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IN VITRO MODELS OF DIABETES - REVIEW AND CRITICAL ANALYSIS

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In vitro models of diabetes - review and critical analysis

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In vitro models of diabetes - review and critical analysis

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Abstract

Diabetes mellitus (DM) is a metabolic disease, and its prevalence has increased over the last decades, affecting more than 8.6% of the world population. Despite extensive research, the mechanism of diabetes development and its complications are still not fully understood. The present review was designed to describe *in vitro* methods used to study diabetes mellitus, with a critical analysis of their advantages and limitations. We searched Pubmed database for free full-text articles that were published between 2014 and 2019 that contained "*diabetes mellitus*" and "*in vitro*" in their content and restricted the result to human studies using the species filter. Clinical reports were excluded. The search and filtering of articles rendered 927 results, of which 25 were used in this review. The majority of studies used glucose modulation models, even though primary cells and serum derived from DM patients were also used, in addition to varied strategies focused on mimicking isolated aspects of the disease. Therefore, different model systems are available for the study of diabetes mellitus *in vitro*, each with its own set of advantages and limitations. The

present review will provide relevant information, which may support the appropriate choice of experimental models in the field.

Keywords: Diabetes mellitus, *In vitro*, Model, Review

1. Introduction

The term Diabetes mellitus (DM) describes a group of metabolic disorders characterized by high blood glucose levels. People with diabetes have an increased risk of developing a number of serious life-threatening health problems resulting in higher medical care costs, reduced quality of life, and increased mortality. The global prevalence of diabetes is increasing over the last decades. It was estimated that in 2017, there were 451 million people (age 18–99 years) with diabetes worldwide, and these figures are expected to increase to 693 million by 2045 [1]. Despite the different etiology of type 1 and type 2 diabetes, no separate global epidemiological data exists [2]. The mechanisms by which elevated glucose levels lead to tissue damage are multifaceted, and nowadays, DM is the leading cause of end-stage renal disease and blindness in Europe and North America. Moreover, it increases the risk of microangiopathies and the development of cataracts, erectile dysfunction, and cognitive impairment, like Alzheimer's disease [3].

Type 1 diabetes (T1DM), which is an insulin-dependent type of diabetes, is a chronic disease affecting genetically predisposed individuals, in which insulin-secreting beta (β)-cells within pancreatic islets of Langerhans are selectively and irreversibly destroyed by autoimmune assault. Type 2 diabetes (T2DM) is characterized by a gradual decrease in insulin sensitivity in peripheral tissues and the liver (insulin resistance), followed by a gradual decline in β-cell function and insulin secretion. Despite their differences, both T1DM and T2DM patients suffer from inadequate amounts of insulin production in order to maintain normoglycemia [4].

The present article aims to provide both an overview of the most relevant *in vitro* models currently in use for diabetes research and a critical analysis of each in regards to its advantages and limitations. The following topics are considered: general discussion of *in vitro* and *in vivo* experimental models for DM research, glucose modulation strategies,

primary cell cultures derived from DM patients, cell culture with DM patients' serum, and strategies focused on isolated aspects of the disease, as shown in table 1 and 2.

2. Materials and methods

We searched Pubmed database for free full-text articles that were published between September 1st, 2014, and September 1st, 2019, that contained "diabetes mellitus" and "*in vitro*" in their content and restricted the result to human studies using the species filter. Clinical reports were excluded. After the filtering, the search rendered 927 papers, of which 25 were used in this review, as seen in table 1. A summary of the models used and their advantages and limitations are shown in table 2.

