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Research Article

Everolimus-Induced Oral Mucositis Can be Prevented by Hippophae Rhamnoides Extract in Rats

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Abstract

Objectives: Oral mucositis is a significant toxicity related to the mammalian target of rapamycin inhibitor everolimus. Oxidative stress and pro-inflammatory cytokines, which contribute to treatment-related mucositis, can be targeted with Hippophae rhamnoides extract (HRE). Herein, we assessed the effects of HRE on everolimus-induced mucositis in rats.

Methods: Eighteen rats were equally divided into healthy, everolimus, and everolimus plus HRE groups. Malondialdehyde (MDA) and total glutathione (tGSH) levels along with interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) gene expression levels were measured in the tongue and buccal mucosa tissues of all groups, histopathological changes were also evaluated. We tested the significance of variations with one-way variance analysis. We also analyzed the differences between groups with Kruskal–Wallis test and Mann–Whitney U-test.

Results: HRE significantly decreased MDA and increased tGSH levels and reduced IL-1β and TNF-α gene expression in both tissues administered everolimus ($p<0.001$ for each). Histological examination revealed that HRE improved epithelial formation and keratinization, disrupted by everolimus, and alleviated everolimus-related mononuclear cell infiltration ($p<0.05$ for each).

Conclusion: In light of these results, HRE may be a promising agent to manage oral mucositis caused by everolimus, given the lack of effective therapeutic options for this type of adverse event.

Keywords: Everolimus, *hippophae rhamnoides*, oral mucositis

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Everolimus is an inhibitor of mammalian target of rapamycin (mTOR) kinase that controls several signaling pathways affecting cancer growth and survival and is widely used for different types of cancers (renal cell cancer, pancreatic neuroendocrine tumors, and breast cancer).[1,2] Oral mucositis is a common adverse event (AE) associated with mTOR inhibitors.[3] During everolimus treatment, the incidence of oral mucositis was reported 58% in the general population and 81% in Asians.[4] The mechanism of mTOR inhibitor-induced oral mucositis is unclear.[5-7] So far, there is no experimental study in the literature showing a relationship between everolimus-induced oral mucositis and inflammation due to oxidative stress. However, several studies reported that tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) could contribute to mucositis associated with conventional chemotherapy.[8] Besides, the increase in the malondialdehyde (MDA) level, and the decrease in the total glutathione (tGSH) level in mucositis, was found associated with oxidative stress and inflammation.[9] Topical, oral, intralesional, and systemic treatments may be beneficial for oral lesions related to mTOR inhibitors.[10] Hippophae rhamnoides extract (HRE) has anti-inflammatory, antioxidant, antiulcerogenic, antimicrobial effects. H. rhamnoides L. plant, which belongs to the Elaeagnaceae family, includes carotenoids (α, β, and γ), riboflavin, Vitamin C, tocopherol, tocotrienol, folic acid, tannin, and fatty acids.[11,12] Kuduban et al. reported that HRE protects buccal mucosa, tongue, and lip tissue through preventing an increase in oxidant and pro-inflammatory cytokines as well as a decrease of antioxidants.[13] To the best of our knowledge, HRE's protective effects against everolimus-induced oral mucositis have not been studied in the literature. In this study, we aimed to assess the histopathological and biochemical effects of HRE on everolimus-induced oral mucositis in rats.

Methods

Animals

A total of 18 male albino Wistar rats, which weighed between 265 and 275 g, supplied by our institution's Medical Experiments Application and Research Center, were used in the study. We equally divided the rats into three groups and kept them in cages in a ventilated room with a 12 h light/12 h dark period. Temperature constantly was 22°C with free access to food and water. We performed our experiment according to the National Guidelines for the Use and Care of Laboratory Animals. Our institution's local animal ethics committee approved our study (Ethics Committee No.: 2020/47, dated April 16, 2020).

Chemical Agents

Everolimus supplied from Novartis-Turkey, thiopental sodium from I.E., Ulagay-Turkey, and H. rhamnoides extract from TOV DKP Pharmaceutical Factory-Ukraine (76002, Ivano-Frankovsk st.30 Nadvirnianska).

