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Physical Stimualtion for Delivery of Curative Liposome to Dermal Diabetic Tissue

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ABSTRACT

Various delivery system were tested as carriers of curative compounds in last 20 years. Subdermal wound tissue and cardiac ischemic tissue are target of many experimental nanoparticles in literature. Our experimental study reports the synthesis of nano-liposomes as vehicles for miR-21, miR-126 and insulin.

The liposomes were not injected directly into the diabetic foot tissue in gel or solution, however loaded to special ultrasound compatible gel body and arranged to surface plane of the diabetic foot. The final gel formulation was able to effectively release of containing liposomes to sorounding space of dermal tissue, the specific ultrasound pulses accelerated the liposome release.

The transport of the liposomes were visualised using confocal microscopy, the kinetic of lipsomes in real tissue were quantified by CT-tomography. The methods have potential to be upgraded for delivery of specific bioactive compounds in cardiology and dermatology.

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Introduction

Even though the pharmaceutical industry has been successful in discovering active compounds for the treatment and prevention of a variety of pathologies connected to diabetes mellitus, the diabeticfoot syndrome (DFS) and especiall chronic foot ulcers (CFU) still remain as global heatlh problem. More than 20 % of patients suffering from diabetes mellitus have some time-period with skin pathology, during their life time, which seriously affect their quality of life [1]. Some recent studies have reported that microRNA and liposomes with specific miRNA may help to stabilisationof DFU and accelerate healing. Molecules of miR-21 [2]. The level of miR-126, correlate to angiogenesis and dermal vascular network rebuilding [3].

Initial stadium of ulcer scan manifestated as recurrent blisters (mostly painful). It occurs with a greater recurrence in lower limbs, mostly on the feet. Although rarer, it can also affect the upper limbs, hands, and fingers [4].

Often, the curing of ulcers and primary blisters is very limited due to inadequate delivery of drugs by circulation (aggravated by decreased blood supply in the ischemic region of diabetics) or inadequate delivery of curative factors by applying a contact bandage (which can be limited by hypertrophic swelling layers of the dermis). The situation is also aggravated by the daily activities of patients. Localization on the surface of the lower limbs is problematic due to the risk of bandage displacement and secondary irritation and infection.

This all knowledge was the starting point for the design of our new curative nano-carriers with a cargo of anti-inflammatory and anti-ulceration compounds. Additive use of electro□sono-stimulation is shown as tool for accelerating of nano-carrier transfer and curative effects.

Methods

Animal and Diabetic Pathology Induction

Adult male Zucker Diabetic Fatty rats (ZDF, 8 weeks, 300 ± 40 g; Slovak Academy of Science, Slovakia) were prepared for experiment. The ZDF rat is an obese diabetic model, which has a mutation in the leptin receptor gene (fa gene) leading to obesity, glucose intolerance and fasting hyperglycemia. Rats were kept in a standard cage with a cardboard box (10x10cm) and a absorbent paper floor (Whatman gel blotting papers, Grade GB005, Merck, Germany). The absorbent paper was changed every second

morning. In next 4 weeks Initial foot blister developed on at least one hind leg of each animal. Glucose level was measured and 8 animals (from 12) with similar glucose level (280-300 mg/dl) and similar diameter of initial blister (8 mm) were selected for the experimental foot curing. All animal experiments underwent under approvement of ethics committee of SAV Bratislava, non-selected four rats were used for another experiments in animal lab via "3R recommendations" of the Council of European Union.

3D Phantom Based on Collagen Matrix

The collagen body (mimicking dermal structure) had to be prepared for preliminary test of basic hypothesis of liposome diffusion from "donor" gel device, before start of animal tests. For collagen body farbrication, we have used the methods of collagen/PEG/Chi/PCL composite synthesis, the 3T3 fibroblasts was arranged into into fresh composite [5,6]. Very briefly: Bovine collagen solution (Acid-soluble collagen; Type I), chitosan (Mn: 234 kDa, deacetylation degree of 95 %), poly-ε-caprolactone (PCL, Mn: 45000), polyethylene glycol (PEG, Mn: 6000), N-hydroxysuccinimide (NHS), 1-Ethyl-3-[3 dimethylaminopropyl] carbodiimide hydrochloride (EDC), Acetic Acid (glacial), tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich.The collagen/PEG/Chi/PCL (CPCP) composite solution was prepared by mix of collagen solution with chitosan (CC) and PEG/PCL (PEPC) solutions and crosslinking was initiated. Double dried by lyophilization steps(freeze-drying) were applied.

