

Biocompatibility and biodegradation of protein microparticle and film scaffolds made from kafirin (sorghum prolamin protein) subcutaneously implanted in rodent models**

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** No benefit of any kind will be received either directly or indirectly by the authors

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ABSTRACT

Kafirin, the sorghum prolamin protein, like its maize homologue zein, can be made into microparticles and films and potentially used as a biomaterial. Zein has good bio- and cyto-compatibility. Kafirin could be advantageous as it is more hydrophobic, more cross-linked, more slowly digested by mammalian proteases than zein and is non-allergenic. The safety and biocompatibility of kafirin implants in two forms was determined in rodent models. One week post subcutaneous injection of kafirin microparticles (size 5 μ m diameter) in mice, chronic inflammation, abnormal red blood cells and gross fibrin formation were observed. This chronic inflammatory response was possibly caused by the release of hydrolysis products such as glutamate during the degradation of the kafirin microparticles. In contrast, films made from kafirin microparticles (50 μ m thickness, folded into 1 cm³) implanted in rats showed no abnormal inflammatory reactions and were only partially degraded by day 28. The slower degradation of the kafirin films was probably due to their far smaller surface area when compared to kafirin microparticles. Thus, kafirin films appear to have potential as a biomaterial. This study also raises awareness that the form of prolamin based biomaterials, (kafirin and zein) should be considered when assessing the safety of such materials.

KEYWORDS

Microparticle, kafirin, histology, film, biocompatibility

INTRODUCTION

Cereal prolamin proteins, such as zein from maize can be formed into bioplastic microstructures, which have shown potential for use as delivery systems, for drugs,¹ nutraceuticals,² antimicrobials,³ essential oils,⁴ as biomaterials in tissue engineering as scaffolds,⁷ and as biomedical coatings for arterial/vascular implants.⁶ Zein has been shown to have good bio- and cyto-compatibility.⁷ In vivo studies of zein scaffolds have shown good tissue response and biodegradability.^{8,9} Further, complexes of zein scaffolds and rabbit mesenchymal stem cells have been shown to undergo ectopic bone formation in the thigh muscle pouches of nude mice.¹⁰

Kafirin is the homologous sorghum protein to zein,¹¹ and may have some advantages over zein for use in biomedical applications. Kafirin is usually considered more hydrophobic,¹² is less digestible,¹² and has a higher glass transition temperature than zein.¹³ Kafirin can be formed into highly vacuolated microparticles, between approximately 1-10 μm diameter,¹⁴ providing a large surface area for binding bioactive compounds such as polyphenolic nutraceuticals,¹⁵ and bone morphogenetic proteins (BMP's).¹⁶ Thin (<50 μm), water stable kafirin bioplastic films can also be formed from these microparticles.¹⁶ Both structures may have potential for scaffold type applications, since kafirin is slowly biodegraded by mammalian digestive proteolytic enzymes,¹² is non-allergenic,¹⁷ and there is good evidence that kafirin is nontoxic to celiac sufferers.¹⁸ However, no published data could be found on the biocompatibility of kafirin or on its safety for application as an implant material.

One of the major concerns with medical devices is inflammatory and immune reaction and implant rejection by the body.¹⁹ Thus, the primary aim of this work was to assess the

biocompatibility of kafirin microparticles using a murine subcutaneous bioassay. For potentially different applications, a subcutaneous bioassay using a rat model was performed to assess the biocompatibility and biodegradability of various kafirin microparticle film implants.

MATERIALS AND METHODS

Materials

Kafirin was extracted from whole grain, milled white, tan-plant non-tannin sorghum, using 70% (w/w) aqueous ethanol containing 3.5% (w/w) sodium metabisulfite and 5% (w/w) sodium hydroxide at 70^o C for 1 h, as described.²⁰ The kafirin was defatted and further processed into kafirin microparticles (1-5 μ m diameter, Fig. 1A) by simple coacervation, as described.²¹ The microparticle suspension was centrifuged at 3150 g for 10 min, the supernatant was removed and the pellet washed with distilled water. This was repeated three times before the resultant pellet was freeze-dried.

