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‘Site of contact genotoxicity’ assessment for implants - potential use of single cell gel electrophoresis in biocompatibility testing of medical devices

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Abstract:	Toxicological risk assessment of medical devices requires genotoxicity assessment as per ISO 10993, Part 3, which is designed to address gene mutations, clastogenicity and/or aneugenicity endpoints. ‘Site of contact genotoxicity’ is a potential genotoxic risk especially for medical implants, that is currently not addressed in biocompatibility standards. We therefore performed initial pre-validation study on the use of alkaline single cell gel electrophoresis (comet assay) for detecting ‘site of contact genotoxicity’ of medical devices, using test items made of acrylic implants impregnated with ethyl methanesulphonate (EMS). Comet assay detected increased DNA migration at the site of implantation, but not in the liver. The same implants also failed to show any genotoxicity potentials, when tested on the standard test battery using Salmonella /microsome and chromosome aberration assays. The study suggested that some medical implants can cause ‘site of contact genotoxicity’, without producing systemic genotoxicity. In conclusion, comet assay will add new dimension to safety assessment of medical devices.

Highlights

- Pre-validation study on comet assay for detecting site of contact genotoxicity.
- Acrylic implants containing EMS (AF+EMS) was used as test item.
- AF+EMS implants showed DNA damage at site of contact using comet assay.
- But these implants were negative on Ames and chromosome aberration tests.
- Implants may cause ‘site of contact genotoxicity’ without systemic genotoxicity.
- Comet assay has potential use in medical device biocompatibility assessment.

‘Site of contact genotoxicity’ assessment for implants - potential use of single cell gel electrophoresis in biocompatibility testing of medical devices

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Abstract

Toxicological risk assessment of medical devices requires genotoxicity assessment as per ISO 10993, Part 3, which is designed to address gene mutations, clastogenicity and/or aneugenicity endpoints. 'Site of contact genotoxicity' is a potential genotoxic risk especially for medical implants, that is currently not addressed in biocompatibility standards. We therefore performed initial validation study on the use of alkaline single cell gel electrophoresis (comet assay) for detecting 'site of contact genotoxicity' of medical devices, using test items made of acrylic implants impregnated with ethyl methanesulphonate (EMS). Comet assay detected increased DNA migration at the site of implantation, but not in the liver. The same implants also failed to show any genotoxicity potentials, when tested on the standard test battery using *Salmonella*/microsome and chromosome aberration assays. The study suggested that some medical implants can cause 'site of contact genotoxicity', without producing systemic genotoxicity. In conclusion, comet assay will add new dimension to safety assessment of medical devices, and this assay can be added to the battery of genetic toxicology tests for evaluating biocompatibility of medical implants.

1. Introduction

Genotoxicity assessment of medical devices is one of the key endpoints to address for the safety of medical devices, especially for devices with prolonged contact (> 24 hours to 30 days) or permanent contact (> 30 days) with blood, bone, mucosa or other tissue, or any novel materials that have not previously been used in the field of medical devices (1,2). Strategy for evaluation of genotoxicity of medical devices is described in the International Organization for Standardization (ISO) 10993, Part 3 (3) and ISO 10993, Part 33 (4). The ISO 10993, Parts 3 and 33 refers to relevant Organization for Economic Co-operation and Development (OECD) guidelines for the conduct of the tests (3,4). The ISO 10993, Part 3 also mentions the use of single cell electrophoresis (comet assay) for genotoxicity evaluation of devices (3).

Implants are among the highest risk medical devices because they are invasive and are in continuous contact with the body tissues and fluids. They can cause local toxicity including irritation, sensitization, and local foreign body reactions in adjoining tissues. They are capable of inducing general and genetic toxicity by releasing chemicals into the systemic circulation and thereby causing toxicity elsewhere in the non-target part of body (5-11). Genotoxicity of implants are assessed by evaluating the extractable or leachable chemicals from the chemical characterization studies (12,13). The genotoxicity of these extractables and leachable chemicals are supported on available genetic toxicology information from reputable toxicology databases, structural activity relationship or threshold of toxicological concern (TTC) (14,15). If genotoxicity endpoint is not supportable based on chemical characterization, ISO 10993, Parts 1 and 3 recommends conducting genetic toxicology tests (1,3). An Ames test and either *in vitro* chromosome aberration (CAbs) assay or mouse lymphoma assay or *in vitro* micronucleus assay are usually recommended (3,4). These tests are conducted on extracts taken from the implant devices exposed to vehicles at up to 70°C for up to 72 h (16). During the extraction process, leaching chemicals are uniformly diluted with the entire volume of vehicle. The strength of chemicals present in the extracts are therefore not realistic representative of concentrations present locally in the microenvironment of the implants and high local concentrations can cause DNA damage. There will be some sort of fibrosis around all implants and therefore the concentration of leachables will be higher in the surrounding tissues compared to that of systemic circulation. We therefore propose that for implants, 'site of contact genotoxicity' testing is essential to establish their genetic toxicology potential. In such situations, application of comet assay will be a realistic and robust method of interest.

To address this issue, we fabricated custom implants made of acrylic filler (AF) containing a range of ethyl methanesulphonate (EMS) concentrations and implanted them in rats. We then studied the 'site of contact genotoxicity' at implant sites and concurrently in the liver using the alkaline comet assay. These custom implants were also subjected to standard battery of genetic toxicology tests as per ISO 10993, Part 3, *Salmonella* microsome assay (Ames test) and *in vitro* CAbs assay (3), to check if they would be identified as genotoxic.

