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Cytotoxicity of 1,4 butanediol diglycidyl ether associated or not with hyaluronic acid in human fibroblasts

Citotoxicidade de 1,4 butanodiol diglicidil éter associado ou não ao ácido hialurônico em fibroblastos humanos

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Abstract
Introduction: *the half-life of hyaluronic acid in the skin is only a few days, so it is stabilised by cross-linking with 1,4-butanediol diglycidyl ether (BDDE). However, BDDE may be toxic and cause hypersensitivity reactions.* ***Aim:*** *this study evaluated the cytotoxicity of different concentrations of BDDE, with or without hyaluronic acid, on human fibroblasts.* ***Methods:*** *human gingival fibroblasts were isolated and added at 1x10⁴ cells per well in 96-well plates. For cytotoxicity analysis, BDDE was tested at concentrations of 1, 2, 20, 70, and 100 ppm relative to the hyaluronic acid hydrogel in the culture medium. Another set was composed of the same amounts of BDDE dissolved only in the culture medium, resulting in concentrations of 0.2, 0.4, 4, 14, and 20 ppm. Control groups included cells in culture media only (positive control), cells in hyaluronic acid without BDDE, and cells with 20% methanol (negative control). Cytotoxicity was measured using the MTT assay at 24 hours and 7 days (n = 3, in duplicate). Cell viability was calculated relative to the positive control group (cells in culture medium only). Data were analysed using one-way ANOVA and the Tukey test (α = 0.05).* ***Results:*** *All groups were not cytotoxic after 24 hours (p > 0.05). However, at 7 days, 20 ppm BDDE without hyaluronic acid (p<0.05) and 70 and 100 ppm BDDE with hyaluronic acid were cytotoxic (p<0.01).* ***Conclusion****: the BDDE cross-linker showed late cytotoxicity (7 days) to gingival fibroblasts at high concentrations.*
Keywords: *Hyaluronic acid; cell viability; aesthetics.*

Resumo
Introdução: *a meia-vida do ácido hialurônico na pele é de alguns dias, sendo estabilizado pela reticulação com 1,4 butanodiol diglicidil éter (BDDE). Contudo, o BDDE pode ser tóxico e causar reações de hipersensibilidade.* **Objetivo:** *avaliar a citotoxicidade de diferentes concentrações de BDDE, com ou sem ácido hialurônico, em fibroblastos humanos.* **Metodologia:** *fibroblastos gengivais humanos foram isolados e adicionados a 1x10⁴ células por poço em placas de 96 poços. Para análise de citotoxicidade, o BDDE foi testado nas concentrações de 1, 2, 20, 70 e 100 ppm em relação ao ácido hialurônico em meio de cultura. Outro grupo foi composto pelas mesmas quantidades de BDDE dissolvido apenas em meio de cultura, resultando em concentrações de 0,2, 0,4, 4, 14 e 20 ppm. Os grupos controle incluíram células apenas em meio de cultura (controle positivo), células em ácido hialurônico sem BDDE e células com 20% de metanol (controle negativo). A citotoxicidade foi medida utilizando o ensaio MTT em 24 horas e 7 dias (n = 3, em duplicata). A viabilidade celular foi calculada em relação ao grupo controle positivo (apenas células em meio de cultura). Os dados foram analisados por meio de ANOVA de fator único e Teste de Tukey(α = 0,05).* **Resultados:** *todos os grupos não se mostraram citotóxicos após 24 horas (p > 0,05). Entretanto, aos 7 dias, 20 ppm de BDDE sem ácido hialurônico (p < 0,05) e 70 e 100 ppm de BDDE com ácido hialurônico foram citotóxicos (p < 0,01).* **Conclusão:** *o BDDE apresentou citotoxicidade tardia (7 dias) para fibroblastos gengivais em altas concentrações.*
Palavras-chave: *Ácido hialurônico; viabilidade celular; estética.*