Polystyrene microspheres (150 μ m) (Corpuscular, NY) were used as a standard in the mouse study. Collagen used as a standard in the rat study was donated by Altis Biologics (Pretoria, South Africa). All other chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO) unless otherwise stated.

Four different film treatments were investigated, including microparticle films treated with two different common cross-linking agents, glutaraldehyde and sorghum polyphenols and rhBMP-2 as a model protein active. Control and 20% glutaraldehyde cross-linked kafirin microparticle films were prepared as described.²¹ Briefly, 4 g of a 2% (w/w) suspensions of kafirin

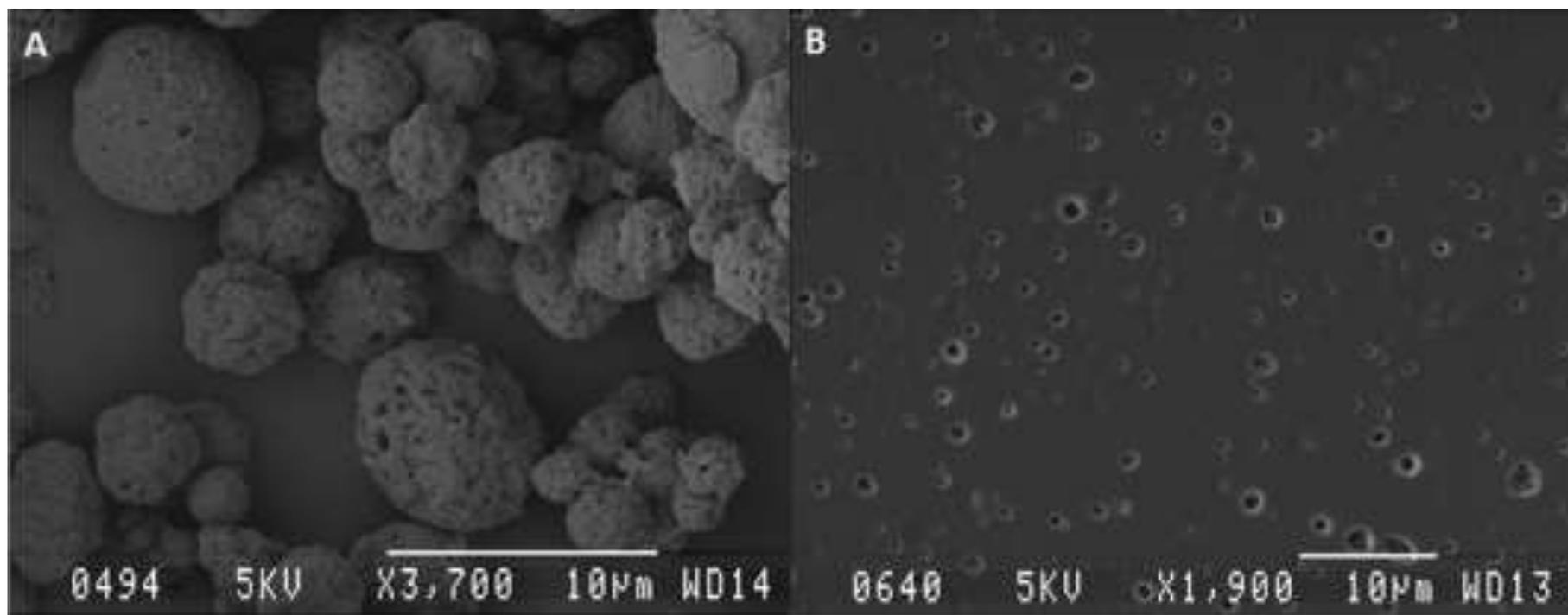


Figure 1. Scanning electron microscopy of kafirin microparticles (A) and kafirin microparticle films (B).

