Carbohydrate derived fulvic acid *(*CHD-FA) inhibits carrageenan induced inflammation and enhances wound healing: An efficacy and toxicity study in rats

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Running title: An efficacy and toxicity study on CHD-FA

Key words: Fulvic acid, CHD-FA, carrageenan, anti-inflammatory, wound healing.

Funding: This research was supported by Fulvimed (Pty) Ltd and the Technology and Human Resources for Industry Programme of the National Research Foundation and the Department of Trade and Industry (THRIP).

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.

A daily oral dosage of CHD-FA at 100mg/kg and higher effectively reduced carrageenan-induced paw oedema in rats which was comparable to an oral dosage of 10mg/kg indomethacin. Furthermore, the topical application of CHD-FA formulated to contain 1.75% active product in an cetomicrogol cream at pH 1.98, compared favourably with fusidic acid cream (10mg/g) in accelerating the healing of excised wounds infected with *S. aureus.* No signs of toxicity were observed in rats during the 6-day acute and 6 month chronic oral treatment with CHD-FA at 100mg/Kg bodyweight. Topical application of CHD-FA, formulated in UEA cream and applied to the right ears of mice at 400mg/Kg bodyweight on days one, and 7 to 38 produced no adverse events. No signs of toxicity were observed in the teratogenicity study where CHD-FA was administered at 100mg/kg bodyweight to pregnant female mice by gavage 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.

Foot note: The corresponding author, CEJ van Rensburg, acts as consultant for the company

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 colour intensity than humic acids.

Most research on the medicinal application of fulvic acid up to date has been done on a fulvic acid product produced from bituminous coal by a controlled wet oxidation process. The structure of this product has been described by Bergh et al. [1997], using GC (gas chromatography) and GC/MS (gas chromatography-mass spectrometry) analyses, to contain many organic acids with carboxylic and phenolic groups of which most of them are common physiological metabolites with 6 or less carbon atoms. The antimicrobial activity of this product was tested on eight microbial pathogens using the macrobroth tube dilution method [van Rensburg et al., 2000]. All eight organisms tested (*Enterococcus faecalis, Staphylococcus aureus, Pseudomonnas aeruginosa, Eschericia coli, Streptocoocus 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]. The fulvic acid cream compared favourably with both the 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, which was done 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 to 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 15min and differed significantly from the 9% cream at 24h. 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 this paper 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., 2010]. It was concluded that the product was well tolerated and can lead to an improvement in their well being.

The main aim of this 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 standardised 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 hour light/dark cycles at 22ºC with ad libitum access to water and normal rat chow. They were allowed to acclimatize for 10 days in the new environment prior to

experimentation starting. Each animal was individually marked and weighed at the time of randomization and grouping.

Animal experiments were carried out in a 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 to 10 weeks old (between 150 and 200g) 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 (obtained from Sigma-Aldrich (Pty) Ltd, Aston Manor, South Africa) administered at 200 mg/kg, dissolved in distilled water and injected as a 200µl bolus) 4 days prior to the beginning of both treatments.

Induction of wounds

The first 20 rats rats were sedated using isoflurane and the hair from the test areas were removed using clippers. Four lesions were produced on each of the rats by cutting a circular area, approximately 4mm, of skin from the test area. The wounds were inoculated with $1X10^{10}$ colony forming units *S. aureus* (ATCC 12600) and the entire area covered with an occlusive dressing (Transpore). The animals were returned to their cages for 48-hours. 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 4 lesions were earmarked as follows; (i) a negative control treated with cetomicrogol cream only, (ii) a positive control treated with fucidic acid cream, (iii) an experimental group treated with CHD-FA/cetomicrogol cream at pH 1.75 and (iv) an experimental group treated with CHD-