3. Results and discussion

3.1. *In vitro* and *in vivo* experimental models for DM research: general discussion

DM is a severe and chronic disease associated with complex comorbidities and high economic impact. Therefore, the search for a better understanding of the disease pathophysiology is currently an unmet need to discover, validate, and optimize preventive and therapeutic strategies. In order to study the diverse DM aspects, different tools and experimental settings are available, all of which present advantages and limitations. Therefore, for each scientific question, it is crucial to analyze and carefully select the most adequate experimental model. For instance, while *in vivo* studies provide physiological details of DM and associated comorbidities, the complexity of the system generates many

variables that are hard to account for and can compromise data analysis. Furthermore, as revised by Kleinert *et al*., non-mammalian models of DM may be attractive alternatives for DM research, due to their low cost and short life cycle, but are limited due to their distinct genetic and physiology. Rodents, on the other hand, are extensively used for research in several fields, due to the apparent familiarity of researchers with those cost-effective models, and the possibility of genetically modifying them, but in the context of DM research, such animals lack genetic diversity and differ in their pancreas architecture from that of humans [5].

Considering the complex etiology of DM and the frequent presence of comorbidities in patients, the possibility of isolating variables is a relevant aspect of *in vitro* experimental models, as condition control is more straightforward in those models.

RNA interference, transcriptional gene silencing, virus-induced gene silencing, microRNAs, and, more recently, CRISPR and base editors technology comprise a series of mechanisms capable of controlling gene activity and cellular phenotypes. This ability to modify and even knockout targeted genes is a valuable tool to assess and confirm hypotheses regarding molecule functions within the cell [6].

For instance, F. Yang *et al*. have investigated diabetic cardiomyopathy (DCM), which is a common complication of DM and has recently been associated with pyroptosis events — a type of programmed cell death related to inflammation. In order to clarify the molecular mechanism behind such a process, and also taking into consideration that the long non-coding RNA (lncRNA) Kcnq1ot1 participates in many cardiovascular diseases, human cardiomyocyte cells (AC16) and primary cardiomyocytes were cultured in vitro with 5.5 and 50 mmol/L glucose. It was found that the expression of Kcnq1ot1 was increased in cardiomyocytes cultured in hyperglycemic conditions, which was in accordance with readings taken from patients with diabetes and diabetes-induced mouse cardiac tissue. The authors have also shown that silencing Kcnq1ot1 inhibits pyroptosis by influencing

miR-214-3p and caspase-1 expression, which reduced cell death *in vitro* and *in vivo* [7].

In vitro models are also convenient to measure kinetics, as changes in pH and colorimetric assays, for example, can be used to monitor cell response to changes in the environment and progression of treatments. An *in vitro* protocol was developed by Varberg *et al*, using fetal endothelial colony forming cells (ECFCs) exposed to maternal DM plated in slides containing basement membrane matrix and a microscope set to acquire shots every 15 minutes so as to stack the images and assess the kinetics of *in vitro* vasculogenesis in order to quantitate rates of network formation and stabilization [8].

The ethical concern of society also renders the search for methods to replace, refine, and reduce animal use, which is a paramount concern for researchers. First promulgated in 1959, the 3Rs — Replacement, Reduction, and Refinement — have evolved as fundamental principles underlying the use of animals and alternatives in science throughout the modern world [9]. As technology advanced and new methodologies were developed, the number of studies that required regulated animal procedures decreased considerably in the last decades, and that is mainly thanks to *in vitro* culture studies [10–12].

Some limitations need to be considered when working *in vitro* rather than *in vivo*. For example, certain cell lines, like neurons and pluripotent stem cells, are difficult to culture, as some require particular media and environmental conditions that are hard to establish and maintain [13]. Furthermore, cellular behavior can be influenced by even small variations in environmental factors such as temperature, CO_2 levels, pressure, and culture confluency [14,15].

3.2. Strategies for modeling DM *in vitro*

3.2.1. Glucose modulation strategies

Modulation of levels of glucose in the culture media was the most frequently used method to mimic diabetic conditions *in vitro* seen in the papers used in this review, as shown in table 1. This is an easy and cheap alternative to emulate the major variable that is associated with the disease. Most basic cell culture media contain 5.5mM of glucose, which is the equivalent of a healthy person's blood sugar level of 99mg/dL [16]. Most studies supplement media with glucose as to mimic hyperglycemia reaching concentrations ranging from as low as 7mM to 50mM, although most are found to use ~30mM as a hyperglycemic "diabetic status."

