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In vitro and *in vivo* toxicity of grapefruit seed extract

Carolina Cardoso Guedes[1], Olga Maria Silverio Amancio[1], Sandra Kalil Bussadori[2], Elaine Marcílio Santos[3], Vanessa Christina Santos Pavesi[2], Manoela Domingues Martins[4]

[1] Department of Pediatrics, Federal University of Sao Paulo/Paulista School of Medicine, Sao Paulo, Brazil
[2] Department of Rehabilitation Sciences, Nove de Julho University, Sao Paulo, Brazil
[3] Department of Pediatric Dentistry, Camilo Castelo Branco University, Sao Paulo, Brazil
[4] Department of Pathology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

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Summary

Background: Grapefruit seed extract (GSE) has been used in clinical practice, but there are no studies in the literature that prove its effectiveness and safe use. The aim of the present study was to assess the *in vitro* and *in vivo* toxicity of a commercial grapefruit seed extract.

Material/Methods: The experimental groups were 0.1% GSE, 0.15% GSE, 0.2% GSE, 0.5% GSE, 0.12% chlorhexidine, and control (*in vitro* study) or sham (*in vivo* study). The *in vitro* analysis was performed on fibroblast cultures (NIH-3T3) at 1, 3, 5, and 7 days to evaluate cell survival. In the test performed on rats, polyethylene tubes containing the substances were implanted in connective tissue; histological analysis of tissue samples was performed at 1, 7, 15, 30, and 60 days.

Results: Both the GSE and chlorhexidine proved cytotoxic, with a greater concentration leading to greater toxicity for fibroblasts. In the connective tissue, GSE caused severe inflammation on the first day at all concentrations. Over time, the inflammation subsided until reaching values similar to the sham group.

Conclusions: At the concentrations studied, GSE exhibits toxicity for cells and connective tissue. Further studies are needed to assess the harm this product may cause to human tissue and determine a safe dosage for its use, as well as to determine if a lower concentration still has the desired antimicrobial potential attributed to GSE.

key words: **toxicity tests • fibroblasts • rats • connective tissue • *Citrus paradisi* Macf. • *Rutaceae***

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Author's address: Olga Maria Silverio Amancio, Department of Pediatrics, Universidade Federal de São Paulo, Rua Botucatu, 703, CEP 04023-062, Sao Paulo, Brazil, e-mail: omsamancio.dped@epm.br

Background

Grapefruit seed extract (GSE) is a product derived from the seeds and pulp of grapefruit (*Citrus paradisi* Macf., *Rutaceae*) and is widely sold as a natural remedy with antibacterial, antiviral, and antifungal properties [1–4]. GSE is sold freely in the electronic market and used for various purposes – as a natural preservative in cosmetics and syrups, as well as an antimicrobial mouthwash for the treatment of the flu, colds, throat infections, and oral diseases [5,6].

Manufacturers indicate GSE as an oral antimicrobial agent that can be diluted in water and used in the form of a daily mouthwash. This chemical action is thought to supplement mechanical oral cleaning procedures that seek to eliminate or limit dental plaque. This supplementation is based on the capability of antimicrobial agents to affect the metabolic activity or adherence of bacteria to dental biofilm.

Among the available commercial brands of GSE, the formulations are not fully natural and contain synthetic preservatives, including benzethonium chloride and triclosan [7–10]. Studies have reported that the exacerbated antimicrobial effect may not be the result of the natural GSE, but also due to the preservatives that are added to the product [10,11].

Few studies have assessed the safety of using this product and the most evaluate the antimicrobial power of the substance. Only 1 study [1] has assessed the *in vitro* toxicity of GSE and no studies have assessed the *in vivo* tissue response.

New products and those with as-yet unknown action should be analyzed for potential toxicity by *in vitro* studies of cell cultures and experiments on animals in order to be then clinically administered to humans. In dentistry, the investigation into the toxicity of materials is of extreme importance, as it reflects on the applicability and clinical follow-up of these materials. One of the requirements for a material to be used in the oral cavity is that it must not cause irritation to the conjunctive tissue or come into contact with vital tissues.

Because GSE has been used in clinical practice but there are no studies in the literature that prove its effectiveness and safety, the aim of the present study was to assess the *in vitro* and *in vivo* toxicity of a commercial grapefruit seed extract.

Material and Methods

Grapefruit seed extract

The commercial GSE used in the present study was the Pure Liquid Gold (Concrete, WA, USA) (batch number not specified by the manufacture), diluted in distilled water at concentrations of 0.1%, 0.15%, 0.2%, and 0.5%, based on the study by Heggers et al [1].