The diameter of the dry phantom scaffold was 35 mm, with 4 mm thickness. Circular scaffold were cuted into 4 micro-blocks (8 x 8 x 4 mm) and set into rectangluar confocal chamber and impregnated by DMEM (3000g/L glucose) solution with 106 live 3T3 fibroblasts. These rectangular microblocks could be called as "phantom body". Another "donor body" with liposomes was moved into the close proximity of the "phantom body" (rectangular too), then these two bodies had one touching wall. The second "donor body" will be described in next text, it is our original collagen sponge which will be used for in vivo experiment (all details in next description text for component #1 on Figure 1 and in Result - Figure 3). Diffusive movement of liposomes was quantified by fluorescence microscopy, details in legend of Figure 3.

The Nano-Liposomes Fabrication and their Incorporation to Original Three-Layer Wound Dressing

The liposomes were created by microfluidic mixing methods. The organic and aqueous phase were rapidly mixed using the Nanoassemblr Benchtop instrument (Precision NanoSystems, Canada). Preparation of organic fraction: we have mixed DSPC, Cholesterol, PE-DTPA-Gd (all from Avanti PolarLipids, USA), fluorescence LissRhod (Macrocyclics, USA), mixed in ratio 60/25/10/1,5 molar % of lipid. Total lipid concentration was 1 mg/ml (in 1:1 solutionofanhydrous ethanol and EtOH: DMSO). Preparation of basic water fraction: aqueous phase (Milli-Q water) (PBS ph 7,4), miR-126, miR-21 (2,3 mg/ml, Biovendor, Czechia), insulin 2,5M (40 IU/ml). The organic and aqueous phase (Milli-Q water, systemIQ 7003, Merck, Germany) were rapidly mixed using the Nanoassemblr Benchtop instrument (Precision NanoSystems, Canada) at Flow Rate Ratios (FRR) 1:3, total flow was set to 6 ml/min to form unilamellar liposomes 1 mg/ml of lipid. During the mixing process, the temperature was controlled using Nanoassemblr Benchtop Heating Controller accessory (Precision NanoSystems, Canada). For preparation of liposomes containing DSPC, the temperature was set to 55 °C.

The vesicle size, polydispersity index, and zeta potential of liposomes recorded in water using dynamic light scattering (Malvern Instruments, Malvern, UK). The measurements were performed in a U-shaped cell with gold-plated electrodes at 37°C.

The three-layer wound dressing was prepared in our lab as modification of patented gel device [7]. The modification consists in small change in central collagen sponge (component #1 on Figure 1). Impregnation of the liposomes (instead of exosomes) fraction to the component #1 was performed. And due to the shape of rat foot, the extension the collagen component #1 was also performed (aim was to achive better direct contact and liposome diffusion to dome-shape of skin surface). Peripheral layer #2 (denser strengthening component) and #3 (PDMS mechanical cover) were almost the same like the patented geometry.

