Effects of Recasting on the Biocompatibility of a Ni-Cr Alloy

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Objective: *To evaluate the effects of recasting on the biocompatibility of a commercially available Ni-Cr alloy.*
Methods: *The alloy tested was cast and subsequently recast four more times. For each cast condition, 24 disk shaped specimens were fabricated (5 mm in diameter, 0.5 mm in thickness). All the recasting was performed without adding new alloy. After the first cast and following each recast, the surface composition and microstructure of the alloy were determined using an X-ray fluorescence spectrometer and optical microscope, respectively. The in vitro cytotoxicity and in vivo mucous irritation potential of the cast and recast Ni-Cr alloy were investigated. The results were statistically analysed at the significance level of 0.05.*
Results: *Recasting neither yielded to cytotoxicity or to changes in the surface composition of the Ni-Cr alloy tested. However, an increase in impurities and porosity of the surface structure was observed with recasting. Also, the segregation of the impurities to grain boundaries was evident after multiple castings. After the fourth recast, the alloys showed significantly greater mucosal irritation than the control.*
Conclusion: *After fourth recast, the alloy of this type may contribute to mucosal inflammation. Furthermore, there is a need for diverse methods addressing different biological endpoints for the evaluation of dental alloys.*

Key words: *recasting, cytotoxicity, membrane irritation, X-ray fluorescence spectrometry, Ni-Cr alloy*

All-ceramic restorations are presently one of the important topics in restorative dentistry[1]. In the category for crown and bridges, hybrid composite resins and ceramics, such as zirconia, are popular materials used without an alloy framework. The advantages of using all-ceramic restorations are highly aesthetic especially in the gingival area. However, there are some problems for all-ceramic restorations in delamination of laminating material. Chipping was more frequent for all-ceramic implant-supported single crowns than the metal-ceramics[2]. If the reasons for the vulnerability of all-ceramic crowns remain unknown, implants with all-ceramic single crowns should generally be recommended with care. Thus, although all-ceramic materials are more and more popular, using a metal framework is still recommended in certain cases.

The cost of precious alloys is getting higher and higher in the dental market and non-precious alloys are being widely used in conjunction with metal frameworks or cast restorations in dental practice[3,4]. The advantage of these alloys include relative low cost, compatible thermal expansion coefficient with the ceramics, and acceptable mechanical and tribological properties[5–7]. As a matter of fact, the demand for non-precious alloys has now resulted in substantial increases in the price. With the increasing costs of the non-precious metal alloys, it would be economically

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advisable to reuse them, as it is the case when using the precious metal alloys[8]. Furthermore, recasting of the alloys might present potential benefits against the over-exploitation of natural resources and metal pollution. Considering the physical and mechanical properties, it has been reported that at least four generations of non-precious alloys can be used[8–10]. However, in fact recasting may have a detrimental effect on the biocompatibility of the non-precious alloys. It has been documented that recasting significantly increased the cytotoxicity of the non-precious alloys[11]. Moreover, Nickel-Chrome (Ni-Cr) alloy was found to elevate genotoxicity when they were reused[12]. On the contrary, cytotoxicity of the recast Ni-Cr alloy was found to be similar to the Ni-Cr alloy ingots[13]. The conflicting findings suggest that this aspect needs further investigation.

In addition to the cytotoxicity and genotoxicity tests, the mucous membrane irritation test is also a useful test for materials that come into contact with the oral mucous membrane[14]. Given that using removable or fixed appliances in small animals is difficult, expensive and time-consuming[15], the mucous membrane irritation test (by means of placing dental materials into hamster cheek pouches for biological evaluation) has been recommended by the Council on Dental Materials and Devices[16]. Although to the best knowledge of the authors, no published study exists that examined the effects of recasting on mucous membrane irritation of the non-precious alloys, it is claimed by the manufacturers that recasting of Ni-Cr alloy increased the amount of Ni release and its cytotoxicity level. Thus, it can be anticipated that recasting of Ni-Cr alloy leads to an increase in mucous membrane irritation. Therefore, the objective of this study was to investigate the effects of multiple recasting on the biocompatibility of a Ni-Cr alloy using an *in vitro* method for cytotoxicity and *in vivo* method for mucous membrane irritation. The hypotheses tested were: *1)* recasting would affect the cytotoxicity of the Ni-Cr alloy; *2)* recasting would create mucous membrane irritation of the Ni-Cr alloy.