INTRODUCTION

Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan polymer of high molecular weight, characterised by its linear, anionic structure[1]. It comprises alternating units of D-glucuronic acid and N-acetyl-D-glucosamine[1]. This configuration endows HA with unique physicochemical and biological properties, rendering it an essential component in various biomedical and cosmetic applications[2]. Hyaluronic acid serves as a crucial component of the extracellular matrix within connective, epithelial, and neuronal tissues throughout the body[1]. Its presence is fundamental to these tissues' structural integrity and function, facilitating cellular processes and contributing to the maintenance of tissue hydration, elasticity, and repair.[1]

The first hyaluronic acid-based dermal filler was approved by the United States Food and Drug Administra-

Correspondente/ Corresponding: *Flávia Gonçalves – Universidade Santo Amaro. Faculdade de Odontologia. – End: Av. Prof. Eneas de Siqueira Neto, 340. 04829-900, São Paulo, SP, Brazil. – E-mail: drafgoncalves@gmail.com; flgoncalves@prof.unisa.br

tion (FDA) in 2003[3]. This marked a significant milestone in aesthetic medicine, offering a novel, non-invasive option for facial rejuvenation and the correction of skin imperfections. According to the International Society for Aesthetic Plastic Surgery (ISAPS), it is the most widely used filler on the world market [4], with a significant increase of 30.3% in hyaluronic acid procedures carried out over the last four years.

Hyaluronic acid is distinguished by its biocompatibility, biodegradability, and hydrophilicity, characteristics that enable it to absorb and retain substantial quantities of water[1]. Nevertheless, in its uncross-linked or unmodified form, hyaluronic acid is rapidly metabolised and eliminated from the injection site, culminating in a transient filling effect that persists for less than a week[5]. This rapid degradation underscores the necessity for chemical modification or crosslinking to enhance its persistence and efficacy as a dermal filler since enzymes such as hyaluronidase and free radicals naturally present in the skin have the ability to degrade it quickly[6].

To overcome the degradation of hyaluronic acid, manufacturers of dermal fillers employ cross-linking agents[7]. These agents are utilised to enhance the durability and stability of hyaluronic acid within the tissue, thereby prolonging its efficacy as a filler. The most common cross-linking agent is 1,4 butanediol diglycidyl ether (BDDE)[7], which significantly increases the stability and duration of the dermal filler, reaching a longevity of six to twenty-four months after injection[8]. The degree of cross-linking significantly improves the rigidity of the hyaluronic acid gel, thereby exerting a profound impact on the material's physical and rheological properties. This modification enhances the gel's structural integrity and influences its behaviour under physiological conditions, making it a versatile tool in medicine and aesthetics[9]. Nonetheless, the process of cross-linking hyaluronic acid chains may result in the presence of unreacted or residual BDDE within the final product[10]. When present in excessive quantities, BDDE can exhibit cytotoxic effects, posing potential risks to cellular health and function[11].

It has been speculated that residual BDDE may be one of the factors responsible for hypersensitivity reactions following dermal fillers with hyaluronic acid[12]. Hypersensitivity reactions can be classified as acute or delayed, depending on the time of onset[13]. Acute hypersensitivity reactions occur within minutes or hours of injections. Late hypersensitivity reactions arise 48–72 hours after injection but can be observed up to several weeks after filling and can persist for many months. When this occurs, these reactions are called Persistent Intermittent Delayed Swelling (PIDS)[14].

In the literature, there is no data to measure the residual BDDE of the fillers available on the market, and manufacturers do not provide data on the total concentration of crosslinking agent added to the product, the degree of crosslinking, or the concentration of residual BDDE in the manuals and package leaflets for these materials.

Given the above, it is important to assess the potential toxicity of crosslinkers at the cellular level, as they may exist as a non-cross-linked residue or a by-product after the filler has been biodegraded. This in vitro study aimed to evaluate the cytotoxicity of different concentrations of BDDE, used in association or not with hyaluronic acid, after one or seven days of contact with human fibroblasts. The null hypothesis of this study is that there is no difference in the cytotoxicity regarding the BDDE concentration used associated or not to hyaluronic acid, and independently of the time evaluated.

