stroke patients specifically with ischemic stroke using cf-mtDNA is critical for early access to interventional therapy and to increase the likelihood of a favorable outcome.

Hence, in this study, quantitative estimation of circulating cf-mtDNA has been performed and validated through quantifying mitochondrial oxidative phosphorylation gene, NADH-ubiquinone oxidoreductase chain 1 (ND1) expression analysis in plasma samples of patients with ischemic stroke. Furthermore, the patients with onset of ischemic stroke have been differentiated from the control population using cf-mtDNA quantity and relative expression levels of ND1 before and after 24 h and 72 h of treatment with either TPA or antiplatelet medications. The diagnostic and prognostic significance of both cf-mtDNA concentration and relative expression levels of ND1 has been evaluated and correlated among the different categories of stroke patients. The results of this study may provide a crucial early and non-invasive tool for diagnosis and prognosis of ischemic stroke patients in real-time for tracking clinical response and improved clinical management.

MATERIALS AND METHODS

Study participants

All study procedures were carried out with the approval of the Institutional Review Board of Deccan College of Medical Sciences, Hyderabad. Informed consent forms were collected from each study participant. Our study group consisted of patients with AIS who were admitted to the stroke unit of our centre within the window period of 4.5 h after the onset of the stroke-related symptoms. A total of 88 individuals including 44 patients with AIS (27 men and 17 women) and 44 healthy controls (29 men and 15 women) with a mean age of 57.46 ± 13.16 years for AIS and 55.46 ± 11.13 years for healthy control were enrolled. All patients underwent a complete analysis of their neurological assessment. Stroke severity was assessed at the time of admission (referred to as baseline) using the National Institute of Health Stroke Scale (NIHSS)[20]. We included only those patients who showed positive treatment responses to either TPA or to antiplatelet therapy. All other patients who were non-responsive to these treatments were excluded. Further, patients with encephalitis, multiple trauma, sepsis, meningitis, hypertensive encephalopathy, migraine, intracranial tumor, post-cardiac arrest, organ failure, endocrine disorders, psychiatric syndromes, shock with hypoperfusion, or drug overdose were also excluded. We also excluded those patients in which the time from the symptom onset to blood collection was more than 12 h.

Sample collection and extraction of circulating cf-mtDNA

A total of 2 mL of venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-coated vacutainers from control individuals, and patients with AIS within 2 h window period (*i.e.*, within 4.5 h, referred to as a disease at onset), and after treatment at 24 h and 72 h. To ensure cell-free plasma collection, EDTA vacutainers were centrifuged at 3000 rpm for 15 min at room temperature. The

circulating cf-mtDNA was isolated from 300 μL of plasma sample using an in-house protocol established in our laboratory. Briefly, 300 μL of plasma was mixed with 1mL of mitochondrial isolation buffer (pH 7.8) and centrifuged at 3000 rpm for 10 min at 4 °C. After centrifugation, the supernatant was collected and again centrifuged at 10000 x g for 10-15min to collect the pellet containing mitochondria. The cf-mtDNA was extracted using an in-house rapid DNA extraction procedure and precipitated using cold 100% ethanol. Finally, the precipitated DNA pellet was air-dried and dissolved in 1X Tris-EDTA solution. The extracted cf-mtDNA was stored at -20 °C and used for quantification using nanodrop reading, and for mitochondrial DNA specific PCR of ND1 gene transcript.

Quantitative analysis of cf-mtDNA

Nanodrop reading: The extracted cf-mtDNA was quantified using a nanodrop reader at 260/280nm absorbance. A 260/280 ratio of approximately 1.8 and less than 2.0 was considered for pure DNA content without protein contamination and used for further analysis. The DNA concentration was recorded for each sample and reported in ng/μL.

RT-qPCR analysis: Cf-mtDNA concentration was further validated using SYBR Green-based real-time quantitative polymerase chain reaction (RT-qPCR). Plasma cf-mtDNA was quantified for the ND1 gene in a CFX-96 Real-time system (1000^{Tm} Thermal cycler, BIORAD) using gene-specific primers (forward: 5′-CTACTACAACCCTTCGCTGAC-3′ and reverse: 5′-GGATTGAGTAAACGGCTAGGC-3′). Mitochondrial 12S gene-specific primers were used as endogenous control. The RT-qPCR conditions included a single step of initial denaturation at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 30 s followed by a melt-curve. All the reactions were performed in triplicates in two different cohort studies with 100% PCR efficiency.

Correlation analysis: The correlation was established between cf-mtDNA concentration as well as ND1 relative fold expression in controls and patients at disease onset, and during the treatment at 24 h, and 72 h by computing Pearson correlation coefficient (r) and identifying *P* values. Further, correlation of cf-mtDNA concentration as well as ND1 relative fold expression was performed with NIHSS score at the baseline in patients with AIS during the disease onset to predict its clinical significance.

Statistical analysis

The statistical analysis was performed and data was recorded using GraphPad Prism software (version 5.0, and 8.4.2). The data were presented as mean ± SD. One-Way Analysis of Variance was used to compare the multiple groups, and a student t-test was used to compare two groups with a significance level $P < 0.05$ at a 95% confidence interval (CI). Relative operative curve (ROC) analysis was performed to predict the diagnostic value of cf-mtDNA concentration and ND1 expression in relative fold change of ND1 gene which was calculated using $2^{-\Delta\Delta Ct}$ method[21]. Correlation analysis was performed by calculating the Pearson correlation coefficient (r) among different groups. The *P* value was also calculated at 95%CI to demonstrate the statistical significance of positive and negative correlation between different groups.

RESULTS

Discriminative analysis and diagnostic value of cf-mtDNA concentration

Comparative analysis of cf-mtDNA concentration in patients at disease onset showed significantly increased levels compared to control individuals (mean difference (MD): -14.16 ± 1.691; CI: -17.55 to -10.76; $P < 0.0001$; Figure 1A). Further, comparison of cf-mtDNA concentration at 24 h (MD: 8.292 ± 2.146; CI: -12.65 to -3.933; $P < 0.0001$; Figure 1B) and 72 h (MD: 3.035 ± 0.6559; CI: -4.378 to -1.692; $P < 0.0001$; Figure 1C) with control individuals also showed significant increased values. ROC analysis of cf-mtDNA concentration in patients at disease onset showed significantly higher predictive value (AUC: 0.9808; $P < 0.0001$; Figure 1D) representing 69.71% sensitivity and 76.34% specificity. Likewise, the ROC at 24 h of treatment also showed significantly higher diagnostic value of cf-mtDNA concentration with 64.68% sensitivity and 55.91% specificity (AUC: 0.7290; $P = 0.029$; Figure 1E); however, it represented 25.18% poor diagnostic value to discriminate patients according to their response to treatment at 24 h. In contrast, at 72 h of treatment cf-mtDNA didn't show positive predicative value for the diagnostic significance and represented only 71.42% sensitivity and 50.18% specificity (AUC: 0.7500; $P = 0.1128$; Figure 1F).

Intergroup analysis and diagnostic significance of cf-mtDNA concentration

Intergroup analysis showed significantly reduced levels of cf-mtDNA concentration in patients at 72 h of treatment compared to the disease onset (MD: 11.12; CI: 0.7827 to 21.46; $P < 0.01$; Figure 2A). However, patients after 24 h of treatment didn't show significant difference in patients at onset (MD: 5.864; CI: -1.059 to 12.79; $P > 0.05$). Similarly, no significant difference was observed between 24 h and 72 h of treatment (MD: 5.257; CI: -5.982 to 16.50; $P > 0.05$). The ROC analysis of cf-mtDNA concentration at

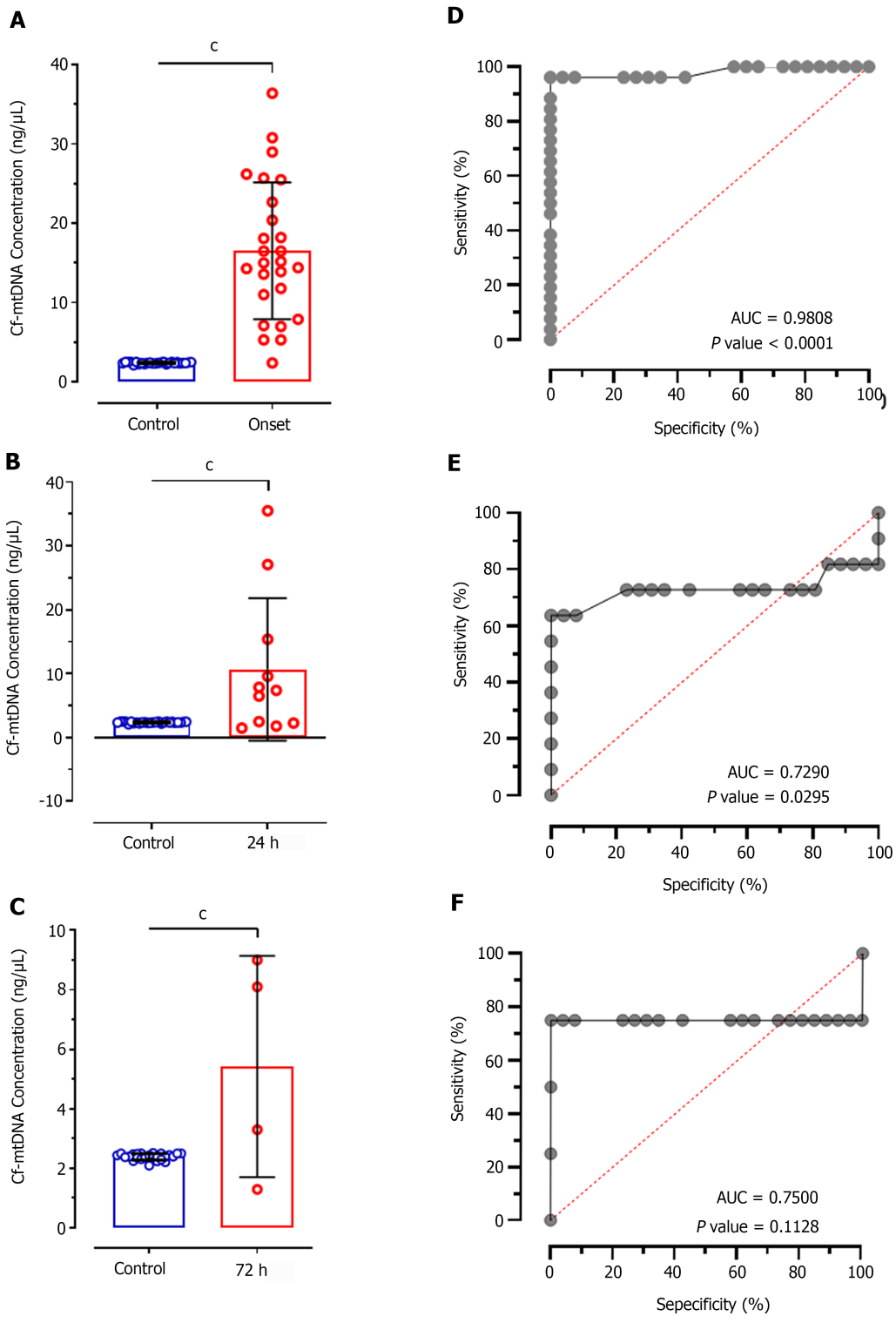


Figure 1 Difference in cell-free mitochondrial DNA concentrations and their diagnostic value in patients after treatment. A-C: Significantly increased levels of cell-free mitochondrial DNA (cf-mtDNA) were observed in patients at disease onset 24 h of treatment and 72 h of treatment compared to control individuals; D-F: Relative operative curve plots showing the diagnostic value of cf-mtDNA concentrations for discriminating the patients at disease onset 24 h of treatment, and 72 h of treatment. °$P < 0.0001$. AUC: Area under curve; cf-mtDNA: Cell-free mitochondrial DNA; ROC: Receiver operating characteristic.