Materials and methods

A commercial Ni-Cr alloy (Uni Metal VH; Shofu), containing 77% Ni, 14% Cr, 4.7% Mo, 2% Al, and 1.8% Be (derived information from the manufacturer), was used in this study. One hundred and twenty disk-shaped (5 mm in diameter, 0.5 mm in thickness) specimens were prepared and randomly divided into 5 groups (n = 24 per group):

- First cast metal (group A1).
- Second cast metal (group A2).
- Third cast metal (group A3).
- Fourth cast metal (group A4).
- Fifth cast metal (group A5).

The recasting (groups A2–A5) was performed without adding new alloy. The surface composition and microstructure of all specimens were analysed using an X-ray fluorescence spectrometer (XRF) (n = 6) and optical microscope (n = 4). For each group, 4 specimens were randomly selected for the cytotoxicity test and the remaining 10 specimens for the mucous membrane irritation test.

Specimen preparation

Fifteen cylindrical patterns (6 mm in diameter, 20 mm in length) were fabricated using a conventional lost-wax technique with an induction-casting machine (Argoncaster-C; Shofu). The casting method was based on vacuum suction and pressure under argon gas where standard dental laboratory procedures were followed. The casting procedure was repeated for an additional 4 times with the metal from the previous casting, thereby producing the second, third, fourth, and fifth cast specimens (each n = 3 patterns). After casting, the investment material was removed and the patterns were cleaned with 50 μm aluminium oxide powder. Each pattern was cut into 8 specimens (5 mm in diameter, 0.5 mm in thickness) using a laser-cutting machine (DN-450/600; Shenzhen Di-energy laser Equipment). Then, the specimens were finished and polished using stone burs.

The polished specimens were cleaned in an ultrasonic cleaner for 30 min each in distilled water and acetone. Before use, the specimens were autoclaved at 121°C for 30 min.

Surface microstructure and composition determination

Six specimens from each group were polished using a series of metallographic abrasives through 0.05 μm Al_2O_3 slurry until a mirror surface was achieved. The specimen surfaces were examined using an optical microscope (XJZ 6A; Jiangnan Optics). The specimens were then etched by aqua regia and the etched surfaces were checked using the optical microscope. Representative images were captured at the magnification of 60×.

The surface composition of the specimens and the alloy ingot (each n = 4) was determined by XRF analysis using a Magix PW2424 spectrometer (Philips) with a rhodium X-ray tube, 2.4 kW generator and five-position crystal charger. The measurement was performed on the whole specimen surface and the obtained

spectra were further analysed using an IQ+ analytical software (Philips).

Cytotoxicity test

Cells used in the cytotoxicity test were mouse fibroblasts, clone 929 of strain L (Cell Bank, Chinese Academy of Sciences, Shanghai Institute of Cell Biology). The L929 cells were grown in a cell culture medium (RPMI 1640; Hangzhou Sijiqing Biological Engineering Materials) containing 10% foetal bovine serum under standard cell culture conditions (37°C, 100% humidity, 95% air, 5% CO_2). Cells were seeded into 96-well plates at 3×10^4 cells/well and incubated for 24 hr to allow adhesion. In the meantime, 4 specimens of each group were randomly selected and each incubated in 10 ml cell culture medium for 24 hr to obtain elute. The cell culture medium without metal alloys was used as a control. Then 0.1 ml elute was added to the cell culture wells. The cells were incubated for 1 day, 3, 5, and 7 days, respectively. The culture medium was changed every 3 days. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay according to the manufacturer's instructions. Briefly, 20 μl MTT (5 mg/ml) was added and the cells were incubated for 4 hr. After the incubation time, 150 μl Dimethyl sulfoxide (Xi'an Chemical Solution) was added into each well. After 4 min, the reaction products were transferred to a 96-well plate and the absorbance was measured at 492 nm using a spectrophotometer (Thermo Multiskan MK3; Thermo Fisher Scientific). The cell viability was calculated as the percentage of the control group.

