Hydroxyproline assay by HPLC-FLD applied for wound healing determination in rat model

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Abstract: In this work, we focused on four types of oils and their response on wound healing of the skin of rats after dietary exposure. A set of samples consisted of 48 adult male rats of *Wistar albino*, which were divided into four groups according to the respective oil. The content of hydroxyproline in the skin tissue, used as a biomarker for wound healing, was determined by high-performance liquid chromatography with a fluorescence detector. There was found out, that the highest content of hydroxyproline was detected in the group of rats, which have been fed with the addition of palm oil (118.4 ± 1.9 µg/g). These results indicate that palm oil had the most positive effect on wound healing.

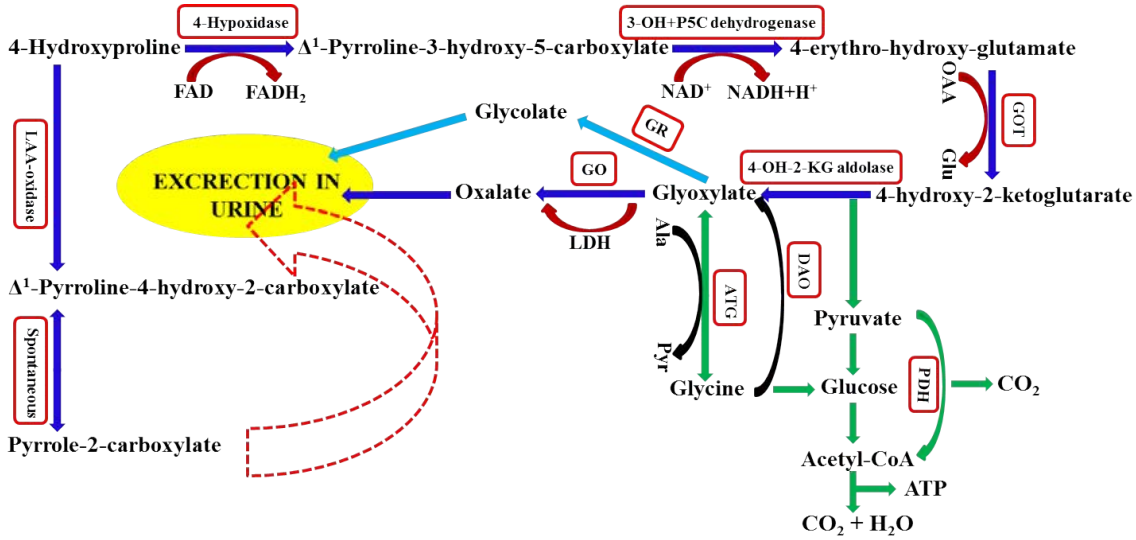
Key Words: high-performance liquid chromatography with fluorescence detector (HPLC-FLD), hydroxyproline, microwave hydrolysis, rat skin

INTRODUCTION

Hydroxyproline is a major part of the collagen of animals (Zhang and Duan 2017) and human (Sugioka et al. 2017) and it is responsible for collagen stability (Shoulders and Raines 2009). Furthermore, the presence of hydroxyproline and its concentration in biological fluids can be used as a marker of collagen catabolism and tissue degradation or bone resorption. Various changes in hydroxyproline metabolism play major role in the pathogenesis of different diseases (Srivastava et al. 2016). The higher level of hydroxyproline is observed in cases of depression and stress, or in cases of poor wound-healing (Srivastava et al. 2016, Dong et al. 2017). In the case of diagnosed fibrosis in hepatitis C, adiposity and cardiometabolic health, hydroxyproline may be used as potential oxidative biomarker. Nowadays, a high-performance liquid chromatography with fluorescence detection, the high-performance liquid chromatography with tandem mass spectrometric detection (LC-MS/MS), a capillary electrophoresis with in-capillary optical fiber light-emitting diode-induced fluorescence detection (Ji et al. 2018) and a hydrophilic interaction chromatography-quadrupole/electrostatic field orbitrap high resolution mass spectrometry (Liu et al. 2017) are used for determination of hydroxy-proline in tissues or body fluids.