Experimental Groups

We divided our albino Wistar rats into three equal groups (n=6) as a healthy group (HG), everolimus group (EVR), and everolimus + HRE group (HREL).

Experimental Procedure

Everolimus was given to EVR and HREL groups at a dose of 2 mg/kg by oral gavage into the stomach. One hour after everolimus administration, two drops of HR extract (each drop contain 0.57 mg of HR extract) were given to the HREL group by gavage into the oral cavity 3 times a day. Similarly, distilled water was given to EVR and HG groups as a solvent. We repeated this procedure once a day for 4 weeks. In the end, all rats were sacrificed with a high dose of thiopental sodium anesthesia. Tongue and buccal mucosa tissues of the rats were removed to measure the MDA and tGSH levels. We also assessed the gene expression of IL-1β and TNF-α.

Biochemical Analysis

For biochemical analysis, tongue and buccal mucosa tissue homogenates were prepared. From each tissue, 0.2 g of the sample was taken. We determined the tGSH and MDA levels obtained from these homogenates based on the literature. We homogenized the tissues in ice-cold phosphate buffers (50 mM, pH 7.4) that were appropriate for the variable to be measured. We centrifuged the tissue homogenates at 5000 rpm for 20 min at 4°C, and subsequently, we extracted the supernatants to analyze tGSH and MDA. All spectrophotometric measurements were performed through a microplate reader (BioTek, USA).

Analysis of MDA

Quantitative analysis of MDA was based on the approach used by Ohkawa et al., related to the spectrophotometrical measurement of absorbance of the pink-colored complex formed by thiobarbituric acid and MDA.[14] The tissue homogenate sample (25 µL) was added to a solution containing 25 µL of 80 g/L sodium dodecyl sulfate and 1 mL mixture solution (200 g/L acetic acids + 1.5 mL of 8 g/L 2-thiobarbiturate). We incubated the mixture at 95°C for 1 h. After cooling, we added 1 mL of n-butanol:pyridine (15:1). Subsequently, we vortexed the mix for 1 min and centrifuged for 10 min at 4000 rpm. We measured the supernatant's absorbance at 532 nm. The standard curve

was obtained by the usage of 1,1,3,3-tetramethoxypropane.

Analysis of tGSH

Complete glutathione analyzes were evaluated using the method defined by Sedlak et al.[15] 5,5'dithiobis [2nitrobenzoic acid]) disulfide (DTNB) is chromogenic in the medium, and DTNB is reduced using sulfhydryl groups without any problems. We measured the yellow coloration in the course of reduction through spectrophotometry at 412 nm. For measurement, a cocktail solution composed of 5.85 mL 100 mM Na-phosphate buffer, 2.8 mL 1 mM DTNB, 3.75 mL 1 mM NADPH, and 80 µL 625 U/L glutathione reductase was prepared. As deproteinization, we added 0.1 mL metaphosphoric acid to 0.1 mL tissue homogenate and centrifuged for 2 min at 2000 rpm. We added the 0.15 mL cocktail solution to 50 µL of supernatant. The standard curve was obtained using GSSG.

Gene Expression of IL-1β and TNF-α

RNA Isolation

We isolated the RNA from the homogenized tongue and buccal mucosa samples using the Roche Magna Pure Compact LC device (Roche, Mannheim, Germany) with MagNA Pure LC RNA Kit (Roche). We assessed the quantity and quality of the isolated RNA with a nucleic acid measurement device (Maestro, Nano). We stored the RNA samples at –80°C.

cDNA Synthesis

We synthesized cDNA from the isolated RNA samples using the Transcriptor First Strand cDNA Synthesis Kit (Roche). For each subject, 1 µl of ddH_2O, 10 µl of RNA, and 2 µl of random primer were combined and incubated in a thermal cycler for 10 min at 65°C. After incubation, we added 4 µl of reaction buffer, 0.5 µl of RNAase, 2 µl of deoxynucleotide mix, and 0.5 µl of reverse transcriptase. The incubation period was 10 min at 25°C, 30 min at 55°C and 5 min at 85°C, and then held at 4°C.

