IN VITRO BIOCOMPATIBILITY OF STAINLESS STEEL BRACKETS

Dr Anju Loomba
Dr Ashish Goel
Dr Poonam Goel
Dr Kapil Loomba

ABSTRACT

To quantify and compare in vitro the leachability of metals (Cr, Ni, Fe) in new and rebonded orthodontic brackets in simulating oral environment and to study the cytotoxic response, if any, of the leachates, in primary cultures of human gingival fibroblast cells (HGFC).New and rebonded stainless steel premolar brackets were suspended in artificial saliva at pH 4.0, 6.8 and 7.4 in a shaker water bath at 37°C for a simulating period of 2 hrs, 2 days and 7 days. Ions released were quantified by atomic absorption spectrophotometry. Mitochondrial Activity (MTT) assay and Neutral Red Uptake (NRU) assay were conducted for the assessment of cytotoxicity in HGFC.Maximum amount of ions (Ni, Cr, Fe) were leached at pH 4.0 and lowest at pH 6.8 for both new and rebonded brackets. A high initial rate of release of ions was observed in all samples. MTT and NRU assay depicted optimum cellular response at pH 6.8. Maximum cytotoxic response is seen at pH 4.0 for all comparable simulating conditions. The results of MTT assay were of highest significance in both new and rebonded brackets when compared for leaching at a simulating period of 2 hours at pH 4.0 and 6.8, pH 6.8 and 7.4; for all exposure periods ($p<0.001$). There is a high initial rate of release of ions which decreases subsequently and is below the level to cause systemic toxicity. Ion release and cytotoxic response are maximum at pH 4.0 and least at pH6.8.

INTRODUCTION:

In the damp oral environment, orthodontic attachments are exposed to a number of potentially damaging physical and chemical agents, such conditions possibly contributing to corrosion of metal, which can modify its properties. The corroded material and leachates come directly in contact with the oral mucosa. A number of studies have shown that metal ions are released by all dental alloys in vitro and *in vivo* [1,2].

International regulations (ISO 10993-5, EU 30993-5) value the biocompatibility of materials; toxic, irritating, inflammatory, allergic, mutagenic or carcinogenic effects. Nickel complexes in arsenides and sulphides have long been known as carcinogenic, allergenic and mutating substances; whereas nickel at non-toxic concentrations has been found to introduce DNA alterations mainly through base damage and DNA strand scission (single strand breaks), which are site specific[3]. The mutative action of nickel maybe derived from its effect on inhibiting several enzymes known to restore DNA breaks, promoting microsatellite mutations, and increasing total genomic methylation, thereby contributing to genetic instability.[4]

Accidental dislodgement of orthodontic bracket caused by occlusal trauma, or intentional removal of a bracket to reposition, is a common orthodontic practice[5]. The analysis of biocompatibility in orthodontic brackets especially recycled or reused materials cannot leave out of consideration the evaluation of ion emission and cytotoxicity studies [6-9]. The aim of the study is to find out the biocompatibility of the rebonded stainless steel brackets at various stages of orthodontic treatment, therefore rebonded brackets with varieties of exposure to the damp corrosive intraoral environment and active orthodontics were studied. New brackets were studied for a comparative analysis of biocompatibility. The study was done in vitro in various simulating intraoral environments. Biocompatibility analysis was determined by studying the cytotoxic effect of leachates on human gingival fibroblasts for different incubation periods.

MATERIALS AND METHODS

A total of 288 stainless steel premolar brackets were selected for the study; these were 144 new and 144 rebonded brackets from patients undergoing orthodontic treatment. The old brackets were reprocessed by flame method and electropolished[10]. The bracket bases were coated with composite adhesive and cured. The new and rebonded brackets were divided into 9 groups each consisting of 8 brackets in every group. All the brackets were washed with distilled water, autoclaved and dried.Artificial saliva was prepared as per the method adopted by Lin[11] and titrated to get three different pH 4.0, 6.8 and 7.4. These media were autoclaved and used as a simulating solution for suspension of brackets and ion release. The vials were kept in shaker water bath at 37°C for a period of 2 hrs, 2 days and 7 days.