It is a great alternative to study the cellular response to high-glucose environment, even though it doesn't mirror chronic effects of the disease, as in this case, it begins to involve other physiological variables, such as hormone imbalances and inflammatory responses observed in patients that have lived with diabetes for an extended period of time. Nevertheless, there are some limitations to the strategy of glucose modulation that needs to be addressed. For example, the exposure of cells to high glucose fails to mimic *in vivo* heterogeneous conditions where cells are exposed to variable glucose levels that fluctuate gradually through time. *In vitro*, these changes occur abruptly each time the culture media is replaced, which raises the possibility that the cell response to the glucose may not be only because of its high level per se, but due to osmotic stress. Ueck *et al*. found that when using hyperglycemic keratinocytes in scratch wound assays to mimic diabetic wound healing, the hyperosmolar aspect of hyperglycemia played a significant role in compromising keratinocyte migration, since the same effect was observed in hyperosmolar conditions [17]. In this sense, the addition of mannitol or fructose in experimental controls may be necessary [18].

Microfluidic systems constitute an attractive alternative in this scenario. Such systems allow for better reproduction of the glycemic fluctuations seen *in vivo*, which can also be designed to mimic and emulate human biology in order to study more complex physiological systems interactions. Bauer *et al*. developed a human microfluidic two-organ-chip model to study pancreatic islet–liver cross-talk based on insulin and glucose regulation, managing to obtain a functional feedback loop between human pancreatic islet microtissues and liver spheroids that can provide an *in vitro* system able to emulate T2DM [19]. Finally and also of note, regardless of the cell culture system, the presence of metals, such as iron in media and calf serum — commonly used as a supplement in cell culture — may exacerbate the effects of high glucose and must be considered when drawing conclusions from each experiment [15].

3.2.2. Primary cell cultures derived from DM patients

Primary cell cultures obtained from patients with diabetes constitute another methodology to study the disease *in vitro*. One great advantage of this method is that the system allows, to a certain extent, to “import” the chronic physiological conditions from the patient into cell culture. This strategy enables the study of the impact of a high-glucose environment on the tissue over a long period. Such temporal “snapshot,” however, needs to be taken into account during results analysis, as some of the conclusions may not apply to younger or older cells, or even to the same cell type harvested from another location or patient, since *in vivo* conditions are highly dynamic [14].

As tissue samples can be obtained and cultured, studies on cellular interaction can be performed *in vitro*. The development of 3D co-cultures that resemble *in vivo* physiology, as well as organotypic cultures, were a great advance and an especially useful tool to study several tissues in the context of DM, such as the corneal nerve dysfunction,

frequently seen in DM patients. The lack of human *in vitro* models that can successfully mimic the complexity of corneal tissue *in vivo*, which is organized in distinct layers devoid of blood vessels, results in very few studies involving the interactions between the corneal stroma and the stromal nerves. The improvement of *in vitro* models based on co-cultures also enables researchers in the field to develop studies on corneal degeneration [20–22].

In addition, this methodology is useful to investigate the inflammatory and immunological responses, which are usually dysfunctional in DM patients. In type 1 diabetes patients, the immune system is primarily involved in beta cell destruction, and in type 2 patients, the systemic inflammation is also a hallmark of the disease [23,24]. Due to organ dysfunction and the inflammatory nature of DM, recent studies aim to explore regenerative medicine alternatives to tackle the disease. In this scenario, the therapeutic potential of mesenchymal stem cells (MSC) on tissue regeneration and insulin resistance stands out as they appear to modulate immune response effectively [25,26].

Importantly, some limitations are observed in primary culture-based DM studies. Primary cells require ethical approval to be assessed, and - for some cell types - may only be obtained from invasive procedures, limiting their availability. After obtention, cellular contaminants, such as microbiological contaminants and undesired cell types may also alter cell culture quality. For instance, some cell lines are difficult to harvest and isolate, rendering them difficult to work with, since after some time in culture, those contaminant cells may be expanded and compromise the experiment. Glial and Schwann cells are an example of such cell lines, as they are difficult to isolate from fresh nerves, due to an abundance of connective tissue and other myelinating cells in the tissue [14,21]. Moreover, cells undergo alteration in some of their properties after culture and successive passages, ceasing to divide and eventually progressing to senescence and death. This limits experiments that require a large number of cells or that are intended to involve long periods of observation [27].