Evaluation of quantitative gene expression by real-time polymerase chain reaction (PCR)

The gene expression of IL-1β and TNF-α and the reference gene (G6PD) was analyzed for each cDNA sample using the Roche LightCycler 480 II Real-Time PCR instrument. PCRs were recorded in a final volume of 20 µl:5 µl of cDNA, 3 µl of distilled water, 10 µl of LightCycler 480 Probes Master (Roche), and 2 µl primer probe set (Real-Time Ready single assay, Roche). The relative quantitative PCRs cycle conditions were pre-incubated at 95°C for 10 min followed by 45 amplification cycles of 95°C for 10 s, 6°C for 30 s, 72°C for 1 h, followed by cooling at 40°C for 30 s. Quantitative PCR analysis and quantification cycle (Cq) values for relative quantification were performed with Light Cycler 480 Software, version 1.5 (Roche). We calculated the quantitative amounts by dividing target genes by the expression level of the reference gene. We used the reference gene for the normalization of target gene expression.

Histopathological Examination

Necropsies of the rats were performed, and the tongue and buccal mucosa samples were taken into 10% buffered formalin solution. Samples were then routinely followed and embedded into paraffin blocks. Sections of 5 µm taken from blocks onto slides were examined under a light microscope after staining with hematoxylin-eosin. The epithelial formation, keratinization, and mononuclear cell infiltration were each assessed as absent (0), mild (1), moderate (2), and severe (3).

Statistical Analysis

We performed statistical analyses using Windows version 19.0 of the Statistical Package for the Social Sciences (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, version 19.0. Armonk, NY: IBM Corp.). We calculated the descriptive statistics for each variable. The mean±standard deviation (SD) is listed as continuous variables. We used one-way variance analysis (ANOVA), followed by the Tukey test to determine the variations between groups. In histopathological findings, differences between groups were determined with the Kruskal–Wallis test and the Mann–Whitney U-test. We used the paired t-test to analyze the statistical differences between groups for body weight changes of the rats. $P<0.05$ was considered statistically significant.

Results

Biochemical Results

The biochemical results of tongue tissue are shown in Table 1. As illustrated in Figure 1, everolimus significantly increased the MDA level in rat tongue tissue than HG ($p<0.001$). HRE also remarkably inhibited the everolimus-related increase of MDA in tongue tissue ($p<0.001$). There was a substantial difference in MDA levels between EVR and HG groups, while levels of MDA were almost the same between HREL and HG groups. Compared with HREL and HG, everolimus substantially reduced tGSH in the tongue tissue ($p<0.001$) (Fig. 1). HRE significantly inhibited the everolimus-induced decrease of tGSH levels in rats' tongue tissue ($p<0.001$). IL-1β and TNF-α gene expression levels were higher in tongue tissue of the rats that received everolimus than the HG and HREL ($p<0.001$) (Fig. 2).

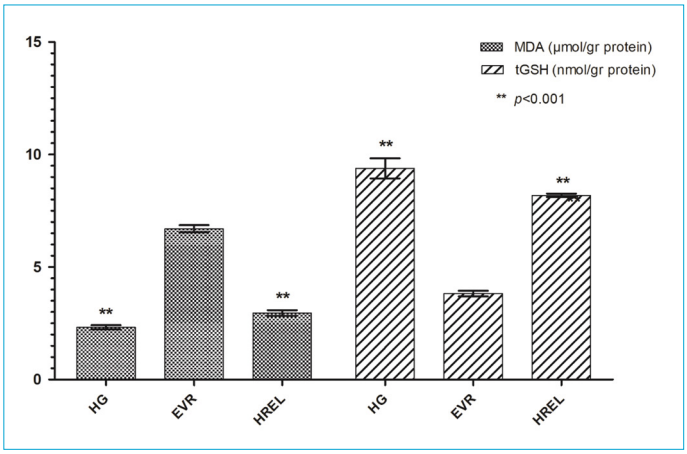

Figure 1. The effects of HRE on MDA and tGSH levels in tongue tissue of rats given everolimus. Bars indicate mean values±standard deviation. The HG is compared with the EVR and HREL groups.