24 h of treatment showed significantly higher predictive value (AUC: 0.7115; $P < 0.044$; Figure 2B) with 65.84% sensitivity and 55.12% specificity. Likewise, the ROC analysis at 72 h showed significantly higher diagnostic value of cf-mtDNA concentration with 84.25% sensitivity and 54.27% specificity (AUC: 0.8750; $P = 0.0173$; Figure 2C). However, no significant diagnostic value was observed for discriminating patients on treatment at 24 h and 72 h which is evident by only 57.14% sensitivity and 52.59% specificity (AUC: 0.5909; $P = 0.6015$; Figure 2D).

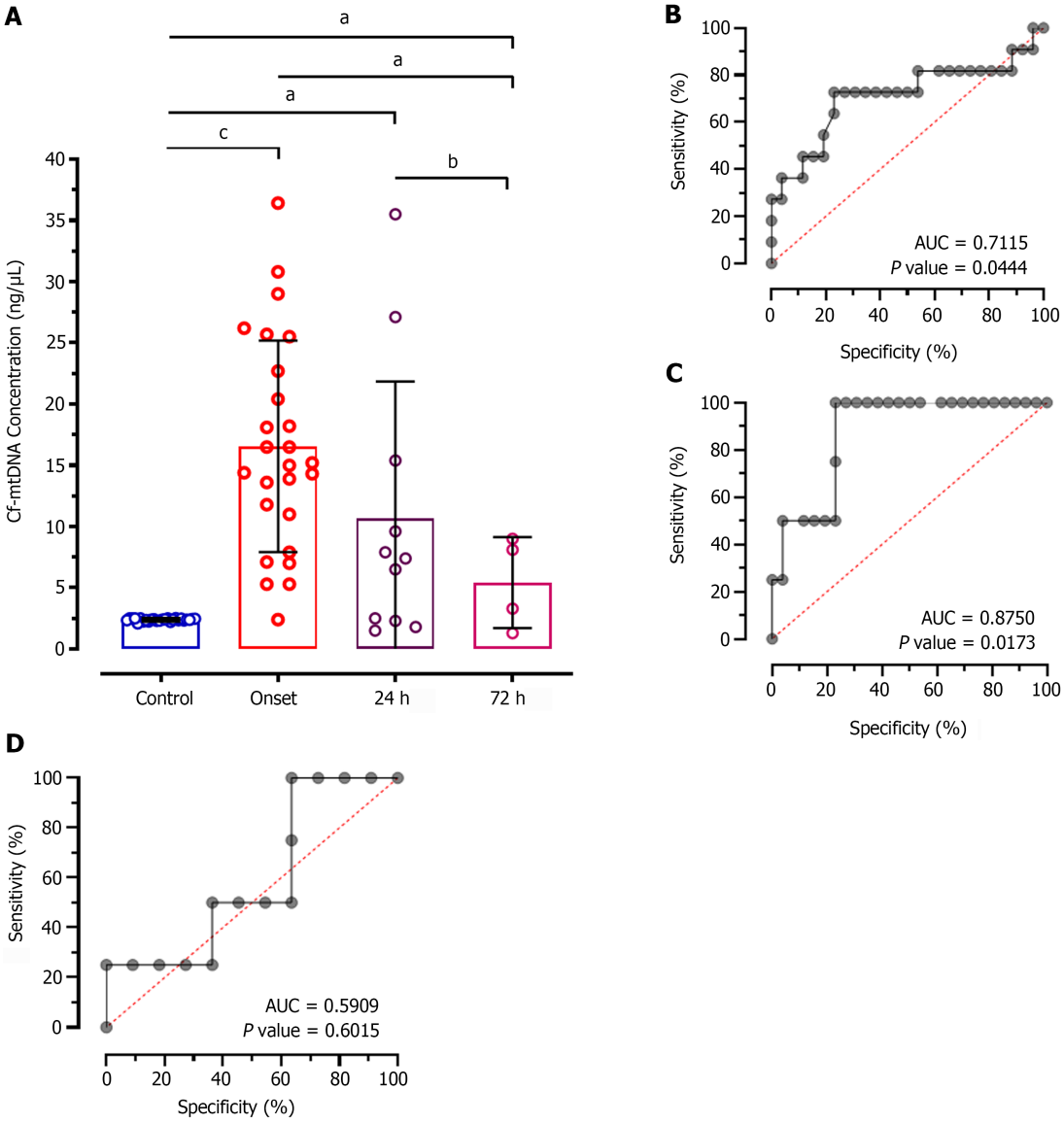


Figure 2 Intergroup differences in cell-free mitochondrial DNA concentrations and their diagnostic value in patients at disease onset and treatment. A: Significantly decreased cell-free mitochondrial DNA (cf-mtDNA) concentration was observed after 72 h of treatment compared to patients at disease onset [a]*P* < 0.01; B-D: Receiver operating characteristic plots showing the diagnostic significance of cf-mtDNA concentrations for discriminating patients at disease onset with 24 h of treatment, and 72 h of treatment. [b]*P* < 0.001; [c]*P* < 0.0001. AUC: Area under curve; cf-mtDNA: Cell-free mitochondrial DNA; ROC: Receiver operating characteristic.

Discriminative analysis and diagnostic significance of relative ND1 expression levels

The concentrations of cf-mtDNA in plasma samples was further validated using SYBR Green-based RT-qPCR analysis of Ct values of mitochondrial DNA sequence ND1 relative to 12S endogenous control. This analysis showed significant difference between Ct values of ND1 between control individuals and patients at disease onset (MD: -7.457 ± 2.030; CI: -11.56 to -3.359; *P* < 0.0001; Figure 3A), at 24 h of treatment (MD: -0.5316 ± 0.1454; CI: -0.8281 to -0.2350; *P* < 0.0001; Figure 3B), and at 72 h of treatment (MD: -0.6572 ± 0.1983; CI: -1.067 to -0.2479; *P* < 0.001; Figure 3C). ROC analysis for relative ND1 expression levels at disease onset showed significantly higher diagnostic value with 68.70% sensitivity and 72.44% specificity (AUC: 0.9021; *P* < 0.0001; Figure 3D). Likewise, ROC analysis at 24 h of treatment showed significantly higher diagnostic value of ND1 expression (AUC: 0.8115, *P* = 0.002) with 69.47% sensitivity and 62.65% specificity; however, it was 9% lower compared to disease onset patients. Almost similar diagnostic value was observed at 72 h of treatment (AUC: 0.9083; *P* = 0.002) with 82.66% and 59.80% specificity for ND1 relative expression.

Intergroup analysis and diagnostic significance of relative ND1 expression levels

Intergroup analysis of relative expression levels of ND1 showed significantly reduced levels at 24 h (MD: 6.926; CI: - 1.874 to 11.98; *P* < 0.001), and at 72 h of treatment (MD: 6.800; CI: 1.614 to 11.99; *P* < 0.001) compared to patients at disease onset (Figure 4A). ROC analysis showed significantly higher

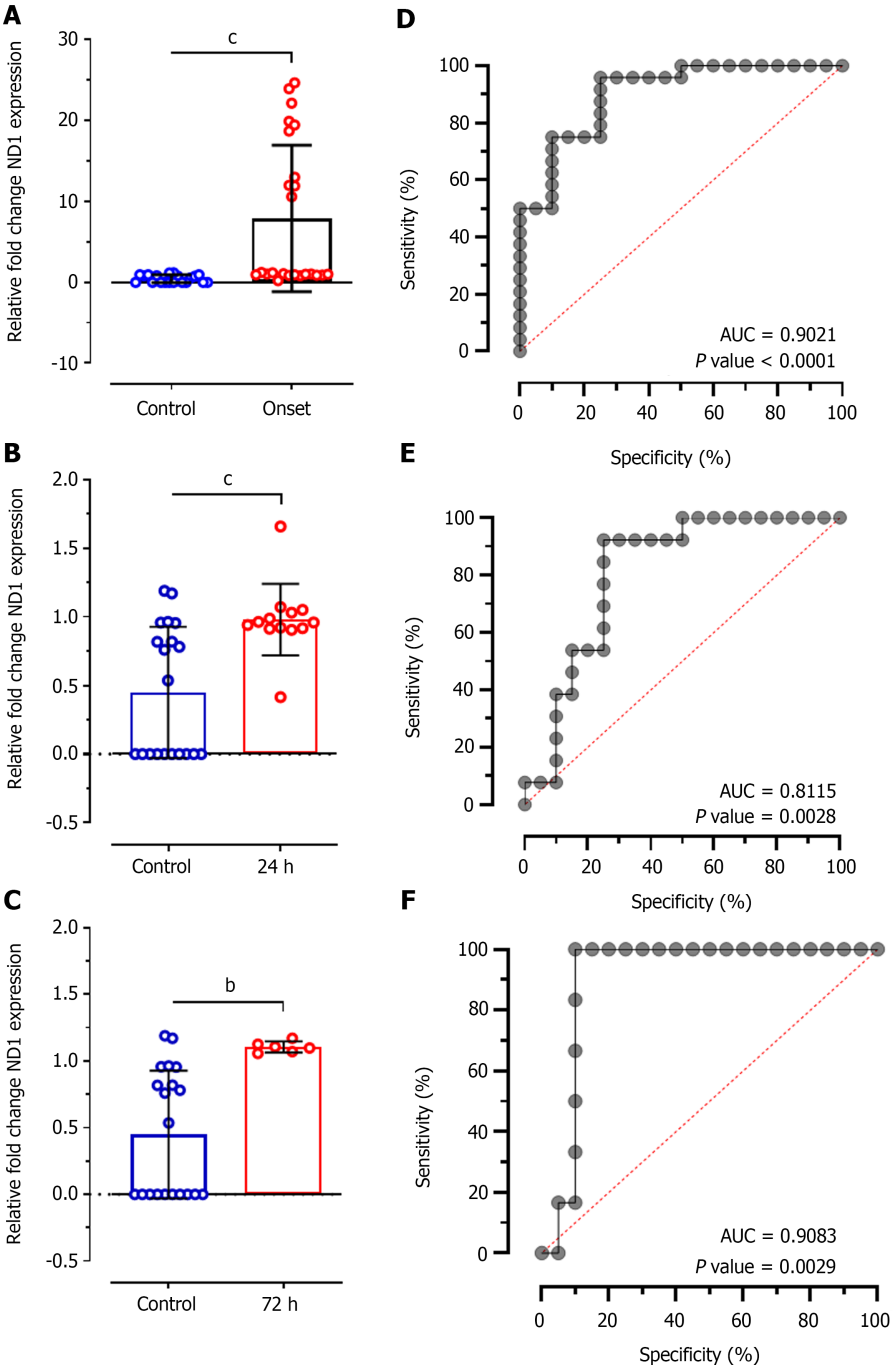


Figure 3 Intergroup differences in cell-free mitochondrial DNA concentrations. A-C: Changes in relative expression levels of NADH-ubiquinone oxidoreductase chain 1 (ND1) compared to 12S expression between control individuals and patients at disease onset, 24 h of treatment, and 72 h of treatment; D-F: Receiver operating characteristic plot showing the diagnostic significance of ND1 relative expression levels in patients at disease onset, 24 h of treatment, and 72 h of treatment. $^{b}P < 0.001$; $^{c}P < 0.0001$. AUC: Area under curve; cf-mtDNA: Cell-free mitochondrial DNA; ROC: Receiver operating characteristic.

diagnostic value of ND1 relative expression to discriminate patients at disease onset and at 24 h of treatment with 64.31% sensitivity and 57.75% specificity (AUC: 0.7147; $P = 0.033$; Figure 4B), and at 72 h of treatment (AUC: 0.8974; $P = 0.0007$; Figure 4C). While, no diagnostic significance of ND1 relative expression values was observed for discriminating patients at 24 h and at 72 h of treatment (AUC: 0.5139; $P = 0.89$; Figure 4D) and represented only 51.14% sensitivity and 50.28% specificity.

