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Research Article

Carbohydrate-Derived Fulvic Acid (CHD-FA) Inhibits Carrageenan-Induced Inflammation and Enhances Wound Healing: Efficacy and Toxicity Study in Rats

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT The objectives of this study were to evaluate the safety and anti-inflammatory and wound-healing characteristics of carbohydrate-derived fulvic acid (CHD-FA) in rats. CHD-FA ($\geq$100 mg/kg p.o.) effectively reduced carrageenan-induced paw edema in rats, which was comparable to 10 mg/kg p.o. indomethacin. Topical application of CHD-FA, formulated to contain 1.75% active product in a cetomicrogol cream at pH 1.98, compared favorably with fusidic acid cream (10 mg/g) in accelerating the healing of excised wounds infected with *Staphylococcus aureus*. No signs of toxicity were observed in rats during the 6-day acute and 6-month chronic treatment with CHD-FA (100 mg/kg p.o.). Topical application of CHD-FA, formulated in UEA cream and applied to the right ears of mice at 400 mg/g body weight on days 1 and 7–38, produced no adverse events. No signs of toxicity were observed in the teratogenicity study, in which CHD-FA was administered at 100 mg/kg p.o. to pregnant female mice 3 days before fertilization to 14 days of pregnancy. In conclusion, CHD-FA is a safe compound with anti-inflammatory and wound-healing properties and merits further evaluation in the treatment of patients suffering from similar conditions. Drug Dev Res 2011.

Key words: fulvic acid; CHD-FA; carrageenan; anti-inflammatory; wound healing

INTRODUCTION

Humic substances are formed during the decay of plant and animal residues in the environment [MacCarthe, 2001]. These substances can be divided into humic acid, fulvic acid, and humin on the basis of the solubility in water as a function of pH. Fulvic acid is the fraction that is soluble in water under all pH conditions and is in general lower in molecular size and weight and lower in color intensity than humic acids.

Most research on the medicinal applications of fulvic acid to date has been done on a fulvic acid product produced from bituminous coal using a controlled wet oxidation process. The structure of this product has been described by Bergh et al. [1997] using gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) analyses, to contain many organic acids with carboxylic and phenolic groups, of which most are common physiological metabolites with $\leq$6 carbon atoms. The antimicrobial activity of this product was tested on eight microbial pathogens using the macrobroth tube dilution method [van Rensburg

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et al., 2000]. All eight organisms tested (*Enterococcus faecalis, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Candida albicans*) were sensitive to fulvic acid at a concentration of 1.5%, with *E. faecalis* and *K. pneumoniae* being susceptible to concentrations as low as 0.5%.

The anti-inflammatory activity of topically applied fulvic acid produced from bituminous coal was compared with that of diclofenac sodium and betamethasone in a murine model of contact hypersensitivity [van Rensburg et al., 2001]. Fulvic acid cream compared favorably with both positive control creams in suppressing the cutaneous inflammatory response. The anti-inflammatory property of fulvic acid applied topically and produced from bituminous coal was also confirmed in a second study, conducted on pyotraumatic dermatitis in cats and dogs [Dekker and Medlen, 1999].

A pilot study was undertaken to establish the safety and efficacy of topically applied fulvic acid cream (4.5%) compared with hydrocortisone cream (0.1%) in healthy volunteers [Snyman et al., 2002]. The 4.5% fulvic acid cream caused inhibition of the elicited inflammatory reaction at 15 min and differed significantly from the 9% cream at 24 h. These changes were similar to that caused by hydrocortisone. Fulvic acid had no effect on any of the safety parameters and did not induce sensitization when applied to the skin.

Because fulvic acid derived from coal contains high levels of toxic heavy metals, an improved fulvic acid product, derived from the oxidation of a metal free carbohydrate source, referred to in the present work as CHD-FA, has been developed. An observational study was undertaken over 90 days with a product (Secomet V) containing high levels of CHD-FA in HIV-positive patients [van Rensburg et al., 2009]. It was concluded that the product was well tolerated and that it can lead to an improvement in their well-being.

The main aim of the present study was to determine whether oral treatment with CHD-FA is effective in reducing carrageenan-induced inflammation and whether topical and/or oral treatment with CHD-FA can suppress a staphylococcal wound infection in rats. The secondary outcome was to evaluate the toxicity of CHD-FA in a standardized animal model, as only the toxicity of fulvic acid derived from bituminous coal has been documented [Dekker and Medlen, 1999].