MDA: Malondialdehyde, tGSH: Total glutathione, HG: Healthy group, EVR: Everolimus group, HREL: Everolimus+Hippophae rhamnoides extract group.

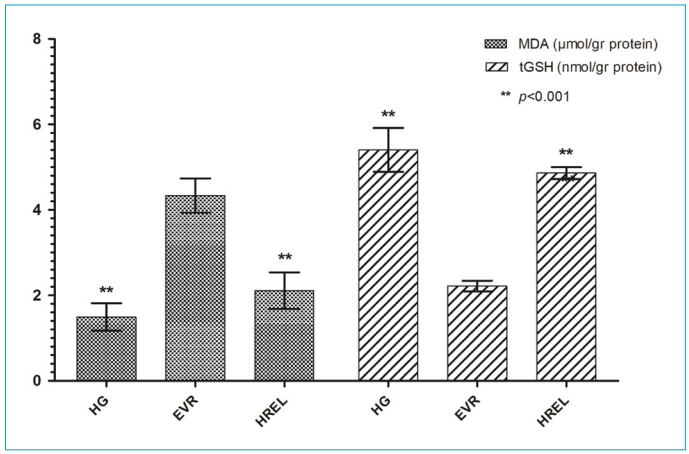

Figure 3. The effects of HRE on MDA and tGSH levels in buccal mucosa tissue of rats given everolimus. Bars indicate mean values±standard deviation. The HG is compared with the EVR and HREL groups.

MDA: Malondialdehyde, tGSH: Total glutathione, HG: Healthy group, EVR: Everolimus group, HREL: Everolimus+Hippophae rhamnoides extract group.

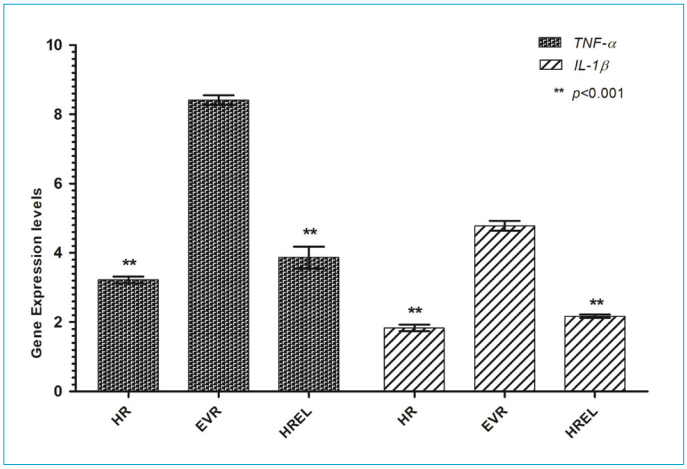

Figure 2. The effects of HRE on TNF-α and IL-1β gene expression levels in tongue tissue of rats given everolimus. Bars indicate mean values±standard deviation. The HG is compared with the EVR and HREL groups.

TNF-α: Tumor necrosis factor alpha, IL-1β: Interleukin-1 beta, HG: Healthy group, EVR: Everolimus group, HREL: Everolimus+Hippophae rhamnoides extract group.

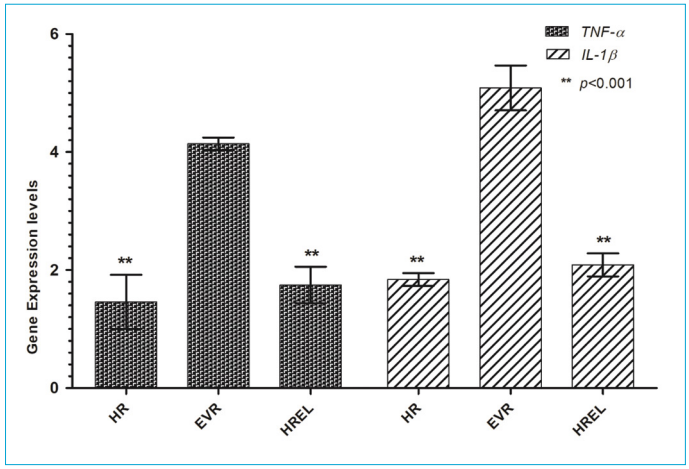

Figure 4. The effects of HRE on TNF-α and IL-1β gene expression levels in buccal mucosa tissue of rats given everolimus. Bars indicate mean values±standard deviation. The healthy group is compared with the EVR and HREL groups.

