The Cytotoxicity of Orthodontic Elastomeric Ligatures in Vitro Comparative Study

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Abstract

The present study was carried out to evaluate the cytotoxicity of orthodontic elastic ligatures in vitro study. Three different colours (transparent, white and yellow) from three different companies were selected. The samples were divided into 9 experimental groups each group had 10 elastic ligatures from the same colour and the same company with control group (cells with no contact with elastomeric ligatures), Also positive control group contain (Phosphate buffer saline) and negative control group contain (Tween 80) were used to verify the results of study.

The cell lines were chicken embryo fibroblast and human lymphocyte of healthy person. These cells were exposed to specific media contain the extracted elutes of elastic ligatures after preserving it in this media for different times interval (1,2,3,7 and 28 days).

Then the cell viability were measured by using the neutral red dye, the optical densities of living cells were measured by ELISA reader devise at wave length 490 nM, The percentage of viable cells was obtained by comparing the mean optical density (OD) in the control group (cells with no contact with elastomeric ligatures) with that obtained from supernatants of cell cultures that had been in contact with elastomeric ligatures.

At the end of study, the results showed that there were increase in the percent of viable cells from the first day of experiment till day 28 and there are highly significant difference between the control group and all the experimental groups at all the periods of experiment.

In conclusion there were significant difference among different manufactures, but there are statically no significant difference among different colors from the same manufacturer.

Keywords: elastic ligature cytotoxicity, fibroblast viability, lymphocyte viability, neutral red dye.

الخلاصة

الروابط البلاستيكية هي عباره عن حلقات مطاطيه صغيره تستخدم لربط سلك التقويم مع البراكيت خلال العلاج التقويمي للأسنان .هذه الحلقات البلاستيكية متوفرة بالوان مختلفة ,تستخدم هذه المواد بصوره واسعه في عيادات تقويم الاسنان لذلك يجب الاهتمام بسميه هذه البلاستيكيات التقويمية بصوره رئيسيه الحلقات التي تستخدم داخل الفم لأنها تكون قريبه جدا من اللثة وباقي الأنسجة الرخوة.

هذه الدراسة أنجزت لتقييم سميه الروابط الحلقية لتقويم الاسنان خارج الجسم. اخترنا ثلاث الوان مختلفة (شفاف, ابيض, اصفر) من ثلاث شركات مختلفة . النماذج قسمت الى تسع مجاميع تجريبيه كل مجموعه تحتوي على عشرة روابط بلاستيكية من نفس اللون ونفس الشركة مع مجموعه التحكم (خلايا بدون تماس مع الحلقات البلاستيكية) ايضا مجموعة التحكم الموجبة التي تحتوي (فوسفات بفر سلاين) و مجموعة التحكم السالبة التي تحتوي(توين ٨٠) استخدمت لايضاح مصداقية نتائج الدراسة.

الزرع الخلوي استخدم لفحص سمية هذه الحلقات البلاستيكية. نوع الزرع الخلوي المستخدم في هذه الدراسة كان من نوع الطبقة الواحدة . خط الخلايا المستخدم كان من نوع الفايبروبلاست لجنين الدجاجة وخلايا الدم (الليمفوسايت) للإنسان السليم . هذه الخلايا تعرضت الى ميديا خاصه تحتوي على مستخلص الروابط البلاستيكية بعد حفظها في هذه الميديا لفترات مختلفة (١, ٢،٣ ,٧ و٢٨ يوم). ومن ثم قمنا بقياس حيوية الخلايا باستخدام الصبغة الحمراء المعتدلة باستخدام جهاز الأليزا بطول موجي ٤٩٠ نانو ميتر. حصلنا على نسبة الخلايا الحيه من مقارنة معدل الكثافة الضوئية في المجموعة التحكمية (خلايا بدون تماس مع الحلقات البلاستيكية) مع معدل الكثافة الضوئية لمستخلص الزرع الخلوي الذي كان بتماس مع الروابط البلاستيكية.

