DOI 10.1007/s10517-021-05083-w

Comparative Study of Wound-Healing Activity of Dihydroquercetin Pseudopolymorphic Modifications

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 170, No. 10, pp. 452-456, October, 2020
Original article submitted July 20, 2020

Wound-healing activity of the crystalline form of dihydroquercetin and its microtubular pseudopolymorphic modification obtained by crystal engineering was compared using the rat model of IIIA degree burn. The rate of wound healing in the group treated with microtubular pseudopolymorphic modification of dihydroquercetin was 4.8±0.1%, which was higher by 11.6% than in the group treated with crystalline form (4.3±0.1%). Bioavailability analysis on MDCK cell culture showed that the apparent permeability coefficient of microtubular pseudopolymorphic modification was higher than that of crystalline form by 31.1% ($19.4±0.2×10^{-4}$ and $14.8±0.3×10^{-4}$ cm/sec, respectively). It was proven that the use of crystal engineering improved the biopharmaceutical parameters of dihydroquercetin and increased its pharmacological efficiency.

Key Words: *dihydroquercetin; burns; polymorphism; biopharmacy; regenerative medicine*

Dihydroquercetin (DHQ), or taxifolin, is characterized by a wide range of pharmacological activity. In Russia, antioxidant drug Diquertin is produced on the basis of this flavonoid [2]. Much attention is now attracted to DHQ due to unique combination of pharmacological effects and its high safety profile [10,12]. Recent studies are focused on the search of new biological targets using *in silico* approach [5,8]; pharmacological properties of this substance [1], mechanisms of antioxidant activity [9], and its metabolism and pharmacokinetics are extensively studied [14]. The development of new DHQ forms characterized by higher water solubility and better bioavailability is a priority research area [13]. Crystal engineering is a new way for the modification of the biopharmaceutical parameters of flavonoids, including DHQ [4].

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DHQ is produced in the form of a pharmaceutical substance (FS-000388-270812) that has a crystalline structure (DHQ-C) according to the data of X-ray powder diffraction analysis. Using crystal engineering approach, a new microtubular pseudopolymorphic modification (DHQ-T) was obtained [6].

The aim of this study was to study and compare the wound healing activity of polymorphic modification of DHQ (microtubular and crystalline) using the rat model of IIIA degree burn.

MATERIALS AND METHODS

The study was carried out on 3-month-old male Sprague-Dawley rats (n=48; mean weight 270 g) provided by Institute of Medical Plant Development (Beijing). The maintenance, feeding, and sacrifice were conducted with strict adherence to GOST 33215-2014 (Guidelines for the Maintenance and Care of Laboratory Animals. Rules for the Equipment of Premises and Organization of Procedures) and GOST 33216-2014 (Guidelines for the Maintenance and Care of Laboratory Animals. Rules for the Maintenance and Care of Laboratory Rodents and Rabbits). The de-

sign of this experiment was approved by the Ethics Committee of the I. M. Sechenov First Moscow State Medical University.

The IIIA burn was modeled by a widely used method [7,11]: the rats were deeply anesthetized with chloralhydrate and a metal plate (m=200 g, d=8 cm^2) heated to 105±5°C was applied to the shaved skin of the back for 20 sec.

In 24 h, the animals were divided up into 4 groups (12 rats per group). Group 1 served as a negative control (application of 1 ml water). Group 2 rats were treated by applying 1 ml suspension of DHQ-C (Lavitol, Ametis) with the concentration of 50 mg/ml on the wound. Group 3 animals were treated with DHQ-T suspension [6]. In group 4 (positive control), the wounds were treated with 1 ml sea-buckthorn oil (Wulumuqi Huajinshan Shipin Co. Ltd.). All preparations were applied daily.

On days 1, 3, 5, 8, 11, 14, 17, 20, and 24, the body weight and temperature were measured. Wound healing was controlled by the Popova's contact method and non-contact method of digital photography with a square standard using GNU Image Manipulation Software 2.10.18 (GNOME Foundation) [37]. The wound healing rate (W) was calculated by the formula:

$$W=\frac{S_0-S_n}{S_0}\times 100\%,$$

where S_0 and S_n are the mean square of the wound before and on day n of treatment. The mean rate of wound healing was determined from the plot using the data of all measuring days.

