Insulin for topical use in wound healing: opportunities and limitations

BEÁTA-MÁRIA BENKŐ[1,2], ISTVÁN SEBE[2*], ZOLTÁN-ISTVÁN SZABÓ[1*]

[1]*Faculty of Pharmacy Department of Drugs Industry and Pharmaceutical Management, George Emil Palade University of Medicine, Pharmacy, Science and Technology of Targu Mures, Gheorghe Marinescu Str. 38, Târgu Mureș 540142, Romania*
[2]*University Pharmacy Department of Pharmacy Administration, Semmelweis University, Hőgyes Endre Str. 7-9, Budapest 1092, Hungary*

Corresponding author: István Sebe, Zoltán-István Szabó
Email: sebe.istvan@pharma.semmelweis-univ.hu, zoltan.szabo@umfst.ro

Received: *11 November 2021* / ***Accepted:*** *24 November 2021*

Aims: The role of insulin in the regulation of energy metabolism, protein synthesis, proliferation, migration, secretion by keratinocytes, endothelial cells, and fibroblasts suggests that its presence is essential for wound healing (WH). The present study aims to explore the opportunities and limitations of topical insulin (TI) formulations.
Methods: To obtain a complete picture of the challenges of the local insulin formulation a chronological review of previous publications in electronic databases was performed, applying data collection and selection criteria.
Results: The opportunity of topically applied insulin has shown active interest over time. According to studies, regular and isophane insulin are suitable for local use, but currently there is no consensus on the appropriate concentration. Insulin can be incorporated into cutaneous liquid, semisolid, and solid dosage forms, either by itself, or by prior nano-or microencapsulation methods. The most important limiting factors to be evaluated are the stability of the peptide and the sterility of the obtained products.
Conclusion: Examination of the balance of opportunities and limitations of TI formulations, it can be concluded that the range of applicable technological methods is wide. A high-quality, safe, and efficacious form of TI would have great value from a socio-economic point of view.

Keywords: topical insulin, local treatment, wound healing, formulation, stability

1. Introduction

1.1 Insulin and wound healing

Insulin is used for the purpose of lowering blood glucose, WH, parenteral nutrition solution, as an anti-ageing agent, for cell culture and organ preservation, prevention of septic shock, etc. [1-9]. Due to its complex mechanism of action, insulin can treat *Diabetes mellitus* (*DM*) and also plays an essential role in preventing the development of its complications [10]. Ischemia, neuropathy, defective WH, and wound infection result in chronic, hard to treat, non-healing diabetic ulcers [11,12]. These are susceptible to infection and may lead to varying degrees of lower limb amputations [11,12]. Foot ulcers are chronic open wounds, affecting 40 to 60 million patients with diabetes globally, requiring prolonged hospitalizations for its management [13,14]. Chronic ulcers and amputations result in a significant reduction in the quality of life and increase the risk of early death [13]. Neuropathy as a complication of *DM* also exposes diabetic patients to burn injuries. Loss of feeling is particularly important because it can allow injuries (higher risk of thermal injury) to go unnoticed, leading to serious infections [15]. Treatment of foot ulcers remains an important clinical challenge as the available therapies have limited efficacy [6], as skin grafts, hydrocolloid dressings, and negative pressure dressings have failed to produce adequate response [16,17], while growth factors and stem cells, are highly expensive and their safety remains to be evaluated [16]. Novel, low-cost and safe strategies to improve WH would be of great social and economic value [11].

The role of insulin in the regulation of energy metabolism, protein synthesis, cell differentiation, and growth suggests that this hormone could also play an essential role in the regulation of WH [17,18]. An important factor responsible for delays in WH in diabetic patients has been postulated to be defective insulin action in the skin [11], as insulin stimulates the growth and development of different cell types and affects proliferation, migration, and secretion by keratinocytes, endothelial cells, and fibroblasts [19].

Numerous preclinical and clinical trials demonstrated the beneficial effects of topical insulin on WH [20]. Besides systemic treatment of *DM*, exog-

DOI: 10.33892/aph.2022.92.3-19

enous administration of insulin is efficient in local treatment of further complications of the disease [21].

1.2 Insulin for topical use: historical background

The first experiments used TI for systemic purposes, but starting with the 1930s insulin began to gain ground in wound management [22]. At an early stage, studies used systemic insulin to improve WH [23], but at the same time the cutaneous absorption problems of insulin were also addressed [24-26].

At the end of the 1950s scientists concluded, that standard insulin containing solutions and ointments do not produce a lowering in blood glucose levels. Insulin absorption occurs only after treating the skin with chloroform or petrol ether (but not ethanol) [27]. Results prompted the exploration of the local effect of insulin on rats [28,29]. Acceleration of WH by the application of TI was described in case reports of human diabetic subjects as early as the 1960s [2,30,31].

From the middle of the 20th century, studies targeted the physiological properties of insulin, which indicated that it might favorably influence WH since it could stimulate the growth of individual cells, as well as cause increased anabolism of the organism [32].

The mechanism and significance of local action of insulin was unknown at that time [29], but it was followed by two decades of experimental data, when a series of papers based on case studies were published, which reported TI successes in the treatment of diabetic WH [30,33,34]. Thereafter, the use of TI for WH purposes decreased and only a few studies have been performed until the late 90s [11,35,36].

Starting with the 2000s the golden decades of TI therapy has begun. In this period the efficacy and safety of cutaneous application of insulin was explored, the local mechanisms of action were described and several TI formulations were reported. The application of local insulin next to diabetic WH reached successes also in the treatment of burn injuries [37]. Although several earlier studies have addressed the healing effect of systemic insulin in burns [36, 37], but only a few have investigated the effect of topical application [11].

Nowadays, the focus has shifted on the technological solutions of TI formulations, to tackle the challenges of protein instability and short duration of action.

1.3 Aim

The aim of this study is to explore the opportunities and limitations of TI formulations by reviewing previous publications. Development of novel external formulations needs to rely on in-depth knowledge of the local mechanism of action of insulin, its physicochemical properties, quality requirements and particularities of the different formulation approaches.

