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In vitro cytotoxicity test of stainless steel arch bar in calf kidney cell culture

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ABSTRACT: The paper reports the quantification of insults due to exposure of stainless steel (SS) arch bar used for maxillomandibular fixation in calf kidney cell culture. The insults due to exposure of the new SS arch wire in the culture system showed no statistically significant difference in the % kidney cell death compared to that in the control group with no metallic material ($P < 0.05$). On the other hand, a significant difference was recorded between the % kidney cell death in the reused and control groups. Cell death due to the effect of ageing prior to use of SS arch wire on the culture system was not significantly different from the result obtained by the use of new wire. The results infer that new arch wire showed no cellular toxicity, while the reused samples were toxic.
Key words: Maxillomandibular fixation; Culture system; Arch bar; Toxicity

INTRODUCTION

In situ degradation of metal-alloy implants may result from electrochemical dissolution phenomena, wear, or a synergetic combination of the two (Tai *et al*., 1992).

Release of metallic elements from almost all types of alloys has been documented (Brune, 1986). The cellular effects of metallic elements do not seem to depend on the type of the cell, which points towards a common mechanism via which cells are affected by metallic compounds (Merritt *et al.,* 1984). Ions released from metals have been thought to be associated with local immune dysfunction, inflammation and tissue cell death. The cytotoxicity of nickel based and titanium implants have been widely studied, especially in the case of nickel, which is a toxic agent and allergen (Costa *et al.,* 1998).

The cytotoxic effects of the corrosion products of AISI (American Iron and Steel Institute) 304 stainless steel and manganese stainless steel (low-nickel SS) brackets on L929 cell culture were compared by two assays, crystal violet, to evaluate cell viability, and MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide), for cell metabolism and proliferation. None of the bracket extracts altered L929 cell viability or morphology, the AISI 304 SS-bracket extracts decreased cellular metabolism slightly. The low-nickel SS presents better *in vitro* biocompatibility than AISI 304 SS brackets (Assad *et al.,* 1998).

Wenyi *et al*. (1999) concluded that the maximum amount of nickel released from three types of nickel-containing arch wires into synthetic saliva *in vitro* was 700 times lower than the concentrations necessary to elicit cytotoxic reactions in human peripheral blood mononuclear cells derived from nickel-sensitive and nickel-nonsensitive individuals.

Subcutaneous implantation of orthodontic SS wire (elemental composition 68% Fe, 19% Cr, 13% Ni) into flanks of rabbits, using polytetrafluoroethylene as control showed that the only local response was the presence of a surrounding collagen capsule containing fibroblasts and fibrocytes (Gjerdet *et al.,* 1987).

In vitro toxicity of arch wires have been investigated using fibroblasts (Ryhanen, 1997; Wever *et al*., 1997; Rose *et al*., 1998), osteoblasts (Ryhanen, 1997; Wever *et al*., 1997), smooth muscle cells (Shih, 2000; Shih, 2001), and cortical cells (Alexis and Doug, 2004). Also, Lori *et al.* (2009) implicated arch bar for releasing the corrosion products, cobalt, iron, manganese, nickel and chromium when immersed in pseudo- and actual-biofluids.

Some of the deleterious corrosion products of SS implants and restorations are mainly found in kidneys, since these elements are eliminated via urine (Juan *et al.,* 2008). A pragmatic evaluation of metal biocompatibility for implantology purpose is an *in vitro* cytotoxic test on kidney cell culture.

The work aimed to study the *in vitro* cytotoxicity of metal ions released from SS arch bar on calf kidney cell culture. The experiment will further examine the effect of ageing prior to use of SS arch wire on the cytotoxic effects.

MATERIALS AND METHODS

Materials and Reagents

All glass wares for the experiment were sterilised by steam autoclaving at 121^{o}C for 15 minutes. Reagents used for the preparations of all solutions were of analytical grade.

