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The Safety and Tolerability of Topically Delivered Kynurenic Acid in Humans. A Phase 1 Randomized Double-Blind Clinical Trial

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ABSTRACT

Scarring is a consequence of biological tissue repair following trauma. Currently, there are no generally agreed ways to prevent scarring. Recently, kynurenic acid has shown to be a potent modulator of extracellular matrix deposition and remodeling. Kynurenic acid can reduce matrix deposition and other fundamental characteristics of fibrosis *in vitro* and *in vivo*. Specifically, kynurenic acid has shown to increase matrix metalloproteinase-1 activity and subsequently reduce collagen deposition in a rabbit ear scar model. In the present study kynurenic acid cream in different concentrations was topically applied on healthy skin on volunteers to assess skin reactions and skin sensitivity in both acute and chronic application settings. Skin reactions were assessed, and concentrations for kynurenic acid were assessed both form serum and urine. Results showed to acute or delayed skin reactions. Kynurenic acid was not detectable in blood at any time point, and only trace elements of kynurenic acid were found in urine. This study supports safety and tolerability of topically administered FS2 when using a liposomal, compounding base carrier.

Introduction

Scarring is a common, devastating consequence of biological tissue repair following trauma. Whether surgically inflicted or resulting from disease or injury, wounds will repair by first creating a "scar," which may then be remodeled into normal tissue. Unfortunately, this sequence of events is rare. Aside from gingival and fetal wound repair, the majority of healed wounds cease to complete the final phase of healing—remodeling. For patients with minimal trauma, the remodeling process continues over years, eventually deminishing the obviousness and mechanical impediments caused by the scar. In contrast, complex, large surface area trauma, such as burns are unable to rely on biological remodeling processes resulting in a need for multiple surgical scar revisions and mitigating modalities that are at best subsatisfactory. This degree of scarring leads to both functional and emotional problems that may significantly decrease quality of life. At present, there are few treatment methods to either prevent or treat scarring. Most of these have proven insufficient or troublesome, including pressure garments, silicone sheets, and intralesional injections. There is a need for preventing scar formation rather than treat existing scars.[1]

Recent findings show the potential of kynurenic acid in preventing scarring in *in vitro* and animal models.[2,3] Briefly, kynurenic acid is the secondary hydrolyzed derivative of kynurenine following the transamination of kynurenine into 4-(2-aminophenyl)-2,4-dioxobutanoate. Kynurenine is an orthosubstituted aminophenyl and amino-containing butanoic acid catabolite of the enzymatic breakdown of tryptophan by Indoleamine 2,3-Dioxygenase and Tryptophan 2,3-Dioxygenase (IDO). Endogenous kynurenine and kynurenic acid are found in the brain, eye, liver, and placenta, where it is produced by trophoblasts as a defense against fetal rejection by the mother. Unlike many other kynurenine derivatives, kynurenic acid is nonredox sensitive, and is generally regarded as biologically safe, either acting as a neuroprotective agent (in an NMDAR antagonist) or otherwise remaining inert and eventually being excreted through the kidneys. In few instances, such as during pregnancy and in certain disease states, tissue-specific increases in IDO activity are known to occur, thereby creating a tryptophan-deficient and kynurenine-rich local environment. Recent research suggests that systemic concentrations of kynurenic acid in adults, in all circumstances, including during pregnancy and in IDO-implicated disease states, generally remain below 1 μM.[4-8]

Aside from its possible beneficial effects as an immunomodulator, recent results have demonstrated that both kynurenine and kynurenic acid are also a potent modulators of extracellular matrix

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deposition and remodeling.[2,3] Kynurenine and its derivatives can effectively tailor the wound healing environment to reduce matrix deposition and other fundamental characteristics of fibrosis *in vitro* and *in vivo*. The most effective dosage for either active compound is approximately 50-150 μg/mL of media *in vitro* and appears to not exert any toxicity to primary skin cells in this range.[3]