METHODOLOGY

Cell Isolation

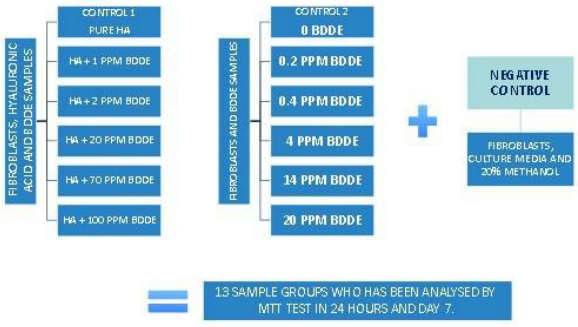
This study was performed in accordance with the Declaration of Helsinki. Human primary cell lines included in this study were approved as part of this study protocol. This human study was approved by the Ethical Committee of the Universidade de Santo Amaro (opinion number 6.572.098). The adult participant provided written informed consent to participate in this study. Human gingival fibroblasts were isolated from one patient using the explant technique from gingival tissue removed for implant reopening. The criteria for inclusion were patients who were adults without any systemic disease, such as autoimmune, inflammatory or cardiovascular, renal, or metabolic. Briefly, the tissue was cut into small fragments and cultured over Peter dishes (6 cm diameter) in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Vitrocell®/Embriolife, Campinas, Brazil), supplemented with 10% fetal bovine serum (Vitrocell®/ Embriolife) and 1% antibiotic-antimycotic solution. The cells were kept in a CO_2 oven at 37°C, and the medium was changed every three days. As the cells migrated from the tissue to the culture plate, they were transferred to culture bottles using the trypsin enzyme (Vitrocell®/ Embriolife). The cells were expanded until they reached a maximum of 90% confluence and then used in the experiment.

Experimental Groups

Thirteen samples were prepared for the cytotoxicity analysis (Figure 1 below). These were: a positive control group with fresh culture medium only and cells; a control group with fresh culture medium, 20% non-cross-linked hyaluronic acid hydrogel without BDDE and cells; and a negative control group in fresh culture medium, 20% methanol and cells, which is known to be cytotoxic. Of the 10 experimental groups, half were made up of 20%

non-cross-linked hyaluronic acid hydrogel (Toskani®/ Mesoestetic®, Barcelona, Spain) associated with BDDE (Sigma Aldrich, São Paulo, Brazil) at concentrations of 1, 2, 20, 70 and 100 ppm in relation to hyaluronic acid, both diluted in culture medium. The other half was made up of the same amount of BDDE, solubilised only in culture medium, which resulted in concentrations of 0.2, 0.4, 4, 14 and 20 ppm in the solution, or 1/5 of the number indicated for the previous concentration. Three samples were made for each experimentally conditioned medium (n = 3), which in turn were measured twice in the cells each time.

Figure 1 – *Sample groups*



Source: own authorship

Cell Viability Assay

Human fibroblasts were added to a 96-well culture plate at passage 5 at a concentration of 1x10^4 cells/well. After twenty-four hours in DMEM medium with high glucose (Vitrocell®/Embriolife), supplemented with 10% fetal bovine serum (Vitrocell®/ Embriolife) and 1% antibiotic-antimycotic solution, the medium was removed, the cells were washed twice with PBS (phosphate-buffered saline), and samples of each material, as well as the control groups, were added to the cells. Half of the culture medium in each well was replaced with samples from the same experimental group every three days. Cytotoxicity was analysed after one and seven days when the cells were washed with PBS, and a solution consisting of 80% DMEM (without serum) and 20% 3-(4,5 dimethylthiazol-2yl)-diphenyltetrazolimbromide (MTT) solution in PBS at 5 mg/mL-1 was added. After three hours in the oven, the solution was removed, the crystals formed were solubilised in isopropyl alcohol, and the absorbance of the solution was measured in a spectrophotometer at 560 nm. For each time, the cells in fresh culture medium were used as a positive control for cell viability, and the cells in culture medium and 20% methanol were used as a negative control for cell viability. The average absorbance of the control group was 100% cell viability, and the cytotoxicity of the other groups was expressed as a percentage relative to the control group.