Materials and reagents used for the study are:

1. Hank's balanced salt solution (HBSS) – The composition is presented in Table 1.
2. Phosphate bovine saline (PBS) solution – The PBS solution was prepared and adjusted to pH 7.4 by using 0.5M Na_2HPO_4 and 0.5M NaH_2PO_4 buffer solutions and drops of 1% HCl solution. The composition of PBS is presented in Table 2.
3. Trypsin Hank's balanced salt (THBS) solution – A 1% (w/v) trypsin (Oxoid Ltd., Hampshire, England) powder was solubilised in 200 mL Hank's balanced salt (HBS) solution to obtain trypsin Hank's balanced salt (THBS) solution.
4. Fetal calf serum (Sigma-Aldrich Co. USA)
5. Minimum essential medium, MEM without Earle's salt (Sigma- Aldrich Co. USA)
6. Streptomycin (Juhel Nig. Ltd., Nigeria)
7. Ampicillin (Juhel Nig. Ltd., Nigeria)
8. Trypan blue (Sigma-Aldrich Co. USA)

All the solutions prepared for the culture were filter sterilised with a membrane filter incorporated into the Millipore filtration apparatus.

Methods

Kidneys from foetus 3 – 5 months gestation were collected from a slaughtered cow immediately after slaughter at the Zango abattoir, Zaria, Nigeria in August 2008. The kidneys were collected into a 250 mL beaker containing cold Hank's balanced salt solution (HBSS). The kidneys were then placed in a Petri dish where the connective tissues, capsule and pelvis were removed; the remainder of the kidneys was then minced finely with paired scalpels.

The minced tissue was then transferred from the Petri dish to a trypsinizing flask and rinsed with 5 mL of phosphate bovine saline (PBS) solution. The minced tissue was further washed with PBS and the supernatant decanted; this was repeated until the supernatant was clear. The washing process was to ensure removal of the red blood cells and some of the connective tissues that were left.

After the tissue has settled, the wash fluid was removed and 50 mL of trypsin Hank's balanced salt (THBS) solution was added in order to remove cells from the culture substrate. The mixture was stirred on a magnetic stirrer at 1200 g for 30 minutes. By using a sieve cloth, the filtrate obtained was centrifuged by using Uniscope laboratory centrifuge (Model SM 800B, Surgifriend Medicals, Essex, England) at 2000 g for 5 minutes. The supernatant obtained was decanted and the cells were harvested.

To 5% fetal calf serum (Sigma-Aldrich Co. USA) and 95% minimum essential medium, MEM (Sigma-Aldrich Co. USA) made up to 50 mL was added a mixture containing 0.1 g streptomycin, 0.1 g ampicillin dissolved in 1 mL PBS. This composition was added to the 250 mL beaker containing the harvested cells. The serum containing medium was added to the cell suspension to inhibit further tryptic activity, which may damage cells. The cell media of 1.5 mL volume were then dispensed into each well plate and a new - as received from the manufacturer - 1.0 ± 0.1 cm SS arch wire (elemental composition 60.05% Fe, 18.35% Cr, 18.62% Ni, 2.94% Mn and 0.03% Co) inserted in a slant position to rest on the well plate, so as to avoid possible cell damage due to physical disruption of the cells. The set up was then incubated in a humidified 5 per cent CO_2 incubator at 37^{o}C for 24 hours. The set up was also carried out for reused SS arch wire. Three independent experiments were carried out in triplicate for each of the experimental group. Control group was obtained by using cell wells having no SS arch wire.

After 24 hours, 0.1 mL aliquots of the cell culture from each of the well plates was collected with a micropipette and transferred into a 12 x 75 mm test tube, 0.05 mL of a 0.2% trypan blue (Sigma-Aldrich Co. USA) dissolved in PBS was added and mixed thoroughly, allowing to stand for 5 min. The suspension was then centrifuged at 1500 g for 5 min and the supernatant fluid was removed. The cover slip of the hemocytometer used

for counting the cells were put in place, and a Pasteur pipette was used to transfer a small amount of trypan blue-cell suspension to both chambers of the hemocytometer. The stained and unstained cells were counted separately on a hemocytometer using an inverted microscope (CK Olympus, Tokyo, Japan). Cells unable to extrude trypan blue were considered nonviable, whereas unstained cells were considered viable. The number of stained cells, expressed as a percentage of total cells, was used as a measure of cell death. Cytotoxicity was quantified relative to control cells having no SS arch wire. Digital images of the stained and unstained cells were taken with a Diagnostic Instruments spot camera at a magnification of X 400.

RESULTS AND DISCUSSION

Data points are average values of % kidney cell death obtained from three independent experiments run in triplicate and is presented in Figure 4. Statistical calculations were performed by a two-way ANOVA, using the Duncan's post hoc test to assess the effects of immersing SS arch wire and ageing of the test material on the cytotoxic effect. Statistical significance was considered to be achieved at a probability of $P < 0.05$.