Correlation analysis of cf-mtDNA concentration and relative ND1 expression

Correlation analysis of circulating cf-mtDNA concentrations showed positive relationship between control and onset ($r = 0.17$; $P = 0.404$), control and 24 h of treatment ($r = 0.06$; $P = 0.863$; Figure 5A and B). However, negative correlation was observed between control and at 72 h of treatment ($r = -0.36$; $P = 0.642$), onset and at 24 h of treatment ($r = -0.40$; $P = 0.228$), and onset and at 72 h of treatment ($r = -0.09$; $P = 0.908$), and at 24 h and 72 h of treatment ($r = -0.82$; $P = 0.181$).

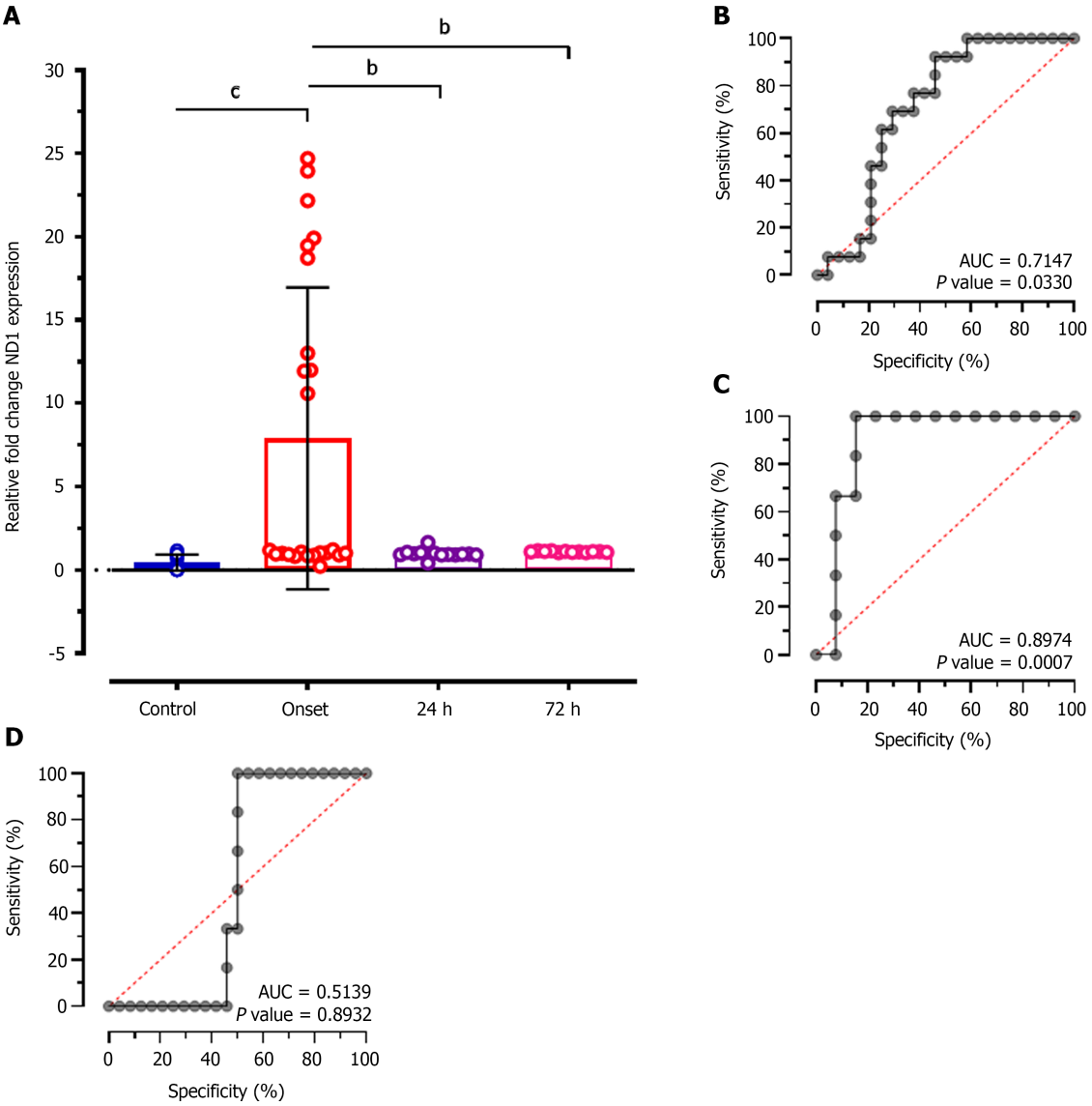
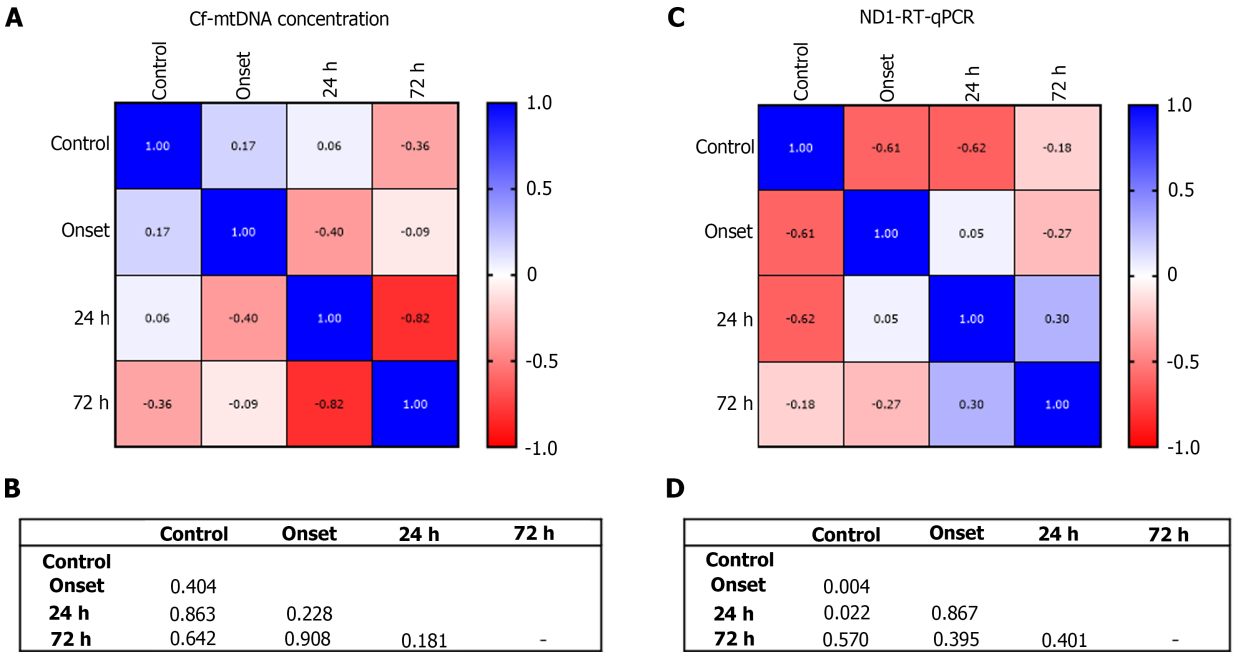


Figure 4 Multiple comparison test showing intergroup differences in NADH dehydrogenase 1 relative expression levels and its diagnostic significance. A: Significantly decreased NADH dehydrogenase 1 (ND1) relative expression levels were observed after 24 h and 72 h of treatment compared to patients at disease onset ($^{b}P < 0.001$) and were comparable to controls; B-D: Receiver operating characteristic plots show the association of ND1 expression levels between patients at disease onset, 24 h of treatment, and 72 h of treatment. $^{b}P < 0.001$; $^{c}P < 0.0001$. AUC: Area under curve; ND1: NADH dehydrogenase 1; ROC: Receiver operating characteristic.

Furthermore, correlation matrix analysis of relative expression levels of ND1 showed significantly negative correlation between control and stroke onset (r = -0.61; P = 0.004), control and at 24 h of treatment (r = -0.62; P = 0.022; Figure 5C and D). Although onset and at 72 h of treatment showed negative correlation; however, no statistical significance was achieved (r = -0.27; P = 0.395). While, correlations between other groups showed positive association including onset *vs* 24 h of treatment (r = 0.05; P = 0.867), and 24 h *vs* 72 h of treatment (r = 0.30; P = 0.401).

Multiple linear regression

Concentration of cf-mtDNA: The disparity of cf-mtDNA values didn't show significant deviation from its linear proportion and demonstrated continuous increasing trend for control *vs* patients with stroke onset (CI: 2.259 to 2.45; P = 0.128; Figure 6A), and control *vs* patients at 72 h of treatment (CI: 1.906 to 3.075; P = 0.83; Figure 6C). However, cf-mtDNA values for control *vs* patients at 24 h of treatment showed significant association in linear proportion with continuous increasing trend (CI: 2.255 to 2.543; P = 0.050; Figure 6B). Similarly, an increasing trend and non-significant deviation of cf-mtDNA concentration values were observed for stroke onset *vs* patients at 24 h of treatment (CI: 7.572 to 24.19; P = 0.356; Figure 6D), onset *vs* at 72 h of treatment (CI: 40.30 to 72.99; P = 0.279; Figure 6E), and at 24 h *vs* 72 h of treatment (CI: 17.01 to 78.58; P = 0.74; Figure 6F).



B

	Control	Onset	24 h	72 h
Control				
Onset	0.404			
24 h	0.863	0.228		
72 h	0.642	0.908	0.181	-

D

	Control	Onset	24 h	72 h
Control				
Onset	0.004			
24 h	0.022	0.867		
72 h	0.570	0.395	0.401	-

Figure 5 Correlation analysis showing differences in cell-free mitochondrial DNA concentration and relative expression levels of NADH dehydrogenase 1 among different groups. A: Heat map showing correlation coefficient r values represented within the heat map; B: *P* values showing differences in the association of cell-free mitochondrial DNA concentration among different groups; C: Heat map showing correlation coefficient r values represented within the heat map of ND1 relative expression; D: *P* values showing differences in the association of relative ND1 expression levels among different groups. Cf-mtDNA: Cell-free mitochondrial DNA; ND1: NADH dehydrogenase 1.

Relative ND1 expression levels: Although significant deviation of ND1 relative expression levels was observed between control and stroke onset patients (CI: 0.4319 to 0.8875; $P = 0.039$; Figure 7A), and control and at 24 h of treatment patients (CI: 0.8292 to 2.550; $P = 0.031$; Figure 7B). The control and at 72 h of treated patients didn't show significant deviation from its linear proportion and demonstrated a continuously increasing trend (CI: -9.746 to 9.316; $P = 0.7664$; Figure 7C). However, a significant linear association with continuous increasing trend was reported between onset patients *vs* at 24 h of treatment (CI: 0.2670 to 1.525; $P = 0.005$; Figure 7D), onset patients *vs* at 72 h of treatment (CI: 35.12 to 87.97; $P = 0.0001$; Figure 7E), and at 24 h *vs* at 72 h of treatment (CI: 7.757 to 4.86; $P = 0.021$; Figure 7F).

Correlation of NIHSS score at baseline with cf-mtDNA concentration and relative ND1 expression

The correlation analysis of baseline NIHSS score with cf-mtDNA concentration in all the enrolled study patients showed positive relationship ($r = 0.6353$; $P = 0.0001$; Figure 8A and B), which was evident with the increasing or decreasing trend of cf-mtDNA concentration values in relation to the higher or lower NIHSS score, respectively. Similar observations were reported for ND1 relative expression values when correlated with the baseline NIHSS score of individual patients ($r = 0.7277$; $P = 0.0001$; Figure 8C and D).