Hamster mucous membrane irritation test

The protocol for the hamster mucous membrane irritation test was approved by the Ethics Committee, School and hospital of Stomatology, Fujian Medical University. In this test, the positive control material used was polyvinyl chloride (PVC) and the negative control material was gutta percha (GP). The control materials were fabricated in the same size as the test specimens (5 mm in diameter, 0.5 mm in thickness). The edges of all the specimens were smoothened. The control specimens were sterilized by immersing in 0.5% iodophor solution (Jiangxi Medical Technology) for 12 hr followed by ultraviolet radiation for 2 hr. Sixty Golden Syrian Hamsters (30 male and 30 female) were acclimated for 7 days and weighed between 100 and 120 g at the start of the study. The cheek pouches were flushed with saline and examined for any abnormalities before treatment. Ten specimens of each group were implanted into the right cheek pouch of 10 hamsters (5 male and 5 female hamsters, 10 sites/treatment) under Nembutal anaesthesia (40 mg/kg body weight). For the control groups, the GP specimens were placed into the right cheek pouch while the PVC specimens into the left cheek pouch of 10 hamsters (5 male and 5 female hamsters, 10 sites/treatment) using the "pouch in a pouch" technique. This technique was described in detail elsewhere[15]. Briefly, an inner pouch (1 cm deep) was created in the hamster cheek pouch. The specimens were placed into the bottom of this inner pouch. Each inner pouch was then closed with a double row of sutures. Fourteen days after implantation, all animals were killed by Nembutal overdose (200 mg/kg) and necropsied. The cheek pouch was removed and examined macroscopically. The tissue was then fixed in 10% neutral buffered formalin, processed in paraffin, and stained with hematoxylin and eosin (H&E). The histomorphological evaluation was performed for the presence of aberrations in the epithelial, connective tissue and muscular layer. One of the authors (ZCY) performed the microscopic evaluation on coded slides (blind procedure) of all the specimens. All adverse tissue responses observed were graded according to the following scores[14]: 1 = minimal; 2 = slight; 3 = moderate; and 4 = marked. If the tissue appeared normal, it was assigned a total severity score of zero.

Statistical analysis

All the results were statically analysed by the SPSS statistical software (SPSS for Windows 15.0) at the significance level of 0.05. The assumption of the approximate normal distribution of the surface composition and cell viability data were investigated by the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was used to investigate the effects of recasting procedure on the surface composition and cytotoxicity of the Ni-Cr alloy. For the scores of histopathological features, the intra-examiner consistency was evaluated by the Cohen's Kappa coefficient. The data were further analysed using Kruskal-Wallis, followed by Mann-Whitney U tests for all histopathological features.

Results

The results of the Kolmogorov-Smirnov test were non-significant, indicating that the assumption of the normal distribution of the data of cell viability and surface composition is not violated.

The result of XRF analysis of the specimen surfaces is shown in Table 1 and Fig 1. The concentration of the major elements remained stable after multiple casts.

Table 1 Mean surface composition (wt%) for the alloys

	Ni	Cr	Mo	Al	Be	Co	Ti	Si	Others
Ingot	77.18	13.44	4.63	1.88	1.80	0.28	0.30	0.26	0.23
First cast	77.77	13.42	4.77	1.65	1.80	0.22	0.22	0.07	0.08
Second cast	77.51	13.45	4.81	1.74	1.80	0.25	0.24	0.10	0.10
Third cast	77.30	13.54	4.81	1.81	1.80	0.23	0.26	0.12	0.13
Fourth cast	77.50	13.44	4.75	1.73	1.80	0.29	0.26	0.14	0.09
Fifth cast	77.22	13.64	4.78	1.79	1.80	0.28	0.25	0.14	0.10

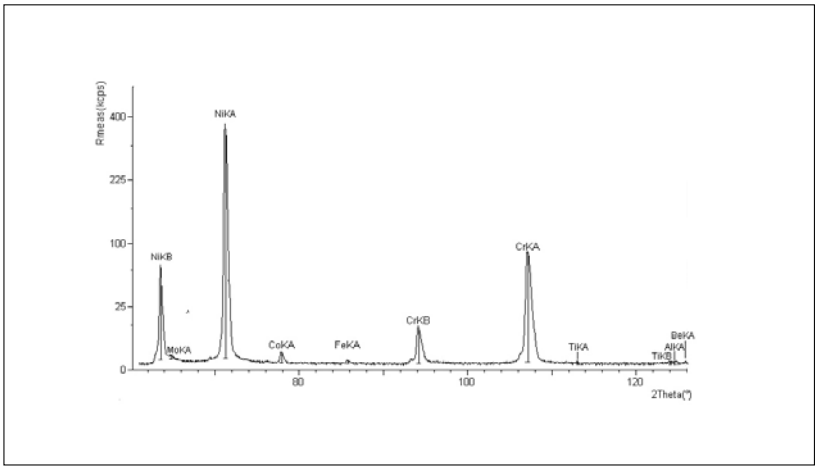

Fig 1 Representative XRF spectrum of the Ni-Cr alloys tested.