Animals

Fifty adult female Wistar rats (*Rattus norvegicus albinus*, Rodentia, Mammalia) approximately 90 days old and weighing 200±20 g were used. The animals were kept in appropriate plastic cages, with controlled room temperature (25°C) and 12-hour light cycle. The animals were fed before and during the experimental periods with a solid Nuvilab® ration (Nuvital Nutrientes S/A, Curitiba, Brazil) and water *ad libitum*. The study received approval from the Ethics Committee of the Federal University of Sao Paulo (# 1143/05).

Experimental groups

Six experimental groups were established:
- 0.1% GSE Group;
- 0.15% GSE Group;
- 0.2% GSE Group;
- 0.5% GSE Group;
- 0.12% chlorhexidine digluconate group. This group was formed to allow comparison of effects of GSE with those of chlorhexidine, which is the most widely used chemical agent in dentistry. The chlorhexidine used in the present study was manipulated by Fórmula & Ação Laboratories (São Paulo, Brasil), 98.27% purity, Origin: Indian (batch number SMAART/CHG/2009);
- Control Group (in the *in vitro* study);
- Sham Group (in the rat study).

In vitro study

The toxicity of GSE and chlorhexidine was assessed in a fibroblast culture using the method of the exclusion of cells stained by Trypan blue.

Cell culture

NIH-3T3 cells were used, which are fibroblasts originating from mouse embryos, a continuous cell line with high contact inhibition (ATCC CRL), which were cultured in Dulbecco's modified Eagle medium (DMEM-Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, São Paulo, Brazil) and 1% antibiotic-antimycotic solution (Sigma). The cells were kept in an incubator at 37°C and a humidified 5% CO_2 atmosphere. Monitoring of cell growth was performed every 24 h and the subculture was performed when the cell monolayer became subconfluent.

Long-term cell survival experiment

1×10^4 cells per plaque were plated on to 60 mm diameter culture plaques. After 4 h of plating, glass slides (15 mm in diameter) containing 1 drop of the substances to be tested were placed in contact with the cell culture. The control group received glass slides with no substance. Every 2 days, 50% of the culture media in the Petri plaques was removed. After 1, 3, 5, and 7 days of contact with the substances, the cells from 3 Petri plaques for each experimental period were trypsinized and counted in a Neubauer chamber using the trypan blue exclusion assay [12].

Rat study

The evaluation of tissue toxicity was performed using histological analysis of the inflammatory reaction in the subcutaneous conjunctive tissue of the rats associated to the implantation of polyethylene tubes containing the substances investigated. Following inhalation of Forane® – isoflurane (Laboratório Abbott – São Paulo, Brazil), the rats were anesthetized by the administration of 1 mL/kg of 1% ketamine

Table 1. Mean count (±standard error) of viable cells ($\times 10^4$) among the groups at the evaluations times studied.

Times	Groups						p-value
	Control (n=3)	0.1% GSE (n=3)	0.15% GSE (n=3)	0.2% GSE (n=3)	0.5% GSE (n=3)	0.12% Chlorhexidine (n=3)	
Day 1	9.9±1.1[a,b]	12.9±0.9[a]	6.4±1.3[b,c]	3.3±0.8[c,d]	0.1±0.1[d]	0.3±1.3[d]	<0.001
Day 3	133.3±28.8[a]	5.2±0.4[b]	7.2±0.5[b]	2.9±0.9[b]	0±0[b]	0±0[b]	<0.001
Day 5	202.7±10.7[a]	1.7±0.2[b]	0±0[b]	0±0[b]	0±0[b]	0±0[b]	<0.001
Day 7	260.7±8.8[a]	1.3±0.6[b]	0±0[b]	0±0[b]	0±0[b]	0±0[b]	<0.001

Different superscript letters denote statistically significant difference ($p<0.05$).

HCl (Dopalen, Vetbrands, São Paulo, Brazil) and 2% xylazine (Anasedan, Vetbrands, São Paulo, Brazil). Trichotomy was performed on the dorsal region, followed by disinfection of the area with 70% alcohol (95%) and iodine (5%). Five-mm incisions were made on the back of the animal and 3 surgical sites in the subcutaneous conjunctive tissue were prepared with surgical scissors. Previously prepared and autoclaved polyethylene tubes (Sondplast®, Sao Paulo, Brazil), 10 mm in length and 1.3 mm in diameter, were implanted in the subcutaneous tissue of each animal. The incisions were sutured with silk thread. Each animal received 3 implants, each with a different substance. The study was carried out in triplicate.

At the end of each period (1, 7, 15, 30, and 60 days), rats were killed with an overdose of anesthesia and the tubes were removed by excisional biopsy. The tissue was fixed in buffered 10% formol. After 48 h of fixation, the surgical pieces were processed in a laboratory and embedded in paraffin. The blocks were sliced on a microtome adjusted to 5 µm. Hematoxylin-eosin staining was used.