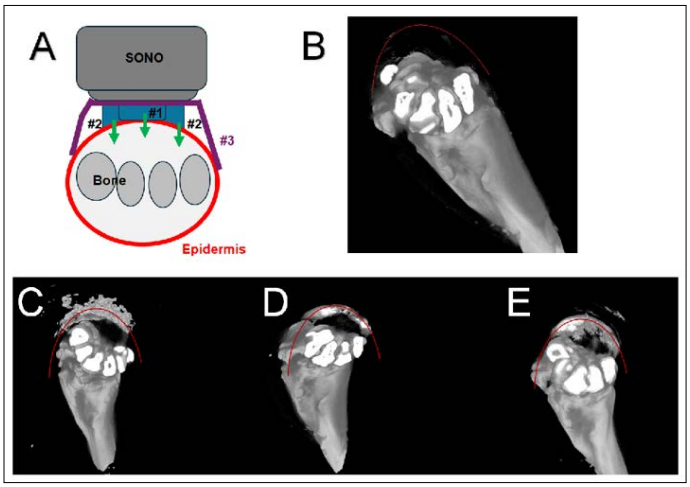

Figure 1: (A) Geometry of complex dressing on animal foot, the initial ulcer on heel is turned upwards, the EMUS applicator is centered on the scar, gently touching the cover (electromagnetic and ultrasonic pulses pulses are fed alternately into the gel and tissue). (B) CT-scan of foot, where no Gd-liposomes are integrated to gel cover. Three scans (C, D, E) are representative illustration of typical foot of fresh gel on epidermis before diffusion, typical foot without sono-stimul (day 3), typical foot after sono-stimul (day 3). In last scan E is well visible Gd-liposomes diffusion to central area subepidermal are. Central white positivity display the bones inside the foot, red lines was added at postprocessing for epidermis geometry.

Apllication of Cover Variant and Evaluation of Liposome Delivery in Vivo

Six + two rats with typical foot ulcers were selected for pilote study. Rats were put under inhalation anesthesia (2% isofluranat a flowrateof 0.4-0.8 liter/min). The selection criterion was size (8 mm in diameter, Figure 1-B), the integrity of upper epidermis and pink color. The foot was softly wash by physiological solution and 10% ethanol and three-layer wound dressing was quickly arranged to foot. Six animal were divided into 3 groups: I - two rats received a complex dressing containing liposome; II – two rats received a complex dressing containing liposomes + 1 hour of EMUS electromagnetic and ultrasound stimulation (details in Figure 1-A); III – two rats received a complex dressing without diluted liposome in central collagen body, EMUS was applied. Additive group IV was later created as "total negative control" (only flexible bandage without dressing and liposomes). Summary of variants are described in Table 1.

The fresh complex dressing with fresh gel was applied for 2 hours on foot topical plane in horizontal position at day 1 and at day 3 (motionless animal under anesthesia), contact of EMUS head was arranged on surface of component #3 (with or without working ultrasound and high-frequency EM generator). After 2 hours, the dressing was recovered by simple elastic bandage (Figure 2-A), fixed by fibrin glue for prevention of mechanical disruption. The same procedure was performed on day 7. The mentioned EMUS applicator consisted of output from electromagnetic generator and ultrasound head. Two types of stimuls were applied foreach foot during 2 hours in contact mode: 15 minutes of electromagnetic harmonic sinusoidal course at the frequency of 5 kHz (selected from our previous work, next 10 minutes of ultrasound (1 - 2 Mhz mode with 2 seconds active time and 2 Seconds pause; 0,8 W) and 5 minutes with no EMUS stimuls. Afte that, new 20 min sequence starts [8].

Table 1: Summary of Animal Group

	Variant	Application of gel + liposomes	EM and ULTRASOUND stimulation
I.	GEL+lipo	Contact of fresh gel (day 1, 3, 7)	No
II.	GEL+lipo +EMUS	Contact of fresh gel (day 1, 3, 7)	Yes, 2 hours (day 1, 3, 7)
III.	GEL-only +EMUS	Contact of fresh gel (no liposomes) (day 1, 3, 7)	Yes, 2 hours (day 1, 3, 7)
IV.	NegCtr	(Only elastic bandage)	No

Evaluation of Liposome Distribution

The evaluation of liposome distribution was scheduled at the initial day 1 and at the end of day 3 by X-ray CT Bruker Skyscan 1276 (Bruker, USA). The fresh gel cargo with Gd-contrast liposomes on skin at day 1 is ilustrated on Figure 1-C; Foot tissue display incorporating Gd-liposomes during next hours and days, the typical distribution for day 3 is ilustrated on representative scan on Figure 2-D and E (EMUS versus no additive stimul). Detail explanation of variant and negative control is in legend of Figure 1 and Teble 1.

Evaluation of Ulcer Development

After opening the elastic cover, the condition of the foot was photographed on day 12. The morphology of the ulceration was compared for individual experimental animals.

Quantification of Growth Factors

On the same day 12, TNF-α and IL-1β levels in serum rats were measured using a kit for an enzyme-linked immune sorbent assay (ELISA), with used antibody TNF-α (kit 13-7326-85, eBiosciences, USA) and IL-1α (kit 13-7111-85, eBiosciences, USA). The cytokine concentrations were measured using a standard curve and expressed as picograms/mL.