DISCUSSION

This study assessed the ability of plasma cf-mtDNA levels to determine its role in diagnosing patients with AIS. For quantitative estimation of cf-mtDNA, we opted two separate widely used, and highly specific and sensitive tools: (1) Nanodrop reading for cf-mtDNA concentration; and (2) RT-qPCR analysis for relative fold change in ND1 expression. These analyses were conducted in patients with onset of ischemic stroke and at 24 h and at 72 h of treatment with TPA or antiplatelet therapy. The findings of our study revealed significantly higher values of cf-mtDNA concentration as well as differences in relative fold expression of ND1 gene in AIS patients at the disease onset compared to healthy control participants. Both the assays had > 64% sensitivity and > 55% specificity. Although both the sensitivity and specificity were higher for ND1 expression, ROC analysis showed the higher diagnostic significance of cf-mtDNA concentration estimated through nanodrop reading (AUC: 0.9808) than RT-qPCR (AUC: 0.9021). While both the tools provide optimum outcomes, quantifying cf-mtDNA through nanodrop reading can provide a more easy-to-use, cost-effective, and sensitive tool for diagnostic implications in patients with AIS. However, such outcome measures can be seen only when we selectively extract and quantify cf-mtDNA rather than total cfDNA (that includes both nuclear and mitochondrial DNA).

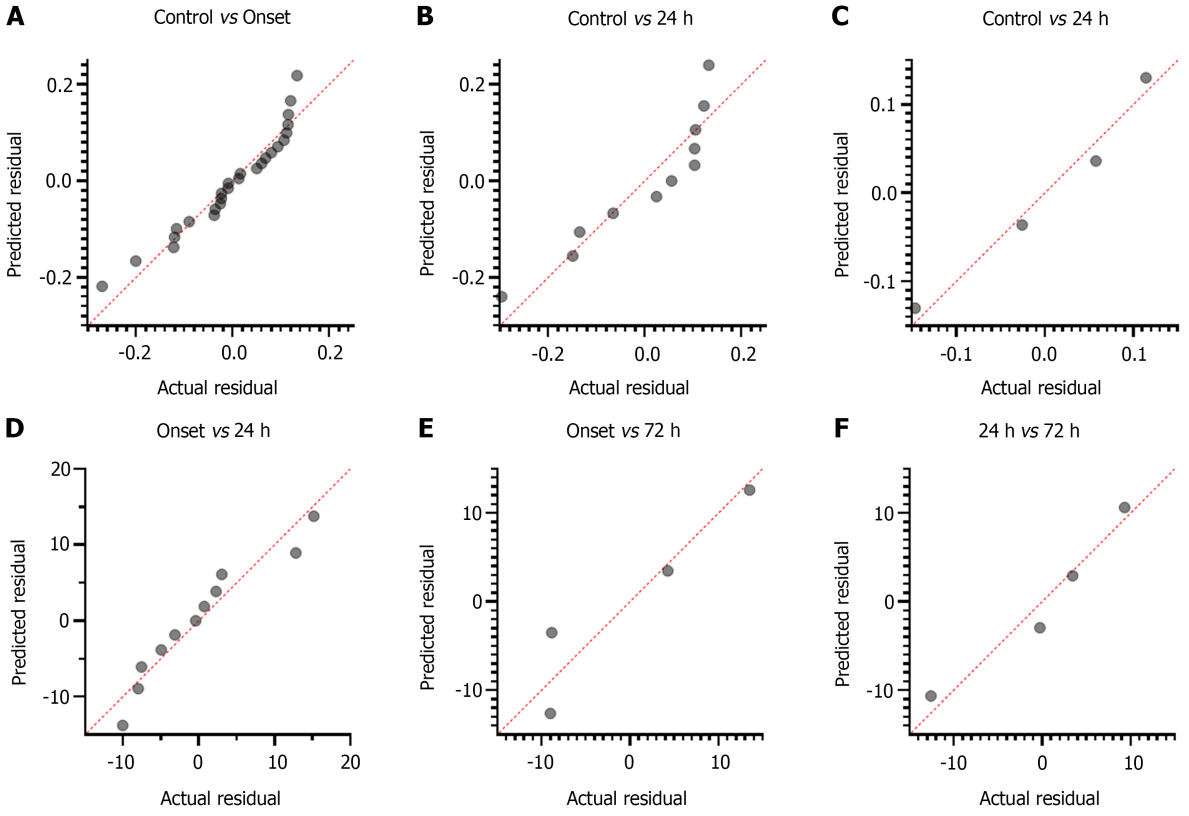


Figure 6 Multiple linear regression analysis. A-F: Multiple linear regression analysis of cf-mtDNA concentration between different groups showing their deviation and relationship with linear response. Cf-mtDNA: Cell-free mitochondrial DNA.

Although some of the earlier studies have reported a significant association of total cfDNA quantity in patients with stroke, they failed to demonstrate the individual role of cf-mtDNA and cf-nuclear DNA in discriminating AIS patients from the general population[7,12,13]. Some of the recent studies have also demonstrated significant involvement of cf-mtDNA in distinguishing stroke from healthy population [19]. Recent studies have also demonstrated that next-generation sequencing (NGS) can provide a more comprehensive tool for the quantification of mtDNA copy numbers[21-23]. However, the output from the NGS dataset depends on the ratio of sequencing reads of nuclear and mtDNA, but it allows analysis of thousands of available data sets which are shared by the research consortia. Although this technique enables high-sensitivity, high-throughput, and accurate assessment of mtDNA levels, a series of normalizations are required to correct for purity, counts, and batch biases[24,25]. Moreover, NGS poses a huge economic burden to the patients or their parents, hence tools presented in our study can be more widely applicable for exploring a non-invasive, simple, and cost-effective assessment with a reduced burden in developing countries.

In addition, we have also explored the usability of a quantitative assessment of cf-mtDNA before and during the treatment of AIS patients. Intergroup analysis of patients at onset and at treatment showed significantly reduced levels of both cf-mtDNA measured by nanodrop reading (84.25% sensitivity and 54.27% specificity), and ND1 relative expression (64.31% sensitivity and 57.75% specificity) assays at 72 h of treatment which was almost similar to the control individuals. These findings represent a highly significant diagnostic value for quantifying cf-mtDNA concentration as well as ND1 expression in prognostication of stroke patients. In accordance with our study results, a recent international, multicenter case-control study conducted on 3,498 cases of acute, first stroke from 25 countries showed buffy coat mtDNA copy number as a robust marker of post-stroke, and determinant of related outcomes [26]. Several other studies have also demonstrated the role of cf-mtDNA in various diseases and different types of cancers[27-29].

During the technical comparison, we observed that ND1 expression also provides a significant difference at 24 h, and at 72 h of treatment (P = 0.0007); however, nanodrop quantification of cf-mtDNA didn't reveal any such difference (P = 0.6015). Furthermore, cf-mtDNA concentration by nanodrop quantification failed to demonstrate a significant difference between onset and at 24 h of treatment patients (P > 0.05). However, RT-qPCR-based quantification of cf-mtDNA through ND1 expression showed a significant difference between values at stroke onset and at 24 h of treatment (P < 0.001) which also predicted significant diagnostic value between the groups during ROC curve analysis (P = 0.0330). This result revealed that RT-qPCR can provide more specific and sensitive information during prognostication of patients with AIS. Furthermore, correlation as well as multiple linear regression analysis in

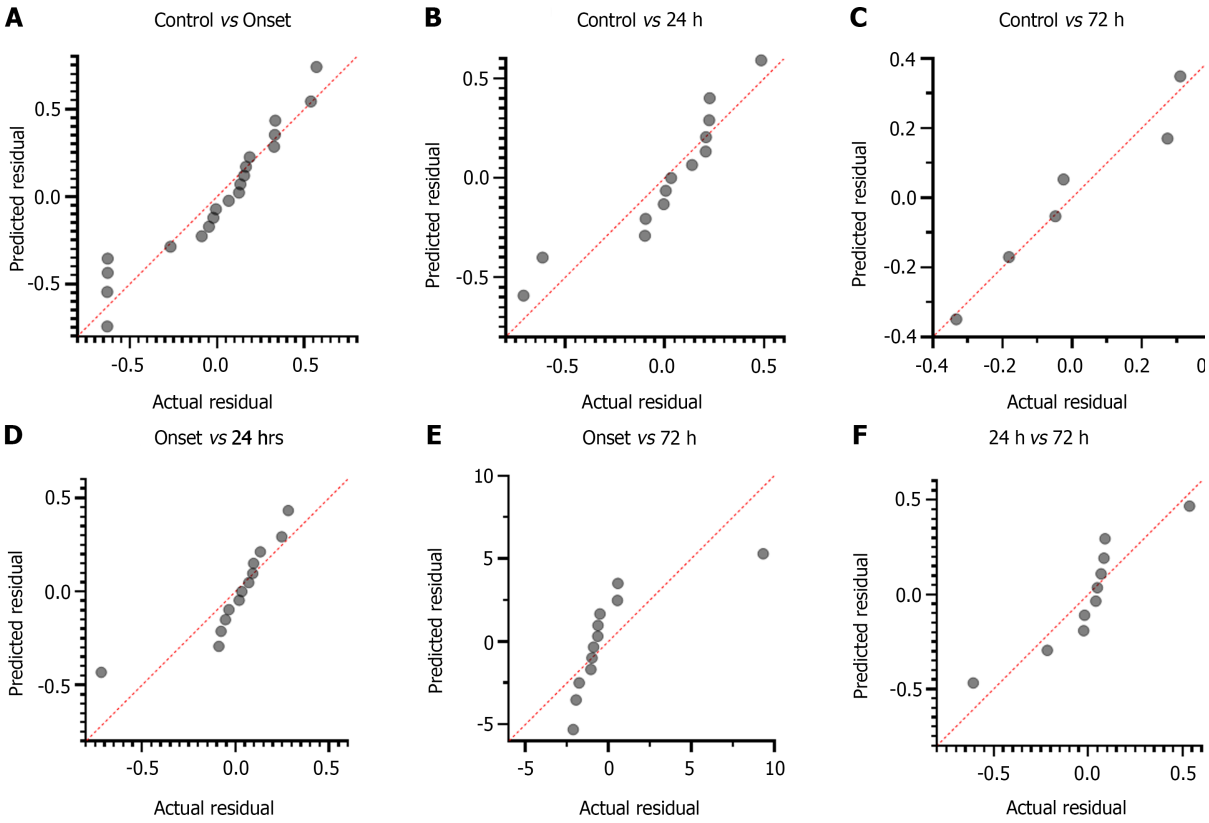


Figure 7 Multiple linear regression analysis. A-F: Multiple linear regression analysis of ND1 relative expression levels between different groups showing their deviation and relationship with linear response. ND1: NADH dehydrogenase 1.

our study showed relatively higher positive and negative predictive values, and diagnostic and prognostic significance of RT-qPCR-based ND1 expression compared to nanodrop-based cf-mtDNA concentration. Similarly, a study by Hernández-Jiménez *et al*[30] showed that mtDNA estimation can predict its applicability in differentiating severe AIS stroke patients with or without infections.

Comparatively lower sensitivity and specificity of cf-mtDNA quantification using nanodrop reading in our study can be explained based upon their source of release and mechanism of cell death. Cell death can be triggered either in form of apoptosis or necrosis which in turn releases cf-mtDNA in circulation. However, changes in cerebral blood flow, ischemia to the brain parenchyma, inflammation, and neuronal cell damage all contribute to apoptosis and neurological impairment in stroke patients and ultimately affect the amount of mtDNA release[8]. Hence, there is a chance of variability from patient to patient in cf-mtDNA concentration using nanodrop-based quantification. In contrast to this, ND1 is a high copy number gene in the mitochondrial genome which is the initial component of the oxidative phosphorylation system. Hence, quantifying ND1 relative expression levels or copy numbers may provide more specific information to predict cellular response in real-time[31]. Several studies support our findings for increased sensitivity and specificity of RT-qPCR-based analysis of cf-mtDNA [32-34].

Besides these findings, the type of cells involved in the release of the source mtDNA must be determined focusing on the molecular mechanisms responsible for the AIS that may provide crucial insights into understanding the pathophysiology and response to the treatment. Furthermore, specifically quantifying cf-mtDNA with associated co-morbidities and patient outcomes in long-term follow-up may produce a more specific response. In our study, 80% of cases had anterior circulation, while the number of cases in the remaining 20% was very less, so we couldn't separate the data based on anterior and posterior circulation. We also couldn't correlate our findings with the primary outcome measure after stroke using a modified Rankin scale score which can categorize independent stroke survivors from dependents. Therefore, further studies with a larger sample size and prolonged follow-up in correlation with more appropriate clinical outcome measures are required to validate cf-mtDNA quantification as a precise and reproducible tool to measure AIS severity and outcome in real-time.