Histological evaluation

The evaluation of the tissue response was graded as mild, moderate, or severe, based on the criteria suggested by Olsson et al [13] and Orstavik & Mjör [14]:

- Grade 1 (no/slight inflammation): reaction area similar or slightly thicker than that near the wall of the tube; few or no inflammatory cells;
- Grade 2 (moderate inflammation): increased reaction zone in which macrophages, plasma cells, or both are present;
- Grade 3 (severe inflammation): increased reaction zone in which macrophages and plasma cells and occasional foci of neutrophil granulocytes, lymphocytes, or both are present.

The reading of the slides was carried out blindly (without identification) by a pathologist.

Statistical analysis

The data were stored in a databank and analyzed using the Statistical Package for the Social Sciences for Macintosh, version 16.0 (SPSS Inc, Chicago, IL, USA). *In vitro* study: mean counts of surviving cells were compared between groups using analysis of variance of 1 criterion, complemented by Tukey's test for multiple comparisons. *In vivo* study: the mean degrees of inflammation were compared between groups using the non-parametric Kruskal-Wallis test. The level of significance was set at $\alpha<0.05$.

Results

The mean cell counts from each group and their respective standard errors are displayed in Table 1. The test groups exhibited a smaller number of viable cells in comparison to the control group in most of the experimental periods. On Day 1, only the 0.1% and 0.15% GSE groups did not exhibit significant differences in mean cell counts in relation to the control group.

Beginning at Day 3, there were statistically significant differences in the experimental groups in relation to the control group, whereas the counts between experimental groups did not differ statistically from one another. Analyzing the groups qualitatively, however, the 0.1% GSE Group still had viable cells at the Day 7 evaluation, whereas there were no viable cells in the Day 5 analysis for the 0.15% and 0.2% concentrations. At the 0.5% GSE and 0.12% chlorhexidine concentrations, this absence in cell viability occurred beginning at Day 3.

The histological analysis, represented by median values (and respective inter-quartile interval) of the degree of inflammation at the different times analyzed are displayed in Table 2. On the first day, the sham group exhibited a moderate inflammatory process, with the presence of neutrophils and intense hyperemia/vascular congestion. The test groups exhibited a severe inflammatory reaction, with the presence of intense hyperemia/vascular congestion. The statistical analysis of the median values at the Day 1 evaluation revealed that the 0.15%, 0.2%, and 0.5% GSE groups, and the 0.12% chlorhexidine group had higher inflammation values, which were statistically different from that of the sham group.

At the Day 7 evaluation, the sham group and chlorhexidine group exhibited a mild inflammatory reaction, characterized by the presence of few inflammatory cells in the area around the extremity of the tube. The GSE groups at 7 days had higher scores and exhibited a moderate inflammatory process, with infiltration of lymphocytes and neutrophils. The analysis of the median values revealed no statistically significant differences between the groups. At the 14, 30,

Table 2. Median values (and respective inter-quartile interval) of the degree of inflammation among the groups at the times studied.

Times	Groups						p-value
	Sham (n=5)	0.1% GSE (n=5)	0.15% GSE (n=5)	0.2% GSE (n=5)	0.5% GSE (n=5)	0.12% Chlorhexidine (n=5)	
Day 1	2 (2–2)[a]	3 (2–3)[a,b]	3 (2.5–3)[b]	3 (3–3)[b]	3 (2.5–3)[b]	3 (3–3)[b]	0.007
Day 7	1 (1–2)	2 (1–2)	2 (1.5–2)	2 (1.5–2)	2 (1.5–2)	1 (1–2)	0.55
Day 14	1 (1–1.5)	1 (1–1)	1 (1–1)	1 (1–2)	1 (1–2)	1 (1–1)	0.24
Day 30	1 (1–1)	1 (1–1.8)*	1 (1–1)*	1 (1–1)*	1 (1–1.5)	1 (1–1.5)	0.69
Day 60	1 (1–1)*	1 (1–1)*	1 (1–1)*	1 (1–1)*	1 (1–1)*	1 (1–1.8)*	0.42

Different superscript letters denote statistically significant difference ($p<0.05$); * n=4 due to the death of one rat.

and 60 day evaluations, all groups exhibited a similar tissue response, characterized by no or mild lymphocyte inflammatory infiltrates and an intense repair process (fibrosis) around the extremity of the tube. The median scores revealed no statistically significant differences between any of the groups at these evaluation times.

