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Research Article

Innovative Wound Dressing Coated with Drug-Loaded Adipose Mesenchymal Stem Cells to Promote Wound Healing in Diabetes

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Abstract

Impaired wound healing is associated with hyperglycaemia in patients with diabetes. Hyperglycaemia induces protein glycation and the formation of Advanced Glycation End-Products (AGEs). The accumulation of AGEs in the body results in the structural and functional modification of tissue proteins. This study was conducted to evaluate compounds with antiglycation activities (S-Ally1 Cysteine (SAC), N-Acetylcysteine (NAC) and the mimic compound A). The extent of glycation in the presence and absence of several inhibitors was assessed via several methods including fluorescence, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)-silver stain, Western blotting, and Enzyme-Linked Immunosorbent Assays (ELISA). Additionally, this research aimed to evaluate and quantify the potential of Human Adipose Mesenchymal Stem Cells (hADMSCs) to uptake and release these drugs as potential therapeutics. To achieve this, hADMSCs were primed with a combination of SAC/NAC and mimic compound A and their concentrations were analysed using High-Performance Liquid Chromatography (HPLC). The SAC/NAC and mimic compound A prohibit the formation of AGEs while the Conditioned Medium (CM) from SAC/NAC- and compound A-loaded hADMSCs induced cell migration and tube formation in BAECs. hADMSCs provide a unique opportunity for the development of an innovative targeting and drug-delivery system which could effectively deliver therapeutics to specific regions of wounds or other damaged tissues. The data provided demonstrate the potential of hADMSCs as a drug delivery method with the potential to improve wound healing, and it may offer potential therapeutic targeting for the development of diabetic complications.

Keywords: Diabetes; SAC/NAC; Mimic compound A; Wound healing

Introduction

The presence of Advanced Glycation End-Products (AGEs) in the wounds of diabetic patients results in continued chronic inflammation which can prevent the wound from progressing to the next stage of healing (matrix deposition and remodelling) and thereby prevents healing from taking place [1].

Many pathological factors affect tissue repair in diabetes mellitus [2] such as atherosclerosis, development of major vascular stenosis or occlusion, and renal failure. Atherosclerosis can lead to an embolism in proximal vessels which results in decreased blood flow, insufficient oxygen delivery and can cause damage to the toes. Diabetic people are more prone to the development of neuropathy and infectious diseases due to immunosuppression. Moreover, in diabetes and venous stasis disease, oedema impairs wound healing.

The organosulphur compounds can be separated into water-soluble and oil-soluble constituents. Water-soluble organosulphur components include SAC (S-allyl cysteine) (Figure-1) and NAC (N-acetylcysteine) (Figure 1) [3]. SAC is a major compound which is primarily observed in garlic extracts from alcoholic and aqueous bases [4]. NAC has a –SH residue donor and a nucleophile [5], deriving from gamma-glutamyl-S-allyl-L-cysteine. A

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typical antidiabetic treatment combined with garlic has demonstrated the ability to improve antihyperlipidemic activity and glycaemic control indicating that garlic could be a beneficial and suitable addition for patients managing hyperlipidaemia and diabetes [6].

The synthetic mimic compound A is a small molecule inhibitor-mimic of SAC and NAC. Mimic compound A [(R) S-Benzyl-L-cysteine], whose molecular formula is $C_{10}H_{13}NO_2S$, is a more fat-soluble compound (Figure 1) [7].

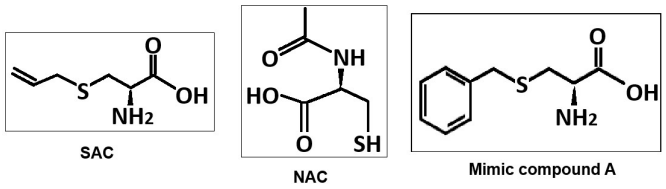

Figure 1:

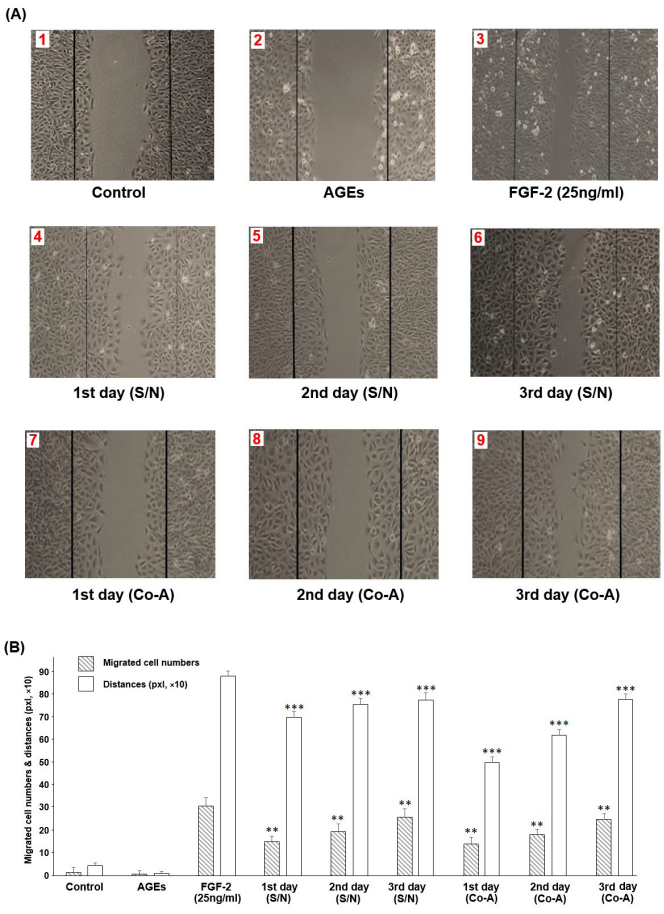

Figure 1:

The Aged Garlic Extract (AGExt) predominantly contains water-soluble compounds and a small number of oil-soluble constituents [8,9]. The process of AGExt increases the concentration of antioxidants and converts allicin, and other unstable compounds, into stable substances that promote health such as SAC and NAC [10]. AGExt is believed to be an excellent natural oxidant that may be capable of protecting the macromolecules that are essential for the survival of cells by limiting the oxygen effects and inhibiting the free radical processes [11]. Additionally, AGExt has received particular attention as many studies have revealed it to be an effective antioxidant with the capacity for free radical scavenging [12]. SAC is the sole reliable marker employed in studies concerning garlic consumption by humans [13] and may be used to compare sources, or for garlic preparation standardisation, as it is found in many preparations. The sole product that is standardised for SAC is AGExt[8]. Various toxicological studies have been able to confirm the safe use and consumption of AGExt [14].