2. Materials and Methods

2.1. Animals

A total of twenty four 8–10 weeks old Wistar rats (90-115 g) were purchased from the National Institute of Nutrition, Hyderabad, India and acclimatized under standard environmental conditions with 12 h light and dark cycles, humidity (40–70%) and temperature (22 ± 3 °C). The animals were fed with a standard pellet diet and water *ad libitum* as described in earlier studies from our laboratory (17,18). Further, the animals were randomly assigned to each group and all the procedures had been performed in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India and approval of the institutional animal ethics committee (IAEC) of GLR Laboratories Pvt Ltd (17,18).

2.2. Implantation study design

Subcutaneous implantation study was conducted in rats as per the study design given in Table 1. In brief, six implantation groups were included in this study: 3 doses of EMS in AF, one AF alone, one high density polyethylene (HDPE) as negative control and one commonly used implant, titanium (19). Each animal had two implants, and therefore a total of six implants were analyzed per group. An EMS treated group was also included to serve as positive control for liver comet assay. For this group, animals were treated 24 hours prior to sacrifice using SC injections. An untreated control group was also included in the experimental design. Samples for comet assay were taken at 2 time points (i.e. 4 and 14 days). First set of samples (aspirates) was taken using a fine needle aspirate from the implant site on Day-4. The second set of samples (implant tissues and liver) was taken after the animals were sacrificed on Day-14 as described elsewhere in details (17,18).

[Insert Table 1 here]

2.3. Preparation of implants

Commercially available acrylic dental filler (Toothfill Plus, UK) applied for root canal filling was used for this study. This AF come as semisolid paste which solidifies on exposure to moisture. Appropriate concentrations of EMS (HiMedia, India) were initially dissolved in ethanol and subsequently diluted in distilled water. They were then added to acrylic filler paste, moulded into shape of rod measuring approximately 10 mm x 4 mm (surface area of each implant approximately 0.3 cm^2). Acrylic implants containing three different concentrations of EMS (10, 20 or 40 mg/ implant) were prepared (i.e. AF+10, AF+20 or AF+40, respectively). Two implants were used per rat (approximate weight of 250 g), resulting in a dose of approximately 80, 160 or 320 mg/kg. Control acrylic implant without EMS was also prepared. HDPE implants were procured from Hatano Research Institute, Japan.

2.4. Implantation study

A subcutaneous implantation study was carried out as per ISO 10993, Part 6 and as previously described by us (20,17). Briefly, the animals were anaesthetised (using ketamine 40 mg/kg, Themis Medicare Limited, India; and xylazine, 5 mg/kg; Indian Immunological Limited, India), one skin incision made on either side of the dorsal midline and subcutaneous pockets made by blunt dissection. The implants were then placed in the subcutaneous pockets and the incisions sutured and appropriate povidone iodine dressing applied. Post-operative anti-inflammatory (meloxicam, 1 mg/kg, Intas pharmaceutical ltd, India) and antibiotics (enrofloxacin 10 mg/kg, Vetoquinol India animal health private ltd., India) was given for four days after surgery.

2.5. Cytotoxicity assessment for comet assay

Samples of aspirates taken on Day-4 were assessed for cytotoxicity using dual staining with fluorescein diacetate (0.125 µg/µl, Sigma Aldrich, UK) and ethidium bromide (0.025 µg/µl, Sigma Aldrich, UK). After staining, the cells were then scored under fluorescent microscope using FITC (green coloured live cells) or ethidium bromide (red coloured cytotoxic cells) filters (21). A total of 100 cells were scored and percentage of cytotoxicity calculated as described by us previously (22,23).

Cytotoxicity assessment of samples (both implant site and liver) taken on Day-14 was also assessed by histopathology. Evidence of necrosis, apoptosis and degeneration were specifically noted in addition to those of inflammation and fibrosis (24).

2.6. Preparation of single cell suspension

Aspirate samples were used as such for the comet assay. Tissue samples from implant site and liver were minced briefly with ice cold mincing solution (Hank's Balanced Salt Solution (HBSS) [Ca^{++}, Mg^{++} free] with 20 mM ethylene diamine tetra acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO; Sigma Aldrich, UK) and allowed to stand for few minutes. Supernatant containing single cells was taken for the comet assay (24).

2.7. Alkaline comet assay

Alkaline comet assay was performed as previously described by us and in line with the OECD 489 guideline (22,25). The critical parameters used in our experiments followed the standard protocol (26,27). Briefly, 5-10 µl of single cell suspension was mixed with 75 µl of 0.5% low-melting agarose (per slide) at 37 °C and spread on the precoated slides and allowed to set on ice. The slides were then placed in ice-cold lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Trizma base, 10% DMSO, 1% Triton-X; pH 10; Sigma Aldrich UK) and cells lysed overnight. after lysis of the cells, the slides were placed in horizontal electrophoresis tanks filled with electrophoresis buffer (300 mM NaOH/1mM EDTA, pH≥13.0; Sigma Aldrich, UK) and DNA in the cells was allowed to unwind for 20 min. The passive recirculation electrophoresis was then started, and the slides were electrophoresed at 25 V, 300 mA for 20 min (0.8 V/cm). After electrophoresis, the slides were neutralized in neutralization solution (PBS, pH 7.4; three times 5 min each) and dried on a slide warmer. The dried slides were stained with ethidium bromide (2 µg/ml) and scored under a fluorescence microscope fitted with a TRITC filter and using in-house image analysis system. The images were scored by a single scorer, unrelated to the study, using same image settings and magnification throughout the study. Two slides were prepared from each sample. Tail length and % DNA in tail (% of light intensity in tail compared to total cell intensity) were noted for 150 comets for each animal (22). Total number of clouds encountered during the scoring of first 100 comets were also noted.

