Effects of Toothpaste on the Gingival Barrier Function *in vitro*

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Abstract

Introduction: Toothpastes (TPs) routinely contain detergents for their emulsifying and foaming properties. The most common detergent sodium lauryl sulfate (SLS) displays irritant effects to skin. Studies suggested that it may also affect the structural integrity of the oral mucosa. Besides detergents protective substances may also be ingredients of toothpastes. The aim of this study was to investigate the effect of different toothpastes on the gingival epithelial barrier function. Materials and methods: Immortalised human gingival keratinocytes (IHGK) were seeded on ThinCert™ cell culture inserts. Slurries from 4 different TPs were applied apically to the cells (1:100 and 1:1000 diluted). One TP contained additionally triclosan, one herbal extracts and one zinc citrate. The Transepithelial Electrical Resistance (TER) was measured hourly (h) until 8h and after 24, 48 and 72h. The cytotoxicity of the slurries was investigated by Lactate Dehydrogenase (LDH) release. Results: All TP slurries in 1:100 dilution caused a decrease of the TER until 8h (8-13 Ohm x cm^2) ($p < 0.05$). The most distinct decrease was observed using the TP without additional components. The 1:1000 dilution of the TP induced a TER increase (5-13 Ohm x cm^2) after 48 and 72h ($p < 0.05$). The most distinct increase was induced by a TP containing zinc. No significant cytotoxic effect caused by TPs was observed. Conclusions: The results of this study showed that TP slurries dose-dependently modulated the gingival barrier function without increased cytotoxicity. High dilutions showed TER enhancing, lower dilutions TER impairing properties. The decreasing effect was reduced by active ingredients like zinc.

Key Words: Periodontal Disease, Toothpaste, Oral Health

Introduction

Toothpastes (TPs) are formulated to supportoral care but there is potential to harm hard and soft tissues mainly from contained abrasives and detergents [1]. While there is strong evidence that normal use of toothpaste produces clinically insignificant effects on hard tissue, little is known about the effects on soft tissues [2]. Besides the cleaning bodies and fluoride, TPs routinely contain detergents for their foaming and provide moderate plaque inhibitory and antibacterial properties [3,4]. The most common detergent sodium lauryl sulfate (SLS) can induce irritant dermatitis as shown on human skin with an occlusion system, analysing the skin reaction by visual scoring [5,6]. As demonstrated in clinical studies, the irritant effects of surfactant could be of importance related to increased inflammation leading to exacerbation of periodontal disease, increased risk of infection and progression of gingival recession and recurrent oral ulceration [7,8]. It has been suggested that SLS could exacerbate conditions with compromised epithelial integrity [9]. In a regenerated oral mucosal in vitro 3D model that was in generated from primary human gingival keratinocytes and fibroblast it could be demonstrated, that SLS in low concentrations caused increased epithelial thickness, proliferation and E-cadherin expression whereas higher concentrations induced decrease of epithelial thickness, E-cadherin expression and proliferation. Furthermore in the central areas of the exposed regions cells detached from each other and underwent cell death [10]. Also in a tri-dimensional engineered human oral mucosa treatment with dentifrices led to desquamation of the cell layers and induced activation of the gelatinase matrix metallopeptidase-9 (MMP-9), up-regulation of IL1β and down-regulation of IL-8 and TNF-α. These results suggested impaired inflammatory response of oral mucosa via cytokine-regulation and affection of normal repair mechanisms via gelatinase activation caused by dentifrices [11].

Epithelial tissues provide a barrier between the body and the environment, where keratinocytes form the first line of defence against the bacterial challenge [12]. Gingival keratinocytes are connected by a variety of specialised transmembrane proteins. In gap junctions aggregations of six connexins form one connexon that joins with a similar connexon in adjacent cells across the intercellular space to establish intercellular pathways for the diffusion of ions and small molecules [13-15]. Adherens Junctions (AJ) are located basally and consist of cadherins, a further family of transmembrane proteins [15-17]. Tight junctions demarcate the border between apical and basolateral membrane domains [18] and function as a semipermeable barrier to the paracellular transport of water, ions and solutes [19-21]. They coordinate a number of signalling and trafficking molecules that regulate cell differentiation, proliferation and polarity [22,23].