It is also worth mentioning the use of differentiated stem cells in research. Some studies are being conducted using induced pluripotent stem cells (iPSCs)-derived cells rather than primary cultures. This methodology holds much promise as research is being done in order to generate stem cell-derived β-cells from patients with type 1 diabetes, as transplantation of exogenous β-cells to replace dead or dysfunctional endogenous β-cells is a potential strategy for controlling blood glucose levels in those patients [28–30].

3.2.3.Cell culture with DM patients' serum

A recent developing area of study in diabetes research is the role of diabetic serum in the pathology of the disease. Studies involving the control of glucose concentration, for example, indicate that the anti-angiogenic properties of T2DM serum are independent of hyperglycemia levels. Thus, it appears that T2DM results in imbalances in other circulating factors, such as growth factors and cytokines, which may have additional impact on endothelial cell behavior and help explain the altered vascular endothelial function observed in DM patients. A study published in 2019 demonstrated that circulating factors present in the serum of T2DM individuals impaired in vitro endothelial cell capillary-like network formation in both primary and telomerized endothelial cell lines. The research suggested that elevation in interleukin-6 and its soluble receptor may be responsible for such alterations and may play a role in the mechanism underlying vascular impairments and reduced angiogenesis often found in T2DM [31].

Also, in recent years, the role of extracellular vesicles (EVs) in T2DM have attracted much attention. EVs are bilayer membrane vesicles secreted from most cells and can participate in regulating various physiological and pathological processes in vivo by being transported between cells. Recently, it was discovered that some abnormal EVs can contribute to the occurrence of T2DM by inducing insulin resistance and can also

participate in the complications of the disease [32]. They also appear to play a role in amyloid formation seen in patients with diabetes [33].

Findings like these introduce a new way to study diabetes *in vitro* as researchers can simulate the effects of the disease culturing healthy cells in culture media supplemented with serum harvested from diabetes patients. It is important to point out that the difficulty of assessing the exact content of the harvested serum and EVs must be taken into account when using this methodology, as mentioned by the International Society of Extracellular Vesicles (ISEV) [34].

3.2.4. Strategies focused on isolated aspects of the disease

Even though the use of primary cells and serum/plasma obtained from DM patients allows for the isolation of some aspects of DM, such strategies still bring a snapshot of various biological alterations that can be observed in the isolated cells and serum. When going through all the experimental approaches of the manuscripts published in the last few years, another possibility of modeling DM in a dish was observed: the isolation and experimentation of one or few specific factors without using a hyperglycemic environment or primary cells harvested from patients with diabetes. That way, the impact of a single factor can be measured and correlated with the clinical aspects of the disease.

Such methodology was used in a study performed to assess the relationship between Parkinson's disease (PD) and T2D in an experiment using only amyloid precursors. In T2D and PD, polypeptide assembly into amyloid fibers plays central roles. In PD, α-synuclein (aS) forms amyloids, and in T2D, amylin (islet amyloid polypeptide - IAPP) forms amyloids. The authors then assessed the cross-reactivity between these amyloid precursors revealing a simple justification as to why T2D is a risk factor for PD, whereas patients with PD are not prone to developing T2D [35].

4. Conclusion and perspectives

The selection of the appropriate experimental approach to apply within a study is an important step in experimental design, and the strengths and weaknesses of each experimental tool must be weighed upon decision. There is a broad variety of experimental systems available for the study of diabetes. *In vitro* models are useful to study individual aspects of the disease as they enable the researcher to assess the intricate interaction of cells and surrounding factors. They are also useful to provide initial exploratory data, since they are usually far cheaper and easier models to work with, when compared to *in vivo* models.