2.8. Experimental units and statistical analysis for comet assay

For 'site of contact genotoxicity', experimental unit was individual implants. Therefore, mean of 150 comets was calculated for each implant and group mean of 6 implants ± SD was calculated for each experimental group. Mean ± SD of number of clouds was also calculated.

For untreated controls and EMS treated groups, experimental unit was individual animals and therefore, mean of 150 comets was calculated for each animal and group mean of 3 animals ±

SD was calculated for each experimental group. Mean ± SD of number of clouds was also calculated.

Two tailed t-test was used to compare comet assay data of various experimental groups as appropriate.

2.9. Positive and negative controls for the comet assay

For site of contact comet assay experiments, HDPE implant served as negative control. No appropriate positive control implant is available for genotoxicity studies. Therefore, AF implants containing EMS which gave positive response, served as positive controls for these experiments. Untreated and 200 µm hydrogen peroxide (H_2O_2) treated blood samples which served as negative and positive controls respectively, were included in the electrophoresis run to demonstrate the assay worked properly. Untreated and 200 µm H_2O_2 treated blood samples gave a clear negative and positive DNA migration response, respectively (4-15% and 46-58% respectively).

2.10. Preparation of extracts for Salmonella/microsome and chromosome aberration (CAbs) assays

Extracts for *Salmonella*/microsome and CAbs assays was prepared by extracting 3 cm^2 of AF+10, AF+20, AF+40, HDPE implants in purified water and DMSO at 50°C for 72 h (16). Only the neat extracts were used in both studies. At the end of extraction, the extracts were clear; no colour change or particulates were observed. Hence, no additional processing such as filtration, centrifugation, pH adjustments or any other processing were made. All extracts were administered to the test system within 12 h of preparation and were considered stable during this time.

2.11. Salmonella/microsome assay

All implants were tested on *Salmonella*/microsome assay as per to ISO 10993, Parts 3 and 33, and OECD 471(3,4,28) to check if their extracts containing leached EMS would causes reverse mutation or not. Based on our experience, TA100 and TA1535 strains of *Salmonella typhimurium* in the absence of S-9 gave reliable response with EMS. Therefore, all device extracts, positive (EMS 100, 300 and 1000 µg/plate) and solvent controls was tested in TA100 and TA1535 strains of *Salmonella typhimurium* (Molecular Toxicology Incorporated, USA) in the absence of S-9, using plate incorporation method. After appropriate treatment and incubation, the background lawn of the plates was examined for signs of toxicity, and revertant

colonies were counted manually. All reagents for the *Salmonella*/microsome assay were procured from HiMedia India, unless otherwise stated.

2.12. Chromosome aberration (CAbs) assay

The *in vitro* mammalian CAbs assay (human lymphocytes) was performed in line with ISO 10993, Parts 3 and 33, and OECD 473 (3,4,29). All reagents for this test was from HiMedia, India, unless otherwise stated. Whole blood cultures, obtained from three healthy volunteers after informed consent, were set up using complete RPMI-1640 medium supplemented with 20% fetal calf serum (Thermo fisher scientific, USA) and 2% phytohemagglutinin. After 48 h, the cultures were treated with device extracts, positive (EMS 300 and 1000 µg/ml) and solvent controls, and incubated for 24 h, without S-9. Duplicate cultures were treated with the implant extracts and positive controls at appropriate concentration and quadruplicate cultures were treated with solvent controls. The aqueous extracts were added 1:10 and DMSO 1:100 with culture medium. Colchicine was added 3 h before harvest to arrest the cells in metaphase. At harvest, the cells were given a hypotonic shock with 0.075M KCl and fixed with fresh, cold methanol/glacial acetic acid (3:1, v/v). Slides were prepared from these fixed cells and stained with Giemsa. The cells were scored for mitotic inhibition (measure of cytotoxicity) and chromosome aberrations.

2.13. Measurement of EMS in extracts using GC-MS

Water and DMSO extracts used for Ames test and chromosome aberrations assays were subjected to EMS estimation using GC-MS using method developed by Ramakrishna et al., 2008 (30). Analysis was carried out on a GC system coupled with mass spectrometer (Model No. 7000D GC/TQ; Agilent Technologies, USA). The compounds were separated on DB-1 capillary column (Agilent Technologies, USA, 30m×0.25mm i.d.×0.25µm film). Two microliters volume with 1:100 split inlet was selected for injection. The GC oven temperature program utilized an initial temperature of 80 °C and an initial holding time of 4 min, then increased at 20 °C/min to 260 °C. The final temperature was held for 4 min. The injection temperature, GC-MS interface and ion source temperatures were 250, 250 and 230 °C, respectively. Helium was used as the carrier gas with a flow rate of 1 ml/min. Concentration of EMS was measured at 79m/z. Standard curves were obtained using 1, 10 and 15 µg/mL and levels of EMS in water or DMSO extracts were calculated from implants containing 10 or 40 mg of EMS. EMS levels were measured in three separate samples each of water and DMSO extracts.