2. Materials and methods

2.1 Data collection procedure

Data sources were obtained from electronic databases, using a bibliographic search in Google Scholar, PubMed, and ScienceDirect, regarding publications from 1920 to 2021 September. The selected time interval was chosen according to the introduction of insulin in therapy. The search was realized using simply the search box and the filter of time periods, overviewing each decade. During the literature retrieving the searched terms were: topical insulin, diabetic ulcers, wound healing. Data collection procedure was simplified by using boolean operators, more exactly „AND" operator was used as conjunctions to combine keywords in the search, resulting in more focused and relevant results, eliminating unsuitable or inappropriate hits, the searched phrases were: insulin AND topical use, insulin AND wound healing, insulin AND ulcers. During the data collection, there was no limitation on the type of publications, the included studies were original, peer-review, review articles, and also patents.

2.2 Data selection procedure

All identified titles were independently screened, by the selection the focus was on the formulation details. The chronological review of previous publications and studies also provides a complete picture of the effectiveness of local insulin use. To evaluate the human evidence, the type of experiment (preclinical study, clinical trial, or *in vitro* experiment) was followed. To explore the opportunities and limitations of formulation it was required to follow the general steps of drug design, according to this, the next characteristics were evaluated: the type of active pharmaceutical ingredient and the appropriate concentration of it, the desired dosage form, and the type of excipients for it.

Therefore, the aspects of data selection were the following: type of insulin, the concentration of insulin, dosage form, type of excipients. If two from this informations were absent the study was excluded from this research. Based on these disclaimers, during the data selection procedure, only original articles and patents were included. Using a PRISMA 2020 flow diagram [38] the most relevant data were extracted to synthesize the results, according to these, the steps of data collection procedure, following the literature research, are summarized on *Figure 1*.

3. Results

3.1 Development of topical insulin use

A chronological review of previous publications delineates the history and development of TI therapy. According to the data collection procedure, 77 studies were found, from which 59 focuses on skin injuries, while in 18 the use of TI was explored in other areas, such as ophthalmology, otology, dentistry, and pulmonology. From the studies dealing with TI applied to the skin, 52 were experimental studies and 7 were review articles (*Figure 1*). *Table I* presents the results of the bibliographic search, overviewing the development of topical insulin in each decade.

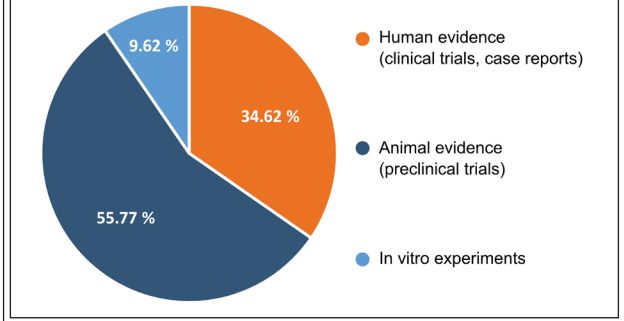

Figure 2 *The type (clinical trial/preclinical study/in vitro experiment) and the proportion of experiments from the 52 evaluated studies*

3.2 Human evidence of topical insulin use

Following the data collection strategy, from the 52 studies identified, 18 studies present human evidence, including clinical trials and case reports, 31 studies were preclinical trials and 8 studies were based on *in vitro* experiments. From the reviewed publications, 5 presented overlapping researches, 2 of them were preclinical trials followed by clinical trials and 3 of them presented *in vitro* experiments combined with studies on animal models. The types and the number of experiments are represented in *Figure 2*.

3.3 Formulation of topical insulin

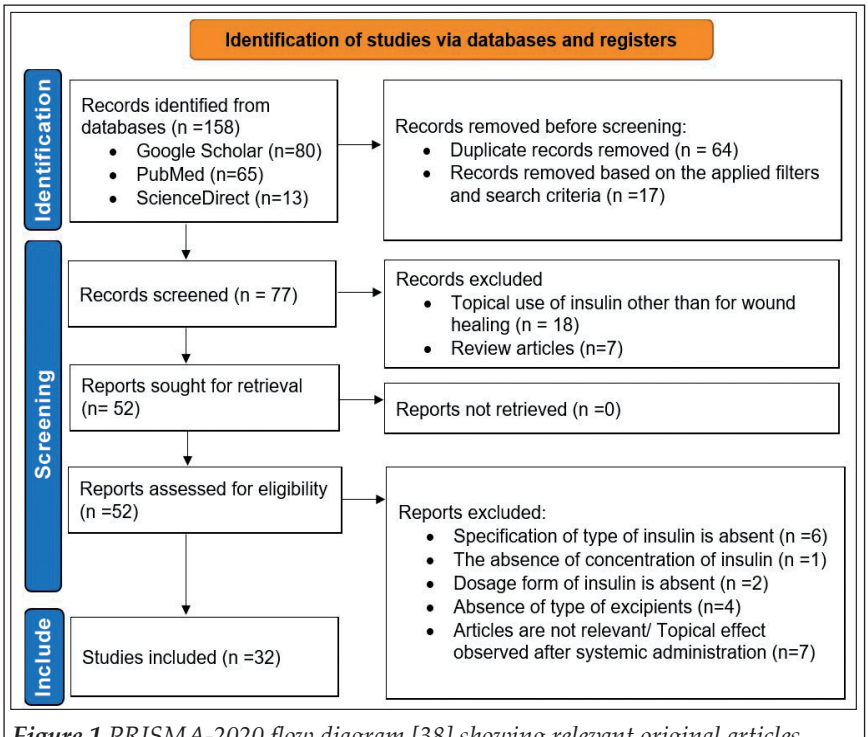

Figure 1 *PRISMA-2020 flow diagram [38] showing relevant original articles included in the study*

From the 77 overviewed articles, 32 were selected according to the selection criteria, focusing on the formulation details. In these studies, corresponding quantitative and qualitative information were found about the formulation technology of TI (*Figure 1*). *Table II* summarizes the most relevant studies about the local application, in which at least two parameters from selection criteria (type of insulin, concentration of insulin, pharmaceutical form, type of excipients) are mentioned. There were two formulations which were not used in the reviewed studies, one of them is a preparation formula from Pharmaceutical Technology Practice Guide issued at the University of Medicine and Pharmacy of