microparticles were centrifuged at 3150 g for 10 min, supernatants were removed and an equivalent weight of 25% (w/w) (4.2 M, pH 2.0) acetic acid containing plasticizer (1:1:1 lactic acid, polyethylene glycol 400, glycerol) was used to re-suspend the pellet. After equilibration for 12 h, films were cast in 90 mm diameter glass Petri dishes and dried overnight at 50°C in an oven (not forced draft). Polyphenol-treated kafirin microparticle films were prepared by soaking the films in an aqueous extract of polyphenols from black non-tannin sorghum grain bran (10 g/100 g catechin equivalents) for 12 h at room temperature. All films were approximately 50 µm in thickness. A plasticizer was included in the film formulation and later washed out by soaking in distilled water for 12 h, in order to act as a porogram, increasing the surface area of the films (Fig. 1B). Water on the film surface after soaking was removed by blotting with paper towel. Films were either dried at ambient temperature or used wet for binding rhBMP-2. To prepare these kafirin films they were plasticised so that they were flexible and could be handled without fracturing. However, to eliminate confounding effects of the plasticiser it was washed out prior to implantation. After washing out the plasticiser, the films retained sufficient flexibility for implantation as they remained partially hydrated.

Two dose levels, equivalent to 8 ng and 160 ng rhBMP-2 per 75 mg implant for low dose and high dose, respectively were prepared by diffusion loading as described,²² using a 400 ng/mL rhBMP-2 (Invitrogen, Carlsbad, CA) solution in 0.01 M phosphate buffered saline (pH 7.4) (Sigma-Aldrich, St. Louis, MO). The implants were incubated at 4°C for 24 h and then air dried.

Animal Husbandry

The University of Pretoria Animal Ethics Committee (H003-12, H016-11) granted approval for all animal experimental procedures. The rodents were maintained at the University of Pretoria Biomedical Research Centre (UPBRC), with ad-lib access rodent pellets (Epol, South Africa) and high-pure water, with a 12 h light/dark cycle at $22 \pm 2^\circ\text{C}$ and relative humidity of $60 \pm 10\%$.

Subcutaneous bioassay using mouse model

Eighteen 8-13 week old male Balb/c mice (SA Vaccine Producers, Johannesburg, South Africa) weighing ≈ 20 g, were used. The kafirin microparticle suspension was sterilized using γ -irradiation (Synergy Sterilisation, Johannesburg, South Africa). Sterile polystyrene microspheres and sterile phosphate buffered saline were used as a standard and a control, respectively. A single dose (5 mg/50 μl) was injected subcutaneously behind the neck of the mouse.

Three mice for each treatment were sacrificed 1 week and 8 weeks post injection and tissue and blood collected. Whole blood smears were made on cover slips. Citrate was added to freshly drawn blood (11 μl citrate /100 μl blood) and the blood centrifuged at 100 g for 2 min to obtain platelet rich plasma (PRP). To study fibrin fiber formation, thrombin (10 μl) was added to 10 μl PRP, mixed immediately and transferred to a glass cover slip to form the coagulum (fibrin clot). The coagula on the cover slips were kept in a humid environment at 37°C for 10 min before washing with PBS for 120 min. Washed fibrin clots and whole blood smears were fixed in 2.5% glutaraldehyde in pH 7.4, stained with 1% Osmium tetroxide and serially dehydrated in ethanol before drying. Scanning electron microscopy (SEM) was performed using a JEOL 6000F FEGSEM electron microscope (Tokyo, Japan).

White blood cell counts were determined from a 10 μ l whole blood smear stained with Giemsa, and evaluated microscopically using an Axioskop 40 double headed microscope (Carl Zeiss, Göttingen, Germany). White blood cells (100) were quantified in terms of monocytes, lymphocytes, eosinophils, basophils and neutrophils.

Tissue samples were fixed in 2.5% formaldehyde/glutaraldehyde mixture and serially dehydrated in ethanol. The tissue was orientated and embedded in LR White resin. Ultra microtome cut sections were stained with Toluidine Blue and viewed with a light microscope (LM) (Nikon Instech, Kanagawa, Japan).