This work was focused on determination of hydroxyproline in rat´s skin using a high-performance liquid chromatography with fluorescence detector. Figure 1 demonstrates the metabolism of hydroxy-proline in animals.

Figure 1 Scheme of metabolism of hydroxyproline in animals. This scheme was inspired by (Wu et al. 2011).



Legend: AA – amino acid, AGT – alanine-glyoxylate aminotransferase, Ala – alanine, Glu – glutamate, DAO D – amino acid oxidase, GO – glycolate oxidase, GOT – glutamate oxaloacetate transaminase, 4-Hyp – 4-hydroxy-proline, GR – glyoxylate reductase, 4-OH-2-KG – 4-hydroxy-2-ketoglutarate, 3-OH-P5C-Δ1-pyrro-line – 3-hydroxy-5-carboxylate, LDH – lactate dehydrogenase, OA – oxaloacetate, PDH – pyruvate dehydrogenase, Pyr – pyruvate

MATERIAL AND METHODS

Chemicals

The chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity, unless noted otherwise.

Animals

Adult male rats of *Wistar albino* (48 pieces) were used as a model animal for this experiment. The average weight of rats was 200 g at the beginning of the experiment. The rats were housed in the plastic boxes in a room maintained at 23 ± 1 °C, humidity of 60% and 12/12 h of light/dark cycle (maximum intensity of 200 lx). The experiment was performed in compliance with the Czech National Council Act No. 246/1992 Coll. to protect animals against cruelty, the amended Act No. 162/1993 Coll., and was approved by the "Commission to protect animals against cruelty" of the Mendel University in Brno (Statement No. 16252-MZE-17214). The animals had the access to food and water *ad libitum* for the whole duration of the experiment. The rats were divided into four groups. Each group contained twelve rats. The rats were fed a base feed (pelleted complete chow for mice and rats, supplemented with 5% of palm oil, 5% of safflower oil, 5% of fish oil and 5% of oil extracted from the *Schizochytrium microalga* (marked as DHA; control). After 56 days of fattening, all animals were anesthetized, and sagittal excision was performed on their back. After an additional 18 days, the animals were sacrificed and immediately subjected to appropriate sampling analysis.

Preparation of the samples and the analysis using HPLC-FLD

The 0.1–0.2 g aliquot of the granulated tissue was weighed (Analytical Weight EP 240A, Precisa, Stare Mesto, Czech Republic) and mixed with 500 µl of 6 M HCl and the microwave hydrolysis (90 min, 120 °C, maximal pressure of 2.5 MPa) was performed (Anton Paar GmbH, Graz, Austria). Hydrolysate was centrifuged (Centrifuge Z326K, Herµe, Gosheim, Germany) 10 min by 24 000 g at 4 °C. After filtration (Mini-UniPrep - Whatman, Maidstone, Stone, UK) and concentrated by nitrogen evaporation (Sample Concentrator, Stuart, UK) at 60 °C the sample was mixed with 200 µl of 0.1 M HCl. Hydroxyproline was determined using a high-performance liquid chromatography HP 1100 Series (Agilent Technologies, California, USA) with a fluorescence detector (FLD; HP, Germany). The column effluent was monitored by a diode array detector (DAD; HP, Germany) at 338 nm and a FLD detector at 266ex/305em nm. For the separation of hydroxyproline, the column Zorbax Eclipse AAA (Agilent Technologies, California, USA) with dimensions of 150 x 4.6 mm and a particle

size of 3.5 µm was used. The column was equilibrated at 40 °C. The mobile phases A consisted of 40 mM sodium phosphate dibasic at pH 7.8 and mobile phase B acetonitrile/methanol/water (45:45:10 v/v/v). The flow rate of the mobile phase was 2 ml/min. The compounds were eluted with a linear upward gradient 0.0 min (0% B) → 1.9 min (0% B) → 18.1 min (57% B) → 18.6 min (100% B) → 22.3 min (100% B) → 23.2 min (0% B).