On the completion of the simulating periods, the leachates obtained were divided into two parts: one part was processed for lyophilization, while the other part was processed for metal analysis by atomic absorption spectrophotometry. A culture of human gingival fibroblast cells (HGFC) was established from gingival biopsy tissues of a healthy human patient following the

Table 1- Nickel, Chromium and Iron ions leached In Vitro

Time Period	ng/ml	pH 4.0		pH 6.8		pH 7.4	
		New	Rebonded	New	Rebonded	New	Rebonded
2 hrs	Nickel	40.46	33.30	32.50	26.53	36.00	29.30
	Chromium	46.26	39.23	36.50	32.40	41.26	35.10
	Iron	43.33	37.16	32.63	29.20	38.20	31.70
2 days	Nickel	42.46	37.33	38.33	34.36	39.33	36.60
	Chromium	47.73	42.33	43.43	38.43	45.26	42.33
	Iron	44.20	39.83	38.46	35.43	41.46	38.50
7 days	Nickel	57.46	48.36	46.43	38.60	50.43	39.26
	Chromium	62.43	53.20	49.40	45.63	54.96	46.30
	Iron	58.56	50.43	46.13	42.06	50.43	43.33

Table 2- MTT Assay (expressed as percent control)

Time Period	Incubation Period	Type of Brackets	pH 4.0	pH 6.8	pH 7.4
Effect from sample of 2hrs of leaching	12 hrs	New	90.36	109.86	91.46
		Rebonded	92.40	105.33	92.36
	24 hrs	New	83.40	117.30	85.43
		Rebonded	90.33	108.56	87.60
	48 hrs	New	77.83	119.33	74.56
		Rebonded	87.33	112.56	83.26
	72 hrs	New	70.56	121.60	71.60
		Rebonded	81.13	116.33	78.30
Effect from sample of 2days of leaching	12 hrs	New	86.43	97.26	89.46
		Rebonded	90.63	98.43	87.43
	24 hrs	New	80.46	92.40	83.46
		Rebonded	86.56	95.93	86.06
	48 hrs	New	73.46	86.33	78.33
		Rebonded	80.56	90.26	80.76
	72 hrs	New	66.26	82.53	68.33
		Rebonded	72.46	86.16	74.56
Effect from sample of 7days of leaching	12 hrs	New	82.50	92.10	86.50
		Rebonded	88.33	94.06	85.50
	24 hrs	New	79.40	88.40	78.60
		Rebonded	82.26	89.36	82.53
	48 hrs	New	70.60	83.26	70.36
		Rebonded	78.66	86.36	78.16
	72 hrs	New	64.43	79.53	62.26
		Rebonded	71.60	80.76	70.33

protocol of Pant *et al*[12]. Phosphate buffered saline was used as an isotonic medium in cell culture. Minimum essential medium (MEM) was used to culture the cells. Giemsa stain was used for staining cultured cells at various time intervals following the exposure to leachates. The lyophilized powder of leachates was reconstituted in the volume as it was prior to lyophilization using incomplete serum free MEM. These reconstituted suspensions were used for the exposure of gingival fibroblast cultures after adjusting the pH 7.4. Trypan blue dye exclusion method was used to see the cell viability by assessing the loss of membrane integrity[12]. This helped to ascertain that the batch of cells used for the study had adequate number of viable cells (95% or above). Mitochondrial Activity (MTT) assay was conducted as one end point and Neutral Red Uptake (NRU) as other end point for the assessment of cytotoxicity in cultures of HGFC, for incubation periods of 12, 24, 48 and 72 hours. The values were then compared with control sets, run parallel under identical conditions without the test compound and the

Table 3- NRU Assay (expressed as percent control)

Time Period	Incubation Period	Type of Brackets	pH 4.0	pH 6.8	pH 7.4
Effect from sample of 2hrs of leaching	12 hrs	New	92.23	96.36	93.26
		Rebonded	97.53	99.30	98.53
	24 hrs	New	89.33	93.43	89.66
		Rebonded	95.50	97.26	96.70
	48 hrs	New	85.43	86.26	84.30
		Rebonded	91.40	96.96	91.26
	72 hrs	New	80.46	78.50	74.36
		Rebonded	89.36	95.13	90.76
Effect from sample of 2days of leaching	12 hrs	New	89.73	95.73	90.50
		Rebonded	94.60	96.36	94.50
	24 hrs	New	83.70	90.33	84.36
		Rebonded	88.33	91.30	90.40
	48 hrs	New	78.46	84.53	78.43
		Rebonded	84.26	89.56	85.63
	72 hrs	New	70.46	79.53	72.23
		Rebonded	80.20	87.50	77.63
Effect from sample of 7days of leaching	12 hrs	New	84.00	91.56	88.43
		Rebonded	91.50	93.60	92.16
	24 hrs	New	79.56	86.40	84.70
		Rebonded	87.50	86.26	88.40
	48 hrs	New	72.40	80.90	74.76
		Rebonded	81.63	84.23	76.40
	72 hrs	New	63.33	73.36	69.60
		Rebonded	76.40	80.36	74.56

results were expressed in percent control.