Statistical Analysis

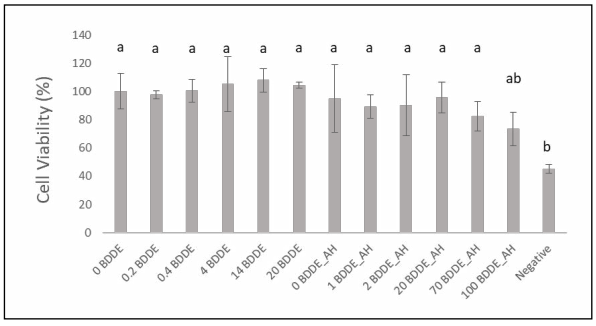
The data was assessed for normality (Shapiro-Wilk test), and homoscedasticity (Levene's test), and it was submitted to a single-factor ANOVA and Tukey's test for each analysis time. The overall significance level was 95% (α = 0.05).

RESULTS

Cell viability at twenty-four hours (Figure 2 and Table 1) was higher than 70% for all the groups evaluated, varying from 108±8 to 73±12%, and similar to the positive control, except for the negative control (43±0.2%), indicating that there was no toxic experimental group.

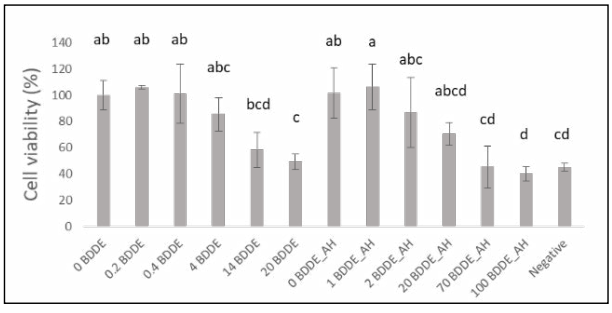
Cell viability at seven days (Figure 3 and Table 1) indicated a cell viability of 20 ppm BDDE in the culture medium of 49±6%, statistically similar to the negative control and different from the positive control. Cell viability was also decreased in the groups with 20% hyaluronic acid and BDDE at concentrations of 70 and 100 ppm BDDE, with cell viability of 45±15 and 40±5%, respectively. There are statistically significant differences between these and their respective positive controls (with or without hyaluronic acid and without BDDE) and statistical similarity with the negative control (Cell viability 43 ± 0.2%).

Figure 2 – *Cell viability (%) in 24 hours*



Source: own authorship

Figure 3 – *Cell viability (%) in 7 days*



Source: own authorship

Table 1- *Mean and standard deviation of cell viability (%) at 24h and 7 days. In the same column, similar letters indicate the absence of statistical difference.*

Groups	Cell viability at 24h (%)	Cell viability at 7 days (%)
0 BDDE	100 ± 13 a	100 ± 11 ab
0.2 BDDE	98 ± 3 a	106 ± 1 ab
0.4 BDDE	100 ± 8 a	101 ± 23 ab
4 BDDE	105 ± 19 a	86 ± 13 abc
14 BDDE	108 ± 8 a	58 ± 13 bcd
20 BDDE	104 ± 2 a	49 ± 6 c
0 BDDE_AH	95 ± 24 a	102 ± 19 ab
1 BDDE_AH	89 ± 8 a	106 ± 18 a
2 BDDE_AH	90 ± 21 a	87 ± 27 abc
20 BDDE_AH	96 ± 11 a	70 ± 9 abcd
70 BDDE_AH	82 ±11 a	45 ± 16 cd
100 BDDE_AH	73 ±12 ab	40 ± 5 d
Negative	45 ± 3 b	45 ± 3 cd

Source: research data

DISCUSSION

The study's null hypothesis was rejected since, in 7 days, the highest concentrations of BDDE, associated or not with hyaluronic acid, were toxic.

Hyaluronic acid is a biocompatible, natural material found in our skin, playing a crucial role in moisturising it and maintaining its elasticity[15]. It is also present in cartilage, in synovial fluid, helping to lubricate joints and reduce friction[15]; in the vitreous humour, a gelatinous substance inside the eyes; and in various connective tissues in the body, providing support and structure[15]. Therefore, it was expected that the control group, in which 20% non-cross-linked hyaluronic acid hydrogel was solubilised in culture medium, would be non-cytotoxic at 24 hours and 7 days. This was indeed confirmed in the present study, validating our methodology in line with other studies[8,16] and indicating that at a concentration of 20%, it is possible to maintain cell viability without a shortage of nutrients for their development.