METHODS AND MATERIALS

Carbohydrate-Derived Fulvic Acid

Carbohydrate-derived fulvic acid (CHD-FA) was supplied as a 4% solution by Fulvimed (Pty) Ltd, Somerset West, South Africa.

Animals

Scientific Procedures and the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (Acts 1986 and 1989, respectively) were strictly adhered to. Animals were purchased from the National Health Laboratories Service, Rietfontein, South Africa, and housed in plastic cages under 12-h light/dark cycles at 22°C with ad libitum access to water and normal rat chow. They were allowed to acclimatize for 10 days in the new environment before experimentation startup. Each animal was individually marked and weighed at the time of randomization and grouping. Animal experiments were carried out in double-blind placebo-controlled fashion at the University of Pretoria's Biomedical Research Centre, Onderstepoort, with the approval of the Animal Use and Care Committee of the University of Pretoria, South Africa. Animals were euthanized by CO_2 asphyxiation at the end of each study.

Wound Healing

Forty female Sprague-Dawley rats of 8–10 weeks old (150–200 g), were divided into two groups, one of which received a topical treatment whereas the other group was treated by gavage. All animals were immunocompromised with cyclophosphamide [Sigma-Aldrich (Pty) Ltd, Aston Manor, South Africa] administered at 200 mg/kg dissolved in distilled water and injected as a 200-µl bolus) 4 days before the beginning of both treatments.

Induction of wounds

The first 20 rats were sedated using isoflurane, and the hair from the test areas was removed using clippers. Four lesions were produced on each of the rats by cutting a circular area, ~4 mm, of skin from the test area. The wounds were inoculated with 1×10^{10} colony-forming units (cfu) *S. aureus* (ATCC 12600); the entire area was covered with an occlusive dressing (Transpore). The animals were returned to their cages for 48 h. At the end of this period, the dressings were removed and the initial measurements of the wounds recorded using Motic Image 2.0 with Multicam 2000 software before commencement of treatment.

Topical treatment

The four lesions were earmarked as follows: (1) a negative control treated with cetomicrogol cream only, (2) a positive control treated with fusidic acid cream, (3) an experimental group treated with CHD-FA/cetomicrogol cream at pH 1.75, and (4) an experimental group treated with CHD-FA/cetomicrogol cream at pH 5.5. All treatments were applied separately

as a bolus of 50 mg to the different lesions as indicated from day 1. The cetomicrogel cream was obtained from Transfarm (Hermanstad, Pretoria, South Africa). Fusidic acid cream was obtained from the same company and contains 1% fusidic acid. The first experimental group was treated with 1.75% CHD-FA made up in cetomicrogel cream (Transform), whereas the second treatment group was treated with 1.75% CHD-FA neutralized with potassium hydroxide to obtain a pH of 5.5 and made up in cetomicrogel as a 1.75% cream.

The wounds were covered after treatment and the animals returned to their cages for 24 h. Wound size measurements were recorded every 24 h and treatments reapplied for the next 6 days. Wound areas were used as an indication of efficacy and wound closures calculated as percentages of the initial readings.

Systemic Treatment

The second group of 20 rats was divided into two groups: (1) an untreated control group that received 1 ml distilled water by gavage, and (2) a group that received 1 ml CHD-FA (buffered to pH 5.5 with sodium acetate and diluted with distilled water) by gavage at a dosage of 100 mg/kg body weight. Each subgroup comprised 10 rats and underwent the same procedures described under the section Induction of Wounds as that of group 1, with the exception that they received only two wounds on their backs instead of four.

Carrageenan-Induced Paw Edema

This study was carried out using previously described methods [Goel et al., 1990; Smith et al., 2000; Recio et al., 2000; Petersson et al., 2001]. In this study, 50 female Sprague-Dawley (SD) rats, 12 weeks old, weighing 145–205 g, were used. CHD-FA was neutralized with sodium acetate to a pH value of 5.5 and diluted with distilled water before administration. The rats were randomized into one of the following five groups and were treated with 1 ml by gavage: (1) an untreated group receiving water, (2) an experimental group receiving 153 mg/kg CHD-FA, (3) an experimental group receiving 100 mg/kg CHD-FA, (4) an experimental group receiving 50 mg/kg FA, and (5) a positive control group receiving 10 mg/kg indomethacin (Sigma Aldrich (Pty) Ltd) p.o. On days 1–5, the experimental groups, i.e., (2), (3), and (4), received a 1-ml aliquot containing the relevant concentrations of CHD-FA, whereas the negative control group (1) received only water by gavage. The positive control group (5) received water on days 1–4, whereas on the fifth day they received indomethacin diluted in 1 ml water at 10 mg/kg/body weight by oral gavage. On the 5th day, 1 h after drug administration, the right hindpaw of each rat was measured with a water displacement plethysmometer to measure the paw volume before inflammation was initiated. λ-Carrageenan was then injected subplanar into the right hindpaw to induce inflammation and paw edema measured from the time of injection hourly for 7 h with a water plethysmometer.