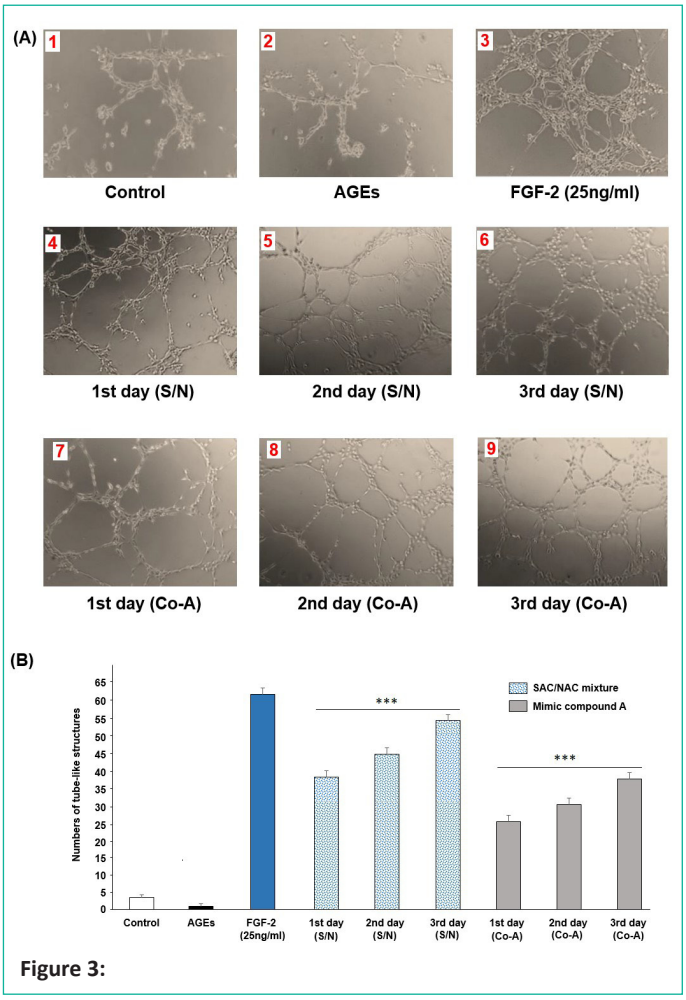

Figure 3:

The innovative approach of utilising stem cells to deliver drugs within the body has been recently researched, with results revealing that MSCs can effectively accept and release treatments [15]. The potential use of delivery vehicles is being explored to improve the method of drug delivery and increase efficiency. A delivery vehicle is a method which encapsulates the drug in a specific material that will release the medicine in a controlled manner and allows the dosage to be optimised for a certain time period [16,17].

The use of MSCs as a delivery vehicle in therapeutic and diagnostic situations is a beneficial option as they are readily available, safe, can travel to specific tumorigenesis and injury sites, and have a small immunogenicity profile [18].

Materials and Methods

Reagents

SAC and NAC were supplied by the Wakunaga Pharmaceutical Company, Japan and the mimic compound A was provided by Dr Alan Jones of the Pharmacy Department at the University of Birmingham, UK. The compounds were tested alone, in mixtures, and in variable concentrations to identify the maximal potential therapeutic effects. All remaining chemicals (unless otherwise noted) were purchased from Sigma-Aldrich at the highest purity (where possible).

Cell Culture for BAECs.

Bovine Aortic Endothelial Cells (BAECs) were prepared as described by Sattar et al. [19] The BAECs were cultured in DMEM supplemented with 10% of Foetal Bovine Serum (FBS), 2 mM

glutamine and 1% antibiotics (100U/ml penicillin and 100µg/ml streptomycin) in a T25 flask pre-coated with 0.1% gelatine and incubated in a water-saturated, 5% CO_2 incubator at 37°C. For each experiment, BAECs were serum starved (incubated with DMEM supplied with % FBS) for 24 hours before the assay. About 80% confluent BAECs were incubated in serum-poor medium (SPM containing 1% FBS) overnight and up to twenty-four hours. The BAECs were used between passages 5-10 for all of the experiments which were repeated three times and samples were prepared in triplicate (n=9) in all cases. Cell viability was optimised by the Alamar blue assay [20,21]. The cells were seeded in 96 well plates (1.6–1.8×10^4 cells per well) for 72 hours, with or without testing compounds. The absorbance of each well was measured at 490 nm using a microplate reader (Multiskan Ascent, Thermo Life Sciences, Hampshire, UK).

Cell Culture for Human Adipose-Derived Mesenchymal Stem Cells (hADMSCs)

Human Adipose-Derived Mesenchymal Stem Cells (hADMSCs) were supplied by Professor Giulio Alessandri and Professor Valentina Cocce (Istituto Neurologico "Carlo Besta"). The cells were removed from liquid nitrogen, thawed, placed in a T-25 flask containing 5 ml Stem Cell Growth Media (SCM) and incubated for four hours. Following this, the medium was exchanged for a fresh growth medium. The cells were grown in SCM composed of 80% Iscove's modified Dulbecco's medium (IMDM - Sigma-Aldrich) containing 5% foetal bovine serum (FBS - Sigma-Aldrich), 10% NeuroCult medium (Stem Cell Technologies), 10% endothelial basal medium (EBM - Lonza), 20µl Epidermal Growth Factor (EGF), 10µl of Basic Fibroblast Growth Factor (b-FGF) and 10µl heparin sodium solution in PBS in a humidified incubator with 5% CO_2 at 37°C.