Fibrosis is characterized by increases in fibroblast proliferation, fibronectin production, and Type I and III procollagen deposition. Also, degradation is hindered with decreased amounts of collagenase, matrix proteases, and matrix metalloproteinase (MMP) activity. Kynurenic acid (Fibrestop2, FS2) has shown to increase MMP-1 activity and subsequently reduce collagen deposition in a rabbit ear scar model.[2] Increased MMP-1 activity leads to decreased collagen production by fibroblasts. Significant reductions in hypertrophic scarring were achieved when a 0.05% w/w FS2 cream was applied once per day to closed wounds (8 mm; 3-5 days after wounding) on the dorsal surface of rabbit ears. Further drug application did not adversely affect healing nor did it produce any adverse or off target effects. Results of subsequent, preclinical toxicity studies (in accordance with preclinical guidelines issued by the International Committee on Harmonization) in mice corresponded with results of preclinical efficacy studies, substantiating the safety of kynurenic acid when administered topically, once per day or for 7 days with intravenous injection. Satisfying preclinical requirements to investigate a new chemical entity in humans, this clinical study sought to examine the safety and toxicity of topically administered dosages of kynurenic acid in healthy volunteers. Dosages were first assessed for tolerance in part A, whereby the highest dose reflects the maximum feasible dose, and second, for chronic toxicity and tolerance over a 30-day period.

As there is a lack of clinical safety evidence for kynurenic acid administration, regardless of the route of administration, our primary objectives were 4-fold: (1) determine the highest feasible dose of topically applied kynurenic acid; (2) investigate if the highest tolerated acute dose illicit acute reactions, (3) investigate if the highest tolerated acute dose elicit any delayed reactions, and (4) determine if the highest tolerated dose can be safely administered once daily for a period of 30 days, thus reflecting a chronic dosing regime for patients receiving the drug in a phase 2 clinical study.

Aim of the Study

To evaluate the safety and tolerability of topically applied kynurenic acid (FS2) to uninjured skin in healthy human subjects.

Methods

This study was granted a Notice of Allowance by the Non-prescription and Natural Health Product Directorate of Health Canada and was approved by the Clinical Research Ethics Board of University of British Columbia (H14-00143). Twenty healthy subjects, aged between 18 and 65 years, were enrolled through advertising. Exclusion criteria included pregnancy, history of a chronic skin disease, the use of anti-histamines, systemic steroids or anti-inflammatory medications, known immunosuppression, known sensitivity to parabens (ingredient in cream) or bandage adhesives. Consent was obtained by a study group member other than the principle investigator. Study itself and data collection took place in the office space in the burn unit.

Kynurenic Acid Cream Preparation

Investigational creams were prepared under Good Manufacturing Practices at a licensed contract facility (Advanced Orthomolecular Research, AB, Canada). The active (pharmaceutical agent or kynurenic acid) was first solubilized in buffered solution and then added, while mixing, to a liposomal dermatological, compounding base (Glaxal Base™). The pH was then adjusted to 6 and verified prior to batch release. The procedure for compounding is as follows in Figure 1.

Standard and Other Solutions

Individual stock solution of kynurenic acid (1 μg/mL) was prepared in sodium chloride (NaCl) 1N. Standard solutions were made by serial dilution of stock solution in phosphate-buffered saline (PBS) (for serum samples) and normal urine (for urine samples), as such dilutions were being 0, 0.05, 0.1, 0.5, 1, 2.5, and 5 μg/mL. All individual standards were initially prepared as neutral solutions, but as perchloric acid, extraction of samples was to be used for standards as well, standards were neutralized using hydrochloric acid (HCL) 1N. The mobile phase was 10-mM sodium phosphate monobasic and methanol (73:27, by volume). The 10-mM sodium phosphate buffer was prepared in ddH_2O and filtered through a sterile 500-mL bottle top polystyrene filter before mixing with methanol. The buffer-methanol mobile phase was kept at room temperature in its reservoir.

Part 1. Double-Blind Acute Sensitivity Study

The purpose of part 1 of the study was to determine the upper limit of the dose with respect to acute sensitivity on 20 healthy volunteers. After obtaining informed consent, 5 test areas per volunteer were drawn with a permanent marker on the volunteer's back (Fig. 2). Creams, in coded 5 g pouches each, containing 0.00% (placebo), 0.15%, 0.25%, 0.4%, and 0.5% of FS2 cream were randomly applied on the test areas and covered individually with a transparent film dressing (Tegaderm™; 3M). The study team member applying the cream chose the application sites by way of a