RESULTS

The results are expressed as a mean and standard error of means for 6 wells of at least three experiments. Student 't' test was employed to detect differences between the treated and control groups, whereas one way ANOVA and post hoc Tukey's test was employed to compare the significant differences between intergroup and intragroup variables.The nickel, chromium and iron ions leached from new and rebonded brackets was lowest for the time period of 2 hours and highest for the time period of 7 days **(Table 1)**, the intergroup and intragroup comparisons were not significant ($p>.05$). Leachability of all the ions studied was maximum at pH 4.0 and lowest at pH 6.8 for both new and rebonded brackets (Table 1), the intergroup and intragroup comparisons were not significant ($p>.05$). The initial rate of release of ions (Ni, Cr, Fe) was high at simulating time of 2 hours for all samples when compared to the progressive increase in simulating time of 2 days and 7 days (Table 1). Cytotoxicity assessment by MTT and NRU assayMTT assay depicted maximum mitochondrial activity and cell viability at pH 6.8 irrespective of the simulating time period of exposure. A reduction in this activity was seen in samples of both pH 4.0 and pH 7.4; more for pH 4.0 than for pH 7.4 **(Table 2)**. The results of MTT assay were significant among new and rebonded brackets when compared for leaching at a simulating period of 2 hours at pH 4.0 for an exposure period of 48 hours ($p=0.020$) and 72 hours ($p=0.011$). The results were of highest significance among new and rebonded brackets when compared for leaching at a simulating period of 7 days at pH 4.0 for an exposure period of 48 hours ($p<0.001$).

The results of MTT assay were of highest significance in both new and rebonded brackets when compared for leaching at a simulating period of 2 hours at pH 4.0 and 6.8, pH 6.8 and 7.4; for all exposure periods ($p<0.001$).

NRU assay depicted optimum cellular response at pH 6.8 irrespective of the type of bracket and simulating time of exposure. The cellular response was seen to decrease progressively in all samples on increasing the exposure period of the cultured cells to the leachates **(Table 3)**. The comparison of NRU assay between new and rebonded brackets were of high significance at pH 4.0 for the simulating period of 7 days ($p=0.001$).

DISCUSSION

The immersion medium used by Huang *et al*[13] were bicarbonate buffers at pH 4.0, 7.0 and 10.0; and artificial saliva. To mimic the intraoral exposure more closely the present study used artificial saliva at pH 4.0, 6.8 and 7.4; these acidic and alkaline limits were determined in the saliva of a volunteer collected by spitting method after intake of

a variety of food stuffs.The present study is in agreement with the study conducted by Huang *et al*[13], Barrett *et al* [14] and Kerosuo *et al*[15], which determined that the measurable amount of ions are released rapidly over the first week. The leachability of ions for both new and rebonded brackets was highest at the time period of 2 hours and did not increase significantly ($p>.05$) for the time period of 2 days and 7 days, depicting a high initial release of ions coupled with a decrease in the rate of release thereafter. This validates the point put forth by Lin[1] and Buchman[10] that surface irregularities are prominent in new brackets, on exposure to the oral environment they become less prominent and the leaching reduces. Bracket recycling causes carbide precipitation, alteration in the surface characteristics and a rearrangement of lattice structure which may cause a transient increase of ion leaching.Systemic toxicity of leached metals is not seen with the use of new or rebonded brackets as the amount of ions leached is much below the daily dietary or environmental exposure. Moreover, the ion release is not instantaneous but over a protracted period. The allergic reaction may occur during the first week of the initial bonding of brackets and may rarely occur if a few brackets are recycled and rebonded during the treatment period. This study tried to analyze the view of Messer et al [16] that a number of cellular functions are altered in response to ions released. The cytotoxic response to leachates may be elicited at levels lower than those for systemic toxicity. Belcastro *et al*[17] were also of the view that low ion emissions do not totally exclude cytotoxic problems.Cell vitality is readily quantified by determining the neutral red assay. Tomakidi *et al*[18] did not report any cytotoxicity from as supplied orthodontic materials on cultured human gingival keratinocytes by neutral red and trypan blue assay. The present study is in agreement to their findings and goes ahead to quantify and determine the cytotoxic effect of leachates at various simulated intraoral pH and exposure times.

CONCLUSION

Within the limitations and the scope of the study the following conclusions can be derived.

- Systemic toxicity has been ruled out.
- A high initial rate of release of ions.
- Prolonged exposure of the brackets to low pH increases the ion release.
- Oral hygiene measures have to be strctly followed to avoid prolonged exposure of brackets to low pH.
- Routine exposure to alkaline pH in mouthwashes should be followed by plain water rinses to prevent prolonged exposure to high pH.
- The in vitro cytotoxic response was highest at pH 4.0 and least at pH 6.8.

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Saraswati Dental College, Lucknow