CONCLUSION

Quantification of cf-mtDNA in circulation using nanodrop reading or RT-qPCR-based assays may

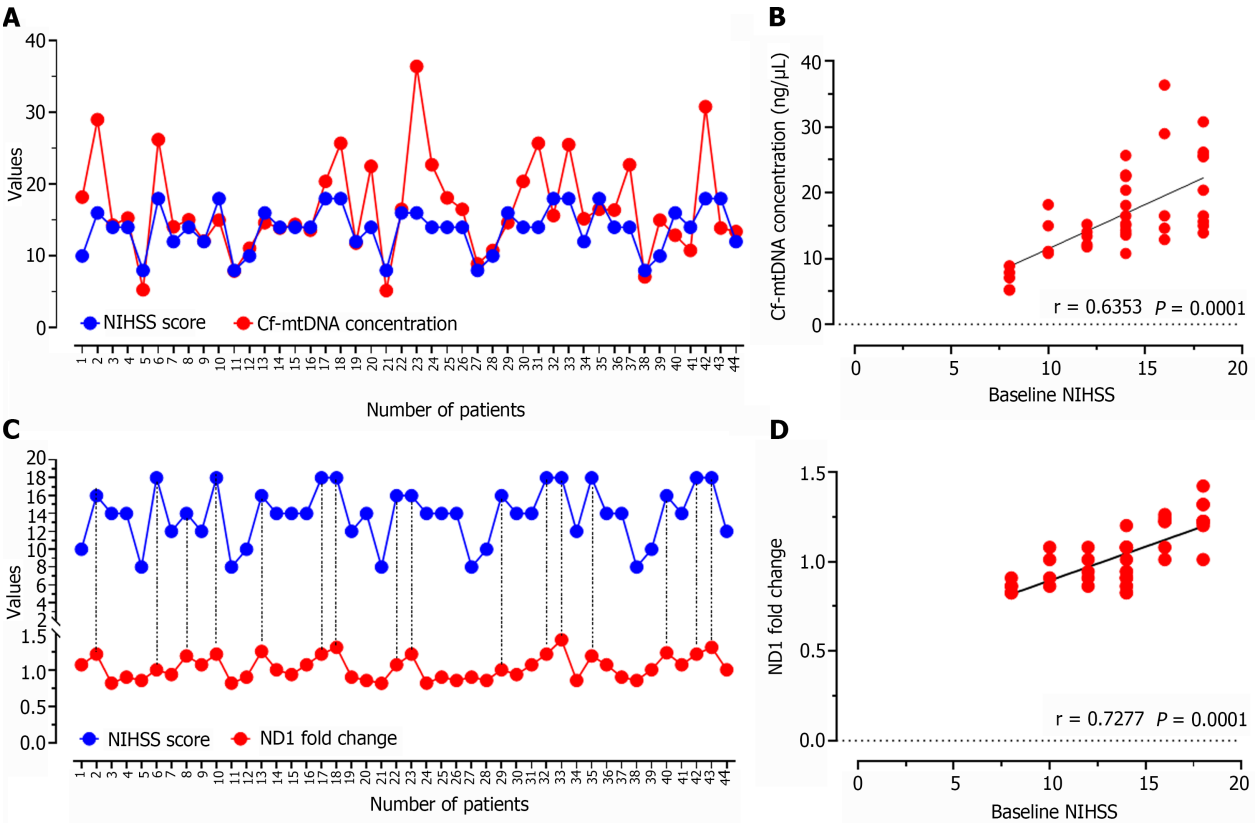


Figure 8 Correlation analysis. A-D: Correlation analysis showing a positive association between baseline NIHSS score and cell-free mitochondrial DNA concentration, and ND1 relative expression. Cf-mtDNA: Cell-free mitochondrial DNA; ND1: NADH dehydrogenase 1; NIHSS: National Institutes of Health Stroke Scale.

provide a simple, highly sensitive and specific, non-invasive, and affordable approach for real-time monitoring and prognostication of AIS patients at onset and during treatment. Hence, this approach may provide a widely acceptable and applicable platform at a relatively lower cost and less time for different clinical conditions other than AIS with further exploration.

ARTICLE HIGHLIGHTS

Research background

Role of circulating cell-free mitochondrial DNA (cf-mtDNA) in assessing disease status and treatment response of acute ischemic stroke (AIS) patients. Quantitative discrimination of AIS patients from the general population using cf-mtDNA. Compared sensitivity and specificity of nanodrop reading and real-time quantitative polymerase chain reaction (RT-qPCR) tools for quantifying cf-mtDNA.

Research motivation

AIS results in a continuously increasing rate of morbidity and mortality, and reduced quality of life worldwide. Cellular apoptosis and necrosis are major events during AIS. The amount of DNA present in circulation is directly proportional to the host cell's death and response.

Research objectives

To validate the quantitative role of cf-mtDNA in discriminating AIS patients from the general population and identifying the treatment response while comparing the sensitivity and specificity of nanodrop reading and RT-qPCR tools.

Research methods

Nanodrop reading and RT-qPCR were used to quantify cf-mtDNA in circulation. The sensitivity and specificity of both the assays were measured using relative operator characteristic (ROC) curve analysis. Correlation analysis of cf-mtDNA was performed with NIHSS score.

Research results

The findings of our study revealed significantly higher values of cf-mtDNA concentration as well as differences in relative fold expression of ND1 gene in AIS patients at the disease onset compared to healthy control participants. ROC analysis showed the higher diagnostic significance of cf-mtDNA concentration estimated through nanodrop reading than RT-qPCR. Intergroup analysis of patients at onset and at treatment showed significantly reduced levels of both cf-mtDNA measured by nanodrop reading, and ND1 relative expression assays at 72 h of treatment. During the technical comparison, we observed that ND1 expression also provides a significant difference at 24 h, and at 72 h of treatment; however, nanodrop quantification of cf-mtDNA didn't reveal any such difference.

Research conclusions

Quantification of cf-mtDNA in circulation using nanodrop reading or RT-qPCR-based assays may provide a simple, highly sensitive and specific, non-invasive, and affordable approach for real-time monitoring and prognostication of AIS patients at stroke onset and during treatment.

Research perspectives

This approach may provide a widely acceptable and applicable platform at a relatively lower cost and time for different clinical conditions other than AIS with further exploration.

FOOTNOTES

Author contributions: Fathima N performed lab experiments, collected the data, performed the statistical analysis, and wrote and formatted the manuscript; Manorenj S provided samples and clinical inputs for the study; Khan AA and Vishwakarma SK provided inputs, designed the study, helped in data analysis and presentation, and wrote and edited the manuscript.

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Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals.

Conflict-of-interest statement: All authors declare that they have no conflict of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: All authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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MINIREVIEWS

Photobiomodulation therapy for osteoarthritis: Mechanisms of action

Fábio Pericinoto Giolo, Gabriel Silva Santos, Victor Fontes Pacheco, Stephany Cares Huber, Kaue Franco Malange, Bruno Lima Rodrigues, Fernanda Bassora, Tomas Mosaner, Gabriel Azzini, Lucas Leite Ribeiro, Carlos Amilcar Parada, José Fábio Santos Duarte Lana

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Abstract

Photobiomodulation (PBM) is a non-invasive therapeutic modality with demonstrated effects in many fields related to regenerative medicine. In the field of orthopedics, in particular, PBM at various wavelengths has demonstrated the capacity to trigger multiple biological effects associated with protective mechanisms in musculoskeletal tissues. The articles cited in this review show that devices operating close to or within the near infrared range at low intensities can provoke responses which favor the shift in the predominant catabolic microenvironment typically seen in degenerative joint diseases, especially osteoarthritis (OA). These responses include proliferation, differentiation and expression of proteins associated with stable cell cycles. Additionally, PBM can also modulate oxidative stress, inflammation and pain by exerting regulatory effects on immune cells and blocking the transmission of pain through sensory neuron fibers, without adverse events. Collectively, these effects are essential in order to control the progression of OA, which is in part attributed to exacerbated inflammation

and degradative enzymatic reactions which gradually contribute to the destruction of joint tissues. PBM may offer medical experts ease of application, financial viability, efficacy and lack of serious adverse events. Therefore, it may prove to be a suitable ally in the management of mild to moderate degrees of OA. This review explores and discusses the principal biological mechanisms of PBM and how the produced effects may contribute to the amelioration of osteoarthritic progression. Literature was reviewed using PubMed and Google Scholar in order to find studies describing the mechanisms of PBM. The investigation included a combination of nomenclature such as: "photobiomodulation", "phototherapy", "laser therapy", "PBM", "osteoarthritis", low level light therapy", "inflammation" and "cartilage". We considered only articles written in English, with access to the full text.

Key Words: Photobiomodulation; Low-level laser therapy; Osteoarthritis; Inflammation; Regenerative medicine

Core Tip: Photobiomodulation (PBM) is a non-invasive therapeutic modality with demonstrated effects in regenerative medicine. In the field of orthopedics, in particular, PBM at various wavelengths has demonstrated the capacity to trigger multiple biological effects associated with protective mechanisms in musculoskeletal tissues. These responses include proliferation, differentiation and expression of proteins associated with stable cell cycles. Additionally, PBM can also modulate oxidative stress, inflammation and pain by exerting regulatory effects on immune cells and blocking the transmission of pain through sensory neuron fibers.

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INTRODUCTION

Photobiomodulation (PBM), often referred to as low-level laser (or light) therapy (LLLT), can be interpreted as a therapeutic modality which utilizes light to promote a wide variety of biological effects such as tissue repair, analgesia and reduced inflammation[1]. These effects can be attained *via* the use of a low-power light source, namely LASER (Light Amplification by the Stimulated Emission of Radiation) or LED (Light Emitting Diodes)[1]. Due to the standard low intensity, the treatment does not cause an expressive increase in the temperature of the target tissue. Additionally, for the same reason, the gross tissue structure is not significantly affected either[2]. One of the distinct features of PBM in comparison to other light-based treatments is that, since it operates within a low-intensity range, it does not provoke ablation. Instead, it is more likely attributed to a photochemical effect, where light is absorbed and chemical reactions in the cellular microenvironment are generated[3]. Furthermore, unlike photodynamic therapy, for instance, PBM does not require the application of photosensitizers. Once photosensitizing agents are activated by the corresponding wavelength, reactive oxygen species (ROS) are released. When ROS levels are not adequately regulated they end up triggering apoptosis and necrosis of target cells[4,5], which is the opposite effect of PBM. Another advantage of this therapeutic tool is that, in addition to being non-invasive, it has a broad range of applications. PBM can contribute in the treatment of various health conditions by playing significant roles in pain relief, promotion of wound healing and thus recovery of injured tissues[6]. It may therefore prove to be of great significance in regenerative medicine for the treatment of many injuries, including musculoskeletal injuries such as osteoarthritis (OA), in particular.