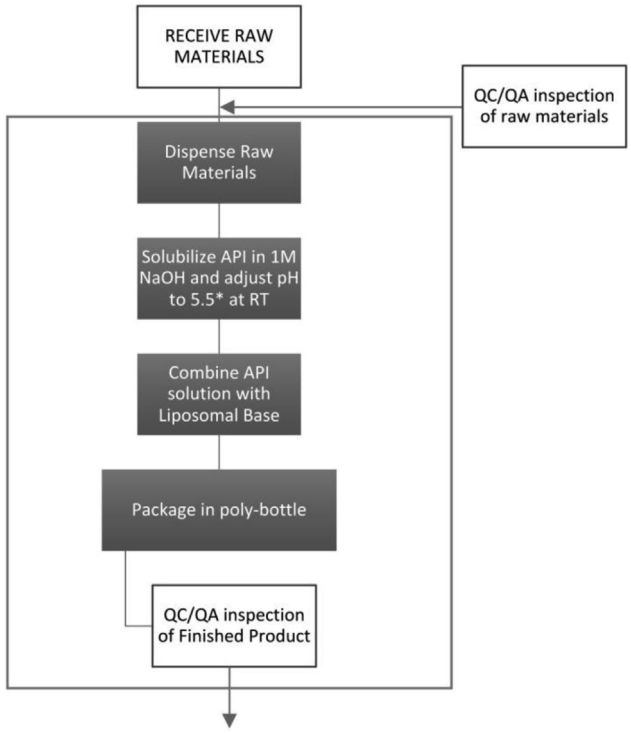

Figure 1. FS2 cream compounding flowsheet.

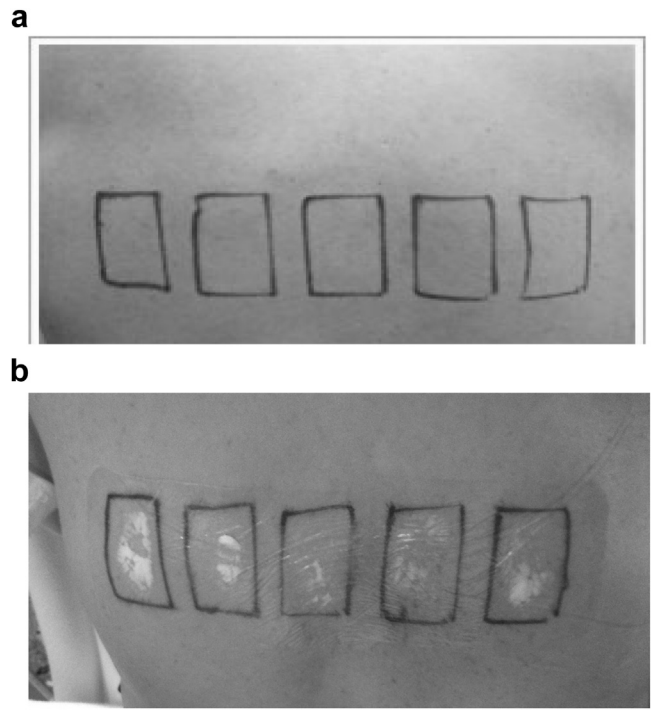

Figure 2. (a) Sample areas marked on patients back. (b) Test cream and Tegaderm® dressings in place.

computerized random-order generator and marked the order on a separate documentation sheet. Patients were evaluated at 24 h postapplication for skin reactions and adverse reactions by a different blinded observer unaware of the order of cream concentration. Subjects returned in 14 days to conduct a repeat challenge test for any potential delayed allergy/sensitivity that may have developed during this period. All subjects were again followed at 24 h for skin and adverse reactions.

Part 2. Double-Blind Chronic Sensitivity Study

The purpose of part 2 of the study was to evaluate the upper limit of the dose for acute sensitivity following chronic use. In this phase, cream containing 0.00% (placebo) and 0.5% FS2 were randomly applied on the forearms of twenty volunteers every 24 h for 30 days. Volunteers were blinded and did not know which cream was placebo or FS2. Adverse reactions were assessed by a blinded observer, photographed, and recorded. The total quantity of drug administered was compared with both urine and serum drug concentrations that were taken on the day prior to applying cream and 2 and 24 h following, as well as on days 15 and 30.

Any reaction seen is scored according to the International Contact Dermatitis Research Group system, as follows:

0 = no reaction
+? = doubtful reaction: mild redness only.
+ = weak, positive reaction: red and slightly thickened skin.
++ = strong positive reaction: red, swollen skin with individual small water blisters.
+++ = extreme positive reaction: intense redness and swelling with coalesced large blisters or spreading reaction.

The distinction between allergic and irritant reactions is of importance. An irritant reaction is most prominent immediately after cream is removed and fades over the next day. An allergic reaction takes a few days to develop; therefore, it is more prominent on day 5 than when the cream is removed.