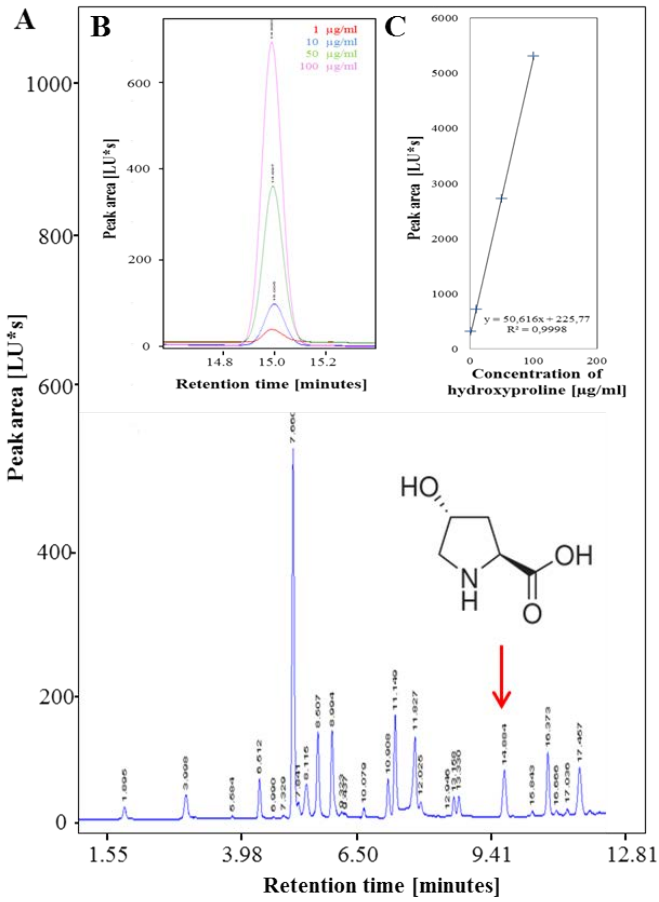
Statistical analyses

The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner (Long and Winefordner 1983), whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

RESULTS AND DISCUSSION

The determination of the presence and of the concentration of hydroxyproline in rat skin samples was done by high-performance liquid chromatography with fluorescence detection (HPLC-FLD). The content of hydroxyproline in a rat skin was expressed as a mean ± standard deviation from three replicates. Calibration curve was prepared in the range 1–100 µg/ml for HPLC-FLD. For analysis of hydroxyproline, the limit of detection (LOD) was 0.33 ng/ml, whereas the limit of quantification (LOQ) was 1.00 ng/ml. The calibration curve showed a good linearity with correlation coefficient R2 = 0.9998 and R.S.D. = 1.5% (Figure 2 B, C). The figure 2A shows the HPLC-FLD chromatographic analysis record of real samples of rat skin.

*Figure 2 (**A**) Chromatographic record of a real sample of rat skin, (**B**) chromatograms from calibration dependence of hydroxyproline determined by HPLC-FLD, (**C**) calibration curve measured within the range from 1 to 100 µg/ml under the experimental conditions.*