The concentration of silicone slightly increased after recasting. There is no significant difference in surface composition between any cast alloy and the alloy ingot.

Fig 2 shows representative surface microstructures of the specimens. A notable increase in surface porosity was found with recasting. Fig 3 shows representative micrographs of etched alloys. Diversity in grain shape was found most noticeably after the fifth cast. An increase in impurities and grain size were found with recasting. In addition, the segregation of the impurities to grain boundaries was observed in later casts, especially in groups A4 and A5.

The cell viability (% relative to control) of all the groups is illustrated in Fig 4. Casting, up to 5 times, had no effect on the cell viability of the alloy at all the tested time.

For the mucous membrane irritation tests, all the hamsters tolerated the procedure well and survived after 14 days wearing the specimens. The retention rate of the specimens was 88% (53/60) at 14 days. No pits were found on the specimens retrieved from the hamsters. Necrotic inflamed mucosa was observed in 1 pouch in group A5 while mucosal hyperaemia was found in 2 pouches in the same group. Histologically, the normal hamster cheek pouch consists of a keratinised stratified squamous epithelium overlying a lamina propria and a layer of skeletal muscle. The intra-examiner consistency for histological features was considered almost perfect agreement (Kappa value range: 0.82–1.00). Representative histological images of hamster cheek pouches from all the groups are exhibited in Fig 5. The adverse tissue responses are demonstrated in Table 2. Scores for the negative control group were significantly different from those for the positive control group. The treated sites of groups A1 to A4 were not significantly different from the negative control group. No statistically significant difference was found between the treated sites of group A5 and positive control group for all adverse tissue responses.