From the *in vitro* cytotoxicity of new as received and reused arch wires investigated in calf kidney cell culture to characterise the effect of ageing prior use on cellular toxicity. Results obtained after a 24 hour exposure in control cultures showed that there was very little trypan blue staining (Figure 1), indicating that most cells were alive; but the experimental settings in figures 2 and 3 had more stained cells.

Quantification of the insults due to exposure of the new SS arch wire in the culture system showed no statistically significant difference in the % kidney cell death compared to that in the control group ($P < 0.05$). On the other hand, a significant difference was recorded between the % kidney cell death in the reused and control groups (Figure 4). Cell death due to the effect of ageing prior use of SS arch wire on the culture system was not significantly different from the result obtained by the use of new wire (Figure 4).

The results imply that new arch wire showed no cellular toxicity, while the reused samples were toxic. Reused SS arch wire may pose a slightly increased risk of a reaction to host due to elevated leaching of metal ions from the surface. However, the fact that reused SS arch wire is toxic to kidney cell culture is not of great health concern regarding the reuse of SS arch wire. Wenyi *et al.* (1999) reported that the concentration of nickel ions released from arch wires into synthetic saliva was 700 times lower than the amount required to produce cytotoxic effects in human peripheral blood mononuclear cells. The present conclusion about SS arch wire is consistent with previous studies that Elgiloy (Elemental composition: Co 39%, Cr 21%, Fe 16%, Ni 14%, Mo 8%) and Unitek stainless steel arch wire (Elemental composition: Cr 18%, Fe 72%, Ni 8%, Mn 1%) were toxic (Rose *et al.,* 1998). Nickel, iron and chromium have been implicated for the toxicity of stainless steel arch wire, because each can enhance free radical production through a Fenton-like reaction (Torreilles, *et al.,* 1990) and each is known to be neurotoxic (Diaz-Mayans *et al.,* 1986; Evans *et al.,* 1995). The mechanism of the cell death due to exposure of SS arch wire to cell cultures is apoptosis, with nickel or chromium being responsible for stainless steel toxicity (Chen *et al.,* 2001; Kim *et al.*, 2002).

The toxicity of reused SS arch wire to kidney cell culture signals increased health risk to patients implanted with consecutively reused arch wires. Though the potentially toxic metal ions leached out are ubiquitous in diet, and ingested in levels far below the human threshold level that can educe health problems.

Table 1. Composition of Hank's Balanced Salt Solution

Salt	Concentration (gL^{-1})
$CaCl_2$	0.14
KCl	0.40
KH_2PO_4	0.06
$MgSO_4.7H_2O$	0.20
NaCl	8.00
$NaHCO_3$	0.35
$Na_2HPO_4.2H_2O$	0.06

Table 2. Composition of Phosphate Bovine Saline (PBS) Solution

Salt	Concentration (gL^{-1})
KCl	0.20
KH_2PO_4	0.25
NaCl	8.00
$Na_2HPO_4.2H_2O$	1.44