Table I *Development of topical insulin in each decade from 1920 to present*

Researched interval	Statements	References
1920-1930	• Systemic insulin treatment reduces infections after surgical prucedures in diabetic patients.	[39,40]
1931-1940	• Healing effect of systemic insulin on postoperative wounds in non-diabetic patients • Experiments about the cutaneous absorption of insulin brought contradictory results.	[22-26]
1941-1950	• The application of standard insulin solutions and ointments on wounds do not produce a lowering of blood sugar levels.	[27]
1951-1960	• The local application of insulin accelerates soft-tissue wound-healing in rats and reduces the inflammation.	[28,32,41]
1961-1970	• Insulin accelerates the healing process via granulation tissue-stimulating effect and helps the clearance of infections. • Insulin produces a local metabolic compensation which, together with the systemic metabolic compensation, lead to rapid healing of diabetic ulceronecrotic lesions.	[29-31]
1971-1980	• Studies resulted conflicting outcomes about the influence of TI on the healing rate of ulcers.	[33,34,42]
1981-1990	• The knowledge about topical insulin growth in an extent way, the possibility of local aplication was introduced in manuals.	[43]
1991-2000	• European Patent: EP0561330A1 "Topical insulin preparation" • Topical insulin accelerates WH by improving wound matrix formation.	[2,36,44]
2001-2010	• Evaluation of the efficacy and safty of topical applied insulin. • Appearance of particulate peptide delivery systems, which are capable of high peptide loading, controlled release, improved stability and are potential for targeting specific tissues.	[19,45-49]
2011-2021	• The local mechanism action of insulin became clearer, the molecular and cellular activity was detalied. • The tedency of technological procedures changed, experiments started to focuse on formulations, which offer long-term delivery of bioactive insulin and extended stability. • Current researches focuse on technological issues and topical application of insulin in the field of ophtalmology.	[11,12,17, 50-78]

Targu-Mures (Romania) in 1981, the other one is an elaboration formula from Corola Pharmacy, Targu-Mures (Romania), mentioning, that the pharmacy insists on composition`s secret, therefore the quality and quantity of ingredients are partially presented. Another exception from original articles, included in *Table II*, is a European Patent with number EP0561330A1, published in 1993.

3.4 Types of insulin used in the topical products

In a few cases, the exact type of insulin used in the formulations was specified: Actrapid® (human insulin produced in *Saccharomyces cerevisiae*), Humulin R® (human insulin produced in *Escherichia coli*), Humulin N® (isophane human insulin produced in *Escherichia coli*), in other cases, when the insulin product was not specified also regular insulin and isophane insulin was used. In the reviewed formulations, there were no cases where insulin analogs were used.

3.5 Concentration of insulin

The different types of experiments applied different doses of insulin and some experiments were based on the preliminary determination of Lima *et al*. [12], however, there is no study that harmonizes in a conventional way the suitable local concentration of insulin. Overviewing the applied quantities, it can be concluded that topical concentration of insulin is lower than the amount administrated systemically. However, the determination of at least an interval of concentration is challenging, because the relevant studies are based both on animal and human evidence. In addition, a large variety of possible dosage forms exist, as dosages differ from those that release insulin immediately from those that release insulin in a controlled manner.

Table II *Formulation types of topical insulin*

Type of insulin	Concentration of insulin	Dosage form	Type of excipients	Formulation strategy:	Year	Ref.
Soluble insulin	20 IU, twice daily	Solution	-	C	1966	[30]
Regular insulin	10 IU, twice daily	Solution	-	C	1976	[34]
Regular insulin	10 IU, twice daily	Solution	-	C	1979	[33]
Regular insulin	60 IU	Oinment	*Unguentum Lanalcooli, Aqua Destillata*	C	1981	[43]
Neutral aqueous insulin solution	100 IU /mL	Emulsion	European Patent: EP0561330A1	C	1993	[44]
Regular pork insulin	100 IU	Solution	-	C	1999	[2]
Soluble insulin	2 U/ 20 mL	Solution	Normal saline	C	2008	[48]
Crystalline insulin	10 IU, twice daily, 1 cc for each 10 cm^2 of wound	Solution	Normal saline	C	2009	[19]
Regular insulin	20 μL, twice daily	Solution	-	C	2010	[17]
Crystalline human recombinant insulin	2.5, 5 and 10% w/w	Suspension	PLGA microspheres	N	2010	[50]
Human crystalline regular insulin	0.5 IU/100 g cream	Cream	Patent number: PI 0705370-3	C	2012	[12]
Bovine pancreas insulin	0.03 IU insulin/20 μL	Solution	Normal saline	C	2012	[51]
Regular insulin	10 IU (0.1 mL)/ 1mL, 1 cc for each 10 cm^2 of wound	Solution	Normal saline	C	2014	[52]
Soluble insulin,	4 IU/1mL, 1cc for each 10 cm^2 of wound	Solution	Normal saline	C	2014	[53]
Isophane insulin	0.1 IU/20 μL	Suspension	Normal saline	C	2015	[54]
Human crystalline regular insulin	0.5 IU/100 g cream	Cream	Patent number: PI 0705370-3	C	2015	[55]
Regular insulin	0.3 IU	Solution	Sterile water , 20 μL	C	2015	[79]
Human recombinant crystalline insulin	125 μg of insulin	Alginate sponge dressing (patch)	PLGA microparticles, alginate	N	2015	[56]
Regular insulin	1 IU/cm^2 wound area, twice daily	Solution	-	C	2016	[57]
Isophane insulin	0.03 IU	Suspension	-	C	2017	[61]
Isophane insulin	0.1 IU/20 μL	Suspension	Normal saline	C	2017	[62]
Porcine insulin	100 mg insulin (27.5 IU/mg)	Silk fibroin sponge dressing (patch)	Silk fibroin microparticles, silk fibroin	N	2017	[64]
Recombinant human insulin, dry powder	5 mg (30-35 μg drug loading per mg nanoparticle)	Suspension	PEG-modified, PLGA nanoparticles	N	2018	[63]
Recombinant human insulin	33.86 μg insulin per milligram of polymer	Gel	PEG-modified, PLGA nanoparticles, poly(vinyl alcohol)-borate	N	2018	[65]
Isophane insulin	5 mL (100 IU/mL)	Ointment	Solid medical grade petroleum jelly (95 g)	C	2018	[66]