The *ex vivo* experiments were carried out on passage 25-28 MDCK cell cultures obtained from the Cell Culture Center of Chinese Academy of Medical Sciences (Beijing). The cells were grown in DMEM (Corning) supplemented with 10% fetal calf serum (Gibco) and penicillin and streptomycin (Gibco) (89:10:1 v/v) in 50-ml plastic flasks at 37°C, 5% CO_2, and 95% humidity. The medium was changed every 48 h.

MDCK cells in complete medium (0.25 ml) were seed on 96-well plates at a final density 4×10^4 cells/ml. After 36 h, the medium was removed, and fresh culture medium was added to 6 wells of the first row (positive control) and 10% DMSO (Xilong Scientific) was added to 6 wells of the last row (negative control). To other wells, culture medium containing DHQ in concentrations from 2000 to 0.002 mg/ml (each polymorphic modification was tested in a special plate). After 2-h incubation in an incubator, the solutions were removed and a cell counting kit CCK-8B (Beijing Bio Dee Biotechnologies) was added for 2 h. The absorbance was measured in a SPARK spectrophotometer (TECAN) at λ=450 nm. The viability (V) was calculated by the formula:

$$V=(A_n-A_0)/(A_{100}-A_0)\times 100\%,$$

where A_n is a mean absorbance of CCK-8 solutions after incubation with MDCK cells in the presence of DHQ in a certain concentration, A_0 and A_{100} are the mean absorbance of CCK-8 solutions in negative and positive control, respectively.

To compare the permeability of polymorphic forms of DHQ, 0.5 ml cell suspension (2×10^6 cells/ml) was seeded on semipermeable membranes (area of 4.7 cm^2), and 1.5 ml of culture medium was added to the inner part of the wells. The medium was refreshed every 2 days until the cells reached confluence; control was carried out by measuring transepithelial electrical resistance using a Millicell ERS voltohmmeter (Millipore). After removal of the culture medium, the wells were washed with Hanks solution (Gibco) warmed to 37°C. Then, 0.5 ml of DHQ-C suspension in culture medium was added to outer parts of 4 wells and 0.5 ml of DHQ-C suspension of the same concentration was added to other 4 wells. The inner part of the wells was filled with 1.5 ml Hanks solution. In 15, 30, 60, 90, and 120 min, 0.2-ml aliquots were taken from the inner part of each well and an equivalent volume of Hanks solution was added to replenish the well volume. The obtained aliquots were mixed with 0.2 ml acetonitrile, the mixture was stirred, centrifuged, and 0.25 ml supernatant was analyzed by HPLC (Waters 600 HPLC with a Waters 2489 UV detector) at λ=290 nm using a preliminary constructed calibration curve (r^2=0.9999). The apparent permeability (P_{arp}) was calculated by the formula:

$$P_{app}=\frac{(dC\times V)/dT}{A\times C},$$

where dC/dT is the change of DHQ concentration in the receiver chamber over time, V=1.5 ml, A=4.7 cm^2, C is initial DHQ concentration in the culture medium (mg/ml).

The data were analyzed using LibreOffice Calc 6.2 (The Document Foundation), including potting and calculation of the mean values, confidence interval, and correlation coefficient.

RESULTS

At the beginning of the study the mean body weight of rats in groups 1, 2, 3, and 4 were 279.4±11.2, 276.1±7.1, 268.2±10.4, and 277.1±7.8 g, respectively; the body temperature was about 38.0±0.9°C, *i.e.* the groups were homogenous and congenerous. After burn modeling, a decrease in body weight (by ~5.4%) was observed in all groups presumably due to distress.

TABLE 1. Wound Healing Rate with the Different Treatment Methods

Parameter		Group 1	Group 2	Group 3	Group 4
Contact method					
Wound area, cm^2	day 1	7.8±1.0	8.7±0.5	9.0±0.5	8.4±0.7
	day 8	6.3±0.6	5.7±0.3	5.1±0.5	5.7±0.2
	day 14	3.2±0.5	2.9±0.5	2.5±0.5	3.0±0.6
	day 24	0.6±0.8	0.6±0.7	0.2±0.2	0.6±0.6
Wound healing, %	day 8	19.2	34.5	43.3	36.0
	day 14	59.0	66.7	72.2	66.3
	day 24	92.3	93.1	97.8	93.3
Non-contact method					
Wound area, cm^2	day 1	8.7±1.3	8.4±1.2	8.9±1.4	8.9±1.3
	day 8	5.8±1.3	5.5±1.6	7.0±1.2	7.5±1.7
	day 14	3.1±0.9	2.5±0.8	2.7±1.0	3.2±1.1
	day 24	0.7±0.8	0.7±0.6	0.6±0.3	0.6±0.7
Wound healing, %	day 8	33.4	34.4	21.0	15.3
	day 14	64.1	70.0	70.2	64.1
	day 24	92.3	92.0	93.4	92.7

Later, this parameter returned to initial values. No significant differences in body temperature were observed.