اظهرت النتائج ان هناك زياده في نسبة الخلايا الحيه من اليوم الاول الى اليوم الثامن والعشرون من التجربة وهناك اختلاف كبير في نسبة الخلايا الحيه بين المجموعة التحكمية وكافة المجاميع التجريبية الاخرى في جميع فترات التجربة.

استنتجنا ان هناك فرق كبير في نسبة الخلايا الحية بين الشركات المختلفة, لكن لم يكن هناك اختلاف في نسبة الخلايا الحية للألوان المختلفة من نفس الشركة.

Introduction

Elastics and Elastomeric are routinely used as active component of orthodontic therapy. Elastics have been a valuable adjunct of any orthodontic treatment for many years. There use combined with good patient cooperation provides the clinician with the ability to correct both Antero-posterior and vertical discrepancies [1].

Both natural rubber and synthetic elastomers are widely used in orthodontic therapy. Naturally produced latex elastics are used in the Begg technique to provide intermaxillary traction and intramaxillary forces [2].

Synthetic elastomeric materials in the form of chains find their greatest application with edgewise mechanics where they are used to move the teeth along the archwire. The links of chain fit firmly under the wings of an edgewise bracket so that chain elastomers also serve to replace metal as the ligating force that holds the arch wire to the teeth. Since they are so positively located on the brackets it is usual for the chains to remain in situ until replaced by the orthodontist at the next visit of the patient. This routine differs from that usually followed for latest elastics, which are changed by the patient every one or two days. The use of latex elastics in clinical practice is predicted on force extension values given by the manufactures for different sizes of elastics [3].

Orthodontic elastics are widely used in orthodontic practice with the purpose of helping orthodontic treatment, therefore need to be inert to oral tissues. Elastics in contact with the oral mucosa for several hours a day is a situation that may continue for months. Therefore, the question arises about the possibility of toxic substances being released by elastomers, which may be capable of harming the cells [4].

As they are widely used materials in the orthodontic clinic, one must be concerned about the cytotoxicity of elastics, particularly the intraoral type that comes into intimate contact with the mucosa, and option for materials that have been proved to be biocompatible from this aspect [7], the protein content of latex is a known allergen. Allergy caused by latex proteins has been well documented [6], including immediate hypersensitivity reactions [5]. Amongst the allergic reactions caused by orthodontic elastics, swelling and stomatitis, erythematous oral lesions, respiratory reactions, and even anaphylactic shock, the most severe

form of allergy , can be cited. Latex allergy occurs in 3-17% of the cases. Because latex allergy is prevalent among occupationally exposed groups and patients, the need for non-latex alternatives is increasing , However, little is known if latex and non-latex products are cytotoxic to oral mucosal cells [4,6 ,8].

Biological tests are important because a material to be used in the oral cavity should be nontoxic and non-absorbable by the circulatory system and should not injure oral tissues. Non-biocompatible materials may be mutagenic or affect inflammation mediators, which may lead to systemic responses, such as toxic, teratogenic or carcinogenic effects. Such materials should be free of agents that may cause allergic responses in sensitive individuals [9].

Cell lines, chicken embryo fibroblasts [10,11,12], have been shown to behave similarly to primary human gingival fibroblasts and are thus a suitable in vitro model to test the toxicity of products used intra-orally during orthodontic treatment [13-15].

Patients and Methods

Three different colored orthodontic elastomeric ligatures from three different manufacturers in addition to control groups were selected for the present study as shown in Table 1 below.

Table 1 Experimental and control groups used for the assays.

Groups	Trademark	Main Composition	Color	Reference number
D1	Dentaurum	Polyurethan	Crystal	774-561-01
D2	Dentaurum	Polyurethan	white	774-561-01
D3	Dentaurum	Polyurethan	Yellow	774-561-01
M1	Morelli	Polyurethan	Crystal	60-06-100
M2	Morelli	Polyurethan	white	60-06-100
M3	Morelli	Polyurethan	Yellow	60-06-100
O1	Orthotechnology	Polyurethan	Crystal	246019
O2	Orthotechnology	Polyurethan	white	246019
O3	Orthotechnology	Polyurethan	Yellow	246019
C+	Tween 80 (Polyoxyethylene-20-sorbitan, Sigma, St. Louis, Missouri, USA)			
C–	PBS solution (phosphate-buffered saline, Cultilab, Campinas, São Paulo, Brazil)			
CC	consisting of cells not exposed to supernatants from the elastomeric ligatures			