Chemicals and Other Materials

Kynurenic acid was purchased from The Sigma-Aldrich Co. Ltd. (Oakville, ON, Canada). Methanol (high-performance liquid chromatography [HPLC] grade) was purchased from Millipore (Canada) Ltd. (Etobicoke, Ontario, Canada). Acetonitrile (HPLC grade), sodium phosphate monobasic, perchloric acid ($HClO_4$) 60%, HCL, and NaCl were purchased from Fisher Scientific Company (Ottawa, Ontario, Canada). HPLC Clear Screw vials, caps and insert Bottom Spring, were purchased from Canadian Life Science (Toronto, ON, Canada).

Standard and Other Solutions

Individual stock solution of kynurenic acid (1 μg/mL) was prepared in NaCl 1N. Standard solutions were made by serial dilution of stock solution in 1 × PBS (10-mM PO_4^{3-}, 137-mM NaCl, 2.7-mM KCl, pH 7.4) for serum samples and normal urine for urine samples, as such dilutions were being 0, 0.05, 0.1, 0.5, 1, 2.5, and 5 μg/mL. All individual standards were initially prepared as neutral solutions, but as perchloric acid extraction of samples were to be used for standards as well, standards were neutralized using HCL 1N. The mobile phase was 10-mM sodium phosphate monobasic and methanol (73:27, by volume). The 10-mM sodium phosphate buffer was prepared in ddH_2O and filtered through a sterile 500 mL bottle top polystyrene filter before mixing with methanol. The buffer-methanol mobile phase was kept at room temperature in its reservoir.

Sample Validation

Detection of FS2 cream in a control sample was done following separation by HPLC using reverse phase, isocratic HPLC with a PBS-methanol mobile phase with pH 7. Following this FS2 was detected identically from the cream used in the study. Both creams had the same wavelength and retention time validating the sample and indicating >95% recovery of FS2 from cream. (Fig. 3).

Preparation of Serum and Urine Samples

To a 0.5 mL portion of human serum and urine, 0.5 mL of 6% (w/v) $HClO_4$ was added. The contents of the tubes were vortexed for 5-10 s and allowed to stand for 5 min before centrifugation at 14,000 × *g* for 20 min, using a Beckman Coulter refrigerated high-speed centrifuge (Mississauga, Ontario, Canada). The supernatant was decanted and neutralized with HCL 1N.

Kynurenic Acid Recovery

After standard solutions were prepared in 6% (w/v) HClO4, 1 μL of standard 2.5 and 5 μg/mL were added to human serum and urine ($n = 3$ each), such that final concentrations of kynurenic acid were 2.5 and 5 μg/mL. Preparation of recovery samples underwent extraction in 6% (w/v) HClO4 as described previously. Extracts of these samples acted as controls. All standards and extracts were chromatographed under the standard conditions described in the following.

Equipment and Data Handling

A Perkin-Elmer LC pump with an autosampler, a solvent manager, a degasser, and a UV/VIS detector in series was used. The

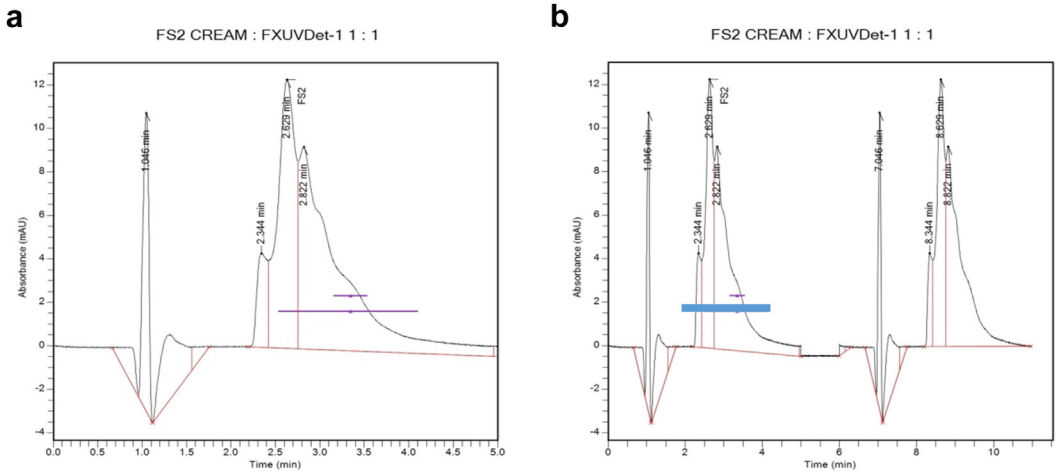

Figure 3. Detection of FS2 at 330 nm (UV) following separation by HPLC using reverse phase, isocratic HPLC with a PBS-methanol mobile phase (pH 7). (a) Detection of a standardized FS2 control sample. (b) Detection of FS2 extracted from cream used in the Phase 1 study. FS2 extracted from cream has the same wavelength and retention time as the standard sample. Results indicate a >95% recovery of FS2 from cream.