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Type of insulin	Concentration of insulin	Dosage form	Type of excipients	Formulation strategy:	Year	Ref.
Human crystalline regular insulin	0.5 IU/100 g cream	Cream	Patent number: PI 0705370-3	C	2019	[67]
Insulin crystals	91.52 μg/cm²/h	Gel	Lipids for liposomes, 2% chitosan gel	N	2019	[68]
Regular insulin	30 IU	Solution	Normal saline, 30mL	C	2020	[21]
Regular insulin	0.2 IU/2 mL	Solution	Normal saline	C	2020	[69]
Recombinant human insulin	0.5 IU	Gel	Chitosan nanoparticles, chitosan gel	N	2020	[70]
Regular insulin	-	Ointment	Vaseline 90g, lanoline 10g	C	2021	*
Regular insulin	0.5 units per 100 g animal weight	Ointment	80% eucerin, 20% liquid paraffin	C	2021	[72]
Insulin human recombinant crystalline dry powder	0.2 IU/g	Gel	Insulin-loaded nanoemulsion (100 g): oleic acid (9 g), tween 80 (37.5 g), polyethylene glycol 400 (17 g), distilled sterile water qs 100 g (36.137 g). Insulin-loaded nanoemulsion gel: *Aloe vera* gel (1.6 g), Carbopol 934 (2.9 g), ethyl cellulose (3.5 g), guar gum (2.5 g), water (qs. 100 g)	N	2021	[73]
Commercial insulin solution (100 I.U./mL)	5 μg/cm²/8h (*in vitro* release)	Gel	Insulin-loaded PLGA-nanoparticles in POLX gel	N	2021	[74]
Insulin	1.5 IU/mg	Xerogel-based dressing (patch)	Alginate-g-poly (methacrylic acid) cross-linked xerogel (AGM2S)	C	2021	[75]

Notes and explanation of abbreviations
C- conventional formulation strategy
N- novel formulation strategy (micro- and nanoformulation)
*Formulation of "Unguent Sharpy" made in Corola Pharmacy, Targu-Mures, Romania

3.6 Dosage forms

The liquid, semisolid and solid dosage forms are generally used for the local application of insulin. The liquid dosage forms are the following: solutions, suspension, emulsions, used under the form of fluids and sprays. The ointments, creams, and gels represent the semisolid dosage form of TI. From all the categories the most frequently used dosage forms are solutions, from the 35 experiments 14 were based on the application of solutions.

The other mentionable dosage forms are semisolids and/or solids. Gels are also frequently used for TI therapy, and in some of these cases insulin is incorporated by novel formulation techniques, which utilize micro- or nano-sized carriers as drug delivery vehicles, such as microspheres, nanoparticles, and liposomes, to enhance cutaneous delivery of topically applied insulin. Innovative drug delivery systems can also be found in the case of solid dosage forms, such as micro- and nanoparticulate topical wound dressings. From the select-

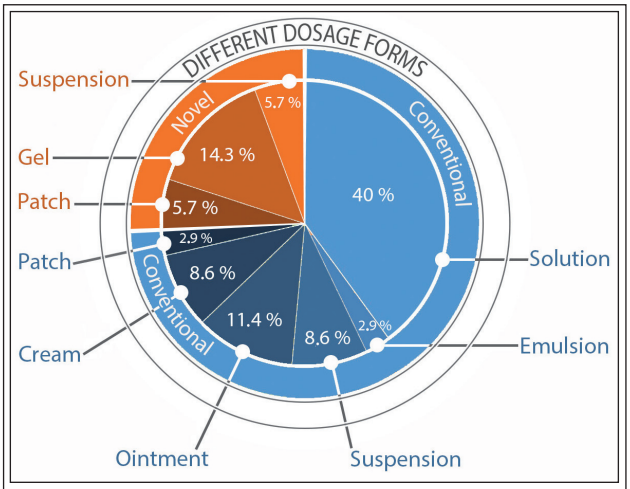

Figure 3 *The frequency of dosage forms of topically applied insulin*

ed studies, 5 experiments evaluated the efficacy of insulin-loaded particle systems with promising results. The frequency of applied dosage forms of TI is illustrated in *Figure 3*.

3.7 Types of excipients

The most used excipient in TI formulations is normal saline, which has been used to dilute the solution of systemically administrated insulin. Other, often found ingredients are zinc and protamine, which are not only excipients, but also active ingredients. Regular crystalline insulin contains zinc in a concentration of 0.4%, which is added to allow the insulin molecules to self-association [80]. Zinc is an essential mineral, involved in numerous aspects of cellular metabolism, immune function, protein synthesis, cell division, and WH [81,82]. Hence, when wounds are treated with insulin, they are therefore also being treated with zinc [52], or with protamine, which takes part in isophane insulin. As a protein, insulin is susceptible to aggregation, precipitation, denaturation, instability at high temperatures, and oligomer formation. In most of the reviewed dosage forms, which release insulin immediately, these issues were not addressed and no attempts were made to stabilize these insulin formulations with excipients. The patent from 1993 emphasized these characteristics and presented an emulsion-type of formulation that is suitable from the stability point of view.

Another key aspect, the issue of sterility of TI products is also scarcely mentioned, with just a few studies mentioning the criteria of preparation in an aseptic manner.

The most recent studies reviewed, focused on the stability of insulin, aided by novel formulation techniques to encapsulate insulin in nano- or microparticles, which protect the active peptide and can also ensure a prolonged release. The list of excipients and their applicability in the variety of TI dosage forms are presented in *Table III*.

4. Discussion

4.1 Opportunities

4.1.1 Local mechanism of action of insulin and the biology of wound healing

Cellular and molecular mechanisms that underline the WH effects of TI were described by different authors [12,51,54,61,67,69], the most complex overviews are given by Emanuelli *et al.* [11] and Abdelkadder *et al.* [58]. Insulin activates its own receptors, which are transmembrane molecules, belonging to a large class of tyrosine kinase receptors in all cell types, including keratinocytes and fibroblasts [83]. In keratinocytes, proliferation, cell differentiation, migration, and glucose transport are regulated by insulin via the insulin receptors (IR), the production of matrix proteins including fibronectin, collagen, and various proteoglycans is also influenced by insulin [11]. Besides regulating glucose and lipid metabolism, insulin is involved in processes including protein synthesis, mitochondrial biogenesis, growth, autophagy, proliferation, differentiation, and migration [84]. WH is a dynamic process, which involves four phases [58,85]: cell adhesion, inflammation, proliferation, and scar formation, and finally restoring the lost or damaged skin layers through the activity of cytokines, growth factors, extracellular matrix molecules, and stimulation of various cell types [58, 86]. Decreased growth factor production [58], angiogenic response [58,87], macrophage function [87], collagen accumulation, epidermal barrier function and keratinocyte migration [58,86] contribute to defective WH [58]. Expression of the IR, insulin receptor substrate-1 (IRS-1), insulin receptor substrate-2 (IRS-2), extracellular signal-regulated kinases (ERK), and serine/threonine protein kinase (AKT) are increased in the tissue of wounds compared to intact skin, suggesting that the insulin signaling pathway has a critical role in this process [58]. Lima *et al.* clearly showed that the use of insulin cream, holding the patent number PI 0705370-3 (University of Campinas, Brazil), is an efficient manner to activate the AKT and ERK pathways, which are essential in the control of WH [12]. At the same time Chen *et al*. demonstrated, that TI application improves healing by regulating the wound inflammatory response, especially the quantity and function of macrophages, increases wound macrophages infiltration, and facilitates the secretion of inflammatory mediators [51]. Li *et al* completed the mechanism of action of TI, revealing that topically applied insulin accelerates vessel maturation of wounds by regulating angiopoietin-1. Blood vessels in insulin-treated wounds showed advanced coverage of pericytes and reconstruction of new vascular basement membrane [54]. Azevedo *et al.* demonstrated that TI reduces the duration of the inflammatory phase, improves wound re-epithelialization, tis-

