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Assessment of the cytotoxic potential of rutin formulations on human oral cells

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Abstract

Rutin is a bioflavonoid found mostly in medicinal plants, but also in food, and other products. Throughout the years, rutin has been proven to exhibit important pharmacological properties, including antioxidant, anti-inflammatory, and anti-cancer activities. Due to its low oral solubility, the flavonoid is preferable to be included in nanoformulas. The main aim of the present study was to assess the cytotoxic potential of rutin and rutin formulations on one healthy cell line (primary gingival keratinocytes - PGK) and two tumoral oral cell lines (tongue carcinoma - SCC-4 and pharynx carcinoma - Detroit 562). The in vitro assay employed was MTT test which revealed that rutin has a cytotoxic effect on cancer cells, without affecting the healthy ones, and its inclusion in liposomes increases its anticancer potential, especially at 10 and 25µM. The results warrant further detailed studies and in-depth anti-cancer mechanistic research.

Keywords: rutin, liposomes, oral cells, cytotoxicity, cancer

1.Introduction

Diseases at the oral cavity level are quite common, and the most frequent lesions can be categorized as follow: change of color (red, white, blue, pigmented, or a combination of these), mucous membrane integrity loss (fissure, ulcer, or erosion, which may be primary or secondary), swelling or growth. Lesions can entail the tooth and bone, combined with other soft tissue lesions, or alone, or the condition may be associated with a syndrome [1]. A retrospective study analysed the history of oral pathology of more than 900 patients. It was observed that patients had experienced symptoms for more than a year before the initial encounter, proof of the fact that patients often neglect oral disorders. Common top complaints were orofacial pain, non-ulcerative mucosal lesions, and dry mouth. In addition, it was observed that patients with cardiovascular disease were predisposed to develop lichenoid lesions, and patients with psychiatric disorders were at higher risk of reporting burning mouth symptoms [2].

According to GLOBOCAN, cancers of the oral cavity and oropharynx were estimated to account for over 98,000 cases of cancer in 2020 with likely more than 48,000 deaths [3]. Incidence rates of oropharyngeal and oral cavity carcinomas have growth by 1.2% for males and 0.5% for females annually from 2012 to 2016 [4]. Central and Eastern Europe region seems to be one of the most affected, with more than 11,000 new cases in 2020, and the male genre remains to be more prone than the female one. A large study reported an overall 5-year survival at around 65% for patients with oral squamous cell carcinoma (SCC) who underwent surgery [5]. Over time it was noticed that over 90% of forms of oral cavity and oropharyngeal cancers have been SCC [6]. Tobacco, alcohol, and high-risk HPV (human papilloma virus) infection are considered major risk factors for SCC as well as for

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oropharyngeal carcinoma [7]. Most of the time, the problem of cancer is poor and aggressive treatment. As a complementary and alternative medicine, many herbs and natural compounds have become increasingly popular in recent decades.

3′,4′,5,7-tetrahydroxyflavone-3-rutinoside commonly named rutin or vitamin P is an attractive phytochemical because of its large pharmacological spectrum. Thereby, it is considered an essential flavonoid in the pharmaceutical industry, being integrated into more than 130 therapeutic medicinal preparations registered worldwide as drugs [8]. Flavonoids are polyphenolic compounds reported as the major dietary constituents of food based on plants [9]. To date, over 4,000 different types of flavonoids have been identified and can be classified into the class of flavones, flavonols, flavanones, anthocyanidins, catechins, and chalcones [10]. Quercetin is the aglycone halfness of rutin after hydrolysis by the microflora in the intestines. The hydrolysis process is catalyzed by glucosidase when rutin produces quercetin and rutinose, so quercetin usually coexists with rutin. Therefore, rutin and quercetin are excellent sources of pharmaceutical products for phototherapeutic drugs [11]. Rutin has shown pharmacological properties, including anti-inflammatory, antioxidant, pro-apoptotic, antiangiogenic, and anti-proliferative potential, all of which may be considered important in the prevention and treatment of cancer. Vitamin P also enhances nitric oxide production and subdues ROS-responsive nucleotide-binding domain-like receptor 3 (NLRP3), thereby reducing the risk of cardiovascular disorders. It has been also reported to combat neurodegenerative problems by abrogating abnormal protein accumulation, neuroinflammation, and apoptosis [12].