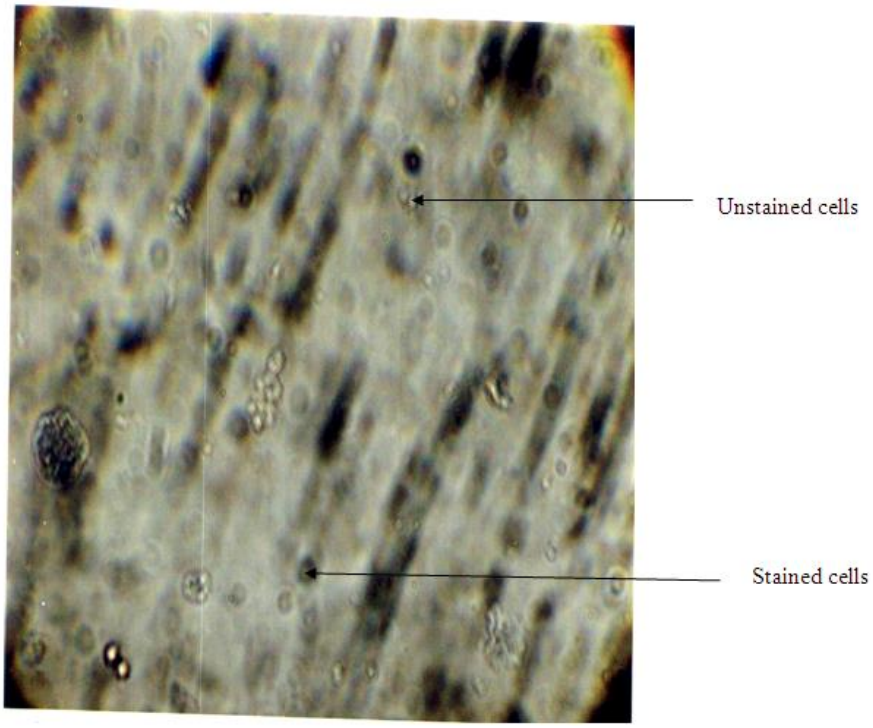


Figure 1. Trypan Blue Staining 24 hours after Incubation in Control Calf Kidney Cell Culture

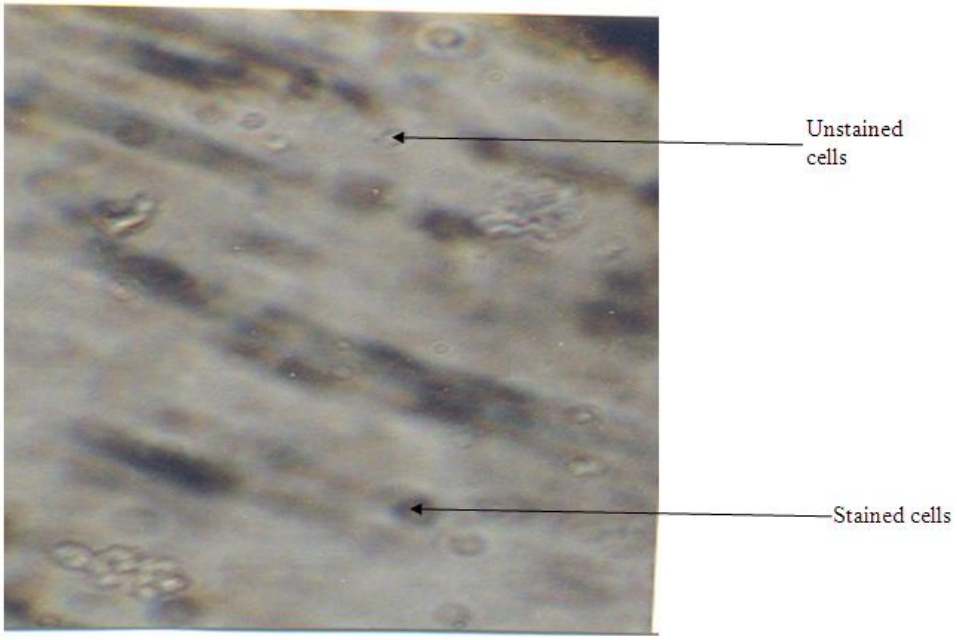


Figure 2. Trypan Blue Staining 24 hours after Incubation in Calf Kidney Cell Culture exposed to New Arch Bar