Cytotoxicity of the Compounds on hADMSCs

The toxicity of SAC/NAC and compound A on hADMSCs was determined in a twenty-four-hour alamarBlue assay (cytotoxicity test - Life Technologies). 96-well plates containing cells in a growth medium were set up. Sub-confluent cultures (2×10^5) of hADMSCs were exposed to different concentrations of SAC/NAC (0.0076 – 500µg/ml) and compound A (0.12 – 250µg/ml) and the final volume was made up to 100µl in each well. Following a 24-hour incubation period, 20µl of Cell Titer-Blue® Reagent was added to each well. The cells were incubated for a further four hours then fluorescence emission spectra were recorded at 560nm after excitation at 590nm.

Endothelial Cell Migration Assay

A migration assay of the BAECs was performed using a standard wound scratch assay (as described previously) [22] and the BAECs were tested in the presence and absence of SAC/NAC and mimic compound A. The BAECs were seeded on 1cm-by-1cm glass coverslips at a concentration of 4×10^5 cells/ml in 1 ml of cell growth medium in each well of 12-well plates and incubated in a water humidified and 5% CO_2 air incubator at 37°C. When the cells achieved about 90% confluence, the growth medium was replaced with serum poor medium [SPM, basal growth medium containing 0.5% (v/v) FBS], plus AGEs (250 µg/ml) and incubated for a further 24 hours. Following this incubation period, each well of the 12-well plates was washed three times with warm Phosphate Buffer Saline (PBS). The adherent cells were scratched in one single continuous line across the glass coverslip using a razor blade and the wells were carefully washed three times with warm PBS to remove any floating cells. The cells were incubated in SPM containing SAC/NAC (at 0.1 µg/ml) or mimic compound A (at 0.1 µg/ml), added to the wells along with AGEs (at 250 µg/ml) and incubated under the same conditions (as detailed previously) for 24 hours. BAECs incubated in SPM were used as a control. BAECs incubated in SPM containing AGEs (at 250 µg/ml) were also used as a negative control while Fibroblast growth factor-2 (FGF-2, at 25 ng/ml) was employed as a positive control.

Following the twenty-four-hour incubation period, 100 µl of 4% (w/v) of Paraformaldehyde (PFA) was added to each well at Room Temperature (RT) for 15 minutes to fix the cells. The medium was then removed, and the wells were washed with PBS, followed by 200 µl of 100% ethanol fixation for five minutes. The wells were left to dry, and the cells were stained with methylene blue for five minutes. The stain was removed, and the wells were washed with Distilled Water (dH_2O). Finally, the cell migration was assessed via phase-contrast microscopy and images were taken using a digital camera (ZEISS, Fisher Scientific, Loughborough, UK). Cells were treated in triplicate and pictures of five areas of each well were taken. The image analysis was performed using Image-J. Both migration distance and the number of migrated cells were measured and the mean ± SD were calculated. Each experiment was performed three times in duplicate and a representative example is provided in Figure 2.

Endothelial Cell Tube Formation Assay

The preparation of BAECs was performed as described above and consisted of 50 µl of Matrigel™ basement membrane matrix reduced in growth factors (BD Bioscience, Berkshire, UK) was added to each well in 96-well plates and incubated at 37°C for one hour to allow the Matrigel™ to polymerise. Following this, 9×10^3/50 µl cells were added to each well including those compounds in low serum (0.5% of FBS, v/v) MV media with 0.1µg/ml of SAC/NAC mixture, or mimic compound A. BAECs incubated in SPM containing AGEs (at 250 µg/ml) were used as a negative control and FGF-2 (25 ng/ml) were used as a positive control. The tests and controls were incubated for 24 hours at 37°C. After this incubation period, BAECs migrated and aligned to form tubes (defined by the enclosure of circumscribed areas). The counts of closed tube-like areas were made in five fields by phase contrast microscopy and images were created by a digital camera (ZEISS, Fisher Scientific, Loughborough, UK) using the ×10 objective. All of the experiments were completed in triplicate, repeated three times and a representative example is provided in Figure 3.

Measurement of SAC/NAC and Compound a Release from hADMSC Cells

hADMSCs were cultured in T75 flasks and, when they were confluenced, the medium was replaced with a plain medium and the SAC/NAC and mimic compound A were added to the cells and cultured for three days. Following the three-day incubation period, the medium was removed, and the cells were washed three times with warm PBS and the plain medium was added to the cells. The medium was collected after 24 (first day), 48 (second day) and 72 (third day) hours. The concentrations of SAC/NAC and mimic compound A were tested and quantified via a standard HPLC protocol (data not shown).