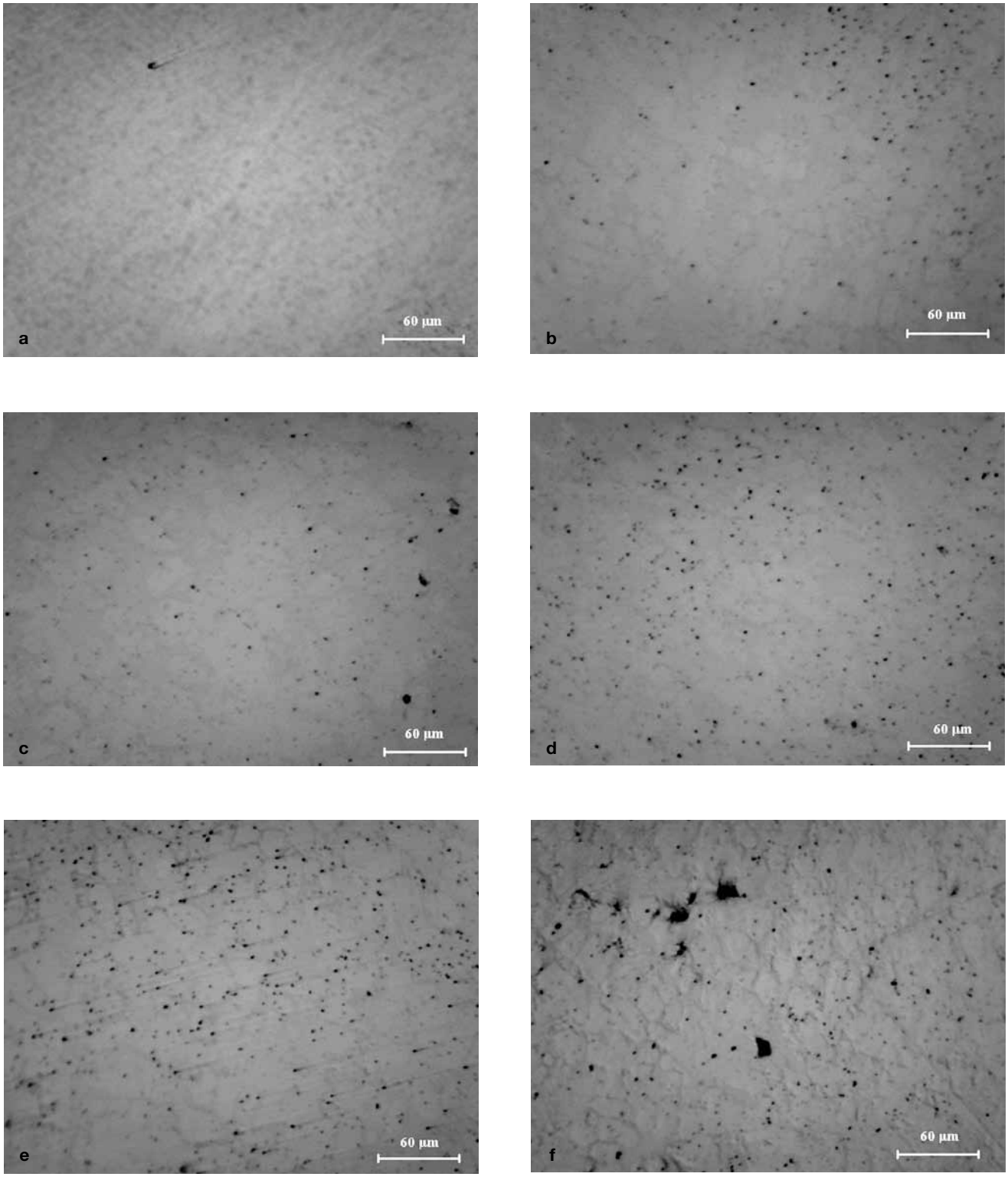

Fig 2 Representative micrographs of the Ni-Cr alloy before etching (× 60): a) alloy ingot; b) first cast alloy; c) second cast alloy; d) third cast alloy; e) fourth cast alloy; f) fifth cast alloy.