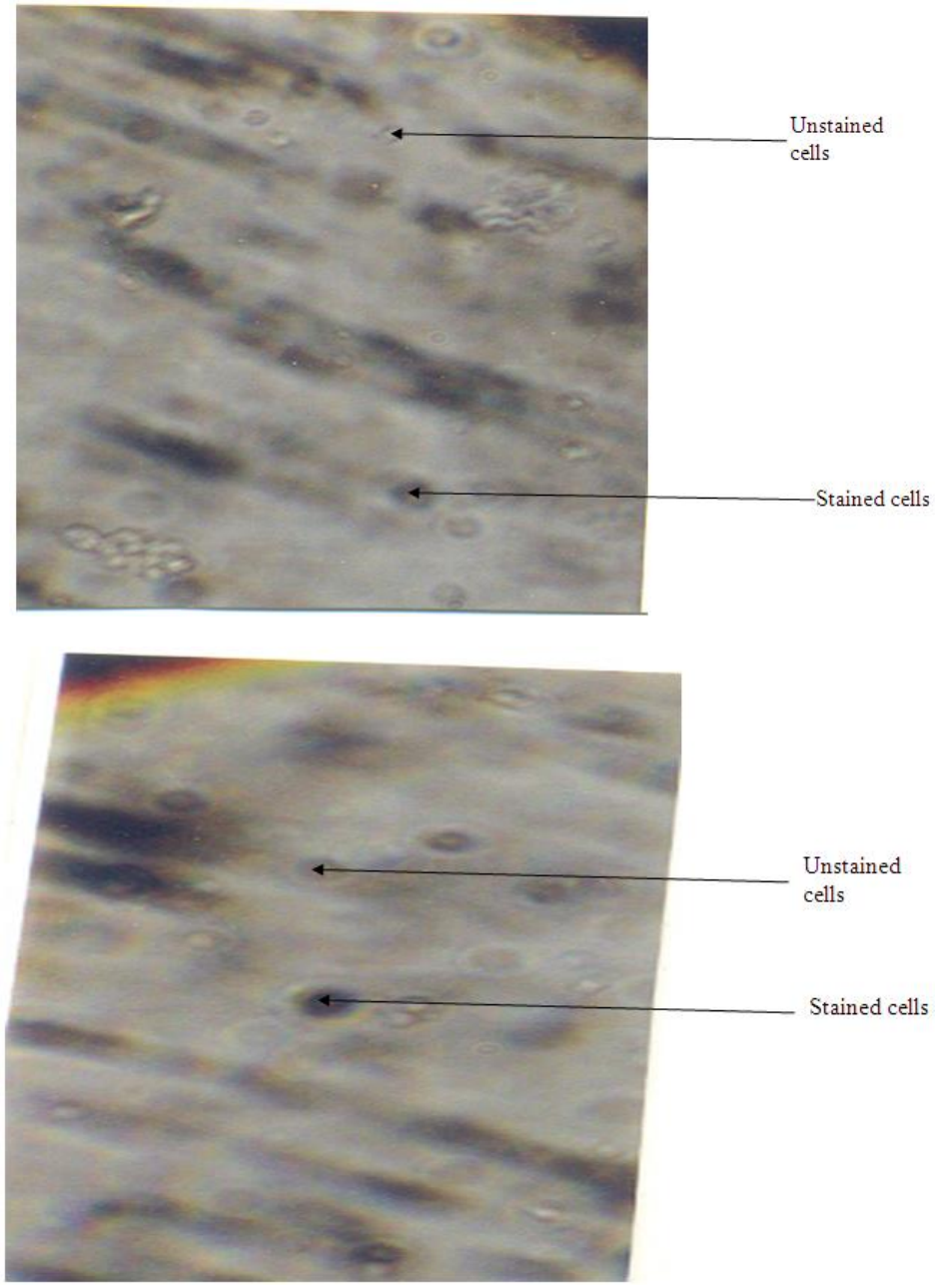


Figure 3. Trypan Blue Staining 24 hours after Incubation in Calf Kidney Cell Culture exposed to Reused Arch Bar

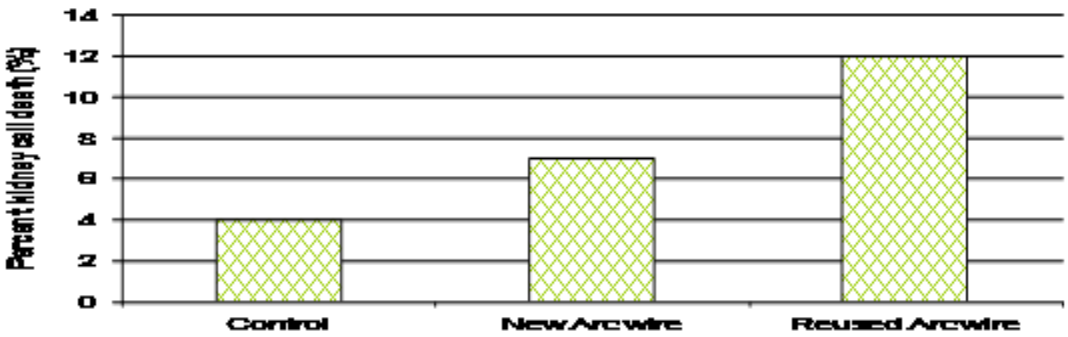


Figure 1. Percentage cell death due to toxicity or arch bar

In Figure 4 above, each column and bar shows the percentage cell death (mean ± standard deviation, n = 9).

CONCLUSION

The insults due to exposure of the new SS arch wire in the calf kidney cell culture system showed no statistically significant difference in the % kidney cell death compared to that in the control group. However, the % kidney cell death by exposure to the reused arch bar was significantly different from that by the control counterpart. Also, the cell death due to the effect of ageing prior to use of SS arch wire on the culture system was not significantly different from the result obtained by the use of new wire. The results infer that new arch wire showed no cellular toxicity, while the reused samples were toxic.

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