FA/cetomicrogol cream at pH 5.5 All treatments were applied separately as a bolus of 50mg to the different lesions as indicated from day one. The cetomicrogel cream was obtained from Transfarm, Hermanstad, Pretoria, South Africa. Fucidic acid cream was also obtained from the same company and contains 1% fucidic acid. The frist 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 1.75 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 hours. Wound size measurements were recorded every 24 hours 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 (i) an untreated control group who received one ml distilled water by gavage and (i) a group who received one ml CHD-FA (buffered to pH 5.5 with sodium acetate and diluted with distilled water) by gavage at a dosage of 100mg/Kg body weight. Each sub-group was comprised of 10 rats each and under went the same procedures described under *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 oedema

This study was done according to previously described methods [Smith et al.,.2000, Recio et al., 2000 and Peterson et al.,. 2001]. In this study fifty female Sprague Dawley (SD) rats, 12 weeks old, weighing 145g-205g, were used. CHD-FA was neutralized with sodium acetate to a pH of 5.5 and diluted with distilled water before administration. The rats were randomized into one of the following 5 groups and were treated with one ml by gavage; (i) an untreated group receiving water, (ii) an experimental group receiving 153mg/kg CHD-FA (iii) an experimental group

receiving 100mg/kg CHD-FA (iv) an experimental group receiving 50mg/kg FA and (v) a positive control group receiving 10mg/kg indomethacin p.o. On days one to five the experimental groups i.e. (ii), (iii) and (iv) received one ml containing the relevant concentrations of CHD-FA whereas the negative control group (i) received only water by gavage. The positive control group (v) received water on days one to four whereas on the fifth day they received indomethacin (Sigma Aldrich (Pty) Ltd) diluted in one ml water at 10 mg/kg/bodyweight by oral gavage.

On the 5th day, one hour after drug administration, the right hind paw of each rat was measured with a water displacement plethysmometer to measure the paw volume before inflammation was initiated. λ-Carrageenan was then injected sub planar into the right hind paw to induce inflammation and paw oedema measured from the time of injection hourly for 7 hours with a water plethysmometer.

Toxicity

Topical application:

Sixty female Balb C mice of 6-7 weeks old 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 400mg/kg body weight. A predetermined amount was applied to the left ears twice on day one 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 creatinin and gamma-glutamyl transferase (GGT) levels.

Oral administration

Forty female Sprague Dawley rats of 8 to 10 weeks old (between 150 and 200g) 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 (behavioural changes). Blood samples (500 μl/rat) were drawn from the animals at the beginning and end of the study for haematological analysis (haematocrit, 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 100mg/kg body weight diluted to an equal amount of distilled water.

The animals were weighed daily and monitored for pain and distress (behavioural changes). Puppies were weighed at birth and monitored daily for clinical and behavioural abnormalities for a period of two weeks after birth. Morphological evaluation was done after termination of the pups which included macroscopical 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 ANOVA to determine significance between the various groups.

RESULTS

Wound healing

Both fuscidic acid (equal to 2mg/kg body weight) and CHD-FA at pH 1.98 (equal to 3.5mg/kg body weight) were effective in the wound healing model (Figure 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 oedema

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

Toxicity

CHD-FA, when applied topically at 400mg/kg body weight on day one and twice a day on day 8 to day 38, does not produce any hypersensitivity or toxic reactions with regards to liver and kidney functions.

The product, at an oral dosage of 100mg/kg, caused a significant increase in serum AST levels after a 6 week treatment. However, this increase was not apparent upon completion of the trial at 6 months (Figure 3). GGT levels were not affected (Figure 4). None of the other safety parameters was affected during the trial (reslults not shown).

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 (Figure 5), the CHD-FA treated group showed significantly greater pup weights (Figure 6). The pathologist concluded that there was no indication of any developmental defects or pathological anatomical abnormalities associated with the CHD-treatment.

DISCUSSION

During the 19th 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 the First World War 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 by gavage, 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 non-irritating, 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 needs to be explored further.

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 oedema 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.

CONCLUSION

In conclusion: CHD-FA, a unique, metal free fulvic acid, inhibits carrageenan induced inflammation in rats 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.