To study the permeability of tight junctions in vitro, the transepithelial electrical resistance (TER) measurement has been widely used, and changes of TER values may directly be related to the function of the paracellular occluding barrier [24,25]. A close correlation between the number of junctional strands and junctional tightness, as judged by transmural resistance values was demonstrated [26]. TER measurements are commonly used to assess the integrity of tight junctions. The correlation between tight junctions and TER was also shown in primary human gingival keratinocytes [27]. TER depends on the intracellular Ca^{2+} concentration, i.e. increasing Ca^{2+} levels are followed by a decreasing NaCl diffusion potential [28].

The aim of this study was to analyze the influence of four commercial available toothpastes on the barrier function of

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gingival keratinocytes in an established in vitro model [29]. Furthermore possible cytotoxic effects of toothpastes on gingival keratinocytes should be investigated.

Materials and Methods

Cells

Immortalised human gingival keratinocytes (IHGK) from the cell line Gie-No3B11 (applied Biological Materials Inc, Richmond, Canada) were cultured like previously described [29]. Briefly the cells were cultured in a serum-free media containing DMEM:Ham's F12 (4:1) Hepes buffer and penicillin/streptomycin (Invitrogen, Karlruhe, Germany) as basal substances. The cells were seeded on ThinCert™ filter inserts in a 24-well plate (Greiner Bio-One, Frickenhausen, Germany) at 3.25x105 cells per insert. After 24 hours the cells are attached to the surface of the insert and polarized upwards. The culture model shows 2 different parts, where the culture medium and the bacteria containing solutions may be added: The apical part above the cells and the basolateral part at the bottom of the wells surrounding the inserts.

The differentiation was induced by adding culture medium containing 1.8 mM calcium and 10 % fetal calf serum (FCS) (Greiner Bio-One, Frickenhausen, Germany) for 24h. The process of differentiation includes the formation of tight junctions and is one prerequisite for the development of the TER. All samples were determined as duplicates.

Measurement of transepithelial electrical resistance (TER)

The transepithelial electrical resistance (TER) was detected daily with a Millicell-ERS-System (Millipore, Eschborn, Germany). Each insert was measured at 3 different sites and the mean values were calculated. Basic values were determined in cell-free inserts. Prior to the start of the experiment cellular confluency was controlled by light microscopy.

Preparation of tooth pastes

Four different tooth pastes were used, Colgate total (Colgate Palmolive, Hamburg, Germany) Rot Weiss (Dental Kosmetik GmbH, Dresden, Germany), Dontodent Kräuter (dm drug store, Karlsruhe, Germany) and Aronal (GABA, Lörrach, Germany). Colgate contains as additional active component triclosan, Dontodent herbal extracts and Aronal zinc citrate. The tooth pastes were provided blinded for the person who did the experiments. The unblinding was performed after the results were analysed- Tooth paste slurries were prepared as follows: 5 g of tooth paste was thoroughly mixed with 5 ml of ddH2O. This mixture was centrifuged with 6500 g at room temperature for 30 min [30].

The supernatants were harvested and diluted (1:100 and 1:1000) in culture medium. The dilutions were used in the test and applied apically (api). The TER was measured before (t = 0h) and after application at t = 1h, 2h, 3h, 4h, 5h, 6h, 8h, 24h, 48h and 72h with the Millicell System (Millipore, Eschborn, Germany) as described above. All experiments were performed at least 3 times.

Measurement of relative cell viability

The Lactate Dehydrogenase (LDH) colorimetric assay system Cytotoxicity Detection KitPlus (Roche-Diagnostics, Mannheim, Germany) was used for spectrophotometric quantification of cytotoxicity. Detection of cytotoxicity was performed according to the manufacturer´s instructions. Briefly, 0.5 x 10^4 cells/well of a microtiter plate were seeded 48h before running the assay. After 8h, 24h, 48h and 72h Culture supernatant was collected and cells were removed from it. The cell-free supernatant was incubated with the substrate mixture from the kit. LDH activity was determined using a coupled enzymatic reaction reducing, the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium salt (INT) to formazan. During the assay, LDH enzyme activity in the culture supernatant increases as the number of dead cells (or cells with damaged plasma membranes) increases. The increase in supernatant LDH activity directly correlates to the amount of formazan formed over time. The formazan dye was detected at 490 nm. Spectrophotometric analysis was performed using a Mithras LB 940 (Berthold Technologies, Gütersloh, Germany). Relative cytotoxicity was shown as comparison of cells in culture medium as negative controls with TP treated cells and relative to a positive control in that cells were lysed by lysis solution from the kit.