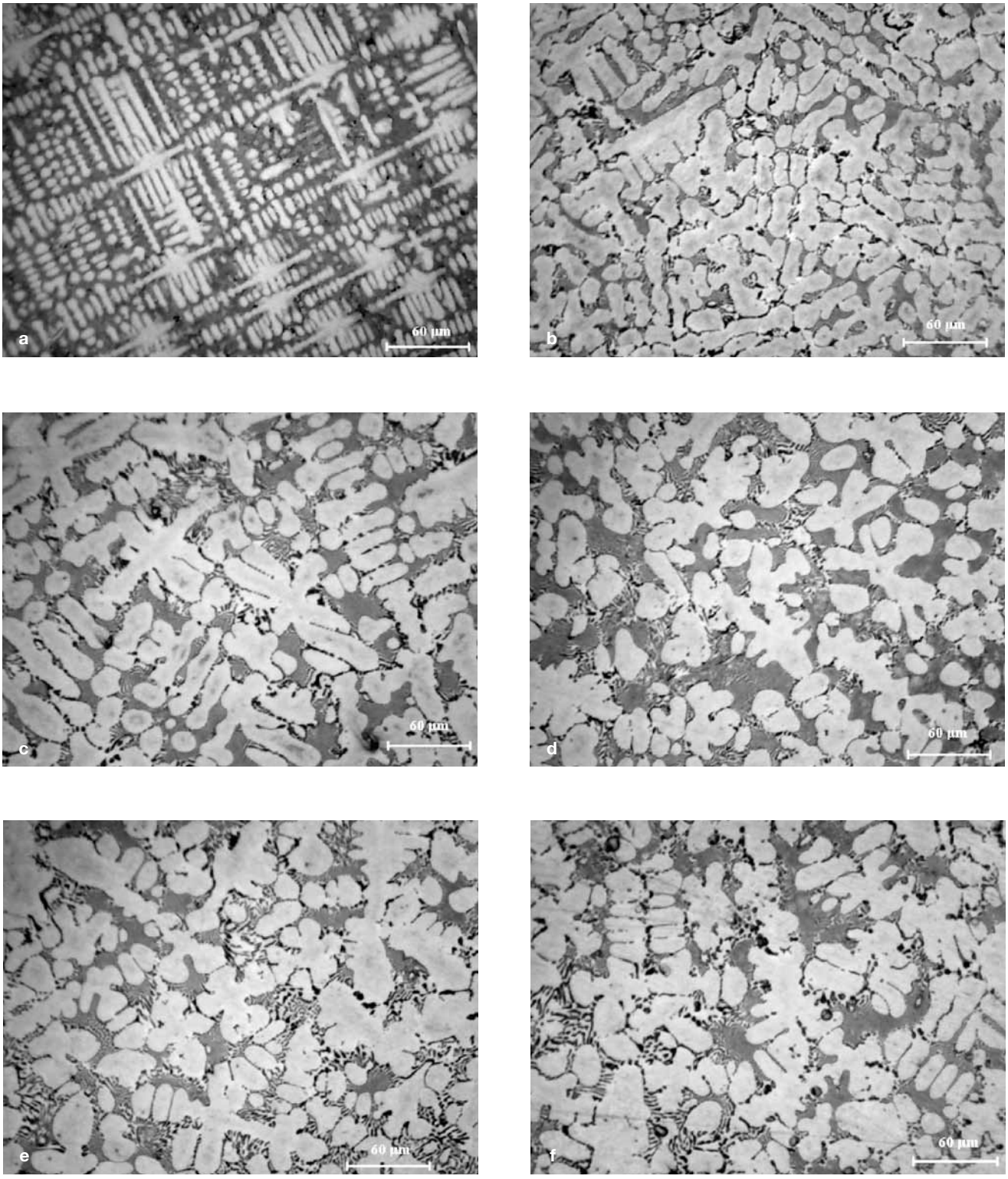

Fig 3 Representative micrographs of the Ni-Cr alloy after etching (× 60): a) alloy ingot; b) first cast alloy; c) second cast alloy; d) third cast alloy; e) fourth cast alloy; f) fifth cast alloy. The black spots in the micrographs represent the impurities of the alloy.

Discussion

Based on the results of this study, the hypothesis that multiple recasting can affect the cytotoxicity of the Ni-Cr alloy was rejected. The hypothesis that multiple recasting has an effect on the mucous membrane irritation of the Ni-Cr alloy was accepted.

For both economic and environmental reasons, recycling of non-precious alloys is desirable, preferably without degradation of the alloy properties. Given that the restorations made from the recycled non-precious alloys might be placed in contact with oral tissues for a certain period of time, it is important to get a better understanding of the possible effects of recasting on the biocompatibility of the metal alloy. The Ni-Cr alloy Uni-VH is readily available in various developing countries, which is the reason for being chosen in the present study.

L929 mouse fibroblasts were chosen in the cytotoxicity test on the basis of its acceptance in previous studies[11,17]. In contrast to a previous study[11], even after the fifth cast, the cytotoxicy of the Ni-Cr alloy remained at the same level (not cytotoxic) as the first cast alloy, indicating that recasting has no effect on the cytotoxicity of the test alloy. Possible explanation could be the differences in the alloy used. However, a further factor to consider might be the different modality of the study (culture fibroblast cells with specimens versus culture fibroblast cells with the elute of the specimens). The metal elements in elute may be either oxidised to ions and co-ordinated with complexes, or precipitated in the solution and are thus become less cytotoxic than respective elements dissolved from the alloy[18].

In addition to the cytotoxicity, the irritation potential of cast alloys was investigated in a hamster cheek pouch irritation model. This *in vivo* test modality was chosen because of the great sensitivity of the buccal mucosa of the hamster cheek pouch[19]. In accordance with the previous study, the retention rate for the specimens using the "pouch-in-pouch" technique was excellent[14]. GP was selected as the negative control according to the recommendation of Council on Dental Materials and Devices and the current literature[16,17]. On the other hand, PVC was used as the positive control analogous to the previous studies[14,17]. For the positive control group using PVC, histomorphological observations were noted with significantly greater severity than those noted for the negative control using GP, suggesting a proper selection of the controls. For the groups exposed to group A1 to A4 specimens, the tissue response was negligible and similar to that noted for the negative controls. However, in cheek pouches exposed to group A5 specimens, more significant histomorphological alterations were observed, demonstrating effects of the fifth casting on mucosal irritation potential of the tested alloy. The current study fails to provide any explanation of this phenomenon. However, it is noteworthy that an increase in impurities and their segregation to the grain boundaries were found with increased number of recasting. The present study did not determine what kinds of impurities were formed during multiple recasting. However, based on the compositional changes of silicone of the alloys, the impurities formed may be related to the contamination from investment and polishing procedure. Moreover, the surface porosity of alloys tested was found to increase after multiple recasting. These structural features are likely to induce an increase in the dissolution rate of metals, which might subsequently increase the mucosal irritation potential of the alloy[20]. The slight reduction in cell viability of group A5 specimens may add some support to this hypothesis. However, further studies are needed to clarify this issue.