Membrane toxic effects of chemicals or other substances can be determined with the neutral red test which provides a colour reaction that can be used to distinguish living and dead cells. Dead and membrane damaged cells cannot accumulate the dye and show reduced staining of these cell cultures. Two cells were used in this study; chicken embryo fibroblast cells and human peripheral blood lymphocyte cells. This study was achieved in tissue culture unit of Pharmacy College laboratory of kufa University during the period from February 2013 till June 2013.

The powder coating of the elastomeric ligatures was removed. The elastics were washed for 15 second with deionized water. Before testing all elastomeric ligatures were sterilized by exposure to ultraviolet light for 30 min [15].

After sterilization the elastic ligatures kept on their original shape and colour after complete the sterilization.

These elastomeric ligatures were distributed as 10 elastic ring in 45 sterile tubes. Each tube contain 10 elastic from specific colour of specific company ,sealed ,labeled and named according to the colour, manufacture , date and arranged in rack . These tubes incubated in Eagles' minimum essential medium (MEM) at 37˚C for 1, 2, 3,7 and 28 days and the extracted elutes were added to the cell line incubated in growth media, this for fibroblast cells test.

Another 45 tubes prepared in the same method above but the elastics incubated in RPMI media which is specific for lymphocyte cells test.

To verify the cell response in extreme situations, three additional groups were included in the study: Group CC (cell control), consisting of cells not exposed to supernatants from the elastomeric ligatures; Group C+ (positive control), consisting of Tween 80 (Polyoxyethylene-20-sorbitan, Sigma); Group C- (negative control), consisting of phosphate-buffered saline (PBS) solution . The elastic ligatures were incubated in MEM maintenance medium (Eagle's minimum essential medium) for 1, 2, 3, 7 and 28 days and the extracted elutes were added to the line cells incubated in the growth medium.

The cytotoxicity of these orthodontic elastics was determined by means of the dye-uptake technique, which is based on the neutral red absorption by living cells [4].

All Study Data were analyzed by SPSS version 17. One way analysis of variance (ANOVA) test was used to analyze the study parameters difference .All parameters of this study were expressed as mean ± S.D.

Results

In this study, there were highly significant difference between control group and all study groups during all different periods of study (1,2,3,7 & 28 days). The results showed that there were a highly significant difference in the optical densities of fibroblast cells as compared with control group i.e. the viability percent of fibroblast cells was increased with time progressing continuously among the three different companies (Dantarum, Morelli and Orthotechnology Companies); Dantarum company groups exhibited more liability percent in comparison with other two companies. While Morelli company groups showed less percent of viability than other two companies. The elastomeric ligatures evaluated in this study showed over 90% cell viability at all experimental periods, except for Morelli elastomeric ligatures at days 1

and 2 and Orthotechnology elastomeric ligatures at the first days.

Lastly, the analysis of study results related to lymphocyte was statistically similar to that of fibroblast as mentioned above and as shown in following tables (Table 2 and 3).