It is also important to bear in mind that results obtained via *in vitro* experiments require further confirmation with *in vivo* studies, as to support the occurrence of similar outcomes in the presence of other interferent factors, and thus help to enlighten the underlying mechanisms of the disease towards the discovery, validation and optimization of preventive and therapeutic strategies for DM. Moreover, as a careful reading of the articles cited in table 1 shows, most published studies do not rely on only a single *in vitro* model, as most of the time different experimental approaches are needed to test and support a hypothesis.

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Declarations of interest

The authors declare no conflicts of interest.

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Appendix

Table 1. In vitro models used for studies on diabetes mellitus

Model used	Major findings	References

Glucose modulation strategies	Topical administration of bosentan prevents retinal neurodegeneration induced by hyperglycemia and tumor necrosis factor α	[20]
	High glucose induce interleukin-1β and soluble interleukin-6 receptor production from macrophages in inflammatory periodontal tissue	[36]
	Silencing KCNQ1 overlapping transcript 1 inhibits pyroptosis and is overexpressed in diabetic cardiomyopathy model	[7]
	Choline and betaine modified some, but not all, determinants of placental transport in response to hyperglycemia	[18]
	Bellis perenis (common daisy) induced insulin-like effect in glucose transporter 4 translocation	[37]
	Diphlorethohydroxycarmalol Isolated from Ishige okamurae represses high glucose-induced angiogenesis	[38]
	Heat shock protein 22 suppresses diabetes-induced endothelial injury by inhibiting mitochondrial reactive oxygen species formation	[39]
	Chrysin, present in bee propolis and herbs, inhibits advanced glycation end products-induced kidney fibrosis and glomerulosclerosis	[40]
	Tissue factor and toll like receptor 4 plays a significant role in high glucose-induced mitochondrial dysfunction, mitochondria-related oxidation, and apoptosis by regulating	[41]

	downstream protein peroxisome proliferator-activated receptor γ coactivator-1α in renal tubular epithelial cells	
	High glucose suppresses the viability and proliferation of trophoblast cells through microRNA-137, protein kinase AMP-activated catalytic subunit α1 and interleukin-6 axis	[42]
	Angiotensin II aggravates lipopolysaccharide induced human pulmonary microvascular endothelial cell permeability in high glucose status	[43]
	Hyperglycemia increase cellular reducing equivalents but reduce survival of 661W photoreceptor-like cell injury	[22]
	Peroxisome proliferator-activated receptor β/δ activation confers vascular protection against hyperglycemia-induced oxidative stress	[44]
	High glucose increases tissue factor and toll like receptor 4 and tissue factor-procoagulant activity in healthy subjects	[16]
	Triterpene extract is a promising candidate to *in vivo* testing in diabetic wounds and environmental influences like hyperosmolarity are important in wound healing assay	[17]
	Development of a novel human T2DM *in vitro* model based on a microfluidic two-organ-chip model to study pancreatic islet–liver cross-talk based on insulin and glucose regulation	[19]
Primary cell cultures derived from DM patients	1,25-dihydroxyvitamin-D3 promotes neutrophil apoptosis through activation of p38-mitogen-activated protein kinase signaling	[45]