Statistical analysis

The changes of the TER were tested by multi-way ANOVA fur multiple testing. Normal distribution could be assumed. The character of the evaluation was explorative and the results of significance test are shown as adjusted p-values.

Results

TER development after incubation with tooth paste slurries

The TER started in all samples with a similar TER between 155 and 157 Ohm x cm^2. After 4h and 6h the control TER increased to 158 Ohm x cm^2 while the samples with the 1:100 diluted tooth paste slurries showed a significant lesser TER around 150 Ohm x cm^2 using Colgate, Dontodent and Aronal ($p < 0.05$). Rot Weiss even caused a decrease to 145 Ohm x cm^2 ($p< 0.01$). After 24h – 72h the effect of Rot Weiss 1:100 was still visible while the TER values using the other tooth pastes induced TER values slightly over the control TER. The 1:1000 diluted slurries showed no significant effect until 8h, then the TER in all TP samples values began to increase to values over 160 Ohm x cm^2 after 24h until 72h, while the control was slightly decreased to 153 Ohm x cm^2 ($p < 0.05$). *Figure 1* shows a representative kinetics of the TER development over the time of the experiment for every tooth paste. In *Figure 2* the mean of the TER (n = 12) for 0h = before treatment with TP slurries, and 4h, 6h, 24h, 48h and 72h after treatment, is shown for every tooth paste in the dilutions 1:100 and 1:1000. The statistical analysis of the two dilutions of every tooth paste in comparison to the non-treated control *(Figure 2)* demonstrated, that the TER decreasing effect of the 1:100 dilution was statistically significant for all tooth pastes after 4h and 6h and the increasing effect of the 1:1000 dilution was significant after 72h using Colgate

(*Figure 2a*), Rot Weiss (*Figure 2b*) and Dontodent (*Figure 2c*) and after 48h and 72h using Aronal (*Figure 2d*) ($p < 0.05$).

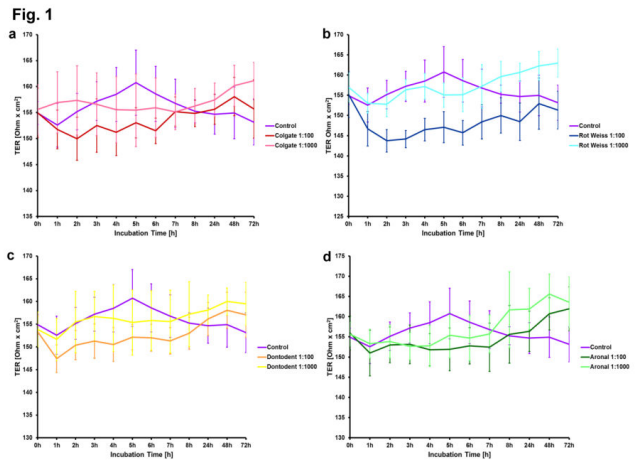

Figure 1. *Kinetics of the TER of the immortalized human gingival keratinocyte (IHGK) cell line Gie-No3B11non-treated and treated with toothpaste (TP) slurries 1:100 and 1:100 diluted (1a: TP Colgate, 1b: TP Rot Weiss, 1c: TP Dontodent, 1d: TP Aronal).*