ACKNOWLEDGMENTS

The authors would like to thank Fulvimed (Pty) Ltd and the Technology and Human Resources for Industry Programme of the National Research Foundation and the Department of Trade and Industry for financial support.

DISCLOSURE

The corresponding author, CEJ van Rensburg, acts as consultant for the company.

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Figures

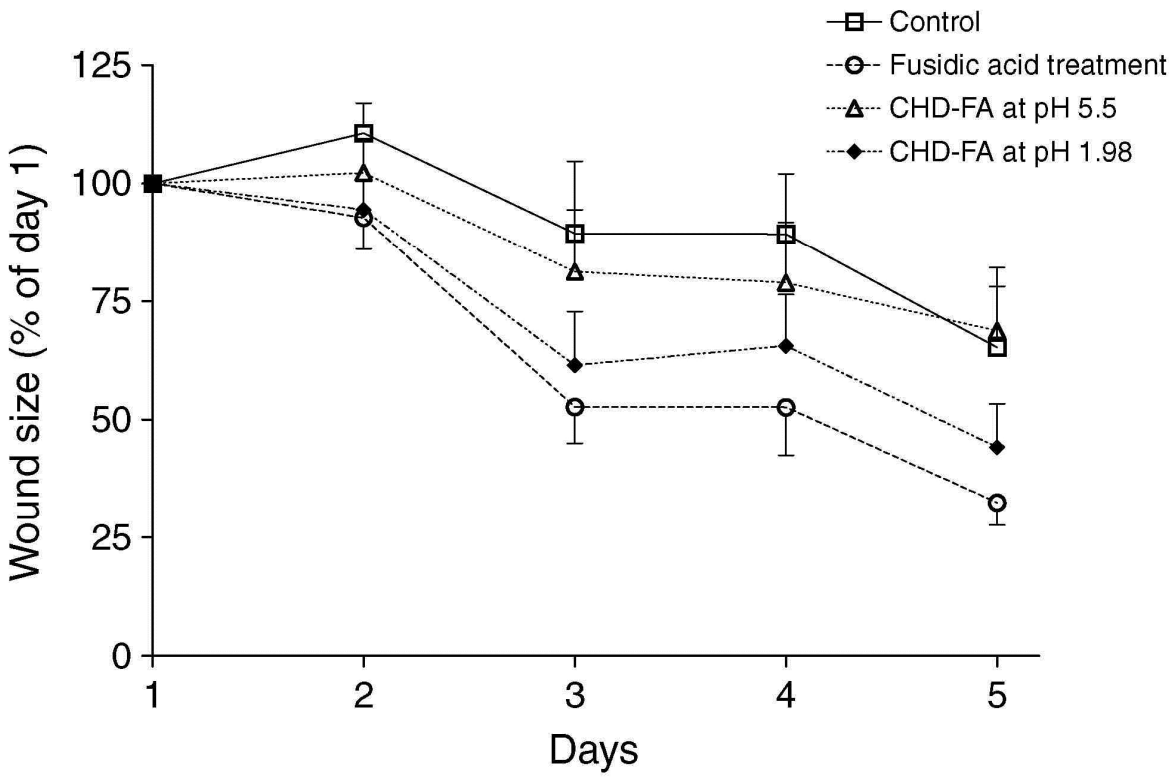

Figure 1. Effects of topical application of cetomicrogol cream alone, fusidic acid based cream at 10mg/g, CHD-FA (1.75%) /cetomicrogol cream at pH 1.98 and CHD-FA (1.75%) /cetomicrogol cream at pH 5.5 on the healing of wounds, infected with *S aureus*, induced in rats. Wound healing was calculated as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