Table III *Type of excipients and other ingredients used in various dosage forms of topical insulin and their function*

Excipients and other ingredients		Dosage forms						
Name of material	Function of material	Liqiud			Semisolid			Solid
		Solution	*Emulsion*	*Suspension*	*Ointment*	*Cream*	*Gel*	*Patch*
Sterile water	Vehicle	x	x	x	x	x	x	x
Normal saline		x	x	x	x	x	x	
Liquid paraffin	Vehicle, ointment base		x		x			
Wool wax alcohol ointment (*Ung. lanalcoholi*)					x			
Eucerin (*Vaselinum cholesterinatum*)					x	x		
Unguentum simplex (vaseline 90g+lanoline 10g)					x			
Vaseline (Petroleum jelly)					x	x		
Lipids (phosphatidylcholine, cholesterol, triacylglycerides, oleic acid)	Vehicle, lipid phase, liposome forming agent		x		x	x	x	
Chitosan	Delivery vehicle, gelling agent, hemostatic control, WH effect						x	
Alginate M or G							x	
Sodium tetrahydroxyborate decahydrate	Gelling agent						x	
Polyacrylamide, C13-14 Isoparaffin and C13-14 Laureth-7 (Sepigel®)	Gelling agent						x	
Poly(vinyl) alcohol (PVA)	Stabilizing agent, gelling agent, emulsifier in the formulation of polymeric particles						x	
PLGA	Delivery vehicle, micro- and nanoencapsulation			x			x	
Poly(ethylene glycol) (PEG)	Matrix former, forming controlled drug delivery formulations, colloidal stabilizer, cellular uptake enhancer, emusificant, cosurfactant		x			x	x	x
Silk fibroin	Delivery vehicle, microencapsulation, matrix former, WH effect							x
Carbopol 934	Gelling agent						x	
Ethyl cellulose	Matrix former						x	
Guar gum	Stabilizing agent						x	
Poloxamer, Pluronic® F127 (POLX)	Gelling agent, stabilizing agent, retain the active sbstance at the target area						x	

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Excipients and other ingredients		Dosage forms						
Name of material	Function of material	Liqiud			Semisolid			Solid
		Solution	*Emulsion*	*Suspension*	*Ointment*	*Cream*	*Gel*	*Patch*
Alginate-g-poly (methacrylic acid) (AGM2S)	Drug-delivery matrix, WH effect							x
Protamine	Complexing agent			x			x	
Tween 80	Emusificant		x			x	x	
Lecithins			x			x		
Palmitin stearyl alcohols			x			x		
Glycerol esters			x			x		
Vitamin C	Antioxidant	x	x	x	x	x	x	
Vitamin E		x	x	x	x	x	x	
Vitamin A	Active ingredient, skin regeneration		x		x	x		
Vitamin B1	Active ingredient, coenzyme		x		x	x		
Vitamin B6			x		x	x		
Aloe vera extract	Active ingredient, WH effect						x	
Negamicin	Active ingredient, antibiotic	x	x	x	x	x	x	
Cliyndamicin		x	x	x	x	x	x	
Glucose	Active ingedient, coeffector, substrate	x	x	x	x	x	x	
Na^+	Electrolyte	x	x	x	x	x		
Ca^{2+}		x	x	x	x	x		
Zn^{2+}		x	x	x	x	x		
K^+	Electrolyte, increases insulin sensitivity	x	x	x	x	x		
Mg^{2+}	Electrolyte, cofactor in the increased protein biosynthesis	x	x	x	x	x		
Aprotinin	Protease inhibitor	x	x	x	x	x	x	
Soy bean trypsin inhibitor (SBTI)		x	x	x	x	x	x	
Lima bean trypsin inhibitor (LBTI)		x	x	x	x	x	x	
Preservatives	Preservative	x	x	x	x	x	x	x

sue granulation, wound contraction, and increases collagen deposition in second-degree burns in healthy and diabetic animals [55]. As an obvious consequence of some review articles [11,58-60], detailed experiments about the cellular and molecular mechanisms appeared, involving insulin-induced chemotaxis of monocyte/macrophage, cells that are critical for proper healing [61], and expression and activation of insulin and insulin-like growth factor (IGF-1) signaling and the regulation of insulin on the inflammatory response of wounds during the healing process [62]. Current researches and review studies suggest the contemporary status of topically applied insulin. The main approaches are to clarify the mechanisms that underlie the modulatory effect of insulin in the inflammatory and proliferative response in diabetic animals [67], the role of insulin in anti-inflammatory macrophage polarization [69], and to prove the effectiveness of TI dressings in the management of diabetic foot ulcers [21]. TI activates IR/SHC/ERK (Src homologous collagen-like protein, SHC) and IR/IRS/PIK3/AKT (phosphatidylinositol 3-kinase, PIK3) signaling pathways [12]. Tissue expression of IR, IRS-1, IRS-2, SHC, ERK, and AKT are increased in WH tissue, the local treatment also stimulates the expression of other proteins, such as endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), and stromal cell-derived factor 1 alpha (SDF-1a) [12,62]. Thereby insulin stimulates the growth and development of different cell types and affects prolifer-

ation, migration, and secretion by keratinocytes, endothelial cells, and fibroblasts [21,88]. In addition, TI has pro-angiogenic and vessel maturating effects on chronic wounds, by restoring impaired insulin signaling, such as the PI3K/AKT and MAPK/ ERK (mitogen-activated protein kinase, MAPK) pathways, and increasing the expression of VEGF and angiopoietin-1 [11,12].