mobile phase consisting of sodium phosphate and methanol were degassed and were pumped from the solvent reservoir into the column. The flow rate of mobile phase was maintained at 0.3 mL/min, and detection wavelength was set at 358 nm with a run time of 6 min. The volume of injection loop was 20 μL. Before injection of each sample, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. The system was run isocratically using Brownlee SPP C18 Column. The Perkin-Elmer software performed all operation of the system.

Results

Part 1

The cream was tolerated well, and there were no treatment-related allergic or irritation reactions on any of the tested sites either after the first 24 h or after the repeated test 2 weeks later. One patient was excluded from the study due to the Tegaderm® cover dressing prematurely detaching while hiking with a backpack.

Part 2

No local skin or systemic reactions were noted. FS2 was not detectable in any blood samples at any given time. Trace elements of FS2 (near or below level of detection) were detected in urine, but the timing of maximum or minimum concentrations were inconsistent among participants. The average baseline of FS2 in urine was 0.585 μL/mL (+/− 1.013). Details of blood and urine concentration finding are presented in Table 1.

Table 1
Detection of FS2 (Kynurenic Acid) in Urine and Blood Using HPLC

Sensitivity or limits of detection (LOD) in urine	2.42 μg/mL (±0.59 μg/mL)
Average recovery in urine	43% (±0.59%)
R^2 standard curve in urine	0.97
Average detection of KYNA in urine	0.932 ± 0.06 μg/mL (1.76˙10-7 μM)
Highest detection above baseline in urine	At 24 h postapplication/2.965 μg/mL
Average baseline of KYNA in urine	0.585 μg/mL (±1.013) (NB: below LOD)
Reported detected range in human urine[7]	4-40 μM
Reported detected concentration in urine of pregnant women[8]	11.7 μM

Discussion

Scarring after a burn, trauma, or surgery is a global problem. As of today, there is no standardized or accepted way to prevent scarring. Various methods have been used to try to control scarring, namely pressure garments, triamcinolone injections, silicone sheets, radiation therapy, systemic interferon-α, and laser ablation, to name a few. Systemic interferon-α2b has shown to decrease angiogenesis in hypertrophic scars.[5] Literature, however, shows little evidence to support evidence of treating scars,[6] and randomized, controlled, prospective studies are missing.

Topically administered kynurenic acid has shown promise in preventing scar formation in a well established experimental rabbit ear hypertrophic scar model.[3] Scar biopsies showed decreased scar elevation index and tissue cellularity in treatment group as well as lower levels of collagen deposition, type-1 collagen, and fibronectin expression and increased MMP-1 expression compared with control group providing evidence that kynurenic acid is a promising antifibrogenic agent candidate for topical scar prevention treatment.

Kynurenic acid has previously shown to be safe regarding wound healing. An *in vitro* wound healing scratch assay showed enhanced keratinocyte migration in response to Kyn and KynA treatment. The enhanced keratinocyte migration may lead to accelerated wound re-epithelialization, reduced wound contraction, and scar formation.[9,10] Also, Poormasjedi-Meibod et al.[3] showed that Kyn or KynA treated rabbit ear wounds did not show any significant delay in wound closure indicating that the application of kynurenines at the midstages of wound healing does not delay the healing process. Topical Kyn and KynA application on the rabbit ear significantly improved the wound healing outcome through increasing the expression of MMP1 and suppressing the expression of Type-I collagen and fibronectin.

A phase 1 study on healthy human volunteers was conducted to provide data on the safety and tolerability of topically administered kynurenic acid cream. The dosage used in the rabbit ear study exceeds 10-fold the amount of the highest dose that was be used in our phase 1 human trial. Our study showed that there were no allergic or sensitivity reactions to skin after acute and chronic application of kynurenic acid cream of various concentrations, and the cream was well tolerated. As expected, there were no detectable levels of kynurenic acid in blood and only trace elements in urine.

Conclusion

This study supports safety and tolerability of topically administered FS2 when using a liposomal, compounding base carrier. With a current wide-spread need for satisfactory fibrotic agents, our data warrant investigating the topical administration of FS2 in a phase 2 study involving scar patients who are currently without satisfactory treatment options.

Acknowledgments

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