3. Results

3.1. Comet assay results on aspirates from implant site on Day-4

The results of % DNA in tail and tail length on aspirates from the implant sites collected by fine needle aspiration on Day-4 are presented in Fig. 1. Cytotoxicity as measured by fluorescein diacetate/ethidium bromide staining ranged between 11% and 27% for all samples and is assumed not to affect the comet assay results. Slightly increase in cytotoxicity may be due to inflammatory response following implantation surgery. As expected, DNA migration (both %DNA in tail and tail length) was low in aspirates from HDPE and titanium implant sites. Aspirate from AF alone implantation site showed some increase in %DNA in tail compared to HDPE. However, the tail length for cells from AF alone implant site was not statistically different from that of HDPE's. AF implants containing 10, 20 or 40 mg of EMS showed a statistically significant dose related increase in DNA migration compared to HDPE. Percentage of clouds in all samples were less than 8% and therefore assumed not to skew the DNA migration results.

[Insert Fig. 1. here]

3.2. Comet assay results on cells from implant site on Day-14

The results of % DNA in tail and tail length on cells collected from the implant sites on Day-14 are presented in Fig. 2. Cells collected from the site of contact with EMS containing AF implants showed a clear and statistically significant dose related increase in DNA migration (%DNA in tail and tail length) compared to negative control HDPE contacting cells. The cells collected from the site of contact with titanium and AF did not show any increase in DNA migration compared to HDPE contacting cells. No cytotoxicity was observed in histopathological examinations of all implant sites. Percentage of clouds in all samples were low.

[Insert Fig. 2. here]

3.3. Comet assay results on liver cells collected on Day-14

DNA migration in liver of all implanted animals were similar and not statistically different to that observed in untreated control animals (see Fig. 3.). The livers from positive control treated animals showed a clear increase in DNA migration. No cytotoxicity was observed in histopathology of liver and the number of clouds were less than 5%.

[Insert Fig. 3. here]

3.4. Results of standard genetic toxicology biocompatibility tests

Results of *Salmonella*/microsome assay and CAbs assay conducted on extracts from EMS containing AF implants are given in Tables 2 and 3, respectively. In the *Salmonella*/microsome assay, mean numbers of revertant colonies in solvent control plates were all within the historical range and were significantly elevated in positive control treatments, hence the assay was considered valid. No thinning of the background lawn was observed in any of the extract treated plates. The mean number of revertant colonies obtained with all implant extracts were comparable to solvent controls.

[Insert Table 2 here]

In the CAbs assay, the proportion of cells with structural aberrations in the untreated negative cultures fell within historical solvent control ranges (95% reference range for aberrations +/- gaps, 0-3). The positive control chemicals induced significant increases in the proportion of cells with structural aberrations. Cultures treated with all implant extract resulted in frequencies of cells with structural aberrations comparable to the concurrent untreated negative controls. The number of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control range. Mitotic inhibition of all extract treated cultures were less than 10%.

[Insert Table 3 here]

3.5. Results of EMS measurements in water and DMSO extracts

Standard reference curves were obtained using 1, 10 and 15 µg/mL of EMS solution. The linearity was expressed using the equation $y = 340254.25x - 28558.15$ and R^2 value was 1. Representative chromatograms are shown in Figure 4. The mean levels of EMS in DMSO and water extracts from implants (containing 40 mg EMS) were 76 and 44 ng/mL, respectively. Similarly, the levels of EMS in DMSO and water extracts from implants (containing 10 mg EMS) were 21 and 19 ng/mL, respectively.

[Insert Figure 4 here]

4. Discussion

To pre-validate the usefulness of comet assay to detect 'site of contact genotoxicity', we looked for DNA damage in tissues surrounding AF implants containing various doses of EMS. By incorporating EMS in AF implants, the release of EMS is reduced such that it will induce

a long term, low level genotoxic stress in surrounding tissues, yet the systemic levels are so low that it cannot cause DNA damage in distant tissues such as liver. Moreover, extracting these implants using standard extraction techniques recommended by ISO 10993, Part 12 (16) did not show any genotoxic potential in the standard battery of genetic toxicology biocompatibility tests as recommended in ISO 10993, Part 3 (3) and 33 (4).

EMS is a mutagenic, teratogenic, and possibly carcinogenic compound that produces random mutations in DNA predominantly by guanine alkylation. This typically produces point mutations by reacting ethyl group of EMS with guanine in DNA, forming the abnormal base O^6-ethylguanine, which results in strand break during replication. EMS is well studied positive control chemical for comet assay and produces consistent DNA migration in many tissues (31-33). Therefore, for this validation exercise, EMS was considered as an ideal choice.

An initial 'site of contact' comet assay assessment was done on Day-4 after implantation. The cells were collected by fine needle aspiration. At this time point, the inflammation reaction to surgery would have substantially subsided, with the use of anti-inflammatory and antibiotics medications. Comet assay on Day-4 showed increased DNA migration in all AF implants containing EMS, suggesting that local tissue levels of EMS were high enough to cause DNA damage. Small increase in %DNA in tail was also observed in AF only, however, no such increase was seen with tail length. This could be due to some residual inflammation, as inflammation is known to cause DNA damage by release of cytokines and reactive oxygen species (ROS) from inflammatory cells (34,35). It should be noted that fine needle aspirates contain several inflammatory cells, such polymorphonuclear cells, lymphocytes, plasma cells, macrophages, mast cells and some fibrocytes. Different inflammatory cells can show different DNA damage, but this was difficult to address this question in our study. Furthermore, no literature is currently available investigating DNA damage by comet assay in various inflammatory cells. No DNA migration was seen with HDPE (negative control) and titanium, a well-known metallic biocompatible implant material (19).

Second set of comet assay was performed on Day-14, after the animals were sacrificed. This time 'site of contact' comet assay was performed by mincing peri-implant tissue and collecting single cells for the assay. Comet assay was also performed on liver, to check for systemic genotoxicity. Like Day-4, 'site of contact' comet assay showed a dose related increase in DNA migration in AF implants containing EMS. No necrosis, apoptosis or acute inflammation was seen in histopathology, suggesting that the DNA migration seen is true DNA

damage. As expected, no DNA migration was seen with HDPE and titanium implants. None of the liver samples from implanted animals showed DNA damage.