Results

Liposome Charactestics and X-Ray Test

The liposomes with encapsulated insuline and miRNAs showed uniform particle sizes around 75 nm. The diagram of size distribution for liposomes is presented in Figure 2. Liposomes had negative surface charge (-9.7 ± 0.6 mV). The liposomes remained intact in the PBS medium for 60 h and without significant aggregation (repeated measuring by light scattering technique on a Zetasizer Nano ZS analyzer, data not shown). The microfluidic methods displayed very well percentages of bioactive factors entrapment efficiency (% BFEE) for miRNAs and insulin in (93 - 97 % in 3 indepdendent tests). The amount of stable s miRNAs and insulin (2 h and 60 h in 4°C) was tested after perforation of liposomes by triton-X - measured by quantitative specific PCR and BCA protein assay (Thermo Fisher Scientific, USA).

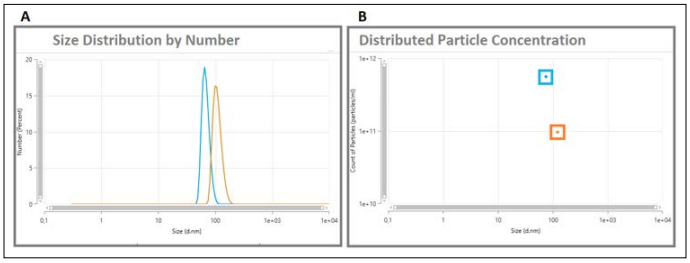

Figure 2: Liposome Size. Blue – Fresh Liposome Containing Insulin and miRNAs. Orange – after 24h (Details in Methods).

Two additive test on liposomes were prepared. The first one was evaluation liposomes shape and number after 24h in physiological solution at 37°C. The results are in the same Figure 3 (organge curve and marker). The stability of liposomes is relatively high, with logical shift of diameter (may be after interaction of liposomes with sorounding solution and aggregation). The second tests was comparison of liposomes with nature and denaturated insulin: negative control type of liposomes with denaturated insulin and non-active RNA (prepared thermaly at 80°C; instead of native insuline) was fabricated by the same microfuidic methods and using the same lipids and chemicals, particle size of the modified liposome was approximately 76 nm, with a negative surface charge (-10.3 ± 1.3 mV). The effect on in vivo was compared in extra additive test with negative result (details bellow in description of results).

In vitro diffusion test brings positive result. After contact of donor collagen body (component #1 on Figure 1, with uploaded contrast liposomes) and collagen phantom body, the liposome could be detected in phantom matrix after 2 or 6 hours (Figure 3 - the curve on left, where representative gradual lighting of fluorescence is visible). The transport of liposomes could be accelerated by EMUS stimul (Figure 3 - curve on the right).

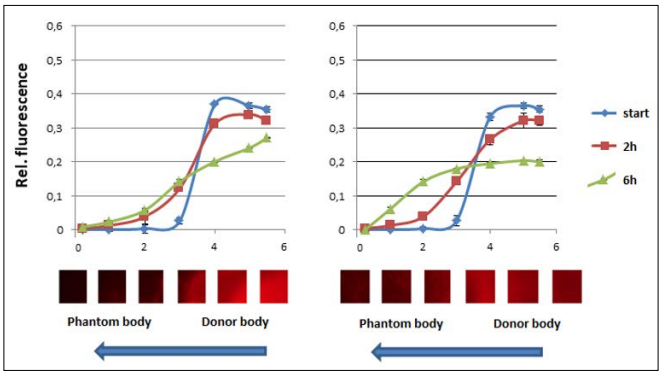

Figure 3: Kinetic of liposomes detected on the basis of fluorescence. Time: blue - starting time, red - 2 hours, green - 6 hours. Red microphotos illustrate the fluorescence intensity of liposomes in specific areas of phantom or donor body (position 3 on horizontal axis is position of phantom – donor contact). Blue axis define the gradient and direct of liposome diffusion. Fluorescence value for each geometrical position and each time-point was expressed as the average of 3 measurements (mean±standard deviation in the graph).