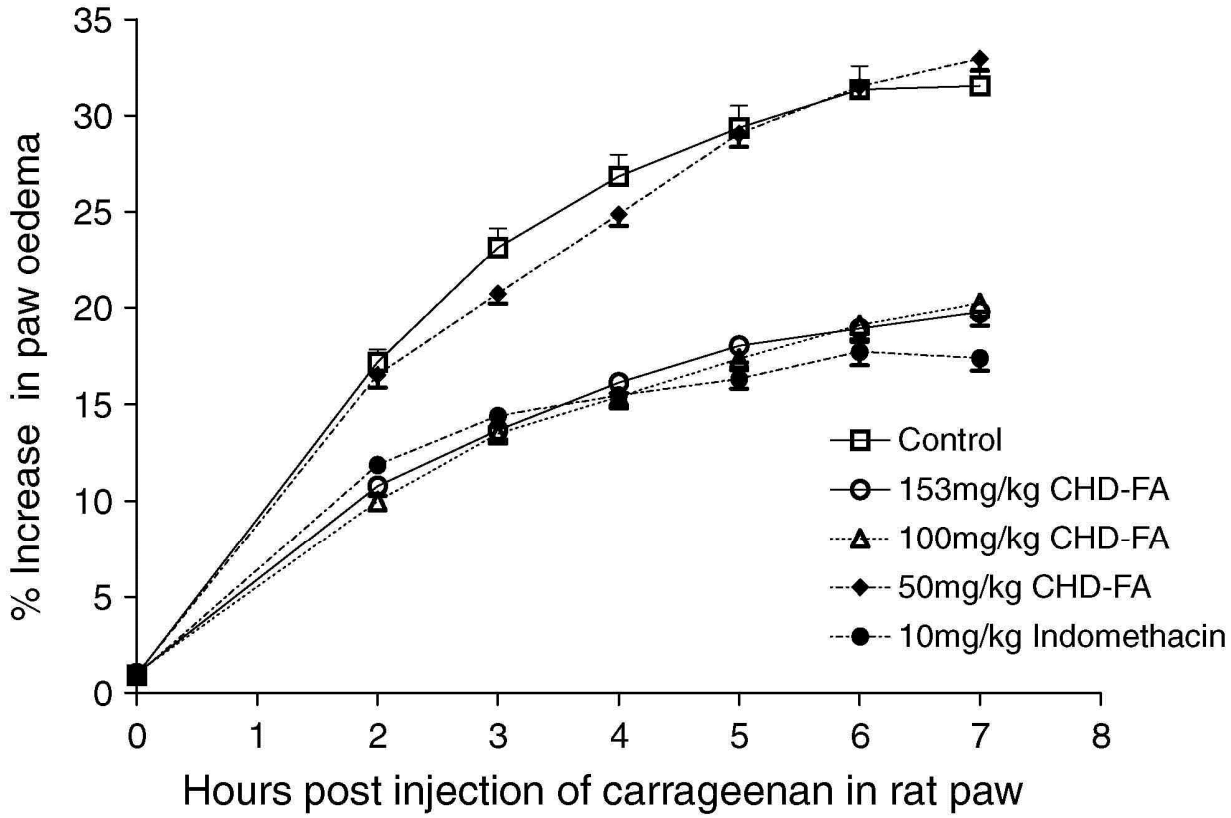

Figure 2. Effects of oral administration of rats with CHD-FA (at 50, 100 and 153mg/kg) compared to 10mg/kg indomethacin on carrageenan-induced inflammation.