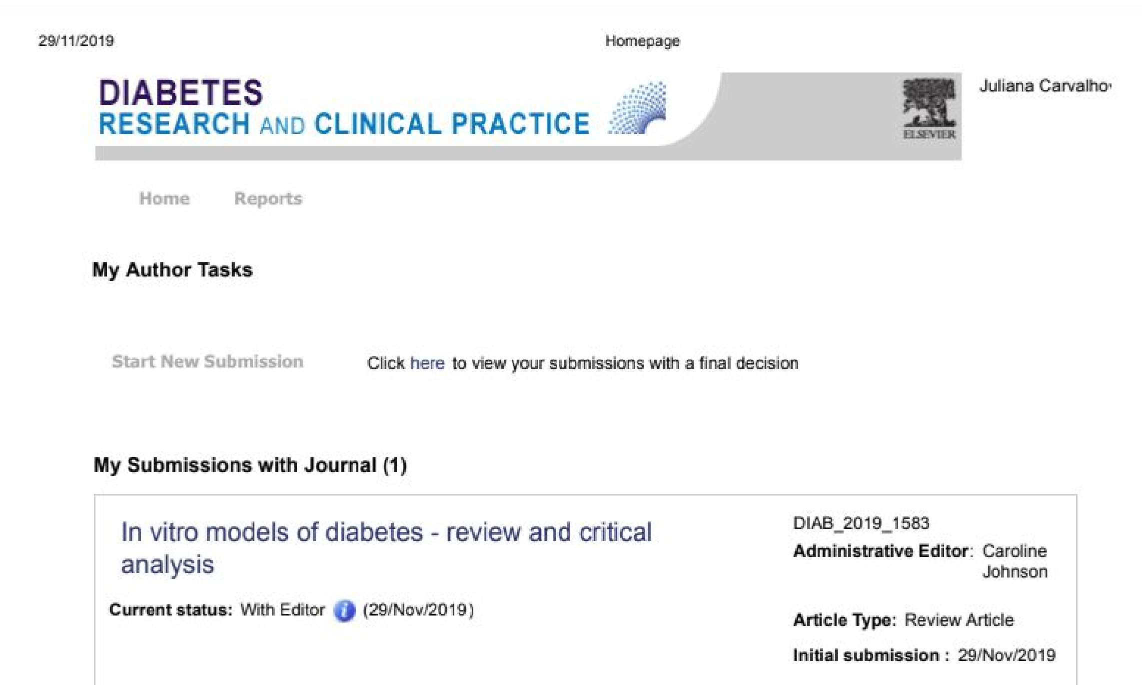
	MicroRNA-31 may be a biomarker for diabetic nephropathy in T2DM patients	[46]
	Programmed death-ligand 1, targeted in cancer immunotherapy, are also expressed in beta cells from T1DM patients	[47]
	Development of a 3D co-culture model of stromal fibroblasts and neuronal cells	[21]
Cell culture with DM patients' serum	Expression of some splicing machinery was associated with risk of developing T2DM in patients with cardiovascular disease	[48]
	Serum interleukin-6 and soluble interleukin-6 receptor in type 2 diabetes impairs endothelial cell capillary-like network formation	[31]
Strategies focused on isolated aspects of the disease	Interleukin-4 boosts insulin-stimulated glucose uptake and lipid synthesis in hepatocytes	[49]
	Poor glycemic control, enhanced platelet activation and oxidative stress increase the content of polyhedrocytes in blood clots generated in T2D patients	[50]
	Human umbilical cord-derived mesenchymal stem cells ameliorate insulin resistance by suppressing NLRP3 inflammasome-mediated inflammation	[25]

Table 2. Summary of advantages and limitations of *in vitro* models used in diabetes mellitus research

Model	Advantages	Limitations
In vitro (general)	• Variable control • Lower complexity as opposed to *in vivo* • Allows the measurement of reaction kinetics and metabolic processes • Compliant with 3R principles	• Some cell types can't be cultured *in vitro* • High impact of small variations in the environment
Glucose modulation strategies	• Easy to work • Controlled conditions	• Osmotic shock influence • Iron and calf serum exacerbates hyperglycemia effects • Constant glucose levels, unlike *in vivo*
Primary cell cultures derived from DM patients	• Great to study cell interaction • "Imports" chronic, as well as direct and indirect effects of the disease	• Temporal/spatial "snapshot" limitations • Certain cell lines are difficult to

		harvest and isolate (purity concern) • Successive passages alter cellular behavior
Cell culture with DM patients' serum	• New discoveries showing serum/extracellular vesicles importance • Brings direct and indirect effects of DM to the experimental setting	• Difficult to determine serum/vesicles composition
Strategies focused on isolated aspects of the disease	• Ideal to isolate and study a specific metabolic pathway	• Doesn't account for complex *in vivo* interactions and metabolics

Picture 1. Submission receipt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