Table 2 Fibriblasts descriptive statistics and groups' differences

Groups	1 day			2 days			3 days			7 days			28 days		
	Mean	S.D.	VC%	Mean	S.D.	VC%	Mean	S.D.	VC%	Mean	S.D.	VC%	Mean	S.D.	VC%
C	0.662	0.049	100%	0.820	0.007	100%	0.770	0.004	100%	0.788	0.005	100%	0.878	0.004	100%
C-	0.649	0.006	98.30 %	0.808	0.004	98.50 %	0.752	0.004	97.60%	0.770	0.004	97.70 %	0.869	0.004	98.90%
C+	0.066	0.002	9.90%	0.065	0.004	8%	0.071	0.004	9.90%	0.077	0.004	10%	0.077	0.004	8.80%
D1	0.609	0.060	91.80 %	0.773	0.004	94.20 %	0.739	0.004	95.90%	0.760	0.004	96.40 %	0.853	0.004	97.10%
D2	0.603	0.003	91%	0.775	0.004	94.50 %	0.740	0.004	96.10%	0.762	0.004	96.70 %	0.853	0.004	97%
D3	0.605	0.005	91.30 %	0.772	0.003	94.10 %	0.738	0.004	95.80%	0.761	0.004	96.50 %	0.855	0.005	97.20%
M1	0.520	0.004	76.40 %	0.669	0.003	81.50 %	0.706	0.004	91.6%	0.739	0.004	93.70 %	0.841	0.004	95.70%
M2	0.507	0.004	76.50 %	0.668	0.004	81.40 %	0.706	0.004	91.50%	0.740	0.004	93.90 %	0.842	0.004	95.80%
M3	0.526	0.045	76.70 %	0.671	0.004	81.80 %	0.703	0.002	91.20%	0.740	0.004	93.90 %	0.843	0.004	96%
O1	0.591	0.004	89.20 %	0.745	0.004	90.80 %	0.729	0.004	94.60%	0.750	0.004	95.10 %	0.849	0.004	96.60%
O2	0.587	0.004	88.60 %	0.746	0.004	90.90 %	0.727	0.004	94.40%	0.751	0.004	95.30 %	0.848	0.004	96.50%
O3	0.582	0.004	87.90 %	0.744	0.004	90.70 %	0.727	0.004	94.40%	0.748	0.004	94.90 %	0.848	0.003	96.50%
F-test	22.699			1223.410			234.159			107.202			57.698		
p-value	0.000			0.000			0.000			0.000			0.000		

Table 3 Lymphocytes descriptive statistics and groups' differences

Groups	1 day			2 days			3 days			7 days			28 days		
	Mean	S.D.	VC%	Mean	S.D.	VC%	Mean	S.D.	VC%	Mean	S.D.	VC%	Mean	S.D.	VC%
C	0.760	0.034	100%	0.862	0.007	100%	0.693	0.004	100%	0.854	0.004	100%	0.742	0.003	100%
C-	0.756	0.041	97.70%	0.853	0.010	98.60%	0.680	0.005	98.10%	0.869	0.013	97.70%	0.720	0.003	97%
C+	0.076	0.003	9.90%	0.076	0.003	8.90%	0.064	0.004	9.30%	0.076	0.004	8.90%	0.060	0.003	8.20%
D1	0.702	0.010	92.30%	0.816	0.005	94.60%	0.666	0.004	96.10%	0.825	0.004	96.70%	0.726	0.002	97.80%
D2	0.701	0.005	92.20%	0.814	0.003	94.50%	0.666	0.004	96.10%	0.828	0.004	97%	0.725	0.003	97.70%
D3	0.700	0.008	92%	0.811	0.007	94.10%	0.665	0.002	95.80%	0.826	0.002	96.70%	0.726	0.003	97.40%
M1	0.586	0.006	77.10%	0.701	0.021	81.40%	0.633	0.003	91.30%	0.800	0.003	93.60%	0.711	0.003	95.80%
M2	0.585	0.010	76.90%	0.698	0.015	81.20%	0.633	0.007	91.30%	0.802	0.004	94%	0.712	0.003	95.90%
M3	0.584	0.006	76.80%	0.698	0.003	80.90%	0.631	0.004	91.10%	0.802	0.004	94%	0.712	0.003	95.90%
O1	0.680	0.006	89.40%	0.782	0.003	90.80%	0.647	0.004	93.30%	0.811	0.003	94.90%	0.720	0.003	97%
O2	0.681	0.005	89.60%	0.787	0.005	91.20%	0.649	0.004	93.60%	0.811	0.004	95%	0.720	0.003	97%
O3	0.682	0.004	89.70%	0.784	0.005	91%	0.649	0.004	93.60%	0.811	0.004	95%	0.718	0.003	96.70%
F-test	180.473			299.171			179.127			164.916			85.691		
p-value	0.000			0.000			0.000			0.000			0.000		