Subcutaneous bioassay using rat model

Twenty male Sprague-Dawley rats (SA Vaccine Producers) (weight range: 155-223 g) were used. The film doses (75 mg, approximately 1 cm square when folded) were sterilised using γ -irradiation (Synergy Sterilisation). Four blind pouches were made subcutaneously on the back of each rat under isoflurane general anaesthesia. Treatments were randomised to the available sites and incisions were closed with cyanoacrylate. For the biocompatibility assessment, eight animals were euthanized on day 7 after implantation and the implants removed for blind independent evaluation by Idexx Laboratories (Pretoria, South Africa). The biocompatibility study was only for 7 days as there is literature which shows that a 1% level of glutaraldehyde cross-linking of protein implants can elicit a severe inflammatory response (REF). In this present work it was necessary to use a 20% level of glutaraldehyde cross-linking to obtain the optimal functional properties since kafirin contains very low levels of lysine. All major organs were subjected to histo-pathological evaluation.

For longer term biocompatibility and biodegradation assessment of some treatments and assessment of potential ectopic bone growth with rhBMP-2 containing implants, the remaining rats were sacrificed on day 28 post implantation. Radiology was used to determine whether bone formation had occurred with rhBMP-2 containing implants before implants were removed and sectioned. Half of each implant per treatment was frozen prior to quantification of alkaline phosphatase (ALP) enzyme activity. The other half was fixed in 10% buffered formalin. After fixation, selected tissue and cross-sections of the implantation sites were embedded in wax and 6 µm sections were cut and stained with haematoxylin and eosin. Implant sites were evaluated for foreign body reaction, granulomatous reaction, osteolysis, osteogenesis (bone morphogenesis) and skin ulceration and scored for tissue response to the implants according to similar studies.²³ Results were graded as follows: negative/none (0), mild (1), moderate (2), severe (3). Standard histopathology was performed on all major organs.

Alkaline phosphatase (ALP) activity of cells in the implants was assayed using an adenosine 3',5'-cyclic monophosphate (cAMP) direct enzyme immunoassay kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Prior to analysis, implants were frozen in liquid nitrogen and ground using a mortar and pestle before homogenizing in 0.1 M HCl to stop endogenous phosphodiesterase activity.

Statistical analyses

One-way analysis of variance (ANOVA) was used to analyse numerical data with Statistica software version 10 (StatSoft, Tulsa, OK). ALP activity tests were performed using four micro-

wells per treatment for at least two replicate experiments. The mean differences were assessed by Fisher's Least Significant Difference test. Histopathology data were obtained by the histological scoring system for tissue response in four replicate implant sites. ANOVA of the white blood cell counts (WBC) was conducted on the rank-transformed data to test for differences between groups and across weeks. A linear model was fitted for each of the five WBC's. Each outcome (WBC rank) was regressed against group and week to pinpoint significant differences. Significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Implant bio-compatibility assessment, mouse model

Light microscopy (LM), 1 week post injection, showed that both the polystyrene microspheres (Fig. 2B) and the kafirin microparticles (Fig. 2D) had caused inflammation. Erythrocyte (RBCs) shape changes, including non-discoid RBCs were visible inside blood vessels. SEM of thrombin added to platelet rich plasma (PRP) from control mice showed that fibrin fibers formed a typical fine fiber net (Fig. 3A). Also SEM of whole blood (WB) smears from the same control mice showed normal RBCs and associated platelets (Fig. 3D). Inflammation is indicated when fibrin fibers form thickened and matted fibrin mats, as was observed by SEM with both kafirin microparticles, (Fig. 3C, I) and polystyrene microspheres, (Fig. 3B, H) week 1 post injection. However, kafirin microparticles (Fig. 3C, I) caused a more severe response than polystyrene microspheres (Fig. 3B, H). WB smears taken from mice injected with kafirin microparticles (Fig. 3F, L) or polystyrene microspheres (Fig. 3E, K) showed RBCs had undergone shape changes and that fibrin was spontaneously formed between the RBCs, again this is indicative of inflammation. Platelets from mice injected with polystyrene microspheres were slightly smaller