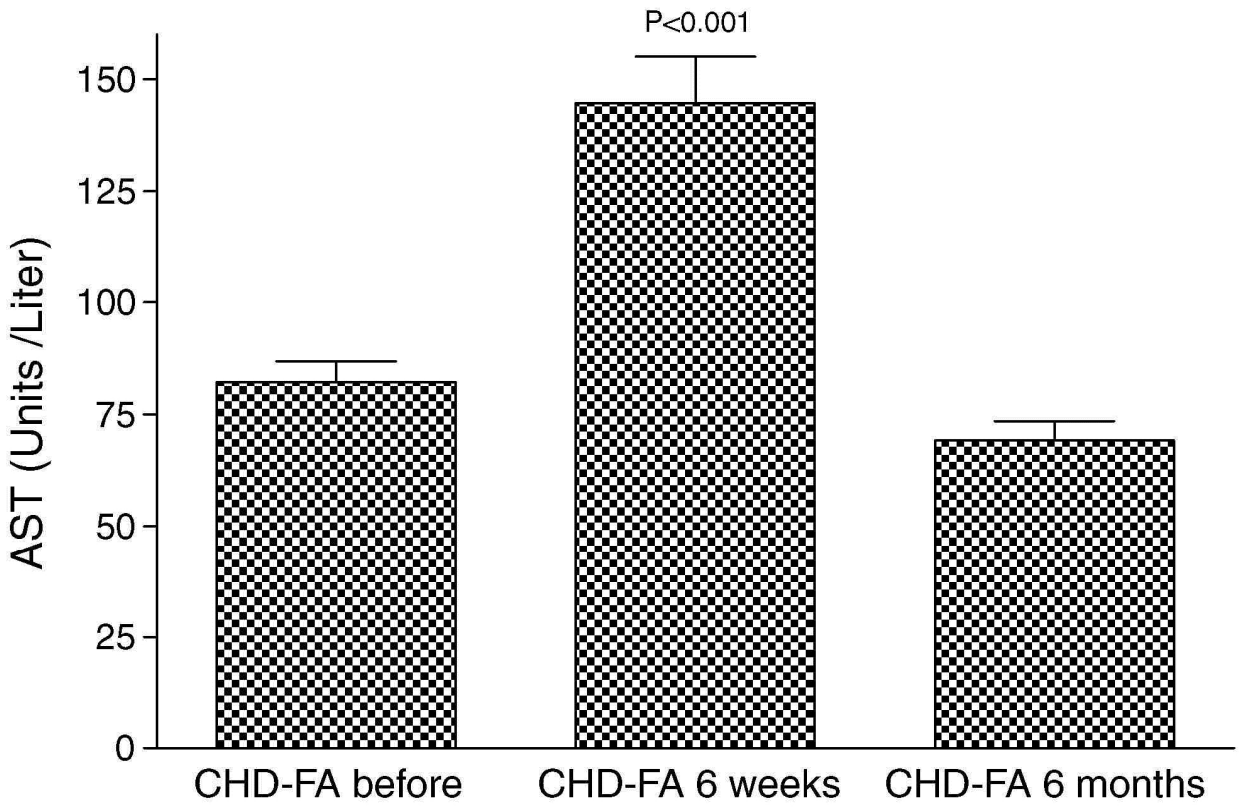

Figure 3. AST levels of rats before and after 6 weeks and 6 months on an oral treatment of CHD-FA at 100mg/kg.