In this study, standard battery of genetic toxicology tests (*Salmonella*/microsome and CAbs assays) as recommended by ISO 10993, Parts 3 and 33 (3,4) was conducted on AF implants containing EMS. These tests were conducted on polar and non-polar extracts from these implants, prepared as recommended by ISO 10993, Part 12 (16). Both *Salmonella*/microsome and CAbs assays gave clear negative results. This is fully explainable as the amount of EMS leaching out from the AF would be extremely low to cause any genotoxic effects. Therefore, the overall conclusion would be non-genotoxic, if we strictly follow ISO 10993, Part 1 (1). It should be noted that the levels of EMS in various extracts as measured by GC-MS was exceptionally low to elicit a positive response in standard battery of genetic toxicology tests. However, prolonged low-level exposure in the vicinity of implants, did show DNA strand breaks.

Our finding suggests that comet assay will be very useful for detecting 'Site of contact genotoxicity' assessment of implant medical devices. 'Site of contact genotoxicity' assessment can easily be bolted-on implantation studies or systemic toxicity studies conducted via implantation route. Moreover, there are several ways of accessing cell samples for comet assay. Fine needle aspiration, needle core biopsy or tissue collected after sacrifice can be used. In addition to alkaline comet assay, use of enzymes such as Fpg or EndoIII can be used to understand the mechanism of DNA damage caused by various medical implants (36-38). Furthermore, as an *in vitro* alternative, *in vitro* direct contact or agar diffusion cytotoxicity assays (39) can be modified to detect site of contact genotoxicity using the comet assay.

Currently, the biocompatibility global harmonized standard, ISO 10993, Part 1 (1), calls only for a comprehensive genetic toxicology assessment for invasive medical devices such as implants. A comprehensive genetic toxicology assessment is applicable for pharmaceuticals and agrochemicals, but medical devices, especially long-term implants have a different route of exposure and pharmacokinetics. In this integrated study using a range of assays, we have shown that implants can cause 'site of contact genotoxicity', in absence of overall genotoxicity. Therefore, in contrast to pharmaceuticals or agrochemicals, risk assessment of implant devices would require an additional genetic toxicology end point to assess. We, therefore, propose that 'site of contact genotoxicity' end point should also be evaluated on a case by case basis for relevant medical devices such as implants.

5. Conclusion

In conclusion, comet assay will add new dimension to safety assessment of medical devices. This will focus on a new biocompatibility end point, 'site of contact genotoxicity' for relevant medical devices, such as long-term implants. Moreover, it is proposed that we explore the possibility of including comet assay to the battery of genetic toxicology tests for evaluating biocompatibility of medical implants.

Author's contributions

Conception and design: T.S.K.; Experimental conduct: S.S.M., N.P., B.B.; Data analysis and interpretation: T.S.K. and A.N.J.; Manuscript writing: T.S.K., B.B., and A.N.J.; Final approval of manuscript: T.S.K., B.B., N.P., S.S.M. and A.N.J.

Conflicts of interest

The authors declare no conflict of interest.

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Table 1. Experimental design used for implantation study.

Treatment groups	Number of animals	Number of implants	Route of exposure	Target tissues examined	
				Day 4	Day 14
Untreated control	3	-	n/a	n/a	Liver
HDPE implant	3	6	SC implantation	Implant site	Implant site and liver
Titanium implant	3	6	SC implantation	Implant site	Implant site and liver
Acrylic Filler (AF) implant	3	6	SC implantation	Implant site	Implant site and liver
AF+10 implant	3	6	SC implantation	Implant site	Implant site and liver
AF+20 implant	3	6	SC implantation	Implant site	Implant site and liver
AF+40 implant	3	6	SC implantation	Implant site	Implant site and liver
200 mg/kg EMS (SC)	3	-	SC injection	n/a	Liver

HDPE, high density polyethylene; AF, acrylic filler; EMS, ethyl methanesulphonate; AF+10, AF+20 and AF+40 represents acrylic filler incorporated with 10, 20 and 40 mg of EMS, respectively; SC, subcutaneous; n/a, not applicable; 3 animals were used per group; in implantation groups, each animals received 2 implants and therefore 6 implant sites were analysed per group.

Table 2. Summary of revertant colony counts of *Salmonella typhimurium* following treatment with polar and non-polar extracts.

Extraction vehicle	Treatment group	Reverse mutation frequencies*	
		TA100	TA1535
Purified water extract	Solvent control	115.33 ± 18.9	32.00 ± 2.6
	HDPE	127.00 ± 6.10	29.67 ± 4.7
	Titanium	124.00 ± 7.50	30.00 ± 6.9
	AF	123.00 ± 14.0	26.33 ± 1.5
	AF+10	122.67 ± 7.00	28.67 ± 7.4
	AF+20	113.33 ± 10.7	33.00 ± 2.6
	AF+40	112.33 ± 23.2	28.67 ± 3.1
DMSO extract	Solvent control	125.00 ± 3.00	28.00 ± 5.6
	HDPE	109.67 ± 13.3	25.33 ± 3.2
	Titanium	116.00 ± 21.3	31.33 ± 6.4
	AF	120.00 ± 16.5	27.33 ± 3.1
	AF+10	109.33 ± 1.20	28.00 ± 3.5
	AF+20	123.00 ± 12.3	28.33 ± 6.1
	AF+40	118.67 ± 15.8	31.33 ± 4.2
-	EMS, 100 µg/plate	152.67 ± 26.8	67.33 ± 13.8
	EMS, 300 µg/plate	296.00 ± 20.1	156.67 ± 13.3
	EMS, 1000 µg/plate	426.33 ± 83.8	285.00 ± 26.9
Historical Control data	Solvent controls (-S9)	100 - 136	19 - 33
	Positive controls (-S9)**	555 - 690	554 - 645

*mean ± SD of 3 plates; ** sodium azide (2µg/plate)

HDPE, high density polyethylene; AF, acrylic filler; EMS, ethyl methanesulphonate; AF+10, AF+20 and AF+40 represents acrylic filler incorporated with 10, 20 and 40 mg of EMS; DMSO, dimethyl sulfoxide.