4.1.2 Efficacy of topical insulin

The efficacy of TI was clearly demonstrated in the last decade. Apikoglu-Rabus S *et al.* revealed that topical insulin application on cutaneous wounds accelerates WH in rats with or without acute diabetes [17]. In 2014 Attia *et al.* observed a possible synergism between insulin and zinc, anabolic actions of insulin as a growth factor and the WH promoting effect of the zinc ions present in its formulation suggests that topical crystalline regular insulin is safe and effective in enhancing acute and chronic WH and hence patients' quality of life [52]. Goenka *et al.* studied the role of topical use of insulin in the healing of the chronic ulcer, following three parameters: rate of wound healing, safety evaluation, and hospital stay. They concluded that TI accelerated WH in chronic ulcers, its use is safe and effective without any systemic side effects, and it also reduces the hospital stay of patients [53]. The effect of TI in pressure ulcer healing was also evaluated in a study, where patients with immunodeficiency, *DM*, pregnancy, osteomyelitis, or peripheral vascular illness were excluded [57]. In this case, TI was found to be safe and effective in reducing pressure ulcer size [57]. A combination of TI therapy with phytotherapy was also explored as the WH potential of *Tinospora cordifolia* and its combination with local insulin therapy in diabetic rabbits was studied by Ajit *et al.* [89]. Özaydin *et al.* performed a detailed clinical, histopathological and immunohistochemical evaluation of the efficacy of isophane insulin on WH in open wounds with tissue loss in diabetic and non-diabetic animals. They observed a facilitated formation of granulation tissue and epithelization, due to faster completion of all phases of the healing process in open wounds [66]. In 2020 after reviewing animal and human evidence Wang *et al.* concluded, that TI improves WH through several mechanisms without causing side effects [20]. Recently Liu *et al.* performed a meta-analysis of animal and clinical studies and reviewed the effects of TI on WH. Results confirmed its conditional applicability in clinical treatment [71]. Mirhoseini *et al.* demonstrated the synergetic effect of TI and clindamycin, and observed that their combination significantly decreased chronic inflammation, increased neovascularization, and collagen deposition [72]. A novel perspective of TI therapy is gaining ground in the field of ophthalmology, as recent publications reveal its efficacy in corneal defects [76-78] and refractory neurotrophic keratopathy [90]. The majority of the gather evidence of TI therapy are mostly based on preclinical trials. In their review articles, Oryan *et al.*, Wang *et al.* and Liu *et al.* also highlighted the supremacy of animal experiments in this field [20,59,71], in contrast to Kannan *et al.*, who focuses only on human evidence [60]. Althought, topical application is an off-label use of insulin, in Romanian pharmaceutical practice the topical formulation of insulin is known under the name of „Sharpy ointment", but there are no statistical data in this regard. TI restores the integrity of damaged skin; therefore, it is of interest in the field of wound repair, particularly owing to its relatively low cost to other growth factors and skin grafts [37].

4.1.3 Diversity of TI dosage forms

Until the year 2000, the conventional pharmaceutical forms were preferable for TI delivery, such as solutions, suspensions, emulsions, ointments, and creams. Formulations with immediate release of insulin exert their effect by insulin receptors, which are situated on the plasma membrane and rapidly mediate the short-term effects on membrane functions [63,65]. In 2008, Wang et al. developed a particulate peptide delivery system, as biodegradable hydrophobic particles were prepared from poly (D,L-lactide-co-glycolide) (PLGA). As a model polypeptide, insulin was chosen since its structure, stability, and physicochemical characteristics have been extensively studied and it is, therefore, a good model of a chemically and physically unstable peptide [47]. The incorporation of insulin into a PLGA matrix was achieved by complexation with protamine [47]. The deposition of the particles within the skin was determined following topical application [47]. In another study, Hrynyk et al. introduced a method of TI formulation, which offered the potential for long-term delivery of bioactive insulin for topical delivery devices [50] and radically changed the tendency of novel technological procedures. In their method,

crystalline insulin was encapsulated into PLGA microspheres by a solid-in-oil-in-water (S/O/W) suspension solvent evaporation technique, which was successful in stabilizing insulin and offered a prolonged release for the bioactive peptide for up to 25 days [50]. Dhall *et al.* continuing the work of Hrynyk *et al.* [50] developed an alginate sponge dressing (ASD) containing insulin encapsulated in PLGA microparticles, which provided a sustained release of bioactive insulin stimulating regenerative healing of burn wounds in rats [56]. Functionalized silk fibroin (SF) dressing with topical bioactive insulin was described by Li *et al.* as they successfully encapsulated insulin in the inner layer of SF microparticles and loaded the insulin-encapsulated SF microparticles into SF sponges, providing a sustained release of the peptide [64]. The development of a nanoparticulate system, with variation of poly(ethylene glycol) (PEG) content, was described by Abdelkader *et al.* [63]. In their formulation recombinant human insulin was encapsulated in PLGA nanoparticles and manufactured with variation in PEG content. The obtained dosage form is capable to release therapeutic levels of bioactive insulin for extended periods of time and supports a particulate uptake mechanism that provides for intracellular insulin delivery, leading to enhanced cell proliferation [63]. Abdelkader *et al.* continuing their previous study, developed a novel poly(vinyl alcohol) (PVA)-borate hydrogel containing human insulin encapsulated in PEG-modified PLGA nanoparticles, which offered a quicker healing on wounds induced on rats when compared to control [65]. Quality by design principles were also applied to obtain insulin containing mucoadhesive liposomal chitosan gels for WH, with sustained release and extended stability for the peptide [68]. Chitosan was also used to form insulin-loaded nanoparticles incorporated within the hydrogel to evaluate its therapeutic activity during WH in diabetic rats [70]. From the point of view of technological issues, nano- and micro-formulations seem to be beneficial. Chakraborty *et al.* formulated an insulin-loaded nanoemulsion gel with Aloe vera and evaluated its synergetic effect on WH in *DM* [73]. Similarly, Quitério *et al.* focused on the preparation and characterization of insulin-loaded PLGA nanoparticles in Pluronic F-127 (poly(ethylene oxide-b-propylene oxide-b-ethylene oxide, POLX) gel. Their results were promising, especially those regarding insulin releasing from nanoparticles and shortening the time of recovery of skin burns [74]. Rajalekshmy et al. developed hybrid active wound dressing xerogels comprising of alginate grafted with poly (methacrylic acid) chains, loaded with insulin to provide a sustained release to enhance the wound healing process [75]. In accordance with stability requirements of the protein, insulin is preferable to be incorporated into carrier particles and in encapsulated form to be embedded in wound dressings, bio-adhesive films, and hydrogels [58].