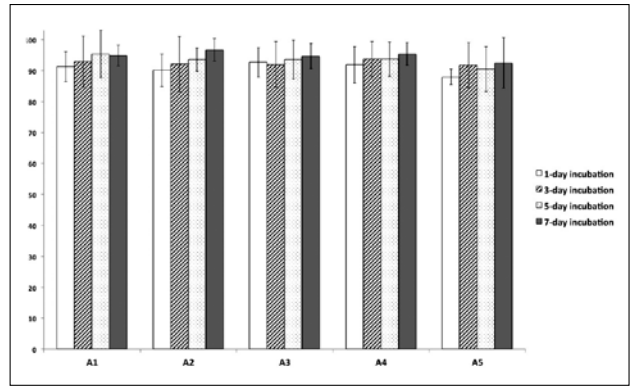

Fig 4 Means and standard deviations (error bars) for cell vitality (% relative to control) of cast alloys.

When the alloy was recast up to 4 times, the results of irritation tests correlated well with the cytotoxicity tests. Similar findings were obtained in another study[21]. On the contrary, this was not the case after the fifth cast. This phenomenon highlights a need for diverse methods addressing different biological endpoints for the evaluation of dental products[22].

It is also important to note some limitations in the current study. Although the surface composition did not change during the five recastings, the phase and impurities on the surface might change after the recasting procedure. This may give hints for possible causes of the detrimental changes in mucosal irritation from multiple