TNF-α: Tumor necrosis factor alpha, IL-1β: Interleukin-1 beta, HG: Healthy group, EVR: Everolimus group, HREL: Everolimus+Hippophae rhamnoides extract group.

The biochemical results of buccal mucosa tissue are illustrated in Table 2. Everolimus increased the level of MDA in buccal mucosa tissue compared to HG. HRE decreased the level of MDA in the HREL group ($p<0.001$). We found a significant difference in MDA levels between EVR and HG groups ($p<0.001$). The MDA levels were similar between HREL and HG groups. Everolimus significantly reduced tGSH levels in buccal mucosa compared to HG and HREL groups ($p<0.001$). HRE significantly inhibited the decrease of tGSH levels in the HREL group ($p<0.001$) (Fig. 3). IL-1β and TNF-α gene expression levels were higher in rats' buccal mucosa tissue that received everolimus, compared to the HG and HREL groups ($p<0.001$) (Fig. 4).

Histopathological Findings

At the time of sacrificing the rats, we detected that body weight was increased from baseline in the HG group. However, bodyweight was decreased from baseline in the EVR and HREL groups (Table 3).

The tongue and buccal mucosa tissues of rats in the HG appeared normal on histological examination (Fig. 5). In the tongue and buccal mucosa tissues of rats from the EVR group, epithelial formation and keratinization were impaired, and mononuclear cell infiltration was severe. Epithelial formation and keratinization were normal, and mononuclear cell infiltration was mild in the tongue and

Table 1. Biochemical results of tongue tissue

	HG (n=6)		EVL (n=6)		HREL (n=6)	
	Mean±SD	**Median (Min-Max)**	**Mean±SD**	**Median (Min-Max)**	**Mean±SD**	**Median (Min-Max)**
MDA (µmol/g protein)	2.32±0.09**	2.31 (2.21–2.48)	6.7±0.16	6.72 (6.47–6.92)	2.96±0.12**	2.91 (2.82–3.13)
tGSH (nmol/g protein)	9.38±0.44**	9.45 (8.87–9.85)	3.82±0.13	3.83 (3.67–4)	8.19±0.07**	8.2 (8.11–8.31)
TNF-α (Pg/ml)	3.22±0.09**	3.19 (3.16–3.41)	8.41±0.14	8.37 (8.3–8.66)	3.86±0.32**	3.76 (3.55–4.3)
IL-1β (Pg/ml)	1.83±0.09**	1.82 (1.7–1.97)	4.78±0.14	4.77 (4.62–4.97)	2.17±0.05**	2.19 (2.11–2.22)

**P<0.001 compared to EVL. HG: Healthy group, EVR: Everolimus group, HREL: Everolimus+Hippophae rhamnoides extract group, MDA: Malondialdehyde, tGSH: Total glutathione, TNF-α: Tumor necrosis factor-α, IL-1β: Interleukin-1β.

Table 2. Biochemical results of buccal mucosa tissue

???	HG (n=6)		EVR (n=6)		HREL (n=6)	
	Mean±SD	**Median (Min-Max)**	**Mean±SD**	**Median (Min-Max)**	**Mean±SD**	**Median (Min-Max)**
MDA (µmol/g protein)	1.49±0.32**	1.5 (1.12–1.88)	4.34±0.4	4.15 (4.05–5.1)	2.11±0.42**	1.93 (1.77–2.82)
tGSH (nmol/g protein)	5.4±0.51**	5.51 (4.66–5.95)	2.22±0.13	2.25 (2–2.35)	4.86±0.14**	4.89 (4.66–5)
TNF-α (Pg/ml)	1.46±0.46**	1.2 (1.12–2.1)	4.14±0.1	4.1 (4–4.31)	1.75±0.3**	1.89 (1.31–2)
IL-1β (Pg/ml)	1.84±0.11**	1.87 (1.69–1.98)	5.39±0.38	4.9 (4.78–5.79)	2.09±0.19**	2.05 (1.83–2.31)