The area of burns and the wound healing rates are presented in Table 1. According the results of contact method, the mean wound healing rate in groups 1, 2, 3, and 4 were 4.0±0.1, 4.3±0.1, 4.6±0.1, and 4.2±0.1%, respectively. These results were consistent with the results of non-contact method (4.1±0.1, 4.3±0.1, 4.8±0.1, and 4.4±0.1% in groups 1, 2, 3, and 4, respectively). In group 3, the wound completely healed by day 22, in group 2 and 4, healing was completed by day 24. In group 1, most wounds did not heal by the end of the experiment.

Based on the results of *in vivo* experiment, we hypothesized that the differences in pharmacological effects of the polymorphic modifications of DHQ are determined by their biopharmaceutical characteristics. To test this hypothesis, *ex vivo* analysis of permeability was performed.

Evaluation of the toxicity of both polymorphic modifications revealed a logarithmic dependence between the concentrations of DHQ samples and cytotoxicity (Fig. 1). The nonlinear dose dependence can be explained by low water solubility of DHQ and formation of suspensions at higher concentrations. Hence, the negative effect on MDCK cells was caused by physical impact of solid DHQ particles,

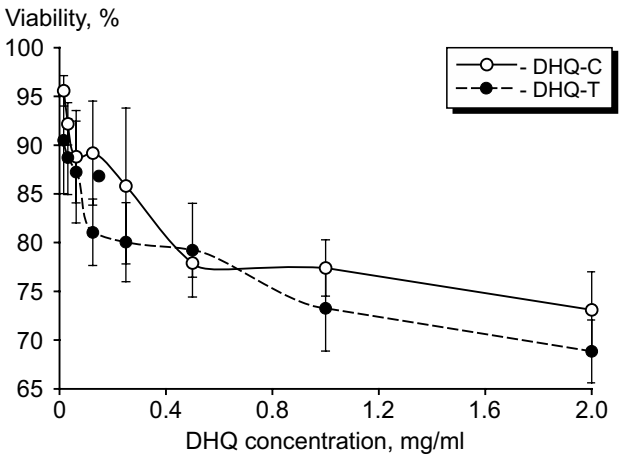

Fig. 1. The dependence on of MDCK cell viability on DHQ concentration.

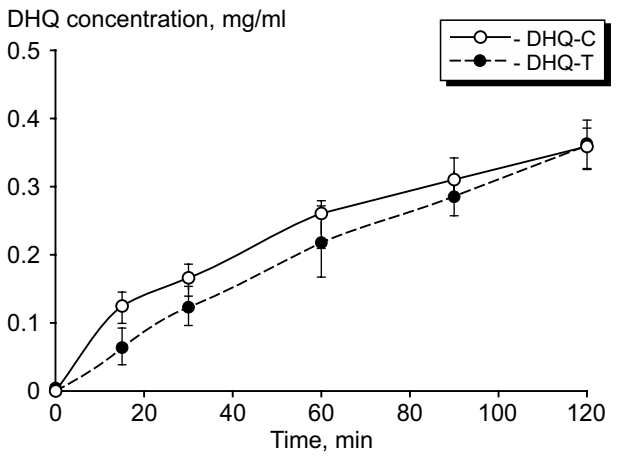

Fig. 2. Time course of DHQ concentration during permeability assay.

rather than DHQ toxicity. The limit of cell viability for both samples was ~75%, so both DHQ forms demonstrated high safety profile. Permeability analysis (Fig. 2) revealed no significant differences between the concentration curves of the studied DHQ forms. The P_{app} of DHQ-T was significantly higher than that of DHQ-C: $19.4\pm0.2\times10^{-4}$ and $14.8\pm0.3\times10^{-4}$ cm/sec, respectively. These findings support our assumption that the differences in wound healing efficacy of polymorphic modifications of DHQ are determined by better permeability of DHQ-T.