4.2 Limitations

4.2.1 Challenges of topical insulin formulation

As early as 1993, the importance of the type of insulin was noted, but in those times the main point of concern was the difference between bovine, porcine, and human insulin [44]. Insulin type is an important factor to consider, especially, because it affects the efficacy of WH and also has an impact on the formulation strategy. However, the majority of publications do not mention whether rapid, short, intermediate, or long-acting insulin has been used during the formulation process. The type of insulin used influences the effectiveness of the topical preparations, modifications to the original recombinant insulin led to analogs with different kinetics and duration of drug action [10]. Insulin analogs can exhibit altered receptor binding characteristics [92] and can change their mitogenic potency [91]. Besides regular insulin, isophane insulin was also used, but there is not enough data about the usage of insulin analogs [93], for this purpose, proving or refuting their effectiveness is the subject of further research. This result can be harmonized with the latest review articles in this domain. Liu *et al.* also confirmed the use of these two forms of insulin [71], while Oryan *et al.* described the deficiency of information about the right insulin type for topical use [59]. Although in their review article, Wang *et al.* followed the type of insulin, they did not focus on the preparation technology [20].

The right concentration of TI is influenced by adjustable factors, like the type of active ingredient and the type of pharmaceutical forms, and non-adjustable factors, like skin integrity and the microenvironment of injuries. For local therapy, for cell regeneration and wound healing, a specific, pharmacodynamically suitable, topical dosage form is required [44]. The dose range for insulin to induce cellular mitogenic or proliferative effects is

significantly lower and is therefore significantly different from the doses, which are used for systemic antidiabetic therapy [92]. Similarly, Kannan *et al.* affirmed that local administration of insulin for WH would generally require lower doses than in the case of systemic administration [50]. Lima *et al.* determined an appropriate concentration of insulin in preliminary experiments. They used different concentrations of insulin in a cream formulation (0.0, 0.1, 0.25, 0.5, and 1.0 IU/100 g), and found that the most promising results in WH were 0.5 IU and 1.0 IU/100 g [12]. The dose of 1.0 IU/100 g, in some animals, induced alterations in plasma glucose [12]. They also confirmed that topical use of insulin at low doses does not result in the alteration of blood glucose levels [12]. Abdelkader *et al.* confirmed the doses of TI in animal experiments, as they varied the insulin dose from 5 to 15 units in mice to 20 units in horses [58]. Although there are studies in which the concentration of insulin was evaluated, however there is no clear data obtained from human clinical studies [11,20,59-71]. The deficiency of a conventional determination of TI concentration is also confirmed by other authors. Oryan *et al.* concluded that the dose of insulin was quite different between some of the *in vivo* studies [59]. Liu *et al.* also observed that the types, usages, and dosages of TI application, as well as methods of measuring outcomes used in the literature, are varied [71]. Topical insulin therapy requires corresponding local dosage accuracy and application skills.

In 1993, a Europan Patent was published, regarding the topical application of insulin, where the following suitable dosage forms were mentioned: emulsion, suspension, cream, ointment, solution, shaking mixture, gel, powder, granules, or plaster systems [44]. The majority of earlier animal experiments and human case reports described the local application of insulin, without focusing on the pharmaceutical formulation strategy necessary to achieve the therapeutic purpose. According to these reports, the most frequent pharmaceutical dosage form is the solution, which is either insulin in the form of conventional aqueous injection solutions for diabetics, either a diluted form with normal saline. The high frequency of using solution for TI therapy may be explained by the hydrophilic nature of the polypeptide molecule, by the ease of use of its application, in the form of dressings or sprays, by the quality and sterility requirements of wound management. The solution of insulin is sterile and the normal saline, used as an excipient, is also sterile, so this form can be easily prepared in an aseptic manner [93]. Here belong also the most of suspensions with a medium frequency, because they are the direct or a diluted form of isophane insulin. However, apart from these aspects, in 1993 it was confirmed that technically is not possible and therapeutically is uncritical to use insulin in unchanged form of injections for topical therapy of tissue injuries, which are used in the treatment of *DM* [44]. Unfortunately, conventional dosage forms do not offer protection for insulin against molecular changes, such as chemical degradation, conformational changes, aggregation, or biological inactivation [44]. In the wound area, the hypoglycemic polypeptide must be protected against enzymatic inactivation by peptidases. In this regard, a suitable topical dosage form must therefore enable a local protective function, but such a protective effect does not exist in the case of topical insulin applications using conventional aqueous injection solutions [44]. Insulin injection solutions often have higher concentrations of preservatives, which can cause local irritation and allergies in wound areas [44]. Abdelkader *et al.* also confirmed that administration techniques can be directly, with drops of insulin solution used in rats and humans [58]. Topical administration of peptide is limited by its short half-life and due to the inactivation effect of peptidases [88]. To eliminate the disadvantages, current topical formulations have utilized micro- and nano-sized carriers as novel drug delivery vehicles [63,65].

In the biologically complex process of tissue regeneration, it is not enough to consider insulin only as a singular active ingredient [44]. Since the basic role of insulin is the increase in cellular glucose transfer, this can also lead to a rapid local consumption of this substrate reserve [44]. Insulin alone can possibly be ineffective if there is no longer sufficient substrate available, therefore it is therapeutically feasible to provide a topical insulin formulation containing also its biological substrates or other cofactors [44]. Based on this same principle, in 1993 a topical insulin emulsion was described, in which the aqueous phase contains: 0.5% crystalline glucose monohydrate and 0.3% aprotinin in 100 ml of a neutral aqueous insulin solution, while the lipid phase is formed of 900 mL of a liquid lipid base (Topolip 492 pharmed) consisting of 80% a mixture of medium-chain triacylglycerides (50%) and medium-chain partial glycerides (30%) of saturated fatty acids with

chain lengths of 6-18 carbon atoms and 20% phosphatidylcholine (soy Lecithin), 0.01% ascorbyl palmitate and 0.05% alpha-tocopherol [44]. The other excipients and ingredients used in various dosage forms are represented in *Table III*. Innovative forms of TI use excipients like PLGA, chitosan, and silk fibroin for the micro- or nanoencapsulation of insulin. Proper selection of excipients is an important aspect for protein or peptide-based formulations, as these may significantly affect the shelf life, pharmacokinetics, and efficacy of the products. In this regard, the retention of bioactivity of the released insulin from diverse dosage forms remains the key challenge.