Table 3a. Summary of mitotic inhibition and types of structural chromosome aberrations following treatment with polar extracts.

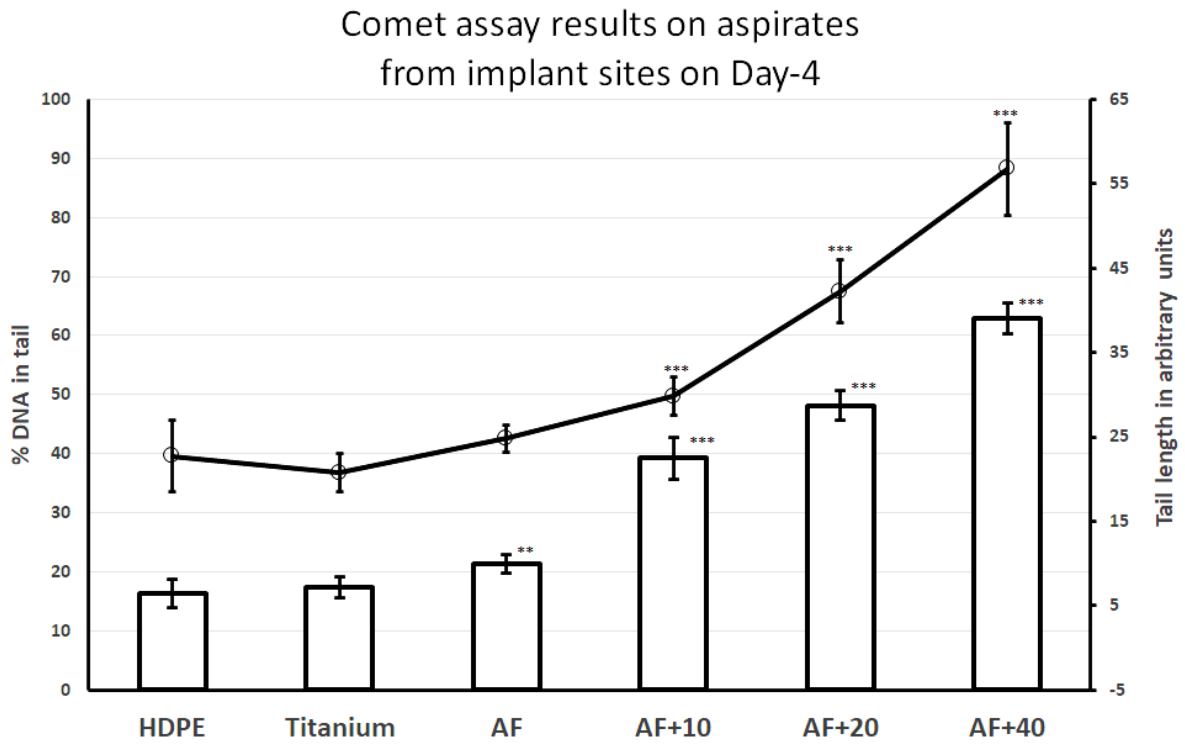
Implant extract purified water	Mitotic Inhibition (%)	Cells scored	Number of cells with aberrations							
			G	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	-	400	5	0	0	2	0	0	7	2
HDPE	7.2	300	2	0	0	2	0	0	4	2
Titanium	2.4	300	2	0	0	0	0	0	2	0
AF	7.8	300	3	0	0	1	0	0	4	1
AF+10	4.6	300	2	0	0	1	0	0	3	1
AF+20	6.7	300	2	0	0	1	0	0	3	1
AF+40	3.1	300	4	1	0	1	0	0	6	2
EMS; 300 µg/mL	11.7	142	6	4	2	5	3	0	20	14
EMS; 1000 µg/mL	43.1	112	6	7	2	11	3	0	29	23

Table 3b. Summary of mitotic inhibition and types of structural chromosome aberrations following treatment with non-polar extracts.

Implant extract DMSO	Mitotic Inhibition (%)	Cells scored	Number of cells with aberrations							
			G	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	-	400	3	0	0	2	0	0	5	2
HDPE	7.9	300	3	0	0	0	0	0	3	0
Titanium	5.9	300	2	0	0	1	0	0	3	1
AF	6.8	300	4	0	0	1	0	0	5	1
AF+10	8.5	300	3	0	0	2	0	0	5	2
AF+20	5.4	300	3	0	0	2	0	0	5	2
AF+40	7.9	300	4	0	0	1	0	0	5	1
EMS; 300 µg/mL	23.7	152	6	4	3	7	4	0	24	18
EMS; 1000 µg/mL	48.3	118	5	7	4	9	3	0	28	23

G, gaps; Chr del, chromosome deletion; Chr exch, chromosome exchange; Ctd del, chromatid deletion; Ctd exch, chromatid exchange, Abs+g, aberrations including gaps; Abs-g, aberrations excluding gaps.
DMSO, dimethyl sulfoxide; HDPE, high density polyethylene; AF, acrylic filler; EMS, ethyl methanesulphonate; AF+10, AF+20 and AF+40 represents acrylic filler incorporated with 10, 20 and 40 mg of EMS, respectively.