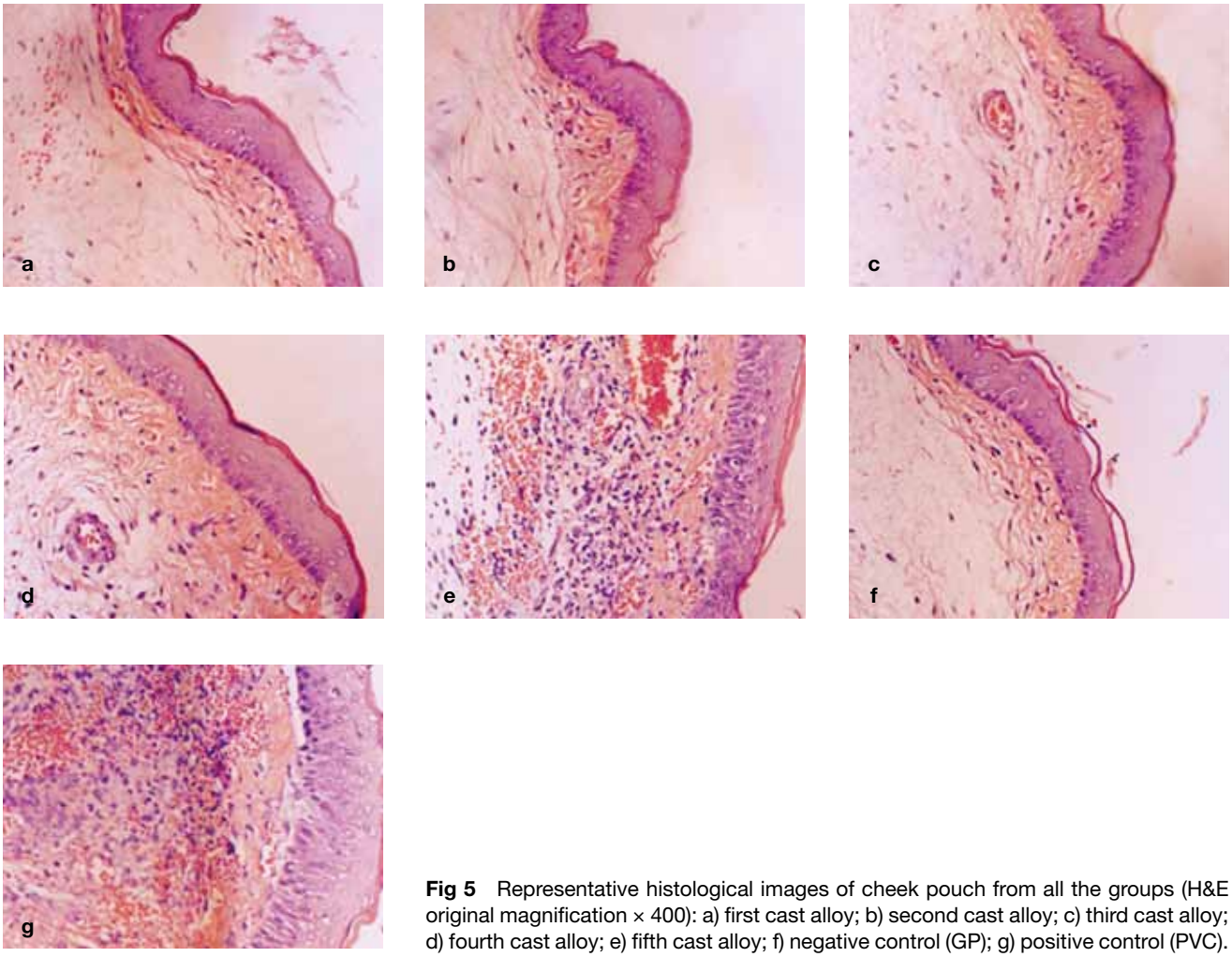

Fig 5 Representative histological images of cheek pouch from all the groups (H&E original magnification × 400): a) first cast alloy; b) second cast alloy; c) third cast alloy; d) fourth cast alloy; e) fifth cast alloy; f) negative control (GP); g) positive control (PVC).

Table 2 Mean scores for histopathological features for all the groups

Histopathological changes	Mean scores for histopathological features of each group						
	Group A1	Group A2	Group A3	Group A4	Group A5	Negative control (GP)	Positive control (PVC)
Epithelial hyperplasia	0.1	0.2	0.0	0.4	1.6	0.0	1.9
Epithelial atrophy	0.0	0.0	0.0	0.0	0.7	0.0	0.5
Focal epithelial down-growth	0.0	0.0	0.1	0.1	1.7	0.1	2.0
Acanthosis	0.0	0.0	0.0	0.0	0.9	0.0	0.9
Submucosal subacute inflammation	0.0	0.0	0.0	0.1	1.2	0.0	1.7
Submucosal fibrosis	0.0	0.0	0.0	0.0	0.3	0.0	0.4
Mucosal ulceration	0.0	0.0	0.0	0.0	0.8	0.0	1.1
Necrotic debri	0.0	0.0	0.0	0.0	1.5	0.0	1.5

recasts. Further studies are needed to verify this issue. Moreover, given the Ni-Cr alloys are a heterogeneous group, the current findings must be interpreted with caution.

The MTT experiment was carried out according to the National Testing Protocol for Dental Materials and published studies[21,23]. Moreover, in agreement of the previous study[17], our pilot study shows that both the direct contact (alloy surface-fibroblasts) and elute methods adopted led to the same results under the present experimental setting. Thus in order to facilitate the testing procedure, the elute of alloys was adopted to evaluate the cytotoxicity of the Ni-Cr alloy.

The present study adopted XRF, optical microscopy, cytotoxicity test, and histological analysis of the membrane irritation test to evaluate the effects of recasting on biocompatibility of the dental alloy. Due to the technical limitation, the accelerated aging, grain crystal structure analysis, and immunohistochemistry were not employed in the present study. In future studies, more fundamental scientific analysis could be considered.

This study raises concern that multiple recasting of the dental alloy away from ideal may yield to mucosal inflammation. When recasting needs to be contemplated, dental technicians could safely recast the alloy tested up to three times.

Conclusion

Under the limitation of the current study, it was concluded that recasting of Ni-Cr alloy had no effects on its cytotoxicity level. However, recasting a fourth time had a detrimental effect on the mucous irritation potential.

Acknowledgements

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