In the case of cancer disorders, rutin targets different inflammatory mediators such as TNF-α and NF-κB, thereby counteracting inflammation. By abrogating the c-met/HGF axis and its downstream cascades, such as Rac-1, paxillin, Akt, and mTOR, rutin has anticancer effects on breast cancer cell lines. Various transcription factors, especially NF-κB and STAT, were identified as capable of directly targeting p38. P38 is then activated by MKK3 and MKK6, inducing inflammation and activating IL-1β, TNF-α, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS). There is evidence that the p38 signalling pathway modulates apoptosis and cell cycle in opposing ways [13]. At the same time, by reducing NF-κB and p38 expression, rutin prevented the development of lung cancer, arresting the cell cycle [14]. On human glioma CHME cells, rutin exhibits an apoptotic effect by abating mitochondrial membrane potential and inducing ROS generation. The apoptotic effect was further endorsed by the upregulation of Bax, cytochrome c, p53, caspases 3 and 9, and the downregulation of B-cell lymphoma 2 [15]. In addition, the flavonoid presents promising in vitro results on colon cancer, leukemia/multiple myeloma/lymphoma, liver cancer, ovarian cancer, etc [16].

On oral and oropharyngeal diseases, rutin has not been sufficiently studied, although the oral administration of this compound is preferred, the compound having direct contact with the oral cavity. In addition, the disadvantage of orally administered rutin is its low solubility and poor oral bioavailability, resulting in limited pharmacological effects. This drawback stimulated the development of new therapeutic nanoformulations. Liposomes are phospholipid and biocompatible vesicles able to incorporate hydrophobic, hydrophilic, and amphiphilic molecules.

The aim of the present work was to obtain a liposomal formulation loaded with rutin and to test it on oral cancer cells (SCC-4 and Detroit 562 cell lines), in comparison with the healthy PGK cell line, in terms of identifying possible beneficial effects on oropharyngeal carcinomas.

2.Materials and methods

Reagents. Rutin, lecithin, cholesterol, chloroform, methylic alcohol, and MTT cell viability kit 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were acquired from Sigma-Aldrich (Darmstadt, Germany analytical grade forms. For cell lines, specific media Dermal Cell Basal Medium (ATCC® PCS-200-030™), DMEM:F12, Eagle's Minimum Essential Medium (EMEM-ATCC 30-2003™), specific kit (Keratinocyte Growth Kit - ATCC® PCS-200-040™), hydrocortisone, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), penicillin/streptomycin mixture, phosphate saline buffer (PBS), trypsin-EDTA solution were purchased from ATCC (American Type Cell Collection, Lomianki, Poland).

Liposome preparation and characterization. Liposomes loaded with rutin were obtained by the thin film dispersion method and involved the following steps: (a) the compounds rutin, cholesterol, phosphatidylcholine (ratio 0.3:1:5) were

subjected to agitation in a mixture of methanol-chloroform (ratio 1:1 v/v) for one hour at a temperature of 42°C, (b) removal of the solvent using a rotary evaporator until a lipid film is obtained, (c) hydration of the lipid film with phosphate buffer for half an hour at a temperature of 42°C, (d) sonication of the reaction mixture for 15 min and (e) filtration and maturation (overnight). Both samples with active compound and control samples (without rutin) were prepared. The characterization of the formulations was done with the help of DLS (Dynamic light scattering) devices and on the Zetasizer (Nano ZS system, Malvern Instruments, Malvern, UK) through which details related to the size and stability are obtained following the previously described methods [17]. The evaluation of the encapsulation of the active substance in liposomes was carried out spectrophotometrically on a T70 UV-Vis spectrophotometer (PG Instruments Ltd., Lutterworth, Great Britain).

Cell lines. All the cells were purchased from American Type Culture Collection (ATCC). *PGK* - Primary human gingival keratinocytes cell line was used as a negative control. Cells are epithelial-like, rounded, not flat, with a high mitotic index, and at nearly 80% confluence, the cells are associated with each other in colonies. *SCC-4*- epithelial-like cell isolated from the tongue of a patient with squamous cell carcinoma. The cells have a slightly elongated shape and develop in colonies. *Detroit 562*- cells representing pharyngeal cancer. The cells are slightly rounded with the tendency to elongate and develop in colonies.