Discussion

There seems to be an important relationship between the manufacturing process of these ligatures and their cytotoxic nature. The quality of elastomeric ligatures is defined by the degree of technology used, the refinement of the technique of production and the quality of raw materials used during manufacture of material (Morton, 1995). Evidence of this cytotoxic feature was shown following exposure of the elastomeric ligatures to cell culture medium. Elastomeric ligatures from Dentarum , Morelli, Ortgotechnology trademarks induced a greater amount of cell lysis at 24 and 48 h compared to the other experimental periods (day 3, 7 and 28). These findings suggest a greater release

of toxic ingredients within the first 48 h due to a possible polyurethane degradation and release of cytotoxic components, which was shown on days 1 and 2, and did not persisted on days 3, 7 and 28. This fact indicates that release of cytotoxic components is neither constant nor continuous.

According to [13] the great danger with the use of intraoral elastics with cytotoxic potential would be the fact that the substances released by these would be ingested by the patient, and over the course of time, cause diseases resulting from the cumulative effect of toxic substances [7]. The exact composition of elastic ligatures was unknown for us because the companies refused that : Prevulcanized latex is produced by mixing pure natural latex, which has the highest molecular weight, with stabilizers such as zinc oxide and chemically vulcanized materials. The resulting mixture is then heated until 70 ˚C [16]. Although zinc is known to be neurotoxic [17] the amount released by orthodontic elastics can be ingested as research studies show no evidence of harm [18].

Anti-ozone and anti-oxidant agents are also added to latex during the manufacture of orthodontic elastics. This process has the advantage of producing latex with higher mechanical properties, thus increasing its strength and elasticity [16,18]. Because natural latex rubber has been increasingly used as dental material, many cytotoxicity issues have been reported as well. Preservatives such as sulfur and zinc oxide as well as antioxidants such as di-thio-carbohydrates, N-nitrosodibutylamine, and Nnitrosopiperidine are all known to be cytotoxic substances.

Allergy to natural latex occurs because of the presence of many types of proteins, and the powder covering the orthodontic elastics works as a transporter for these proteins. Therefore, the development of non-latex elastics has become increasingly important for clinical usage [8].

Allergy caused by latex proteins has been well documented, including immediate hypersensitivity reactions [6]. Among the allergic reactions caused by orthodontic elastics, swelling and stomatitis, erythematous oral lesions, respiratory reactions, and even anaphylactic shock, the most severe form of allergy can be cited. Latex allergy occurs in 3-17% of the cases [19].

As an alternative to latex, different types and compositions of elastomers, such as polyurethane and silicone elastics have been launched on the market, in order to decrease the risk of allergic reactions caused by latex orthodontic elastics. Elastics derivatives of polyurethanes, are thermoplastic polymers processed currently by injection molding and by sintering. After the chemical reactions of polymerization that the originate, appear as amorphous masses, whose polymeric chains have relatively weak traction forces between them and chemical bonds randomly located along these chains. To improve its mechanical properties, must occur to union between the side chains through cross covalently bonds using the process known as vulcanization [20].

Depending on how elastic is stored, alterations may occur in its composition, as its major limitation is sensitivity to ozone or other systems generating free radicals, such as sunlight which weakens the latex polymer chain [21].

However, as in vitro experiments cannot reproduce the oral environment in all its aspects, those elastics should not be considered clinically inert. Although

orthodontic elastics are cytotoxic under in vitro conditions, this effect is not always reflected in the clinical practice. Most of the allergic reactions have been associated with the use of orthodontic elastics, which is characterized by presence of small vesicles or acute edema and complaints of itching and burning and this in agreement with the study of [22, 23].

As sterilization is a prerequisite for cytotoxicity assay, ultraviolet light was used in the present study for sterilizing both sides of the elastics during 30 minutes. All the elastics were found to have the same color and malleability following UV light sterilization and this in agreement with the study of [24].

Autoclave sterilization may be used, however, elastics have been shown to darken and harden after this type of sterilization due to the heat liberated which may cause degradation and the release of substances that are toxic to cells [25].

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