**P<0.001 compared to EVL. HG: Healthy group, EVR: Everolimus group, HREL: Everolimus+Hippophae rhamnoides extract group, MDA: Malondialdehyde, tGSH: Total glutathione, TNF-α: Tumor necrosis factor-α, IL-1β: Interleukin-1β.

Table 3. Body weight changes of the rats during the study

Groups	**Body weight (g) – initial**	**Body weight (g) – end of experiment**	**p**
HG	268.7±2.8	275±1.9	<0.001
EVR	270.5±1.5	264±3.2	<0.001
HREL	269	272.5±4.5	0.161

HG: Healthy group, EVR: Everolimus group, HREL: Everolimus+Hippophae rhamnoides extract group.

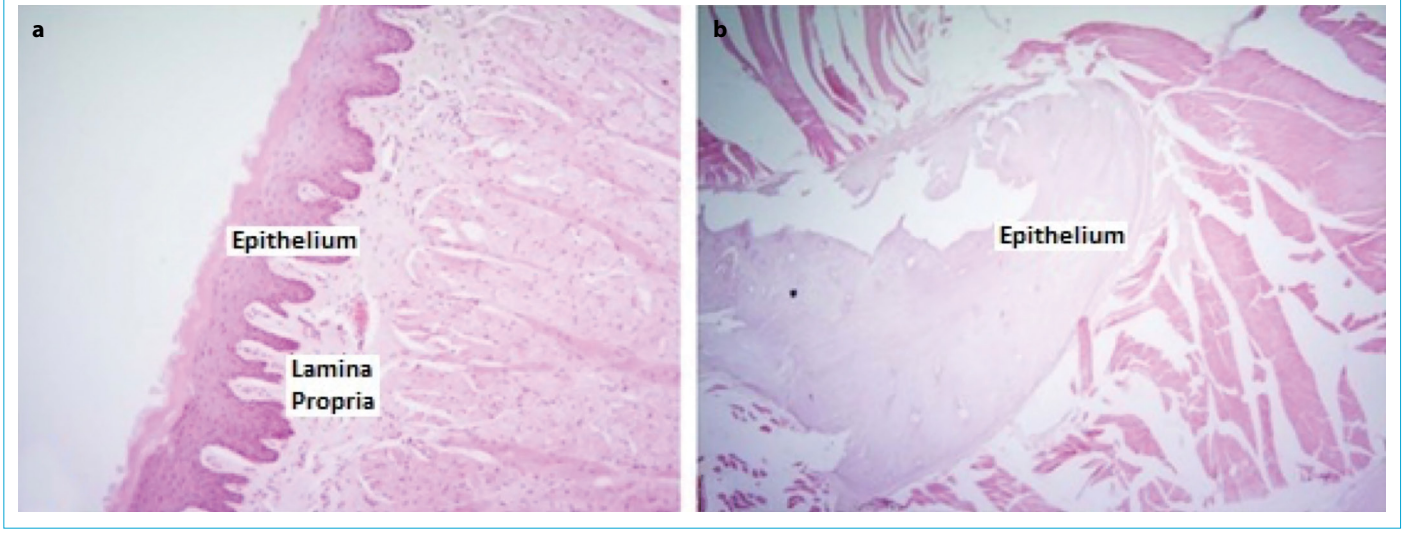

Figure 5. Normal histological appearance of (a) tongue and (b) buccal mucosa tissue in healthy group.