OA is still listed as one of the most common degenerative and progressive joint diseases and a major cause of pain and disability in the adult population, affecting 7% of the global population[7]. According to the Global Burden of Disease 2019 study results, the number of individuals affected by this condition increased globally by 48% from 1990 to 2019, classifying OA as the 15th most common cause of years lived with disability, in the same year[8]. This significant increase is likely attributed to extrinsic factors such as aging of the population and the incidence of poor dietary habits, especially in terms of metabolic syndrome[9-11]. This disease appears to be influenced by the complex interplay between local, systemic and external factors, which consequently dictate disease progression and the manner in which patients

respond to treatments[12]. It is typically characterized by a specific set of features encompassing the formation of osteophytes, continuous loss of articular cartilage, thickening of the subchondral bone, unbridled synovial inflammation, degenerative alterations of ligaments and menisci as well as joint hypertrophy[10]. Recent evidence[13] suggests that osteoarthritic progression is not exclusively linked to biomechanical trauma. The pathophysiology of the disease also appears to be closely associated with other biochemical processes, especially the imbalanced overproduction of oxidant molecules, such as ROS, which further aggravate oxidative stress and inflammation[13]. Several management strategies have been proposed albeit with not very optimistic results. Conservative methods, such as the administration of pharmacological agents, only promote temporary alleviation of pain but do not address the etiological source of the disease[13,14]. Non-pharmacological strategies have limited potential since they are usually limited to regular physical therapy, postural education, implementation of physical aids, nerve ablation and low impact exercise and weight loss programs, for example. In more severe cases, however, such as end stage OA, surgical intervention with joint replacement procedures may be inevitable and therefore extremely detrimental to the patient[12,13]. These hurdles motivated researchers to design more convenient non-operative alternatives, such as the application of PBM. Although optimistic results have been revealed, there is still a significant lack of consensus in the literature in regards to dose, power density, wavelengths, exposure duration, area irradiated, manual technique and even a minimum number of sessions for optimal clinical outcomes. The heterogeneity of reported studies makes standardization of PBM for the treatment of musculoskeletal diseases a challenging task. Although various mechanisms of action have been hypothesized and proposed, researchers have barely scratched the tip of the iceberg; the potential of PBM in regenerative medicine remains to be further explored. There may be multiple signaling pathways and mechanisms underpinning this technique, each playing a specific role and contributing to the regenerative processes, collectively. In this review we explore and discuss the biological potential of PBM and how its effects may contribute to the amelioration of osteoarthritic progression.

THE ORIGINS OF PBM

What is known today as 'photobiomodulation' first emerged almost 50 years ago in early experiments conducted by Hungarian physician Endre Mester at the Semmelweis Medical University[6,15]. Mester's goal was to shave the back of mice, implant a tumor *via* an incision and evaluate the effects of applied light from a ruby laser, with a wavelength of 694 nm. This was one of the first attempts to reproduce the studies described by Paul McGruff in Boston, in 1965[6,15]. At that time, ruby lasers were used to treat malignant tumors in rats and were later tested in human patients. Unbeknownst to him, Mester's equipment was only delivering a small fraction of the power recorded in McGruff's parameters. Due to this inaccuracy, the Hungarian physician was unable to successfully treat tumors. Interestingly, on the other hand, he noticed that the rate of hair growth in the treated mice was faster compared to the control group[16], naming this effect "laser biostimulation". Years later, Mester applied HeNe (helium-neon) lasers (632.8 nm) to stimulate wound healing in animals and, subsequently, in clinical studies[17]. It was long thought that coherent laser light was necessary, however, in recent years researchers found that non-coherent light sources such as LED carry as much potential as lasers in PBM therapy[18,19].

PARAMETERS

LLLT typically employs the use of light in the red or near-infrared region, where the wavelengths fall between 600 to 700 nm, and 780 to 1100 nm[6]. Due to the fact that this therapeutic modality can be applied to a wide variety of tissues and anatomical sites and every individual is unique, complications arise. The lack of standardized protocols in the literature generates much variance among reported results, and reproducibility therefore becomes difficult, further confounding an already complex field of study. Practitioners working with LLLT should ideally have a checklist in order to better understand and report all the necessary parameters for a reproducible scientific study (Table 1). Previously, Jenkins & Carroll[20] proposed the eight most important beam parameters to help researchers better report clinical and laboratory studies involving the application of photomedicine. According to the duo, these indispensable parameters encompass: power, wavelength, irradiation time, beam area (at the skin or culture surface), pulse parameters, number of treatments, anatomical location and interval between treatments[20].

Regarding dose, the three most commonly used parameters are time, energy and energy density. The authors further propose additional factors to consider, which may include coherence, application technique (projection, contact, scanning and pressure), spectral width and beam profile[20]. It is worth noting that beam power tends to decrease as a consequence of heat generation by the device itself and the inevitable aging of the equipment. This is why such pieces of technology must be routinely checked and calibrated accordingly before any experimentation is carried out. Measurement of beam area and power require precision and special equipment as well as experienced individuals in order to ascertain

Table 1 Technical specifications

Device	Considerations
Manufacturer	Important to consider well known sellers in the market, making wise cost-effective decisions
Device ID	For reference and tracking of malfunctioning equipment
Year produced	It is always best to choose the latest and most recent models to guarantee long-term success
Beam delivery system	Light can be delivered into the tissue *via* manual probe apparatus, fiberoptic or free air/scanned
Number of emitters	Left to the practitioner's decision
Emitter type	There are different types of laser such as KTP, LEDs, InGaAlP, and GaAlAs
Spatial distribution	Number of emitters and the distance between them as well as the pattern of distribution

KTP: Potassium-titanyl-phosphate; LEDs: Light emitting diodes; InGaAlP: Indium gallium aluminum and phosphorus; GaAlAs: Gallium-aluminum-arsenide.

more accurate data. Power readings must be monitored consistently throughout the applications procedure, which means that this should be done before and after each session, at frequent intervals. Selection of the correct wavelength is rather obvious but also important, because when erroneously configured, poor absorption occurs. According to the Grotthus-Draper law, without absorption there can be no reaction[21]. Additional observations include technical specifications of the device itself. A more complete and clear set of parameters has been created for ease of comprehension, as illustrated by Tables 1, 2 and 3, according to the variables and concepts proposed by Jenkins & Carroll[20].

Considering these variables, it is therefore evident that inappropriate parameters are likely to denigrate the therapeutic value of this modality. The concept of biphasic dose response curve (hormesis) is well-established for PBM. When variables such as radiant exposure, irradiance, delivery time and number of repetitions are either too high or too low, the desired results can often be negligible; sometimes, in the case of excessive photostimulation, the response can generate undesirable inhibitory effects[3]. This concept is based on the Arndt-Schulz law, where weak stimuli slightly accelerate vital activity. Conversely, stronger stimuli raise it further until a peak is reached, and even stronger stimuli suppress it until a negative response is achieved[22].

The relevance of these observations has been previously demonstrated. For instance, an *in vitro* study led by Karu and Kolyakov[23] revealed that stimulation of DNA synthesis rate depends on light intensity at a constant energy density of 0.1 J/cm^2 with a clear maximum at 0.8 mW/cm^2. A murine model of myocardial infarction proved that constant energy density and different irradiances after induced heart attack promoted beneficial effects at 5 mW/cm^2, whereas irradiances as low as 2.5 mW/cm^2 or as high as 25 mW/cm^2 had less significant results[24]. A similar study evaluated the reciprocity of exposure time and irradiance on energy density during photoradiation on wound healing in a murine pressure ulcer model. The authors learned that a fixed energy density of 5 J/cm^2 with only 8 mW/cm^2 irradiance enabled improvements in pressure ulcers in the treated mice[25]. It is worth noting that the proliferation rate of some cell types, such as fibroblasts, can be suppressed with inadequate energy delivery. Zhang and colleagues observed a significant increase in human fibroblasts after irradiating these cells at 628 nm with an energy density of 0.88 J/cm^2, an attenuated proliferation rate occurred at a staggering 9 J/cm^2, a parameter approximately 10 times more intense[26]. Despite these interesting findings, the World Association Laser Therapy guidelines recommend medical experts to use a dosage of at least 1 joule per target point, on 4 to 6 points for knee irradiation, specifically[27].

BIOLOGICAL EFFECTS OF PBM

PBM is rapidly growing and gaining recognition from many experts in the medical field due to its reported stimulatory effects on healing, tissue regeneration, attenuation of oxidative stress and reduction of pain and inflammation without causing severe side-effects[28]. Since osteoarthritic progression is in part correlated with an imbalanced overproduction of ROS and subsequent oxidative stress, application of PBM might prove to be a feasible tool in shifting equilibrium and managing some of the detrimental effects that is generated by this condition.

In addition to these effects, the literature also contains many studies reporting more interesting results arising from the application of this tool at varying wavelengths which may further assist in the fight against OA pathophysiological processes.

Proliferation

In numerous mammalian cells and tissues, light is absorbed by internal photoreceptors of the

Table 2 Photobiomodulation treatment parameters

Parameters	SI units	Additional notes
Exposure duration	Seconds (sec)	Some tissues may require more or less exposure duration, depending on the physical traits of the patient. For instance, obese individuals
Radiant exposure	Joules per centimeter squared (J/cm^2)	Intensity of the equipment must be adequately regulated depending on the different points to be irradiated. If the power density is too low, extending the irradiation time to reach the ideal energy density may not give an adequate final result. This should not be confused with "dose"
Number of irradiated points	-	Left to practitioner's decision depending on the treatment plan
Area irradiated	Centimeter squared (cm^2)	Area of target tissue must be carefully measured with precision for optimal results
Manual technique	-	Physicians must keep consistent pressure against the target point to ensure optimal delivery and penetration into the target tissue
Total number of sesssions	-	Number may vary depending on how the patient responds to the treatment
Session intervals		
Irradiance at target point	Milliwatts per centimeter squared (mW/cm^2)	This parameter must be adequately regulated depending on the different points to be irradiated, otherwise, the absorption of photons will not be sufficient to attain the desired result. Additionally, very high intensities may generate excessive heat
Beam spot size at target point	Centimeter squared (cm^2)	This must be carefully measured with precision for optimal results
Radiant energy	Joules (J)	Different tissues may require more or less energy according to the patient's unique physical attributes (*e.g.* skin pigmentation and mass)
Total radiant energy	Joules (J)	The total accumulated energy delivered per session and over all sessions

SI Units: International System of Units.

respiratory chain in the mitochondria, such as cytochrome c oxidase, subsequently inducing the activation of this organelle within cells[29]. The photons transmitted from a low-power laser, for instance, have been shown to be absorbed by mitochondria (Figure 1), causing an increased production of ATP, especially in mesenchymal stem cells (MSCs)[30]. When adequately stimulated with specific biochemical signals these cells are able to exert many biological roles (Figure 2) which are of great benefit in injured tissues. MSCs can secrete various cytokines and growth factors which, in turn, allow them to modulate neighboring cells *via* paracrine signaling effects. Moreover, they are also highly appreciated for their ability to perform self-renewal and further differentiation into specific mature cell lineages; therefore, enhancing tissue repair mechanisms[31].

A recent study has shown that adipose tissue-derived MSCs, in particular, display a substantial increase in proliferative and secretory activity when irradiated with a power density of 5 J/cm^2[30].

Low-level lasers (LLL) have demonstrated the ability to induce activation of several cell signaling pathways associated with proliferation and migration. Once induced, the tyrosine-protein kinase receptor, for example, can subsequently activate the MAPK/ERK signaling pathway, therefore promoting cell proliferation[32]. LLL can also cause phosphorylation of the PHAS-1 (protein heat and acid-stable) protein, which in turn up-regulates the expression of EIF4E (eukaryotic initiation factor 4E) and Cyclin D, further enhancing proliferative cycles. The EIF4E is a major regulator of cap-dependent mRNA and is known to respond to various biological stimuli including growth factors, hormones and mitogens[32]. Interestingly, LLL can also increase phosphorylation of the PI3K/AKT pathway, subsequently inducing the phosphorylation of mTOR, which ultimately leads to the phosphorylation of PHAS-1 in order to boost proliferative and migratory cell activity[33].

Nitric oxide and its associated signaling pathway also play a pivotal biological role by increasing angiogenesis and vasculogenesis. The expression of endothelial nitric oxide synthase, for instance, can be significantly increased in endothelial cells when irradiated with LLL set at a wavelength of 632.5 nm [34,35]. As a consequence, the enhanced proliferative and migratory capacities of these cells can then contribute to angiogenesis[34,35], which is an essential component that medical experts highly envisage when treating musculoskeletal diseases. In additional studies, LLL at 632.5 nm has also demonstrated the ability to activate the PLC-gamma pathway, which is responsible for catalyzing phospholipids and increasing the concentration of DAG and IP3. IP3 increases calcium levels from the endoplasmic reticulum in order to activate the PKC pathway. This signaling pathway is effective in cell proliferation, differentiation and apoptosis[36].