X-ray tests with plastic vials demonstrated that the used 1014 Gd-liposomes in 0.5 ml of gel are contrast on X-ray scan against animal soft tissue. The lowest detection limit in collagen box was evaluated as 105 Gd-liposomes in 0.5 ml (data not shown).

Liposome Detection and Kinetic Evaluation

After the phantom tests, the X-ray detection on live animal was initiated, used liposomes in fresh gel were able to detect on surface of the skin as contrast "cube-shape" drop (Figure 2-C). The gel without Gd-liposomes (Figure 2-B) was used as a non-contrast control. Evaluation of liposome redistribution in short times was investigated by X-ray, the situation of positive pixels redistirbution was perfectly visible, when we compare initial situation Figuer 2-C and Figure 2-D or Figure 2-E. "Cube-shape" contrast area is redistributed to "envelope-shape" (simple diffusion to upper layer of skin of relatively big part of liposome cargo). The ultrasound stimulation caused the significant larger redistribution forward to internal are the foot at the end of day 3.

Evaluation of Ulcer Development

The morphology of the ulceration was compared for individual experimental animals, the variant GEL-LIPO+EMUS display the best healing result and alignment with the original skin plane, representative photos for all variant on Figure 4.

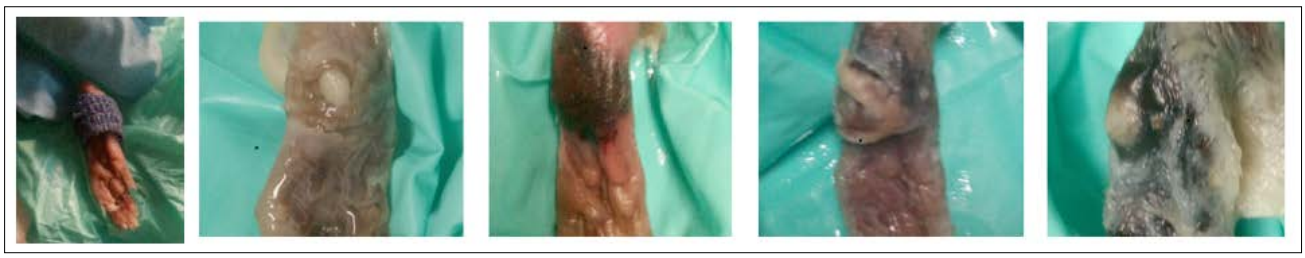

Figure 4: Mechanical elastic bandag on foot (on the left) and final state of skin of foot at the end of experiment (4 photos from the left: GEL+lipo, GEL+lipo+EMUS, GEL-only+EMUS, NegCtrl).

Quantification of Growth Factors and Inflammatory State

To explore the potential correlation between particular growth factors levels and the foot healing after curative liposome appliaction, the levels of TNF-α and IL-1β were quantified. Variant GEL+lipo+EMUS is significantly better for TNF-α and IL-1β tests than negatives control variant (Neg-Ctrl) and laso than GEL+LIPO without EMUS stimulation. We have additionaly prepared the extra variant Neg-Ctrl2, to see the in vivo effect for GEL+lipo+EMUS variant, where denaturated insulin and mRNA were applied (see details of variant denaturating in Methods). Curative effect of negCtrl2 was very far from results of original liposomes with functional cargo.