Cell culture. All the cells were cultivated in their appropriate medium, thereby PGK in Dermal Cell Basal Medium plus one Keratinocyte Growth Kit. SCC-4 in DMEM:F12 supplied with 400 ng/mL hydrocortisone and fetal bovine serum (FBS) to a final concentration of 10%, and Detroit 562 in Eagle's Minimum Essential Medium supplied with 10% FBS. All the media were completed with 1% penicillin/streptomycin, to avoid microbial contamination. The cells were grown under standard conditions: 5% CO_2 and a temperature of 37°C in a humidified incubator.

Cell viability. The cell viability was assessed by applying the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) technique. So, cells were cultured in 96-well plates (10^4 cells/200 µL/well) and upon reaching a confluence of approximately 80%, cells were treated with different concentrations of liposomes with rutin (1, 5, 10, 25 µM) followed by 48 h of incubation. Following the treatment period,10 µL/well of MTT solution (5 mg/mL) was added and the plate was incubated after 3 h, the formazan crystals formed were dissolved during 30 min in the dark with 100 µL of solubilization buffer provided by the manufacturer. Finally, the reduced MTT was spectrophotometrically measured at 570 nm, using the Cytation 5 (BioTek Instruments Inc., Winooski, VT, USA) microplate reader. All experiments were performed in triplicate.

Statistical analysis. The experimental data are presented as means ± standard deviation. One-way ANOVA analysis and Dunett's multiple comparisons post-test were used to compare the differences between data. The used software was GraphPad Prism version 9.0.0 for Windows. The statistically significant differences between data were noticed with * (* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

3.Results and discussions

Oral and oropharyngeal cancers are some of the commonest cancers worldwide and present an alarming health problem. Available treatment options are radiation, surgery, and chemotherapy with cisplatin or cetuximab [18]. However, these treatment options induce severe acute toxicities, swallowing dysfunctions, and long-term morbidities, and request improvements and/or replacement. The aim of this research was to test rutin liposomes on tongue carcinoma and pharyngeal carcinoma cells and compare with the effect on the healthy gingival keratinocytes.

Liposomes loaded with rutin are characterized by a negative zeta potential, located around -23 mV, medium sizes and distributions, and polydispersity indices below 0.2. They prove such homogeneous distribution and stability. Regarding the encapsulation efficiency, it was about 62%.

At all four tested concentrations, rutin did not manifest a cytotoxic effect, and even a slight stimulation of cell growth is observed. In the case of liposomes charged with rutin, results were similar, the exception being the two highest concentrations (10 and 25µM), which presented a slow reduction of cell viability. The same tendency was observed in the case of liposomes without rutin, at 5,10, and 25 µM (Figure 1).

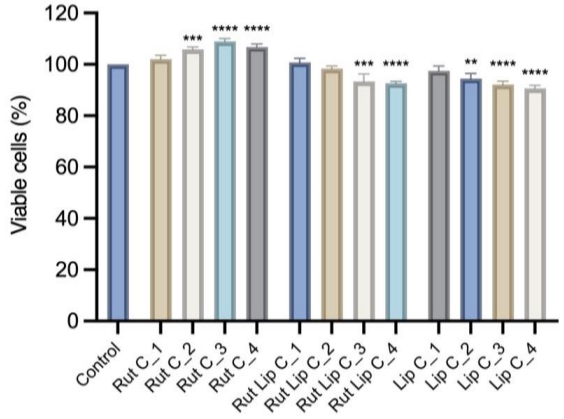

Figure 1. Percentage of viable primary gingival keratinocytes PGK after 48h of treatment with Rutin, Liposome-Rutin, and Liposome Blank (1,5,10,25 µM). The results are expressed as cell viability percentage normalized to control cells (unstimulated). The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was applied to determine the statistical differences in rapport with control cells followed by Dunnett's multiple comparisons post-test (** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$).

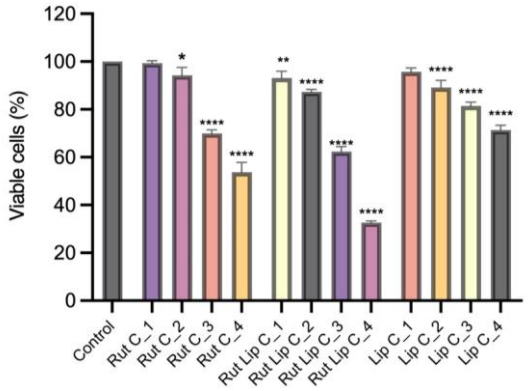

Figure 2. Percentage of viable oral tongue carcinoma cells after 48h of treatment with Rutin, Liposome-Rutin, and Liposome Blank (1,5,10,25 µM). The results are expressed as cell viability percentage normalized to control cells (unstimulated). The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was applied to determine the statistical differences in rapport with control cells followed by Dunnett's multiple comparisons post-test (*$p < 0.1$, ** $p < 0.01$, **** $p < 0.0001$).