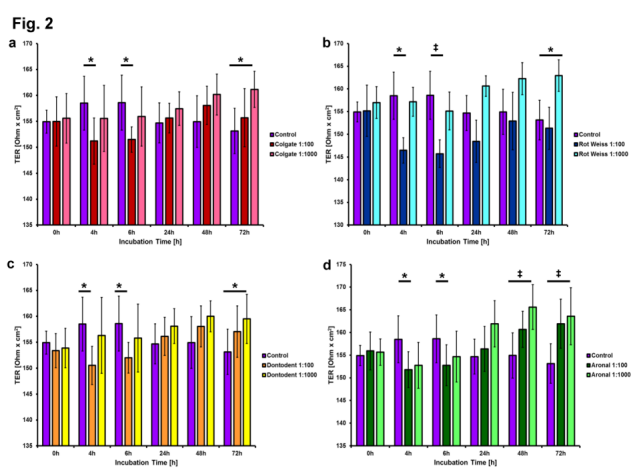

Figure 2. *Mean TER values of the IHGK cell line Gie-No3B11 treated with TP slurries 1:100 and 1:100 diluted before treatment (0h) and after 4h, 6h, 24h, 48 and 72h compared to the non-treated control. (1a: TP Colgate, 1b: TP Rot Weiss, 1c: TP Dontodent, 1d: TP Aronal, * = $p < 0.05$, ‡ = $p < 0.01$.)*

Comparison in between the toothpastes (*Figure 3*) showed that Rot Weiss (1:100 diluted each) caused the most distinct TER decrease after 6h compared to the 3 other TPs ($p < 0.01$). This effect was still detectable after 48h (Rot Weiss to Aronal $p < 0.01$) and 72h (Rot Weiss to Aronal $p < 0.01$, Rot Weiss to Colgate and Dontodent $p < 0.05$).

The TER increasing effect of Aronal (1:1000 diluted each) was the most distinct and significantly higher in comparison to Dontodent and Colgate after 48h ($p < 0.01$) and to Dontodent after 72h ($p < 0.05$).

Figure 3 demonstrates the mean of the TER values ($n = 12$) of the samples of every tooth paste 1:100 and 1:1000 diluted, in comparison with each other after 0h, 24h, 48h and 72h.

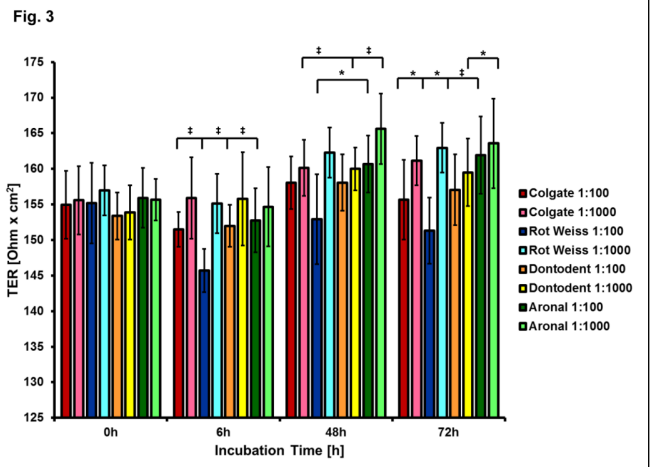

Figure 3. *In between comparison of mean TER values of the IHGK cell line Gie-No3B11 treated with the different TP slurries 1:100 and 1:100 diluted before treatment (0h) and after 6h, 48h and 72h, * = $p < 0.05$, ‡ = $p < 0.01$.*

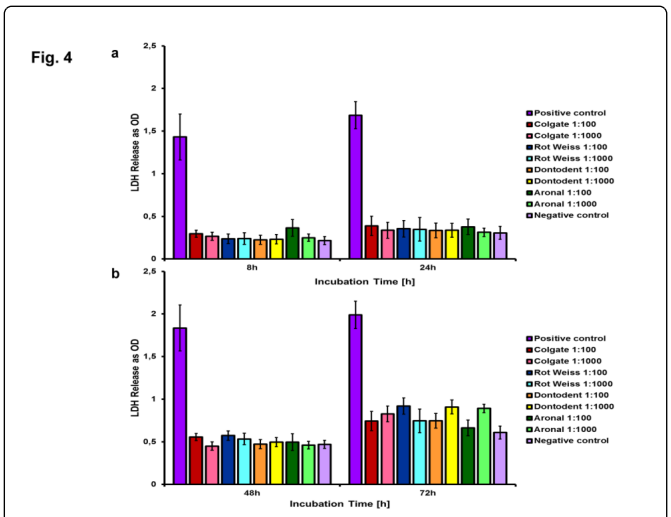

Figure 4. *Cytotoxicity of TP slurries 1:100 and 1:1000 diluted, investigated by LDH release shown as optical density (OD) of the IHGK cell line Gie-No3B11, Figure 1a LDH release after 6 and 24h, Figure 4b LDH release after 48 and 72h.*

Cytotoxicity of the tooth paste slurries

The cytotoxicity quantified over the LDH release showed no significant cytotoxic effect of any of the tooth pastes, neither after 8h and 24h nor after 48 and 72h. The measured LDH release was only slightly above the corresponding negative control. In *Figure 4* the mean values of three experiments with the samples compared to negative and positive control are shown, in *Figure 4a* 8h and 24h are depicted, in *Figure 4b* 48h and 72h ($n = 12$).