Table 4. Histopathological results. Significant statistical difference was observed between all three study groups (*,**,***p<0.05)

Group	Epithelization	Keratinization	Mononuclear cell infiltration
HG	2.83±0.40*	2.83±0.40*	0.00±0.00*
EVR	1.33±0.51**	1.33±0.51**	2.66±0.51**
HREL	2.66±0.51***	2.66±0.51***	1.16±0.40***

HG: Healthy group, EVR: Everolimus group, HREL: Everolimus+Hippophae rhamnoides extract group.

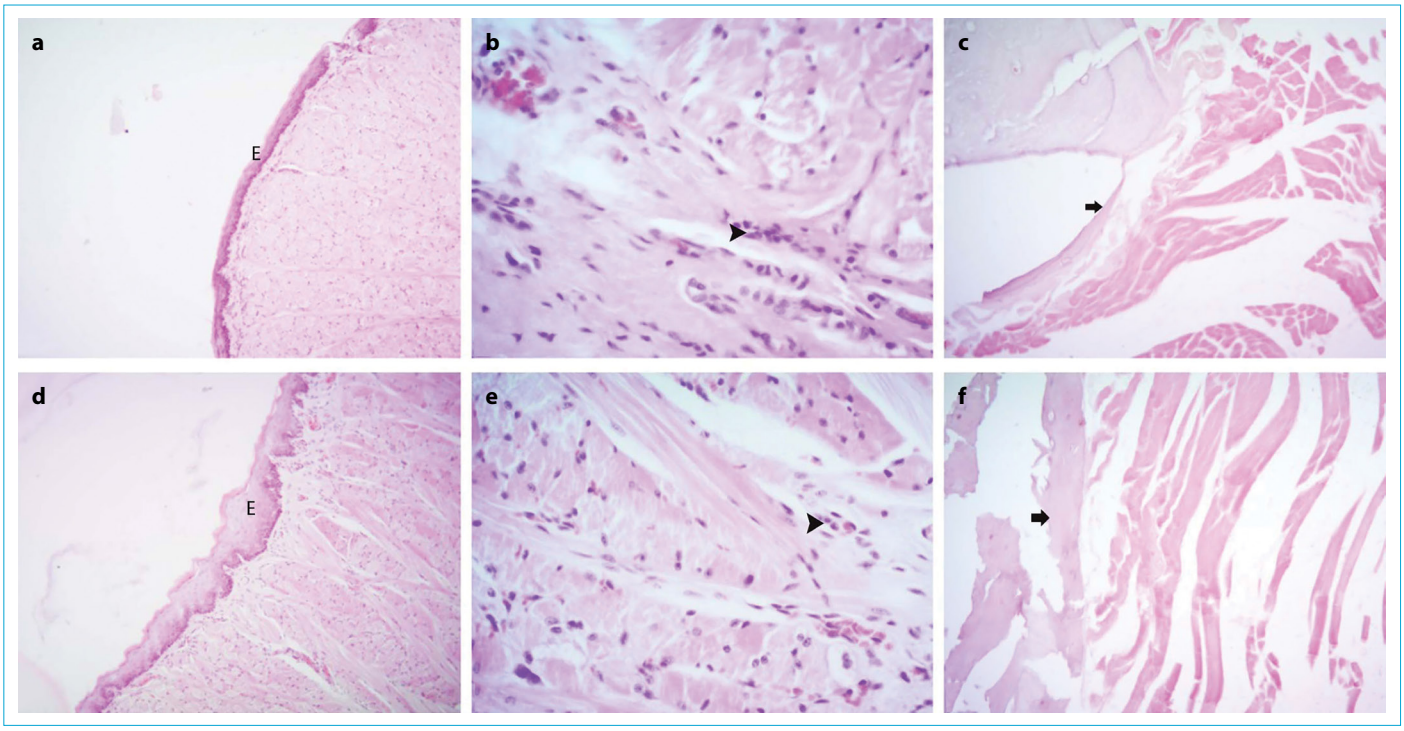

Figure 6. (a and b) Mild epithelization (e) severe mononuclear cell infiltration (arrowhead) in tongue tissue (EVR group). C. Mild keratinization (arrow) in buccal mucosa tissue (EVR group). (d and e). Severe epithelization (e) and mild mononuclear cell infiltration (arrowhead) in tongue tissue (HREL group). (f) Severe keratinization (arrow) in buccal mucosa tissue (HREL group).

buccal mucosa tissues of rats from the HREL group (Fig. 6). Statistically significant difference was found among groups in terms of all histopathological features (p<0.05) (Table 4).