Thus, DHQ-T demonstrates the same safety profile as DHQ-C and better wound healing activity. The increase of pharmacological efficacy of DHQ-T correlates with the better biopharmaceutical characteristics.

This work was supported by the Russian Academic Excellence Project 5-100.

REFERENCES

1. Plotnikov MB, Aliev OI, Sidekhmenova AV, Shamanaev AY, Anishchenko AM, Fomina TI, Chernysheva GA, Smol'yakova VI, Arkhipov AM. Dihydroquercetin Improves Microvascularization and Microcirculation in the Brain Cortex of SHR Rats during the Development of Arterial Hypertension. Bull. Exp. Biol. Med. 2017;163(1):57-60. doi: 10.1007/s10517-017-3737-7
2. Plotnikov MB, Tyukavkina NA, Plotnikova TM. Diquercetin-Base Drugs. Tomsk, 2005. Russian.
3. Savchenko YuP, Fedosov SR. Methods of determination of sizes of the wound surface. Vestn. Khirurg. im. I. I. Grekova. 2007;166(1):102-105. Russian.
4. Selivanova IA, Terekhov RP. Crystal engineering as a scientific basis for modification of physicochemical properties of bioflavonoids. Russ. Chem. Bull. 2019;68(12):2155-2162.
5. Terekhov RP, Selivanova IA. Molecular modeling of the interaction of the dihydroquercetin and its metabolites with cyclooxygenase-2. Byull. Sib. Med. 2019;18(3):101-106. Russian.
6. Terekhov RP, Selivanova IA, Zhevlakova AK, Porozov YuB, Dzuban AV. Analysis of dihydroquercetin physical modification via in vitro and in silico methods. Biomed. Khimiya. 2019;65(2):152-158. Russian.
7. Alemzadeh E, Oryan A, Mohammadi AA. Hyaluronic acid hydrogel loaded by adipose stem cells enhances wound healing by modulating IL-1β, TGF-β1, and bFGF in burn wound model in rat. J. Biomed. Mater. Res. B Appl. Biomater. 2020;108(2):555-567. doi: 10.1002/jbm.b.34411
8. Fischer A, Sellner M, Neranjan S, Smieško M, Lill MA. Potential Inhibitors for Novel Coronavirus Protease Identified by Virtual Screening of 606 Million Compounds. Int. J. Mol. Sci. 2020;21(10):3626. doi: 10.3390/ijms21103626
9. Ilyasov IR, Beloborodov VL, Selivanova IA. Three ABTS•+ radical cation-based approaches for the evaluation of antioxidant activity: fast- and slow-reacting antioxidant behavior. Chem. Papers. 2018;72(8):1917-1925.
10. Muramatsu D, Uchiyama H, Kida H, Iwai A. Cell cytotoxity and anti-glycation activity of taxifolin-rich extract from Japanese larch, Larix kaempferi. Heliyon. 2019;5(7):e02047. doi: 10.1016/j.heliyon.2019.e02047
11. Sayar H, Gergerlioglu N, Seringec N, Ozturk P, Bulbuloglu E, Karabay G. Comparison of efficacy of topical phenytoin with hypericin in second-degree burn wound healing: an experimental study in rats. Med. Sci. Monit. Basic Res. 2014;20:36-46. doi: 10.12659/MSMBR.890337
12. Schauss AG, Tselyico SS, Kuznetsova VA, Yegorova I. Toxicological and Genotoxicity Assessment of a Dihydroquercetin-Rich Dahurian Larch Tree (Larix gmelinii Rupr) Extract (Lavitol). Int. J. Toxicol. 2015;34(2):162-181. doi: 10.1177/1091581815576975
13. Shikov AN, Pozharitskaya ON, Miroshnyk I, Mirza S, Urakova IN, Hirsjärvi S, Makarov VG, Heinämäki J, Yliruusi J, Hiltunen R. Nanodispersions of taxifolin: impact of solid-state properties on dissolution behavior. Int. J. Pharm. 2009;377(1-2):148-152. doi: 10.1016/j.ijpharm.2009.04.044
14. Yang P, Xu F, Li HF, Wang Y, Li FC, Shang MY, Liu GX, Wang X, Cai SQ. Detection of 191 Taxifolin Metabolites and Their Distribution in Rats Using HPLC-ESI-IT-TOF-MS(n). Molecules. 2016;21(9):1209. doi: 10.3390/molecules21091209