Discussion

Toxicity tests are fundamental for biological evaluation, safety of use, and acceptance of new materials that have a therapeutic and rehabilitation purpose in healthcare. For a product to be used with a therapeutic purpose, an *in vitro* toxicity test is the first test to be carried out to assess the safety of its use [15,16]. In the literature, there is only 1 previous study that assessed the *in vitro* toxicity of GSE. Heggers et al. [1] assessed the Citricidal® GSE (BioChem Research, Lakeport, CA, USA) in a culture of fibroblasts from human skin, and concluded that the product is toxic within the dilution range of 1:1 to 1:128. However, at a dilution of 1:512, the product allowed the maintenance of cell viability after 3 days while also maintaining its antimicrobial effect. In the present study, which assessed another commercial brand of GSE [Pure Liquid Gold (Concrete, WA, USA)], only the 0.1% dilution was not cytotoxic on the first day and, at 3, 5, and 7 days, all groups caused cell death and a significant reduction in the population of viable cells. The cytotoxic effect of GSE was dose-dependent, with a greater concentration causing greater toxicity. As the composition of commercial GSE is not defined and the extraction methods are not specified by the manufacturers, these extracts may contain additives [8]. The GSE tested in the present study claims to be 100% pure, but only a chemical composition test could ensure that the product is truly natural and thereby rule out the possibility that some other additive is responsible for the toxicity of the product.

The results of the cytotoxicity of chlorhexidine demonstrate that it is as aggressive toward the cells as GSE. A number of authors have stated that chlorhexidine may negatively affect the healing of oral wounds and is highly toxic to different types of cells, causing damage to gingival fibroblasts, endothelial cells, and alveolar osteoblasts [17,18]. The dose-dependent effect observed for GSE is also characteristic of chlorhexidine [19], found in Listerine® and povidone-iodine [17], which are well-known mouthwashes.

Some products used in dentistry, such as calcium hydroxide, have proven cytotoxic in culture models, but have an excellent therapeutic, anti-inflammatory effect on tissues [20–22]. According to Saw et al. [23], to ensure the innocuousness of a product, it is necessary to test different cell culture models and perform *in vivo* studies, which better approximate clinical situations.

A second test for the assessment of toxicity is the implantation of the material in the conjunctive tissue of animals. In the literature, no previous study was found that assessed the *in vivo* toxicity of GSE, which is why the decision was made to use rats in the experiment described in the present study. On the first day, the results revealed an acute, severe inflammatory reaction in all the test groups and a moderate reaction in the sham group. This situation was expected, as an acute inflammatory response is the first line of defense of vascularized conjunctive tissue in the face of aggression. Moreover, there was the local damage inherent to the operation, followed by the implantation of the tubes and initial contact with the product. GSE and chlorhexidine caused more intense acute, suppurative inflammation than that found in the sham group, thereby demonstrating the greater injurious capacity associated with intense tissue response. In this respect, there was no difference in tissue response between the different concentrations of GSE, which demonstrates that either the extract itself or some additive in the product is likely aggressive to conjunctive tissue.

At Day 7 all groups exhibited a reduction in inflammatory response. Histologically, the sham and chlorhexidine groups exhibited a less intense tissue reaction than the GSE groups. Chronification of the inflammatory process and a tendency toward tissue repair occurred in the sham and chlorhexidine groups, whereas neutrophil infiltrates were present in the GSE groups, demonstrating the perpetuation of the acute condition. These findings are corroborated by the basic concepts of the inflammatory process, which portray the acute inflammatory response as non-specific, rapid, and lasting minutes, hours or even a few days, whereas chronic inflammatory response is characterized by a long duration, has a proliferative nature, and establishes a close relationship with the repair [24]. With the passage of time, the inflammation became milder. At 14, 30, and 60 days, all groups tested were similar to the sham group, with chronification of the inflammatory process and tissue repair.

The criteria established by the Féderation Dentaire Internationale [25] for the analysis of tissue compatibility indicate that no or mild reaction between 2 and 12 weeks, or a moderate reaction for 2 weeks that diminishes by 12 weeks, are acceptable. A mild reaction that evolves into a moderate to severe reaction by 12 weeks, a moderate reaction between 2 and 12 weeks, or a severe reaction at any time studied, is not acceptable. GSE at the concentrations studied caused a histologically severe reaction in the first week and is therefore not acceptable as a material compatible with oral tissues. Statistically, there were no differences between the groups studied on the Day 7 evaluation. At 14, 20, and 60 days, GSE was found acceptable based on the FDI criteria.

Conclusions

The results demonstrate that the grapefruit seed extract in the Pure Liquid Gold commercial brand at the different concentrations tested is cytotoxic to fibroblasts (*in vitro* test) and causes an intense inflammatory response in the first few days after contact with conjunctive tissue (*in vivo* test). Thus, there is a need for further studies to assess the harm this product can cause to human tissues, to determine the safe dosage for its use, and to discover if GSE has the antimicrobial potential that is attributed to the product at a low concentration.

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Declaration of interest

The authors declare that there were no conflicting financial interests.

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