Toxicity

Topical application

Sixty female Balb C mice, aged 6–7 weeks, were divided into three groups. One group received only aqueous cream (UEA; Transfarm), whereas the other two groups received UEA cream containing either CHD-FA or CHD-FA neutralized with sodium acetate to a pH of 5.5, both at a dosage of 400 mg/kg body weight. A predetermined amount was applied to the left ears twice on day 1 and once on day 7, after which they were examined for signs of sensitization. From day 8, they received the products applied topically twice a day for 30 days (up to day 38). All animals were observed on a daily basis, and blood was drawn upon termination to determine creatinine and γ-glutamyl transferase (GGT) levels.

Oral administration

Forty female Sprague-Dawley rats, aged 8–10 weeks old (150–200 g), were divided into two groups, one of which received an oral dose (by gavage) of 100 mg/kg body weight/day of CHD-FA (potassium salt), neutralized to a pH of 5.5 and diluted in distilled water, as a 1.0-ml bolus per day for 183 days. The second group received an oral dose (by gavage) of 1.0 ml distilled water per day to control for the procedure used in the experimental group. The animals were weighed daily and monitored for pain and distress (behavioral changes). Blood samples (500 μl/rat) were drawn from the animals at the beginning and end of the study for hematological analysis (hematocrit, red blood cells, white blood cells, and platelet counts) and kidney and liver enzyme levels (creatinine, urea, aspartate aminotransferase [AST] and GGT).

Teratogenicity test

For the teratogenicity experiment, a group of 10 pregnant Sprague-Dawley rats received 1.0 ml distilled water by gavage 3 days before fertilization to 14 days of pregnancy, whereas the treatment group of 10 pregnant rats received CHD-FA, neutralized to a pH of 5.5, by gavage on the same days at a dosage of 100 mg/kg body weight diluted to an equal amount of distilled water. The animals were weighed daily and monitored for pain and distress (behavioral changes). Puppies were weighed at birth and monitored daily for clinical and behavioral abnormalities for a period of 2 weeks after birth. Morphological evaluation was done after termination of

the pups and included macroscopic necropsy and histological examination of the adrenal glands, brains, hearts, gonads, intestines, kidneys, livers, lungs, spleens, stomachs, and thymuses.

Statistical analysis

Statistical analysis was done using analysis of variance (ANOVA) to determine significance between the various groups.

RESULTS

Wound Healing

Both fusidic acid (equal to 2 mg/kg body weight) and CHD-FA at pH 1.98 (equal to 3.5 mg/kg body weight) were effective in the wound-healing model (Fig. 1). CHD-FA at pH 5.5, on the other hand, had no effect in this model. Oral treatment with CHD-FA showed no improvement over placebo (results not shown).

Carrageenan-Induced Paw Edema

Throughout the 7-h observation period, the level of edema induced by carrageenan injection increased in all the rats, as determined by the increase of paw volume (Fig. 2). Indomethacin as well as CHD-FA at dosages of 100 and 135 mg/kg reduced the inflammation significantly. However, CHD-FA, at a dosage of 50 mg/kg, had no effect on the development of edema.

Toxicity

CHD-FA, when applied topically at 400 mg/kg body weight on day 1 and bid on days 8–38, did not produce any hypersensitivity or toxic reactions with regards to liver and kidney functions. At 100 mg/kg p.o., CHD-FA caused a significant increase in serum AST levels after 6 weeks. However, this increase was not apparent upon completion of the trial at 6 months (Fig. 3). GGT levels were unaffected (Fig. 4). None of the other safety parameters was affected during the trial (data not shown).