In the case of Detroit cells, the viability was also dose-manner reduced, and the highest activity was observed in the case of liposomes with rutin at 25µM, which reduced cell viability by around 30% (Figure 3). Blank liposomes presented a slight viability reduction at the highest concentration.

Similar results were obtained on the human epidermal keratinocyte line (HaCaT), highlighting the safe profile of the bioflavonoid [19]. In addition, another study suggested that rutin protects HaCaT cells against oxidative damage by inhibiting reactive oxygen species (ROS), nitric oxide, and malondialdehyde secretion, increasing total superoxide dismutase (SOD) activity and restoring glutathione peroxidase (GSH-Px) activity [20].

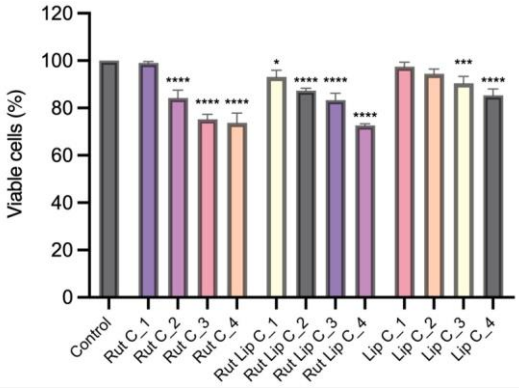

Figure 3. Percentage of viable pharynx carcinoma cells after 48h of treatment with Rutin, Liposome-Rutin, and Liposome Blank (1,5,10,25 µM). The results are expressed as cell viability percentage normalized to control cells (unstimulated). The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was applied to determine the statistical differences in rapport with control cells followed by Dunnett's multiple comparisons post-test (*$p < 0.1$, *** $p < 0.001$, **** $p < 0.0001$).

In the case of tumoral cell lines, rutin tends to decrease cell viability starting with 5µM, thereby at 25 µM, the viability is reduced almost by half (Figure 2). The insertion of rutin into liposomes seems to be much more aggressive with tongue cancer cells, reducing viability up to 35%. Liposomes Blank slowly reduce viability, so at the highest concentration, it tends to be around 80%.

Similar results were obtained in the study done by Dziedzik et al, where Detroit 562 line were exposed to a range of concentrations (5–100 µM) of quercetin, hesperidin and rutin for 24 and 48 h. The viability reduction of the pharynx cells treated with the three flavonoids for 48 h revealed a significant dose-dependent trend, relatively equal for the three substances. It was observed that rutin exposure increased the proportion of apoptotic SCC cells, either during necrosis or late apoptosis [21].

Both in vivo and in vitro experiments on the anticancer mechanisms of rutin have shown multiple properties, such as regulation of different cellular signaling pathways (Wnt/β-catenin, p53, p53-independent pathway, PI3K/Akt, MAPK, JAK/STAT) which make him a good candidate in the oncology field [22]. The insertion in nanoformulas, besides the fact that they improve its absorption properties, also increases its therapeutic potential. Thereby, in a study, phyto-sterosomes with rutin were prepared and reduced the cell

viability of the hepatocellular carcinoma HepG2 cell line in a significant and dose-dependent manner [23]. Paudel et al. reported similar results on the A549 human lung epithelial carcinoma cell line. So it was observed that Rutin-loaded liquid crystalline nanoparticles have promising anti-proliferative and anti-migratory activities against lung cancer [24].

Our results suggest that liposomes with rutin have better activity than simple rutin against SCC-4 and Detroit 562 tested cells and identifying the mechanism of action of this could be a promising solution in order to improve anticancer therapy in this field.

Conclusion

The present study highlighted that rutin is a flavonoid with good integration capabilities in liposomes. Blank liposomes containing cholesterol and phosphatidylcholine did not present cytotoxic effects, but rutin nanoformulations manifest a significant potency against tongue carcinoma and pharynx carcinoma viability, better than the pure phytocompound.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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