Table 3 Irradiation

Parameters	SI units	Additional observations
Operating mode	-	Physicians may select a continuous or pulsed wave, for example
Pulse on duration	Seconds (sec)	It is important to equally distribute time intervals between pulse on and pulse off cycles
Pulse off duration	Seconds (sec)	
Irradiance at aperture	Milliwatts per centimeter squared (mW/cm^2)	Irradiance can be significantly affected by the angular aperture of the light guide. For instance, irradiance measured with an aperture is greater than that without an aperture. Physicians should always keep this in mind
Aperture diameter	Centimeters (cm)	Values may vary significantly across different manufacturers and specific devices are better suited for different application objectives
Beam divergence	Radians or degrees (rad/deg)	Beam divergence may be an important variable depending on the nature and localization of the target tissue
Beam shape	-	The beams may be circular or elliptical, for instance
Laser beam polarization	-	The electric field vibration can be simple, with only one direction along the beam path (linear polarization) or it can be complex
Beam profile	-	Depending on the scenario (clinical or laboratory study), a specific profile may be indicated, such as Gaussian or Top Hat
Peak radiant power	Milliwatts (mW)	This variable must be carefully adjusted according to the target sample being irradiated
Average radiant power	Milliwatts (mW)	
Center wavelength (CW). And Spectral bandwidth (FWHM – range of wavelengths)	Nanometers (nm)	Practitioners must carefully select a suitable device with the appropriate wavelength and bandwidth specifications for the intended objectives. The FWHM (Full Width at Half Maximum) filter is important because outside the ideal bandwidth range light can be significantly attenuated
Frequency	Hertz (Hz)	The operator should always be aware of the frequency being applied to the area
Energy per pulse	Joules (J)	This parameter must be adequately regulated depending on the different points to be irradiated. Different tissues may require more or less energy per pulse. In clinical scenarios, the corporal density of each patient may vary significantly. In three-dimensional tissue cultures there are fewer layers of materials impeding light penetration and less scattering

SI Units: International System of Units.

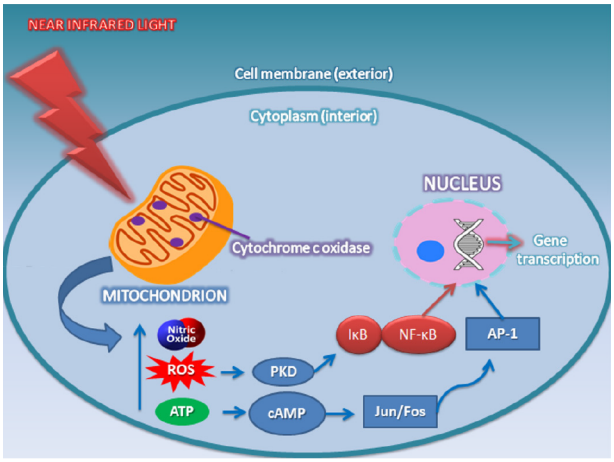

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Figure 1 Photobiomodulation at near infrared light stimulates a subset of biochemical reactions in the mitochondrion in order to trigger transcription of genes associated with positive biological effects. ROS: Reactive Oxygen Species; ATP: Adenosine Triphosphate; PKD: Protein Kinase D; cAMP: Cyclic Adenosine Monophosphate; IkB: IkappaB kinase; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; Jun/Fos: Proto-oncogenes; AP-1: Activator protein 1.

Another important signaling pathway stimulated by LLL is the JNK/AP-1, which is also illustrated in Figure 1. The laser can cause elevation in cAMP levels and subsequent JNK phosphorylation, increasing the production of AP-1. This protein, in turn, can augment the expression of target genes involved in proliferation, survival and angiogenesis, especially in MSCs[37].

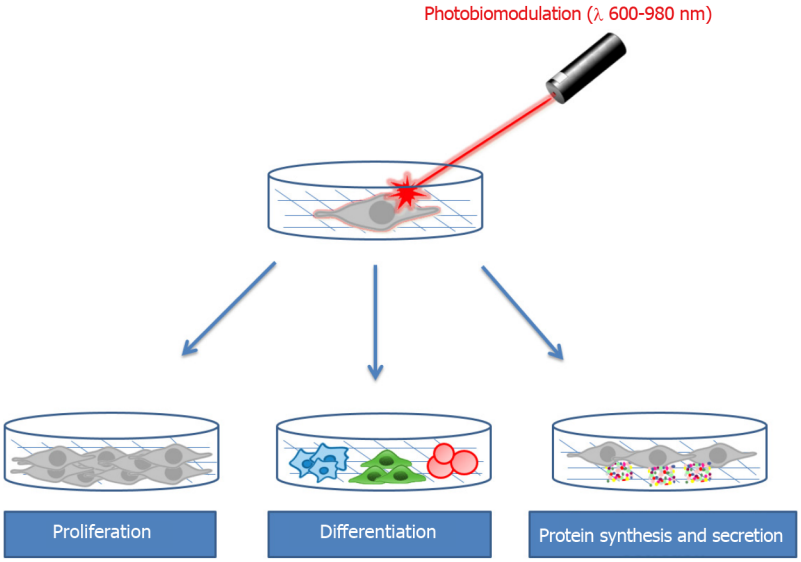

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Figure 2 *In vitro* application of wavelengths between 600 – 980 nm stimulates differentiation, proliferation and the secretion of specific cytokines and growth factors for further modulatory roles.

Interestingly, the ROS/Src pathway is also important. LLL can increase ROS, which regulate the activity of different protein kinases, especially Src. These kinases serve as a target for ROS, and this specific interaction triggers proliferation, migration and influences cell survival[37]. Although ROS are often linked to inflammation and degradation, it is worth noting that they are a "necessary evil" since they do play an essential role in natural redox signaling for the maintenance of physiological functions [13]. Under adequate regulation, low levels of ROS trigger the activation of other signaling pathways and initiate the cascade of various biological events. It only becomes a major problem when ROS are overproduced and surpass the amount of antioxidant compounds within the cells, and attack biological components such as proteins, lipids and DNA[13].

In the case of degenerative disorders such as OA, it is important to establish and maintain adequate stimulation of local cells in order to reverse or at the very least prevent aggravation of incessant apoptosis and degenerative effects. Activation of the p53 gene, for example, can inhibit cell growth and induce apoptosis by up-regulating the expression of BAX and P21 genes[37]. BAX is a pro-apoptotic protein of the BCL2 family, and is involved in numerous physiological and pathological processes. In fact, a recent study[38] revealed that chondrocytes from the articular cartilage of OA patients exhibit increased levels of BAX. These proteins are responsible for the delivery of apoptotic signals to the mitochondria, leading to the activation of Caspase-3 and, inevitably, chondrocyte apoptosis. However, LLL has been found to increase the expression of the BCL2 anti-apoptotic orthologues. BCL2 has been implicated in the regulation of apoptotic pathways, especially due to the fact that its increased expression appears to reduce the levels of BAX proteins[36,37,39].

Differentiation

In addition to generating multiple biological effects which favor the proliferation of cells, PBM also stimulates cell differentiation under various circumstances. According to previous research, the application of this tool on stem cells *in vitro* generates promising results in the regenerative medicine context. LLLT irradiation, for instance, is capable of activating intracellular and extracellular chromophores and the subsequent initiation of signaling pathways associated with differentiation events[40, 41]. More specifically, PBM or LLL set at the red or near-infrared wavelengths has been reported to trigger proliferation of stem cells and their differentiation towards the osteogenic lineage[42]. Interestingly, Wang and colleagues[42] in 2016, investigated the effects of PBM (blue and green light) on human adipose-derived stem cells (hASC). The authors examined the effects of four specific wavelengths (420 nm, 540 nm, 660 nm and 810 nm) on hASC cultured in osteogenic medium, at a dose of 3 J/cm^2 for five times with two-day intervals over a period of three weeks. Following RT-PCR assays, increases in the expression of RUNX2, osterix and osteocalcin proteins were observed. The blue and green (420 nm and 540 nm) wavelengths, in particular, caused significant increases in intracellular calcium, exerting a more significant effect in osteoblast differentiation when compared to 660 m and 810 nm. These results appear to be mainly attributed to the stimulation and activation of light-gated calcium ion channels by blue and green light, which suggests that these specific wavelengths may be useful in stimulating the differentiation of hASC towards a more specific cell lineage. In the case of certain musculoskeletal disorders, this feature may prove to be more or less attractive to medical experts,

depending on the individual needs of each patient.

Similarly, Fekrazad *et al*[43] investigated the application of single and dual combinations of laser wavelengths on bone marrow-derived MSCs from rabbits. In their set-up, the authors allocated the samples into one control and eight experimental groups as follows: infrared (IR, 810 nm), red (R, 660 nm), green (G, 532 nm), blue (B, 485 nm), IR-R, IR-B, R-G and B-G, respectively. The samples were irradiated every day for 21 days and then examined for cell proliferation and differentiation into osteogenic or chondrogenic lineages *via* analysis of RT-PCR biomarkers such as SOX9, aggrecan, COL2 and COL10 for cartilage; and ALP, COL1, and osteocalcin proteins for bone differentiation.

Proliferative activity was found to be increased in all PBM treatment groups except G. IR and IR-B promoted significant increases in the expression of all cartilage-associated genes but not COL10, which was actually attenuated by IR-B. In terms of osseous differentiation, a significant increase in ALP expression was observed in the R and IR treatment groups, whereas IR-R, IR-B and G diminished ALP expression. Furthermore, whilst COL1 expression was strongly stimulated by R and B-G, it was suppressed in the IR-B, IR-R and G groups. Osteocalcin expression was significantly increased only in the IR group and decreased in the B, B-G and G groups.

Overall, these recent findings show that cartilage differentiation appears to be significantly stimulated by the IR and IR-B wavelengths. Although in this study the effects of single or combined PBM irradiation procedures were not fully clear on osseous differentiation, osteogenesis appeared to be stimulated by the R and IR spectrum. Conversely, green light exhibited inhibitory effects. Therefore, at least in an *in vitro* experimental model of OA, PBM may convey beneficial effects. It must be considered that MSCs do not always display high proliferative capacities in culture, especially due to the fact that these cells may undergo replicative senescence during repeated passages *in vitro*, which would hinder potential clinical applications[44]. In addition, their ability to differentiate into more specific and mature cell types is quite restricted to a wide variety of biochemical signals, be it in the form of growth factors, proteins, physiological stress or even a combination of external sources of stimuli which can be induced with the application of PBM therapy (Figure 2).

In more recent developments, George and colleagues further elucidated other effects of PBM on the differentiation capacity of immortalized adipose tissue-derived stem cells (iASCs) into a specific cell line [44]. To elaborate, the authors determined the biological effects of low-intensity lasers on neurospheres generated from iASCs *in vitro*. NIR diode laser (825 nm) set at 5, 10 and 15 J/cm^2 with an average power output of 104 mW was applied in continuous waves to the respective culture plates *via* an optical fiber and adjusted to cover the entire area of the 35 mm diameter plate, achieving a spot size of 9.1 cm^2. The researchers learned that the irradiation procedure was capable of enhancing the differentiation of neurospheres into neurons. In particular, the power density of 5 J/cm^2 generated statistically significant increases in the early neuronal marker but not the expression of late neuronal markers. George *et al*[44] proposed that PBM is responsible for enhanced stem cell differentiation and, in this scenario, an increased yield of neurons by specifically modulating cellular metabolism and redox status. This is in parallel with similar results previously reported by other authors. These findings hold particular significance as PBM itself is more of a stimulatory tool (Figure 2) rather than an invasive technique. It has the capacity to guide the desired clinical outcomes towards differentiation of stem and progenitor cells into more specialized cell types without causing major alterations to the original tissue properties [44]. This allows medical experts and researchers to explore the clinical potential of these cells towards musculoskeletal health without raising major drawbacks linked to regulatory compliance[45].