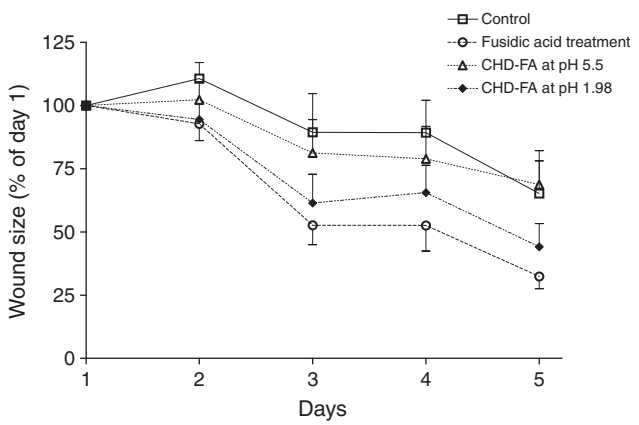

Fig. 1. Effects of topical application of acetomicrogol cream alone, fusidic acid-based cream at 10 mg/g, CHD-FA (1.75%)/acetomicrogol cream at pH 1.98 and CHD-FA (1.75%)/acetomicrogol cream at pH 5.5 on the healing of wounds, infected with *S. aureus,* induced in rats. Wound healing was calculated as a percentage of wound size before treatment.

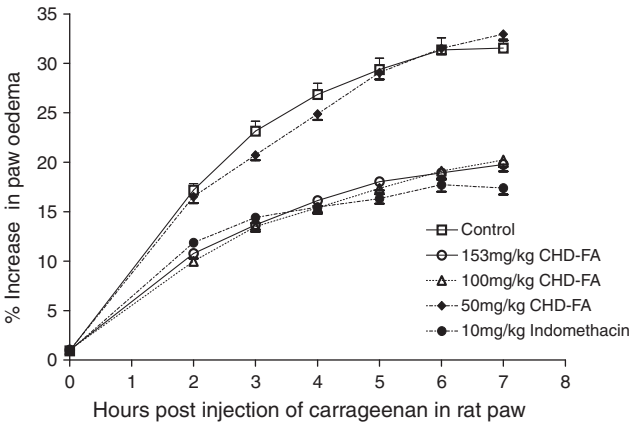

Fig. 2. Effects of oral administration of rats with CHD-FA (at 50, 100, and 153 mg/kg) compared with 10 mg/kg indomethacin on carrageenan-induced inflammation.

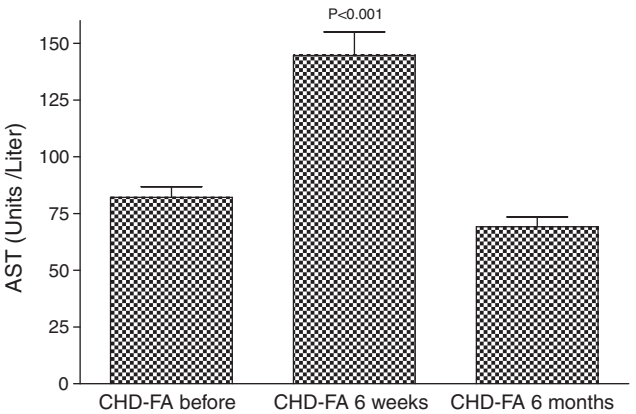

Fig. 3. AST levels of rats before and after 6 weeks and 6 months on an oral treatment of CHD-FA at 100 mg/kg.

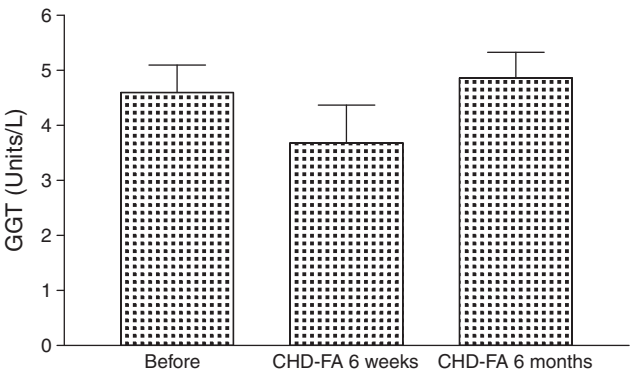

Fig. 4. GGT levels of rats before and after 6 weeks and 6 months on an oral treatment of CHD-FA at 100 mg/kg.