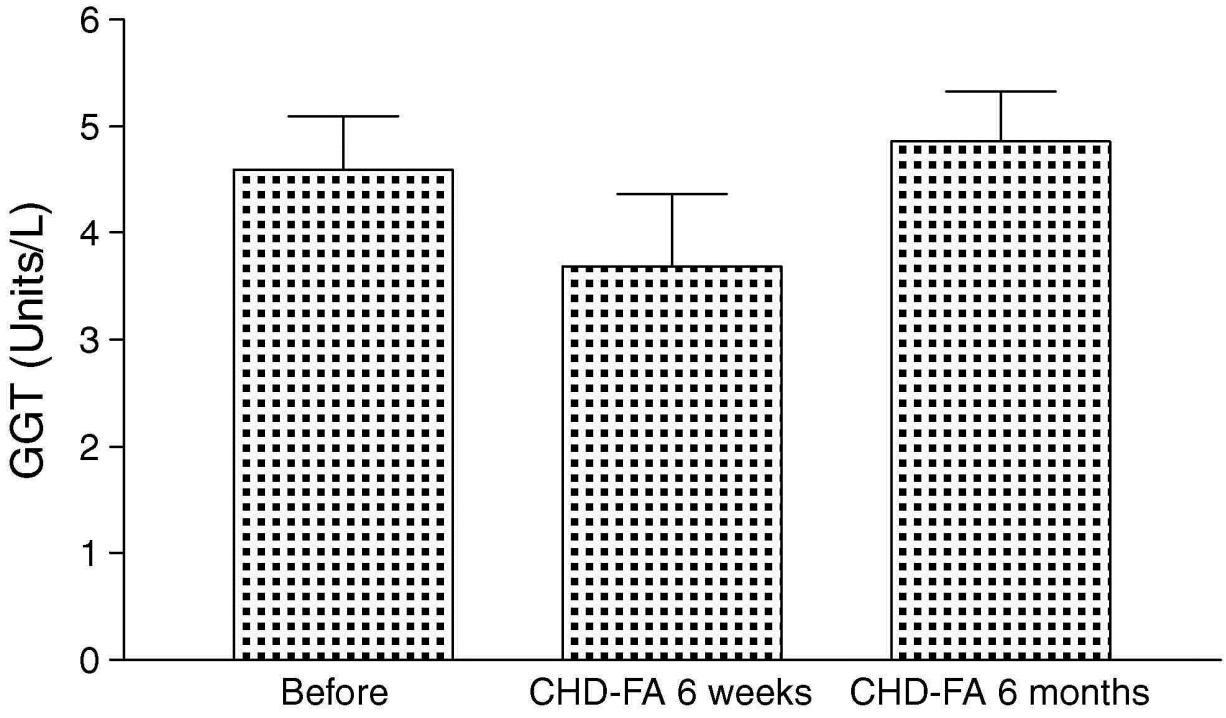

Figure 4. GGT levels of rats before and after 6 weeks and 6 months on an oral treatment of CHD-FA at 100mg/kg.