NOCICEPTIVE MODULATION

Photoneuromodulation

PBM is also renowned for its analgesic effects and may therefore be indicated for mitigation of pain associated with different pathologies. For this very reason, application protocols may then vary in terms of appropriate wavelength, irradiance and fluence depending on the physiological properties and anatomical location of the specific target tissues[46]. Although not entirely clear, the mechanisms of action underpinning this technique have been previously hypothesized; it appears that PBM is capable of directly modulating the nociceptive response, thereby reducing pain transmission[46]. Zupin *et al*[46] recently conducted an animal study aiming to investigate the probable analgesic effects of 800 (WL1) and 970 (WL2) nm wavelengths on processed dorsal root ganglia of male mice at 6-8 weeks of age. Before induction of nociception, the mice were allocated into 4 groups as follows: Vehicle injection (saline) without PBM treatment; capsaicin injection without PBM; and capsaicin injections with PBM treatment protocols at WL1 and WL2, respectively. It was found that both wavelengths were effective in decreasing the production of ATP in neurons of murine dorsal root ganglia whilst increasing the intracellular levels of ROS and anion superoxide. ATP molecules are essential for proper functioning of the Na^+/K^+–ATPase system and subsequently the generation of action potentials[46]. Elaborating these concepts further, the PBM-mediated inhibition of this system may block the transmission of pain through sensory neuron fibers, promoting an analgesic effect, especially in painful conditions such as OA (Figure 3). Zupin and colleagues observed that the rodents pre-treated with PBM at 970 nm

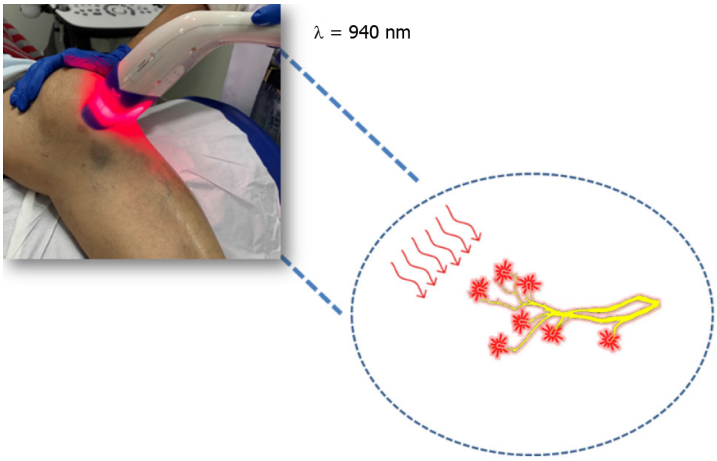

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Figure 3 Photobiomodulation at a wavelength of 940 nm blocks the transmission of pain through sensory neuron fibers, thus promoting analgesic effects.

exhibited a far less reactive response to nociceptive stimuli after having their paws inoculated with capsaicin. This was mainly expressed by minimal licking, biting or shaking of the treated limbs in comparison to the other treatment groups. These observations are also in alignment with the findings of the systematic review conducted by Chow *et al*[47] where ROS were shown to cause axonal varicosities, subsequently leading to the blockade of fast axonal flow in the small diameter Aγ and C fibers, which are involved in nociceptive mechanisms. Also, in a similar study conducted by Wang *et al*[48], a wavelength of 980 nm was able to promote a thermal response and activation of the TRPV1 in adipose tissue-derived stem cells. The TRPV1 is a specific type of receptor mainly found in nociceptive neurons of the peripheral and central nervous system, thus is involved in the transmission of pain and integration of diverse painful stimuli[49]. Temperature increase at the cell membrane is a possible mechanism whereby TRPV1 is activated, suffers reduced activity upon the given stimuli and then subsequent desensitization toward capsaicin stimulation[46]. In simple terms, PBM at wavelengths close to or within the NIR range can reduce TRPV1 activity and calcium flow, thus is more effective in diminishing nociceptive responses (Figure 3), at least *in vivo*. Interestingly, other authors have shown that 830 nm continuous wave lasers are also capable of reducing the velocity of action potential conduction, increasing latencies in the median and sural nerves, and producing analgesic effects[50]. In additional rodent studies, PBM also suppressed nerve conduction in myelinated Aδ and unmyelinated C fibers[51,52].

Inflammatory photoregulation

PBM can also exert 'inflammomodulatory' roles by influencing secretory activity in cells (Figure 4). This is also essential in the attenuation of nociception in many disorders where inflammatory stress is escalated due to exacerbated pro-inflammatory cytokine secretion, especially in OA patients. Yamada *et al*[27] recently investigated the effects of PBM on the knees of rats after inducing OA with intra-articular injections of MIA (monosodium iodoacetate). In comparison to the control group (saline), MIA mice had significantly higher levels of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukins (IL)-1β and IL-6. The authors then designed a PBM treatment with a wavelength of 904 nm at doses of either 6 J/cm^2 or 18 J/cm^2 and applied them to the rat knees 3 d per week, for eight sessions. Capsule, menisci and cruciate and collateral ligaments samples were collected from knees and the corresponding biochemical assays were performed. The researchers then observed that the dose of 18 J/cm^2, in particular, proved to be far more effective in reducing the levels of the aforementioned pro-inflammatory cytokines. The effects of this specific parameter appeared to be more expressive from the fourth day of treatment and continued until the eighth day, when the researchers noted that the protective behavior in MIA mice was significantly diminished, indicating reduced pain. This observation is most likely attributed to a decrease in the levels of inflammatory mediators as well.

Furthermore, PBM can also increase antioxidant capacity and dampen oxidative stress damage at distant sites, which also contributes to attenuation of pain by peripheral and central sensitization reduction[53]. This tool has been shown to be responsible for increasing the levels of the enzyme superoxide dismutase (SOD), which provides an essential antioxidant defense system against oxidative stress. SOD acts as a good therapeutic agent against ROS-mediated diseases[27]. In the case of OA, in particular, SOD is often found to be scarce and in association with increased levels of nitric oxide, leading to mitochondrial dysfunction and subsequent chondrocyte apoptosis[54]. Cartilage degeneration alone is not the principal culprit in OA-associated pain as this structure has no sensory innervation. However, activation of the primary afferent nerve fibers of the knee joint might be. The

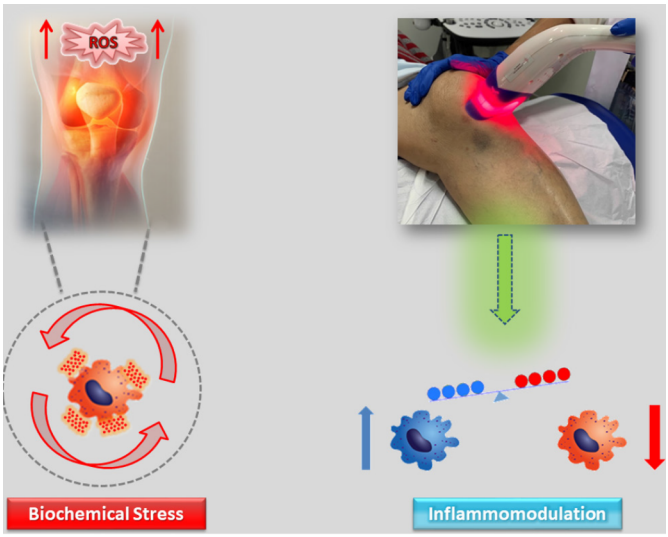

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Figure 4 The application of photobiomodulation between 600 – 980 nm can attenuate inflammation by decreasing the production of pro-inflammatory agents and regulating the activation of immune cells.

predominant pro-inflammatory microenvironment in osteoarthritic knees is outlined by extensive cellular damage, mast cell degranulation, and unbridled secretion of inflammatory mediators and enzymes which sensitize nociceptors[55]. Nociceptors usually respond to noxious stimuli with more intensity, however, they may also 'perceive' and interpret non-harmful stimuli as painful due to peripheral sensitization. Overexposure of the periphery to nociceptive signals in turn increases excitability and activity in the spinal cord, giving rise to central sensitization[27,56].

This is why resolution and control of inflammatory stress is of utmost importance in the treatment of these degenerative disorders. Synovitis, for instance, is a relevant source of both pro-inflammatory and anti-inflammatory agents. Synoviocytes are known to secrete TNF-α, IL-1β, and IL-6, facilitating cartilage degeneration and joint hyperalgesia, which contribute to OA progression[57,58]. TNF-α does not only activate sensory neurons but also stimulates the production of interleukins, giving continuity to an inflammatory cascade. IL-6, in particular, is highly detrimental as it stimulates the synthesis of matrix metalloproteinase-1, an enzyme involved in the breakdown of extracellular matrix and cartilage degradation[59]. Unresolved inflammation progresses towards chronic biochemical stress, where cytokines may trigger ROS overproduction, aggravating pain and oxidative stress, generating a complicated negative feedback loop (Figure 4) which is difficult to break[9,13,60,61]. PBM has demonstrated the potential to contribute to the alleviation of exacerbated biochemical stress and chronic inflammation through its capacity to downregulate the expression of various pro-inflammatory mediators in different scenarios[27,62,63].

Regulation of immune cell activity is also another robust attribute of PBM (Figure 4). Neutrophils, in particular, are well-known for their immunological properties as well as their inflammatory and hyperalgesic reactions that arise from their increased activity[64]. Migration of these cells from circulation to the peripheral tissues, for instance, is significantly intensified in patients with knee OA; their infiltration into synovial tissues can be quite harmful to the knee joint due to the release of proteolytic enzymes[27]. This means that, upon receiving nociceptive stimuli, neutrophils react and further amplify inflammation by subsequently producing more biochemical signals to recruit more neutrophils and additional immune cells into the site of tissue injury, which is especially true for osteoarthritic patients[27,65]. In comparison to their inactivated states in circulation, neutrophils are more reactive with increased activity in peripheral tissues, where they release more cytokines and inflammatory mediators that contribute to the aggravation of pain and inflammation[66,67]. More specifically, the leukocyte-derived enzyme myeloperoxidase is released by these immune cells, catalyzing the formation of ROS and therefore contributing to extensive tissue damage during inflammation[68,69]. These detrimental effects can and have been reversed *via* the application of PBM in the study by Yamada *et al*[27], where the dose of 18 J/cm^2 successfully reduced the increased MPO activity associated MIA-induced OA in the knees of mice. This study provides sufficient evidence to indicate that PBM may effectively reduce neutrophil migration and MPO release, consistent with reported changes in pain, which then again, may reflect attenuated oxidative stress. Lastly, this may also suggest a secondary systemic effect associated with PBM.

CONCLUDING REMARKS

The application of PBM could prove to be a feasible tool in managing some of the detrimental effects generated by mild to moderate degrees of OA. PBM has recently shown interesting results in the literature, especially in regards to the proliferation and differentiation of MSCs towards osteogenic and chondrogenic lineages, for example. This could be particularly beneficial in the treatment of some musculoskeletal disorders, such as OA, under specific circumstances. This technique has multiple comparable benefits, at least in terms of ease of application, non-invasiveness, no serious adverse effects, financial viability and efficacy particularly for pain alleviation. These are perhaps the most prominent features which a debilitated patient primarily seeks when he or she walks into the clinic, as OA pain alone is often the most expressive symptom and driver in clinical decision making. Although photomedicine is slowly expanding and showing some optimism in the medical community, additional studies are highly warranted in order to further elucidate and support its regenerative medicine potential in musculoskeletal disorders.

FOOTNOTES

Author contributions: Santos GS and Giolo FP wrote the manuscript; Pacheco VF and Huber SC proposed the research subtopics; Rodrigues BL, Malange KF and Bassora F were responsible for navigating the literature and sharing the relevant studies included in this review. Mosaner T formatted the citations and compiled the references; Parada CA revised and formatted the body of the manuscript, correcting spelling, punctuation and grammatical errors. Azzini G and Ribeiro LL created the figures and tables. As the leader, Lana JFSD was responsible for reviewing and approving all the modifications made to the manuscript, from draft to final version.

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