Fig. 1. Results of %DNA in tail and tail length on aspirates from the implant sites collected on Day-4.

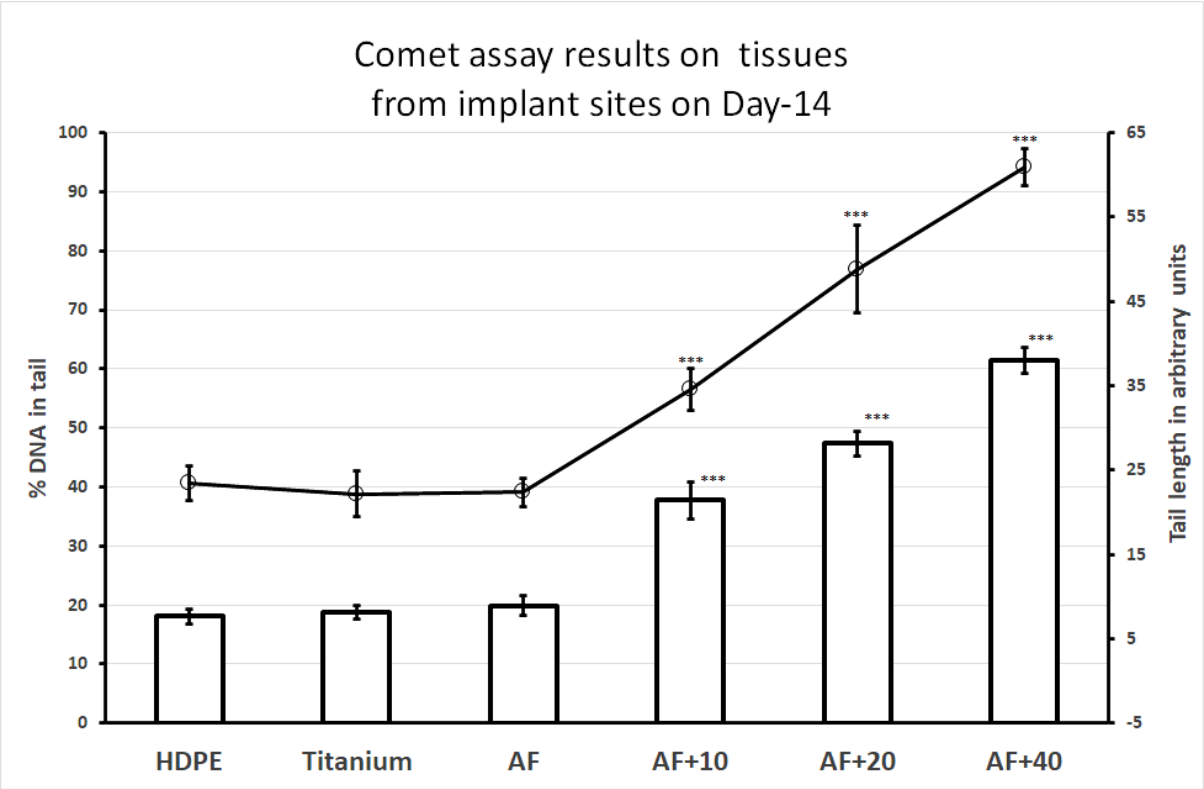


Bar chart represents % DNA in tail; line chart represents tail length in arbitrary units. Each experimental point represents mean ± SD of 6 implants.

**, P<0.01; ***P<0.001 based on t-test, compared with HDPE.

HDPE, high density polyethylene; AF, acrylic filler; EMS, ethyl methanesulphonate; AF+10, AF+20 and AF+40 represents acrylic filler incorporated with 10, 20 and 40 mg of EMS, respectively.

Fig. 2. Results of %DNA in tail and tail length on cells collected from the implant sites on Day-14.