Results

This research work has examined the potential benefits of the SAC/NAC mixture and mimic compound A released from hDAMSCs to inhibit AGE's potential damage to the endothelial

cells. SAC/NAC mixture and mimic compound A were tested by BAECs proliferation assay which revealed that there were no differences between the cells with and without SAC/NAC and mimic compound A on cell growth (data not shown).

A BAEC cell migration assay indicated that the SAC/NAC mixture and mimic compound A provided effective protection for cell migration in the presence of AGEs and was significantly higher when compared to the control and cells incubated with AGEs alone (both by the cell numbers and migrated distances). Both the cell numbers and the migrated distances were measured using 10×object (Figure 2).

In the BAEC cell tube formation assay, the SAC/NAC mixture and mimic compound A demonstrated protection against the monolayer damage caused by the high concentration of AGEs (250.0 µg/ml) (Figure 3).

The bar graph in Figure 3 depicts the significant difference between the control and the test conditions.

Discussion

There is evidence of the involvement of AGEs in complications associated with long-term diabetes and AGExt plays a protective role against the potential damage of endothelial dysfunction [23-26].

As diabetes is a major public health concern, alongside atherosclerosis that results from damage to the macro-vasculature, it is considered that AGExt could be a potential nutraceutical for anti-atherosclerotic therapy while also protecting against protein glycation in diabetic patients [6,27,28].

This study has demonstrated that the most active components (the combination of water-soluble cysteinyl moieties, SAC, NAC, and mimic compound A) are exceptionally powerful antioxidant phytochemicals which protect against oxidative stress and inhibit subsequent cellular damage *in vitro* which was supported by a previous study[6]. SAC and NAC are sulphur-containing amino acids derived from garlic which possess antioxidant, anti-cancer, neurotrophic and other properties. Mimic compound A is synthesised according to the SAC/NAC structure and is more fat soluble.

To the best of the author's knowledge, this is the first work to investigate the uptake of AGExt-activated ingredients and their release by hDAMSCs protecting against the potential damage caused by AGEs *in vivo.* These significant findings indicate that the SAC/NAC and mimic compound A could potentially prevent the ECs' oxidation-induced protein damage. Therefore, the combination of SAC and NAC (SAC/NAC) and mimic compound A, carried by hDAMSCs, could be of potential therapeutic value in the treatment of diabetes wound healing and cardiovascular disease in patients.

The use of MSCs as a delivery vehicle in therapeutic and diagnostic situations is an appealing option as they are readily available, safe, can travel to specific tumorigenesis and injury sites, and have a small immunogenicity profile [29]. However, there are studies to be carried out and challenges to master before adopting the clinical use of MSCs to deliver drugs within the body. One such challenge is how to effectively load the drug on the cell. The cells need to be altered to manufacture elevated levels of the agent, or loaded with enough drugs to produce healing effects, without harming the viability of the cell or the necessary properties needed for effective delivery of the drugs such as the migration potential of the cell. Studies carried out on animals have demonstrated that it is possible to generate modified and therapeutically effective MSCs and human clinical trials will be necessary to determine if these outcomes can be effectively translated. Another challenge is to develop a method for the drug to be released effectively which requires mechanisms that initiate the release from the MSCs to enter or access the target cell. Overall, the use of MSCs as a drug delivery system is a promising prospect. The use of these cells in this manner will only become widespread if an effective method for loading and releasing these drugs at the right times and locations can be developed and ensured [30].

A chronic wound is defined as one that has persisted for a minimum period of three months and is commonly classified as being diabetic, a pressure ulcer or vascular [31,32]. Multiple factors can play a role in delaying the process of healing a wound including diabetes, arterial or venous insufficiency, renal disease, local pressure effects, old age, and trauma.

Diabetic Foot Ulcers (DFUs) are experienced by a considerable percentage of diabetic patients (15%) and may result in amputation of the lower leg [33]. Wound care currently focuses on recognising any factors that can aggravate the wound and removing these to lower the inflammation and encourage the healing process to continue [34].