The American regulatory agency, the Food and Drug Administration (FDA), recommends that the residual level of unreacted BDDE should be less than two ppm to be safe[17]. This would be equivalent to <0.002 mg of BDDE in 1 ml of hyaluronic acid gel. In fact, in the present study, this concentration did not prove cytotoxic in the 24 h and 7-day readings in the groups in which BDDE was diluted in culture medium without hyaluronic acid association, and the cytotoxic dose was around 35 times higher than the limit, indicating the safety of keeping within the regulatory limits.

When BDDE is associated with hyaluronic acid, they are expected to react with each other. The epoxy group of BDDE reacts with the hydroxyl group of hyaluronic acid, forming a covalent bond. If both ends of BDDE react with hyaluronic acid, it is considered fully reacted. However, if only one epoxy group reacts, it is considered partially crosslinked or a pendant group. If BDDE does not react with hyaluronic acid, it can be hydrolysed and excreted as glycerol and butanediol, or it may retain its original structure and be considered residual BDDE[7,8]. The molecular forms with cytotoxic and immunological potential are the pendant or residual forms of BDDE[11].

We have not yet found any studies in the literature that actually quantify the concentration of residual BDDE in cross-linked hyaluronic acid hydrogels, which limits the comparison between different brands and the control of commercial materials.

In the present study, although none of the concentrations were cytotoxic within 24 hours, the concentrations of 14 and 20 ppm BDDE in culture medium not associated with hyaluronic acid and 70 and 100 ppm associated with hyaluronic acid were cytotoxic within 7 days, indicating delayed cytotoxicity. Many studies speculate that the delayed hypersensitivity reactions observed clinically after filling with hyaluronic acid are due to the high concentration of cross-linked BDDE associated with short chains of hyaluronic acid[18], resulting in high concentrations of non-cross-linked BDDE. The data from this study corroborates these hypotheses, although controlled in vivo studies are needed to establish this cause-and-effect relationship properly.

One study evaluated the cytotoxicity of hyaluronic acid as a vehicle in an autologous cell compound for bone grafting with 20% Teosyal® cross-linked hyaluronic acid hydrogel (the concentration of BDDE was not informed by the manufacturer) in culture medium and cells of the mouse pre-osteoblastic lineage called OFCOL II[16]. The MTT test was carried out 48 hours after adding hyaluronic acid to the culture medium and cells. The average cell viability was 72%, concluding that the product was not cytotoxic to the cells analysed, corroborating the results of the present study.

The current study also corroborates with the results of a study in which only BDDE in a culture medium did not induce toxic cellular responses in fibroblasts at low concentrations (0–25 ppm) but was cytotoxic at a concentration of 100 ppm after 7 days of culture[19].

However, I would emphasise that some authors observed that the hyaluronic acid filler cross-linked with 10 ppm BDDE already decreased cell viability within 24 hours[20]. This is possible because it was a different cell line, i.e., human foreskin fibroblasts, and because it was evaluated with a different proportion of hyaluronic acid in the culture medium, which was not reported in the study.

This is a laboratory study that allows us to understand the dose-dependent cytotoxicity of BDDE associated or not with hyaluronic acid hydrogel, but it should be noted that this data cannot be taken directly into clinical practice. More in vitro and in vivo studies are needed because, as we work with commercial non-cross-linked hyaluronic acid, other product formulation components may interfere with the results. Since BDDE was added but the cross-linking obtained was not analysed, the concentration of residual

BDDE was also associated with the volume of hydrogel added, the degradation rate, the cell type analysed, and the hyaluronic acid viscosity[19,20].

CONCLUSION

We can conclude that BDDE, whether associated or not with hyaluronic acid, was not cytotoxic within 24 hours at the concentrations evaluated. However, it was cytotoxic at 14 and 20 ppm in the BDDE-only group and at 70 and 100 ppm in the BDDE and hyaluronic acid groups after 7 days.

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