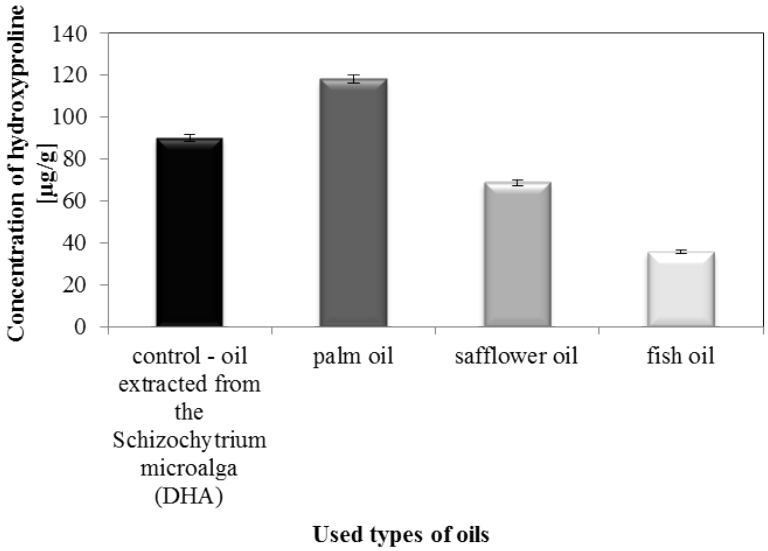


Sample analysis was performed by calibrated high-performance liquid chromatography with fluorescence detection (HPLC-FLD). As a control, an oil extracted from the *Schizochytrium microalga* (DHA) was taken up with a value of 90.2 ± 1.7 µg/g. In Figure 3 is obvious, that the highest

content of hydroxyproline was recorded in samples of rats, which have been fed with palm oil (118.4 ± 1.9 µg/g) compared to other oil supplements. The results indicate that the most positive effect on wound healing had a palm oil. Results of content of hydroxyproline for safflower oil (68.9 ± 1.3 µg/g) and fish oil (35.7 ± 0.9 µg/g) are lower than control results.

Only one third of hydroxyproline content in wound was determined against the control group after 18 days after excision of rats fed with fish oil (36 µg/g vs. 118 µg/g; Figure 3). This result agrees with the data of Gercek and co-workers (Gercek et al. 2007) who reported lower hydroxyproline content (99.3–99.8 µg/g) compared to control (saline; 152.9 µg/g) in wounds of rats treated five days after wounding with parenteral fish oil emulsion. Similarly, Rosa and co-workers (Rosa et al. 2014) found out lower hydroxyproline content (ca 0.25 µg/g) fourteen days post-excision in wounds of mice ingesting (by gavage) fish oil in comparison with both olive oil (ca 0.50 µg/g; high content of 18:1 n-9 oleic acid) and a control (water; ca 1 µg/g). Otranto and co-workers (Otranto et al. 2010) fed rats thirty days before cutaneous wounding with a diet supplemented with (among others) a fish oil and sunflower oil and found out two times higher hydroxyproline content in wounds fourteen days later in the fish oil group (about 2.2 µg/g) compared to the sunflower oil group (ca 1.1 µg/g).

Figure 3 Hydroxyproline content 18 days post-excision in wounded skin of rats fed with a diet supplemented with 5% of Schizochytrium microalga extract (control), 5% of palm oil, 5% of safflower oil and 5% of fish oil.



Legend: The values are expressed as the mean of twelve independent replicates (n=12). Vertical bars indicate standard error p<0.05.

All the above-mentioned experiments of (Gercek et al. 2007), (Rosa et al. 2014) and (Otranto et al. 2010), used a "basic" spectrophotometric determination according to (Woessner 1961), while HPLC method was applied in this work. Moreover, considering the fact that collagen fibrils begin to appear by the fifth day and collagen continues to accumulate within the second week (Hashimoto et al. 2002), the absolute amounts of hydroxyproline measured at different time intervals after the excision are practically incomparable. Only a relative comparison of dietary interventions is meaningful.

CONCLUSION

The aim of this experiment was to determine the concentration of hydroxyproline in the rats' skin wound after supplementation with 5% of palm oil, 5% of safflower oil, 5% of fish oil and 5% of oil extracted from the *Schizochytrium microalga* (marked as DHA; control) in the feed. The results showed the highest concentration of hydroxyproline in samples of rats that have been fed with palm oil (118.4 ± 1.9 µg/g) compared to the others. The results suggest that the most positive effect on wound healing had palm oil. Content of hydroxyproline for safflower oil (68.9 ± 1.3 µg/g) and fish oil

(35.7 ± 0.9 µg/g) is lower than control (90.2 ± 1.7 µg/g). This data will serve as a pilot study for a larger experiment.

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