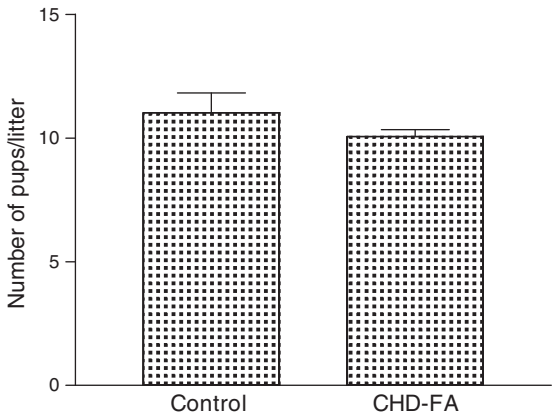

Fig. 5. Effects of oral administration of female rats with CHD-FA (at 120 mg/kg) on the number of pups per litter compared with the average number of pups per litter of untreated female rats.

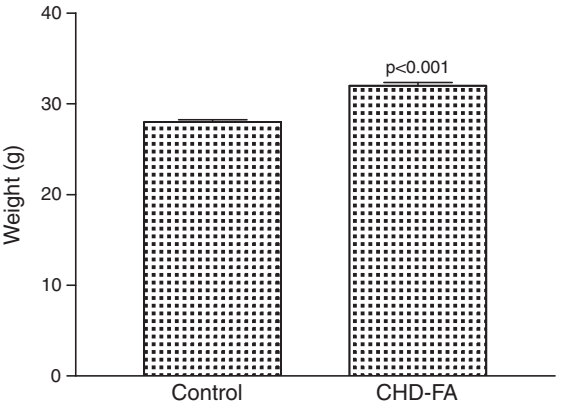

Fig. 6. Effects of oral administration of female rats with CHD-FA (at 120 mg/kg) on the average weights of their pups compared with the average weights of pups of untreated female rats.

None of the animals died in the teratogenicity study, and no abnormalities were observed in the puppies. The weight growth patterns of the females during the pregnancy period between the groups were almost identical (results not shown). Although having smaller litter numbers on average (Fig. 5), the CHD-FA-treated group showed significantly greater pup weights (Fig. 6). The pathologist concluded that there was no indication of any developmental defects or pathological anatomical abnormalities were associated with CHD treatment.

DISCUSSION

During the nineteenth century, the healing effects of mud baths, rich in humic and fulvic acids, were used to treat rheumatic conditions [Kleinschmidt, 1988]. Peat was also used during World War I to treat wounds and amputations in field hospitals to prevent infections, relieve pain and facilitate healing [Van Beneden, 1971]. Jansen et al. [1996] claimed that humic acid can promote wound healing.

In this study, topically applied CHD-FA at pH 1.98 effectively enhanced the healing rate of wounds infected with *S. aureus*. No such effect was seen when CHD-FA was administered p.o., possibly due to the fact that the concentrations necessary to reduce the growth of *S. aureus* [Van Rensburg et al., 2000] was not reached at the site of infection.

CHD-FA cream, applied topically to the left ears of mice, was well tolerated and nonirritating, confirming in vivo animal toxicity data obtained with oxyfulvic acid [Van Rensburg et al., 2001]. Snyman et al. [2002] demonstrated that oxifulvic acid cream possesses anti-inflammatory properties similar to that of 1% hydrocortisone cream. A possible mechanism of action for fulvic acid might be due to its free radical scavenging properties [Wang et al., 1996], as well as inhibition of interleukin-2 production [Snyman et al., 2002]. However the mechanism of action requires further exploration.

Although it has been demonstrated that brown coal-derived humate, administered by gavage, inhibits the cutaneous hypersensitivity reaction [Van Rensburg et al., 2007] as well as the carrageenan-induced edema and graft vs host reaction in rats [Naudé et al., 2010], no research has been done to prove the anti-inflammatory effects of fulvic acid administered in a similar fashion. The results obtained in this study indicate that CHD-FA inhibits the carrageenan-induced inflammatory response as effectively as indomethacin, indicating that this product is systemically available. This is indeed an exciting result, as it has not been possible to date to determine the pharmacokinetic profile of this product due to the complexity thereof.

In conclusion. CHD-FA, a unique, metal-free fulvic acid, inhibits carrageenan-induced inflammation in rats in a manner similar to indomethacin but with no signs of systemic toxicity. Furthermore, it is effective in accelerating the healing of *S. aureus*-infected wounds in mice when administered topically. This warrants further evaluation of this product in humans.

DISCLOSURE

The corresponding author, C.E.J. van Rensburg, acts as consultant for Fulvimed (Pty) Ltd.

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