Discussion

A commonly used component of dentifrices is the anionic detergent sodium lauryl sulfate (SLS) which emulsifies plaque deposits, solubilizes lipophilic substances in tooth pastes and mouth washes and confers foaming characteristics [31,32]. In Europe and North America the used concentrations for SLS in

toothpaste range from 0.5% to 2% [33]. Applying toothpastes with different SLS concentrations from 0.01 to 1.5% directly on mucosa of volunteering individuals by a cap splint it was shown, that 1.5% SDS caused desquamation of the epithelium in 60% of the subjects [34]. In patients that suffered from recurrent aphtous ulcers it was demonstrated that a significantly higher frequency of lesions occurred when the patients brushed with an SLS containing toothpaste [8]. In a subsequent study that investigated the effect of SDS on a reconstructed human three-dimensional cell culture model generated from primary normal human oral keratinocytes and fibroblasts it was found that epithelial thickness increased, proliferation was enhanced and a more distinct expression of E-cadherin throughout all epithelial cell layers were observed in cultures exposed to low concentrations (0.015%) of SLS. At exposure to higher SLS concentrations (≥ 0.15%), epithelial thickness, cell proliferation and E-cadherin expression gradually decreased and cells detached from each other or underwent cell death. These findings suggested dual effects of various concentrations of SLS on reconstituted human oral mucosa and lead to the conclusion that the increased epithelial thickness, proliferation and E-cadherin expression induced at lower concentrations might be associated with a protective mucosal response, whereas higher concentrations displayed a more destructive reaction [10]. In concordance with this study we could demonstrate this dual effect of lower and higher SLS concentrations on the barrier function of a human gingival in vitro model as well. While the higher concentrations of all used toothpaste slurries induced a significant decrease of the TER in the first 24h, the lower concentrations caused a TER increase beginning after 24h and lasting until the end of the experiments after 72h. An increase of the TER as marker for the strength of the epithelial barrier indicates a rather protective effect of a low concentration of SLS while higher concentrations impaired the epithelial barrier. These results are conclusive since 3 of 4 toothpastes that were used for the study contained a SDS as detergent. The forth toothpaste contained sodium C14-16 olefin-sulfonate, a tenside that is used mainly in shampoos and bath and shower products because of its cleaning and foaming properties. Using dermal irritation studies mild to moderate skin irritation in guinea pigs and rabbits after repeated exposure to >2% olefin-sulfonates were reported [35,36].

The barrier impairing effect was different in between the 4 toothpastes which may be explained by the different composition of the toothpastes that contained different ingredients which possibly modulated the TER decreasing impact. One toothpaste (Colgate total) contained triclosan. The antibacterial agent triclosan, which has been introduced to toothpastes and mouthrinses, is a compound that shows anti-inflammatory properties [33,37,38]. Triclosan has been supposed to alleviate the undesireable effects due to SLS exposure [32,33,39], an effect that at first was reported by Waaler et al., who observed that triclosan added to SLS-containing mouthrinses reduced the mucosal desquamation and pain caused by this detergent [32]. A subsequent study which showed that that triclosan in combination with 1% SLS inhibited the expected skin reaction to SLS supported those findings [33]. Triclosan in toothpastes has also been demonstrated to reduce the severe desquamations of the oral mucosa caused by SLS when it was tested in a cap splint model system [9]. The mechanism of the protective properties of triclosan against the damaging effects of SLS, of the oral mucosa is still not understood. Triclosan is contained in the hydrophobic interior of SLS micelles, possibly this alters the damaging effect of SLS. The reductive effect on oral desquamation was confirmed by a further study that used human ventral tongue mucosa that was exposed with SLS. SLS treatment caused a significant increase in water permeability compared to control tissue and histological examination revealed that tissue exposed to SLS had a marked disruption of the epithelial surface. Treatment with a SLS/ triclosan/zinc mixture had no effect on the permeability to water and the histological examination showed that tissue treated with a SLS/triclosan/zinc mixture was comparable to the controls [40].