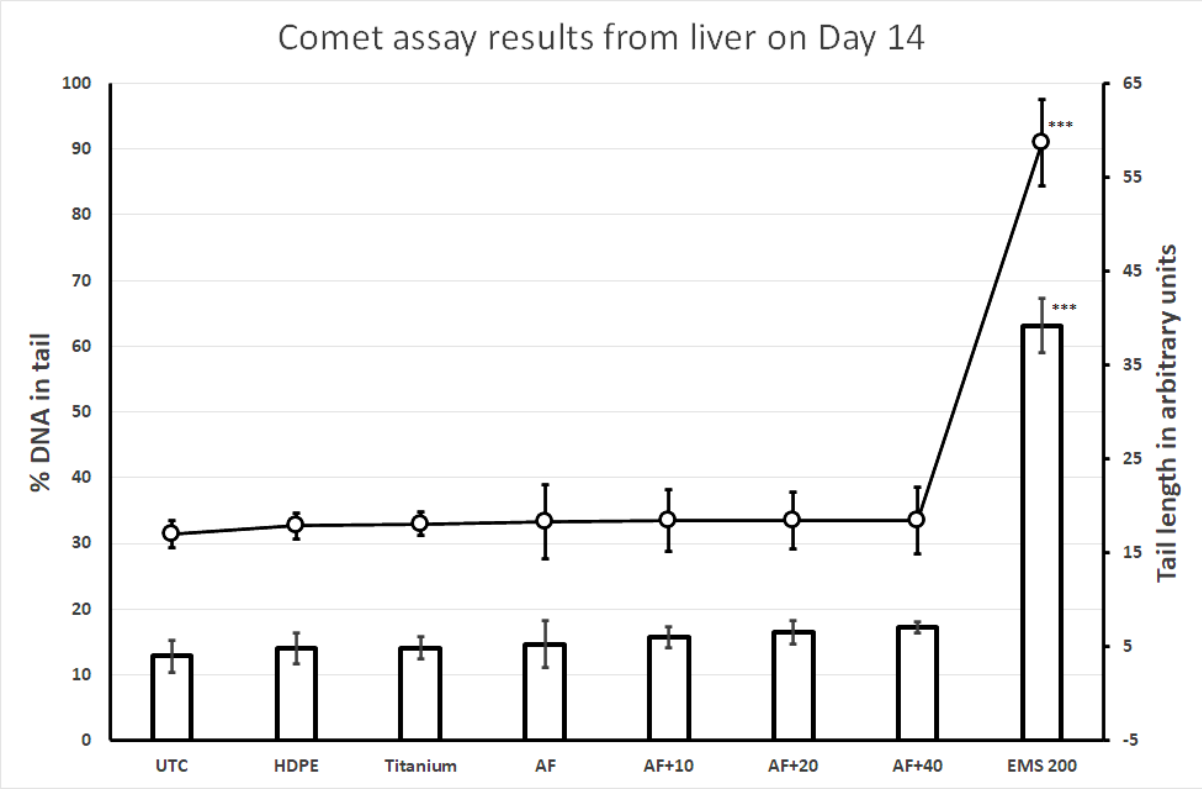


Bar chart represents % DNA in tail; line chart represents tail length in arbitrary units. Each experimental point represents mean ± SD of 6 implants.

***P<0.001 based on t-test, compared with HDPE.

HDPE, high density polyethylene; AF, acrylic filler; EMS, ethyl methanesulphonate; AF+10, AF+20 and AF+40 represents acrylic filler incorporated with 10, 20 and 40 mg of EMS, respectively.

Fig. 3. Results of %DNA in tail and tail length on liver cells collected on Day-14.

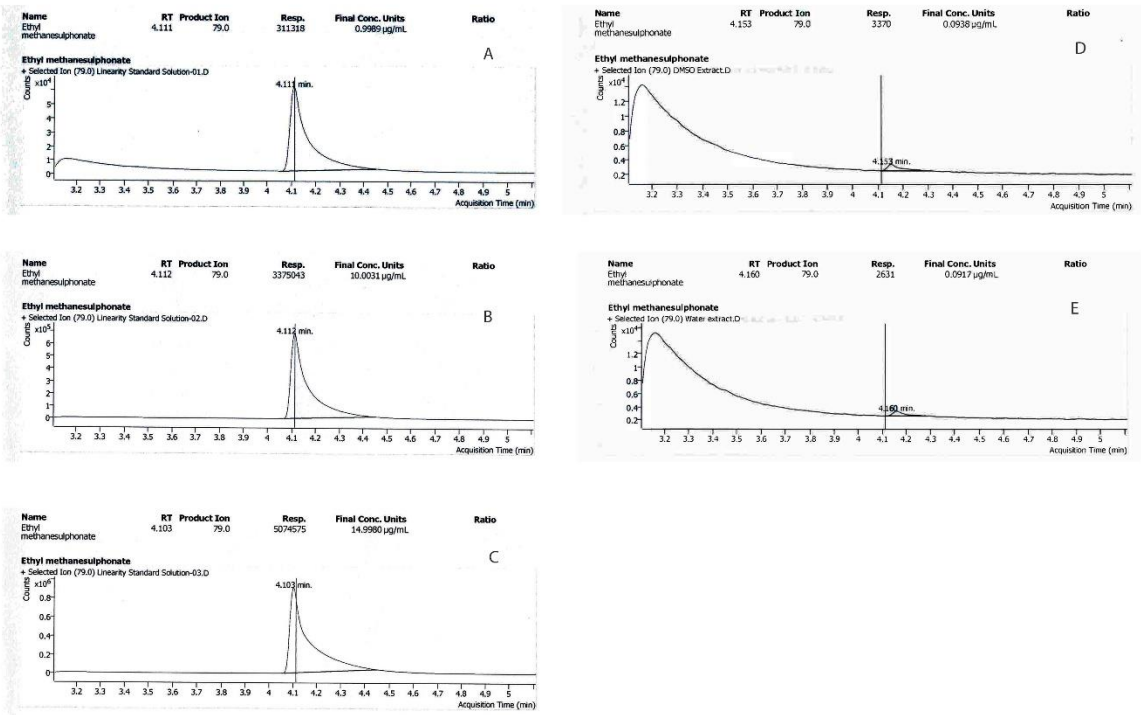


Bar chart represents % DNA in tail; line chart represents tail length in arbitrary units. Each experimental point represents mean ± SD of 3 animals.

***P<0.001 based on t-test, compared with HDPE.

HDPE, high density polyethylene; AF, acrylic filler; EMS, ethyl methanesulphonate; AF+10, AF+20 and AF+40 represents acrylic filler incorporated with 10, 20 and 40 mg of EMS, respectively.

Fig. 4. Representative GC/MS chromatograms from EMS estimation in water and DMSO extracts.



GC/MS chromatograms of EMS in standard solutions 1 µg/mL (A), 10 µg/mL (B), 15 µg/mL (C), DMSO extract (D), and Water extract (E).