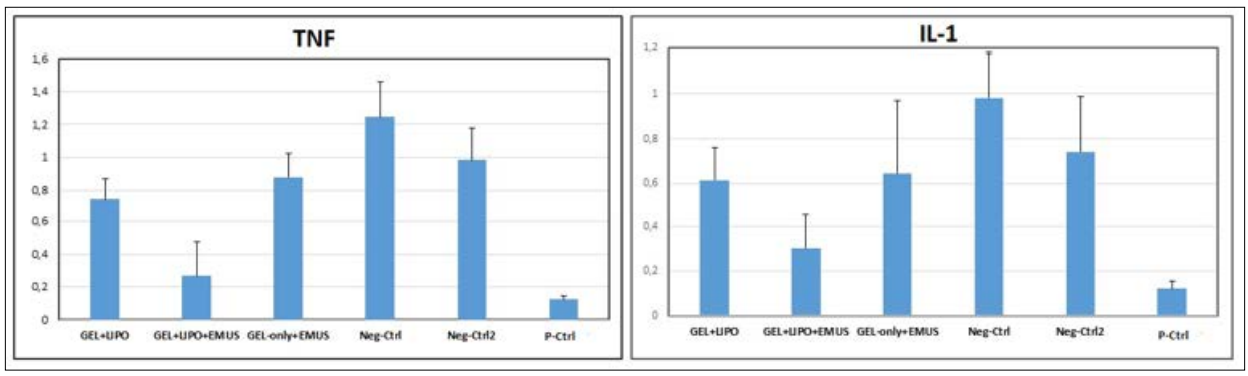

Figure 5: Result of TNF and IL-1 quantification. Abbrevations GEL/lipo/EMUS for variant are described in the Table 1, P-Ctrl is positive control (rat before any ulcer initiation). The horizontal axis described detected protein amount in nanograms expressed per mililiter. Each variant value was computed as average value from 6 measurement (2 animals x 3 sample collection), mean±standard deviation is visualized in the graph.

Discussion and Conclusion

Our investigation has three basic outputs. Firstly, the proposed liposomes were evaluated, and their important shape, charge, and cargo characteristics were quantified. Secondly, the contrast of the liposomes was sufficient for noninvasive X-ray tracking of liposome delivery kinetic to deeper layer of the skin during the hours after topical application to the skin. Thirdly, liposomes applied in the form of the original collagen cup (previously presented as a patent by our lab-group displayed significant effects on in vivo diabetic skin pathology and prevented ulcer development and skin instability in diabetic rats [9].

The two bioactive agents, insulin and two specific miRNAs, and their respective mechanisms of action were taken into account before designing our current liposomal system and methodical steps that could affect the stability of the liposome cargo. The model, the target pathology, was an undeveloped rat leg ulcer on adult diabetic rats . The negative control tests were based on variant GEL+lipo (application of collagen cover with liposomes, however without accelerating EMUS stimulus) and variant GEL-only + EMUS (application of collagen cover without curative liposome under EMUS stimulus). The collagen with liposomes itself or EMUS stimulus itself did not caused blister reduction or well prevention of dermal instability. The „total negative" control was administrated as foot without any gel cover, only elastic bandage for prevention of mechanical irritation. This negative control

control showed the development of most lrgest inflammation.

Liposomes containing miRNA were used in a number of articles in the last years. Similar diabetic pathology and liposome delivery were used in experiments by Kiani and Wang, where slightly different liposomes with miRNA21 were used [10,11]. However, the main difference is the location of the pathology and the timeline of the curing strategy. Kiani or Wang both used a model of induced open ulcers and a longer time period for covering the wound. A very similar diabetic foot model was used by Kaliamurthi [12]. The physical properties of the nanodelivery system were similar to our liposomes; however, the cargo of his nanodelivery system was only Rutoside, not miRNA or insulin. Our study also had a principal difference in the use of the acceleration ultrasound device and electromagnetic harmonic sinusoidal costimulation. This brings the great advantage of quick loading of liposomes into the epidermal and mostly subepidermal tissue, where a direct action on endothelial cells, peripheral cells of vessels, and keratinocytes can occur.

Insulin is a factor that was mainly used for intravascular injection, for complex redistribution to all tissues of the body. However, beside VEGF and TGF-beta, insulin is also one of the very effective growth factors for local topical therapeutic use in a set of preclinical and clinical studies [13-15]. Only one big disadvantage of insulin is its short half-life in vivo due to proteases in the wound bed. Hence, insulin encapsulation in liposomes is a chance to ensure proteolysis. Encapsulation into liposomes and acceleration of liposome trasfer by ultrasound or another biophysical force is also a chance to accelerate the distribution of a higher dosage of insulin in the internal part of the dermis and foot tissue.

Therefore, our miRNA carrier liposomes connected with Gd-conjugates could be considered as a promising miRNA delivery platform, where additive biophysical forces can significantly increase the influx of the liposomes into the dermal and subdermal tissue. Thereby, this complex system could expand in the use of modern synthetic exogenous miRNA mimics and specific bioactive factors to various therapeutic areas for topical treatments.

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