However, the formulation of innovative dosage forms, which contain insulin incorporated in particulate delivery systems, requires equipment that is not a common feature of hospitals and retail pharmacies.

4.2.2 Stability of insulin

At the time of writing this review there is no approved dosage form for TI. This can partly be explained by the instability of the peptide. The major problem of topical administration of peptides is their short half-life and loss of bioactivity in the peptidase-rich wound environment [95]. A specific topical dosage form requires a physiochemically defined consistency, the preference of adsorption or adhesion to damaged tissue and must also take into account the biochemical conditions in the tissue damage, e.g. the local pH value, osmotic pressure, and features such as the presence of wound exudates and potential infections, etc. [44]. In 2004 Duckworth *et al.* also confirmed insulin-degrading activity in wound fluid, similarly to other proteases in chronic wound fluid, the insulin degrading enzyme decreases the number of growth factors in addition to insulin and an excess of this enzyme could contribute to delayed wound healing [49]. Insulin as a polypeptide hormone represents all the classic properties of proteins. The formulation and delivery issues of therapeutic proteins can contribute to difficulties in pharmaceutical development such as stability, immunogenicity, pharmacokinetics- and pharmacodynamics-related problems [10]. The complexity develops from the hierarchical nature of its structure [10]. In general, chemical instability (hydrolysis, deamidation, oxidation, proteolysis, incorrect disulfide formation, beta elimination etc.) is related to the primary structure of the protein, whereas physical instability (denaturation, adsorption to surfaces, aggregation, precipitation) is associated with the global fold of the molecule.[10] Proteins like insulin present an increased tendency for self-association, dimers and hexamers have to cleave to monomers for receptor binding and exert its effect [10]. Factors such as presence of salts, organic solvents, heat, shear forces associated with mixing, interfacial tension can lead to unfolding and deactivation of insulin and a highly acidic microenvironment of the wound can cause peptide denaturation [50]. In the case of TI products, the production of large batches is not advisable, as the mixing effect enhances the aggregation potency. The larger batch to be produced, the greater mixing force exerted on it, leading to a deterioration of the quality of the final product. Insulin is a well-known example of agitation-induced instability, and patients are advised to avoid vigorous shaking of the insulin preparation [10]. Shaking is known to accelerate the degradation of insulin by way of covalent dimerization [10]. This limitation can be observed not only on industrial production, but also on hospital and retail pharmacy elaborations. The major challenges for the use of TI, like molecule instability and sustained delivery mechanisms, have recently been overcome with the development of micro- and nano-encapsulated insulin that can be incorporated in various pharmaceutical forms [11]. Localized skin therapy can be achieved with the particulate carrier systems, which minimizes the systemic exposure, maximizes the local drug concentrations, enhances drug stability, protects peptides from skin peptidases, targets their delivery to or within the skin, and reduces the skin irritation associated with some drugs [47]. However, most of the methods for the preparation of insulin containing microparticles, such as emulsification and the solvent extraction method [50], need processing in organic solvents, at extreme pH values, or mechanical stress, potentially challenging the bioactivity of insulin [96,97]. Preclinical and clinical studies have demonstrated positive effects of insulin on WH, but no suitable method for routine clinical use of topically applied insulin has been reported [19].

4.2.3 Wound management requirements

In the development of TI products, some important aspects should be taken into accounts, such as the structure and the integrity of the skin and the pathophysiological characteristics of wounds. The

main target of topically applied insulin is WH, therefore sterility is an important consideration in the preparation of various formulations. The current guidelines provide the sterility of the drug product if it is to be used on large open or deep wounds or on severely injured skin, and products used prior to invasive procedures (e.g. preoperative skin antiseptic) and for preparations for irrigation [98]. From the point of view of containers and closure systems drug products having sterile requirements should be packaged in single-use containers [98]. In the case of biopharmaceuticals, product sterility is achieved by performing the last manufacturing step in an aseptic environment and by ultrafiltration (through 0.2 μm pore size filters) or, if possible, by terminal heating of the product before filling. However, this latter process cannot be used for heat-sensitive proteins like insulin, because of protein inactivation by denaturation at elevated temperatures [10]. Preparation in an aseptic manner requires special equipment, which could be economically burdensome for hospital and retail pharmacies.

5. Conclusions

Although the idea of TI therapy dates way back to the years 1930s, it has yet to achieve its full therapeutic potential. The present review reveals that this is mostly due to deficient data and parameters. The mechanism of action, the signaling pathways, and the metabolic effects of insulin are well known at different levels, such as the central nervous system, liver, muscles, and adipocytes. However, the role of insulin in healthy skin and in injured tissues is a less studied area. Summarizing the results of preclinical and clinical studies, it can be concluded that the data presented supports the WH effect of local insulin administration, which acts through a complex mechanism, without hypoglycemic side effects. Topically applied insulin enhances the restoration processes for skin integrity and it is favorable in wound treatment because of its relative cost-effectiveness. The range of formulation strategies is large however, a special attention should be accorded to the stability of insulin and sterility of the product. For the moment there is no approved insulin-containing product on the market intended for topical use. Generally accepted formulations are described in patents PI 0705370-3 [12] and EP0561330A1 [44], therefore these products might be accessible in hospitals and retail pharmacy elaborations, despite the fact that sterility assurance still remains a challenge. Examining the balance of opportunities and limitations of TI therapy, it can be concluded that novel innovative technologies could overcome the formulation difficulties. Drug delivery systems using particulate carriers are promising approaches for sensitive biologicals. Encapsulation of insulin into micro- and nanoparticles, enables controlled drug which could accelerate WH. A high-quality, safe, and efficacious form of TI would be of great value from a socio-economic point of view. According to the best of our knowledge, the present study is the first research, which does not just assess the efficacy of TI therapy, but also explores the opportunities and limitations of its formulation.

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