Zinc salts are ingredients of oral health products to provide control of the plaque and calculus formation and to reduce malodor. Zinc salts show antibacterial properties because they are able to inhibit bacterial adhesion, metabolic activity and growth [41-43].

A study that determined the effect of oral zinc sulphate supplementation on radiation-induced oropharyngeal mucositis in patients with head-and-neck cancer, observed that the degree of mucositis in the patients in the zinc sulfate treatment group was significantly lower than that in the placebo group ($p < 0.05$), concluding that zinc sulfate is beneficial in decreasing the severity of radiation-induced mucositis and oral discomfort [44]. The results of our study in addition suggest a possible protective effect of zinc on the oral epithelial barrier. The toothpaste that contained zinc (Aronal) induced the most distinct TER increase in the 1:1000 dilution after 24 until 72h and even showed an elevated TER using the 1:100 dilution after 48h and 72h. Its increasing effect on the barrier after 48 and 72h differs significantly from the three other toothpastes. The toothpaste on the other hand, that contained no further possible protecting agent (Rot Weiss) showed the most distinct TER decreasing impact in the 1:100 dilution and the less TER increasing effect in the 1:1000 dilution.

The fourth toothpaste that was used in ours study contained as additional components a mixture of herbs consisting of chamomilla recutita flower extract, salvia triloba leaf extract, commiphora abyssinica resin extract, mentha arvensis herb oil. In a single blind study that investigated the effectiveness of medicinal herbs in both a toothpaste and oral rinse on dental plaque, sulcus bleeding, and the pH of total saliva it was found that compared with the placebo preparations, the herbal ingredients significantly reduced both the approximal plaque index and the sulcus bleeding index. The pH of the total saliva was significantly displaced into the alkaline range by the application of the herbal products, whereas the placebo products had a contrary effect. The results of this study suggested that herbal ingredients can be employed supportively in the therapy of periodontal diseases and for routine prophylaxis [45]. The effects of herbal components on the gingival barrier have not been investigated yet. However, the results of our study analyzing a toothpaste slurry containing a herbal extract mixture on the TER did not indicate that the herb mixture in the toothpaste provided distinguished protective effects on the gingival barrier. The

effects did not differ significantly from the toothpastes containing triclosan or SLS alone.

To investigate cell death after exposure to detergents, TERT-1 keratinocytes were exposed to varying concentrations of the detergents adinol, SDS, tego betain and pluronic and the cells viability was analyzed using an tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide test. It was found, that except for pluronic, cell viability was markedly reduced for all detergents at all increasing concentrations, what suggests, that some detergents may have the potential to cause soft tissue damage in the mouth [46].

In contrast to this study our results demonstrated no significant cytotoxic effect of the tooth paste slurries on the gingival epithelial cells. These divergent results probably are due to different experimental settings and SLS end-concentrations that were used.

Consistent to our study Mostefaoui et al. did not detect increased cells death in a tri-dimensional engineered human oral mucosa after treatment with dentifrices as determined by trypan blue exclusion. This group furthermore showed that dentifrices up-regulated IL-1β but down-regulated IL-8 and TNF-α secretion, thus indicating that, via cytokines, dentifrice contributes to the modulation of the inflammatory (pro-inflammatory/anti-inflammatory responses) process [11].

Conclusion

In conclusion the results of this study showed that SLS containing toothpaste slurries dose-dependently modulated the barrier function of a human gingival keratinocyte in vitro model without increased cytotoxicity. High dilutions showed TER enhancing properties while lower dilutions decreased the TER, an effect that was diminished by protective toothpaste ingredients like zinc citrate and triclosan.

Ethical Considerations

All experiments followed the guidelines of good clinical/ laboratory practice (GCP/GLP) and the WHO declaration from Helsinki 1964, latest update Seoul 2008 (59th WMA General Assembly, Seoul, October 2008).

Acknowledgements and Competing Interest

There are none to declare. The study did not receive financial support.

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