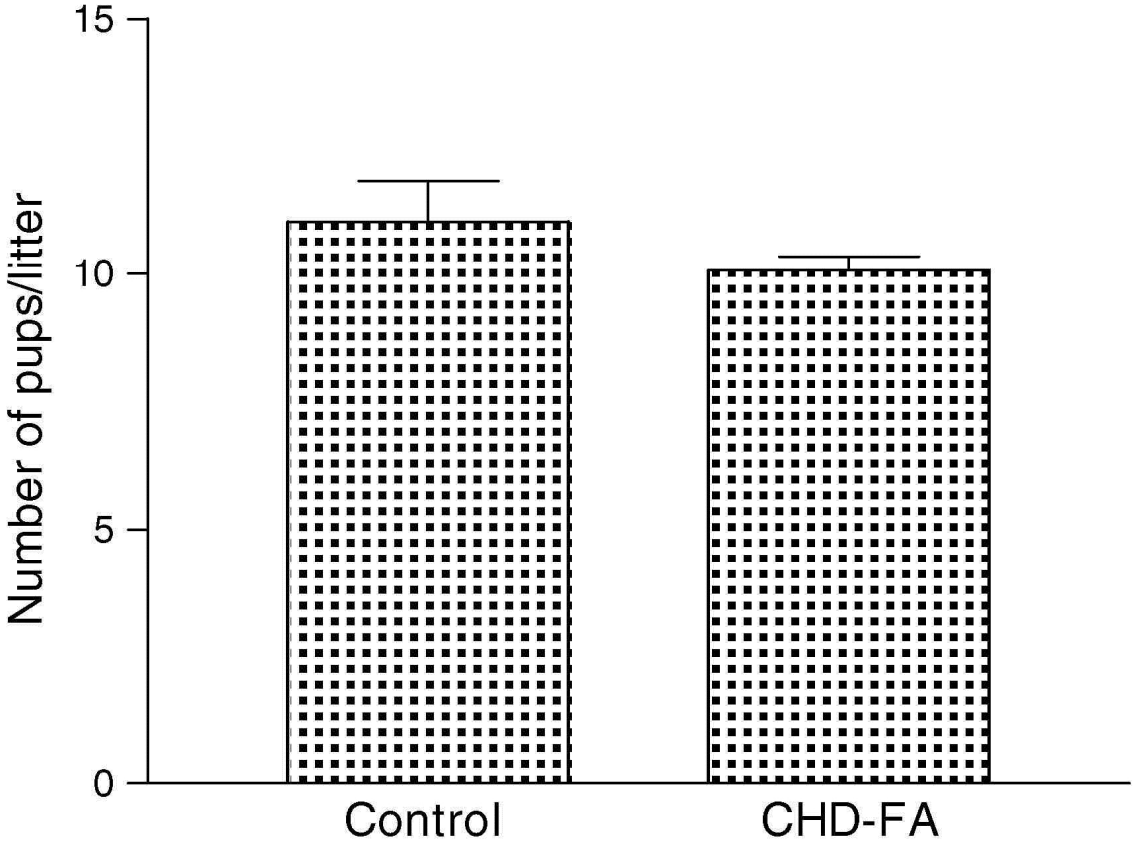

Figure 5. Effects of oral administration of female rats with CHD-FA (at 120mg/kg) on the number of pups per litter compared to the average number of pups per litter of untreated female rats.

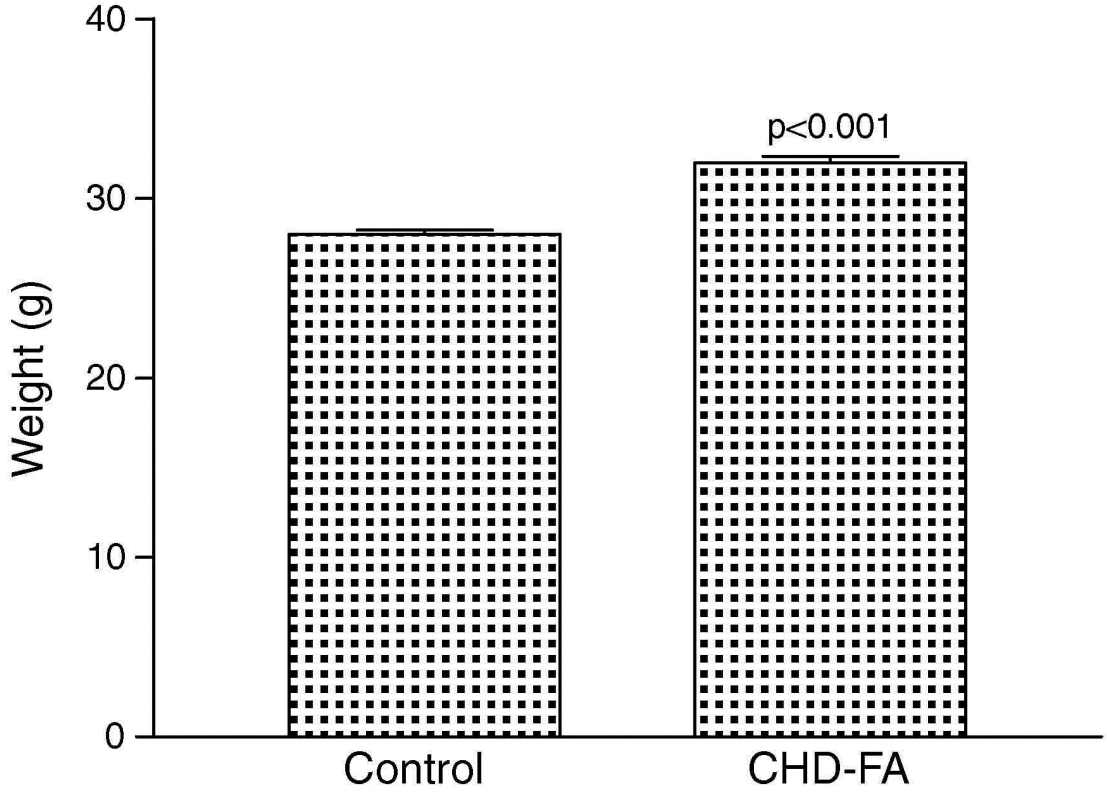

Figure 6. Effects of oral administration of female rats with CHD-FA (at 120mg/kg) on the average weights of their pups compared to the average weights of pups of untreated female rats.

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