Multiple chronic wound therapies have been developed although the success rates have been varied [33]. One type of therapy that has been proposed and demonstrates the potential for use in enhancing tissue regeneration concerns the use of stem cells. Using MSCs as a wound treatment results in the increased formation of granulation tissue, increased angiogenesis, and an improved rate of wound closure. However, it is not the MSCs replacing the damaged cells by differentiating that causes the positive outcomes; it is the MSCs' ability to produce a therapeutic properties agent via the secretion of soluble factors. This secretion helps to regulate the cells' responses to a cutaneous wound [35,36]. Angiogenesis is promoted, cell death is reduced and the formation of scars at the area of the injury is reduced with the MSCs cytokine and growth factor secretion [37]. Additionally, immunosuppressive factors are released by MSCs and these help to prevent the increase of immune cells including natural killer cells, B-cells and T-cells [38]. Administering exogenous MSCs to wounds accelerates the closure of the wound and increases angiogenesis and the formation of granulation tissue [35]. It is suggested, by a further study, that the secretions from MSCs can produce such results due to their ability to regulate the responses to injury by the main cell types that are present within the wound: keratinocytes, endothelial cells, macrophages and dermal fibroblasts [35]. In another study, topical allogeneic MSCs were used to treat full-thickness wounds and resulted in reduced inflammation and enhanced healing. These results were probably due to the release of immunosuppressive factors within the wound which prevented an increase of immune cells [39]. A recent study revealed that MSCs used on bioengineering scaffolds encouraged the enhancement of re-epithelialisation which is characterised by the enhanced formation of blood vessels alongside the reappearance of hair follicles, multi-layered epidermis and sebaceous glands [40]. In a study conducted on animals, adipose MSCs were used on an acellular dermal matrix and resulted in improved angiogenesis, enhanced healing of the wound and played a role in the newly formed vasculature [41].

MSCs are an effective therapy for wound healing and the healing of scars despite warnings concerning their source or

identity [32]. Bone Marrow Mesenchymal Stem Cells (BMSCs) *in vitro* could have limits in terms of their long-term potential for differentiation and growth [42]. Therefore, it is vital to identify other MSC sources in humans which can be used for therapies to heal wounds. Various MSCs, including dermal, adipose-derived stromal cells and MSCs from the umbilical cord and amniotic fluid, have been involved in preclinical investigations into wound healing [43]. The dermal MSCs and adipose-derived stromal cells can be harvested using minimally invasive procedures and the cells are abundant within skin and fat. The use of these cells presents an ethical alternative to other, more controversial, harvest sites which makes them a notable alternative.

The use of dermal MSCs and adipose-derived MSCs (ASCs) demonstrate promise as, when compared with BMSCs, they exhibit similar immunogenicity, differentiation potential and biological characteristics [44]. In studies concerning wound healing, adipose-derived stem cells (ASCs) display promising outcomes *in vitro* [45] and, in animals, *in vivo* [46] and are being tested in clinical trials for their ability to help heal ulcers and burn wounds. An elevated level of success has been observed in the healing of chronic radiation wounds using human ASCs taken from the debridement of the burned artificial dermis [47]. One study noted that the paracrine factors' secretion from Amniotic fluid MSCs (AF-MSCs) was related to cell survival and proliferation was enhanced by hypoxia [48].

Additionally, the MSCs alter a macrophage's phenotype into more of an anti-inflammatory M2 phenotype characterised by an upregulation of interleukin-12 and other similar anti-inflammatory cytokines as well as TNF-α and an increase in phagocytic ability [49]. MSCs also demonstrate bactericidal properties as they secrete an antimicrobial substance and increase phagocytosis by immune cells which enhances the eradication of bacteria [50].

The therapeutic benefits of MSCs are primarily a result of their capability to secrete pro-regenerative cytokines which makes them a potentially successful option for chronic wound treatment [51]. Current studies and research are being conducted using stem cells obtained from various sources to determine their abilities to regenerate tissue and heal wounds. These clinical and preclinical trials have proven that therapy using stem cells is a safe and tolerable option [52] and has demonstrated positive results.

Author Statements

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The authors Yinglin Bao and Feng Liu had contributed equally to this work.

Conflict of Interests

All the authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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