Discussion

Mucositis is an inflammatory process that is a common AE during cytotoxic chemotherapy and radiotherapy, but it can also be caused by targeted agents and, in particular, mTOR inhibitors. Our study revealed that everolimus-induced oxidative stress caused an increase in the expression of pro-inflammatory cytokines and led to histopathological decomposition in the tongue and buccal mucosa tissues of rats. We also demonstrated that these effects were antagonized by HRE, dosed as two drops 3 times a day. To the best of our knowledge, this is the first study showing everolimus-related oxidative stress and the protective effect of HRE in rats. In the literature, the pathogenesis of chemotherapy or radiotherapy-induced oral mucositis is well described.[16] From the perspective of chemotherapy, this involves changes in levels of oxidant and antioxidant products MDA and tGSH in mucositis related to agents such as 5-fluorouracil and irinotecan.[17,18] In contrast, mTOR inhibitors were associated with a rare form of oral mucosal injury, which is becoming frequent AEs related to anticancer therapies.[6,19] In an experimental study, Kezic et al. demonstrated that everolimus might decrease cytoprotective capacity in kidneys and causing ischemic reperfusion injury due to the promotion of oxidative stress.[20] Biochemical results of our study supported this view, indicating that everolimus increased levels of MDA, which is an oxidant product, and decreased the levels of tGSH known as an antioxidant product, in the tongue and buccal mucosa tissues of rats. On the other hand, HRE was shown to reduce MDA levels and augment tGSH. Thus, HRE may be a therapeutic tool for everolimus-induced oral mucositis, which un-

derstandably impairs quality of life in treated patients and for which interventions have only been palliative so far.

Inflammation can contribute to the mucosal damage caused by anticancer therapies. This occurs mainly through stimulation of pro-inflammatory cytokines TNF-α and IL-1β, which was demonstrated with irinotecan and everolimus in animal models.[20,21] Our results were in line with this observation as everolimus caused an increase in expression of TNF-α and IL-1β genes in the tongue and buccal mucosa tissues of rats. These were ameliorated with HRE, which implies that it can exert anti-inflammatory effects by decreasing pro-inflammatory cytokines and therefore carries the therapeutic potential for everolimus-related stomatitis.

Histopathological alterations can be seen in chemotherapy- and radiation-induced mucositis, mainly in the form of epithelial thickness change and lymphocyte infiltration.[22] Mucositis in the oral cavity generally damages non-keratinized mucosa (i.e., cheeks and the floor of mouth), but it can also affect keratinized mucosa (loss of filiform papillae in dorsal tongue).[23] In our study, remarkable histological changes associated with everolimus were impairment of epithelization and keratinization along with severe mononuclear cell infiltration; all of them were significantly reversed by the addition of HRE.

Conclusion

We found that HRE protects buccal and tongue tissue against mucosal damage related to everolimus, principally reducing oxidative stress and inflammation. HRE can be a potential treatment for everolimus-induced mucositis, especially considering that there is no effective intervention established yet. Additional studies may be useful in understanding the generalizability of our results.

Disclosures

Ethics Committee Approval: Our institution's local animal ethics committee approved our study (Ethics Committee No: 2020/47, dated April 16, 2020).

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors have no conflicts of interest.

Authorship Contributions: Concept – B.A., M.O., N.C.D., F.O., H.S.S., A.G.; Design – B.A., M.O., I.C., B.S., H.S.; Supervision – B.A., M.O., B.S, H.S.; Materials – B.A., M.O.; Data collection &/or processing – B.A., M.O., N.C.D.; Analysis and/or interpretation – B.A., M.O., F.O.; Literature search – B.A., M.O., Writing – B.A., M.O., N.C.D., F.O., H.S.S., A.G.; Critical review – B.A., M.O., B.S, H.S.

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