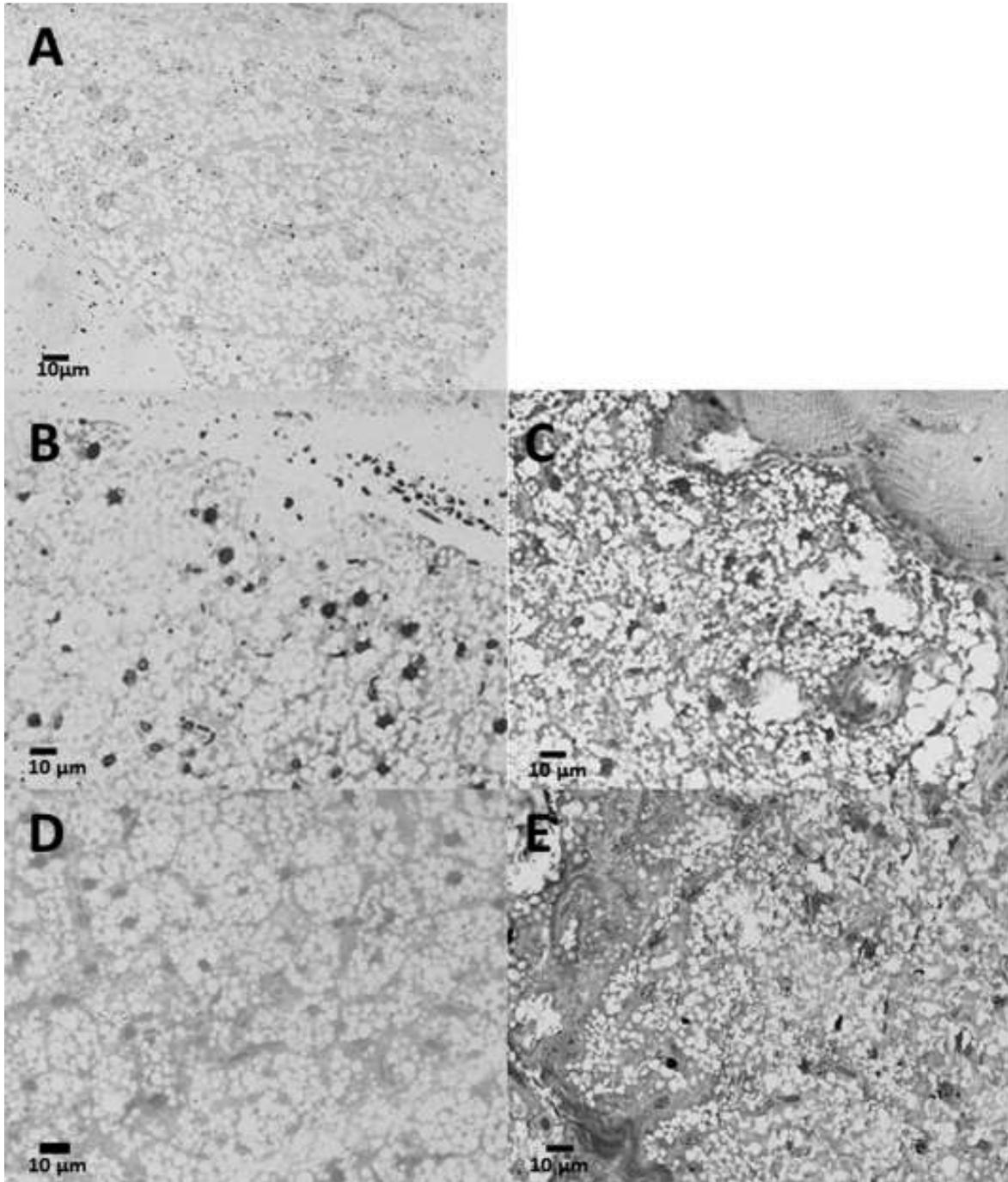


Figure 2. Light microscopy of subcutaneous implants into mice: control day 0 (A), polystyrene microspheres week 1 (B), polystyrene microspheres week 8 (C), kafirin microparticles week 1 (D), kafirin microparticles week 8 (E).

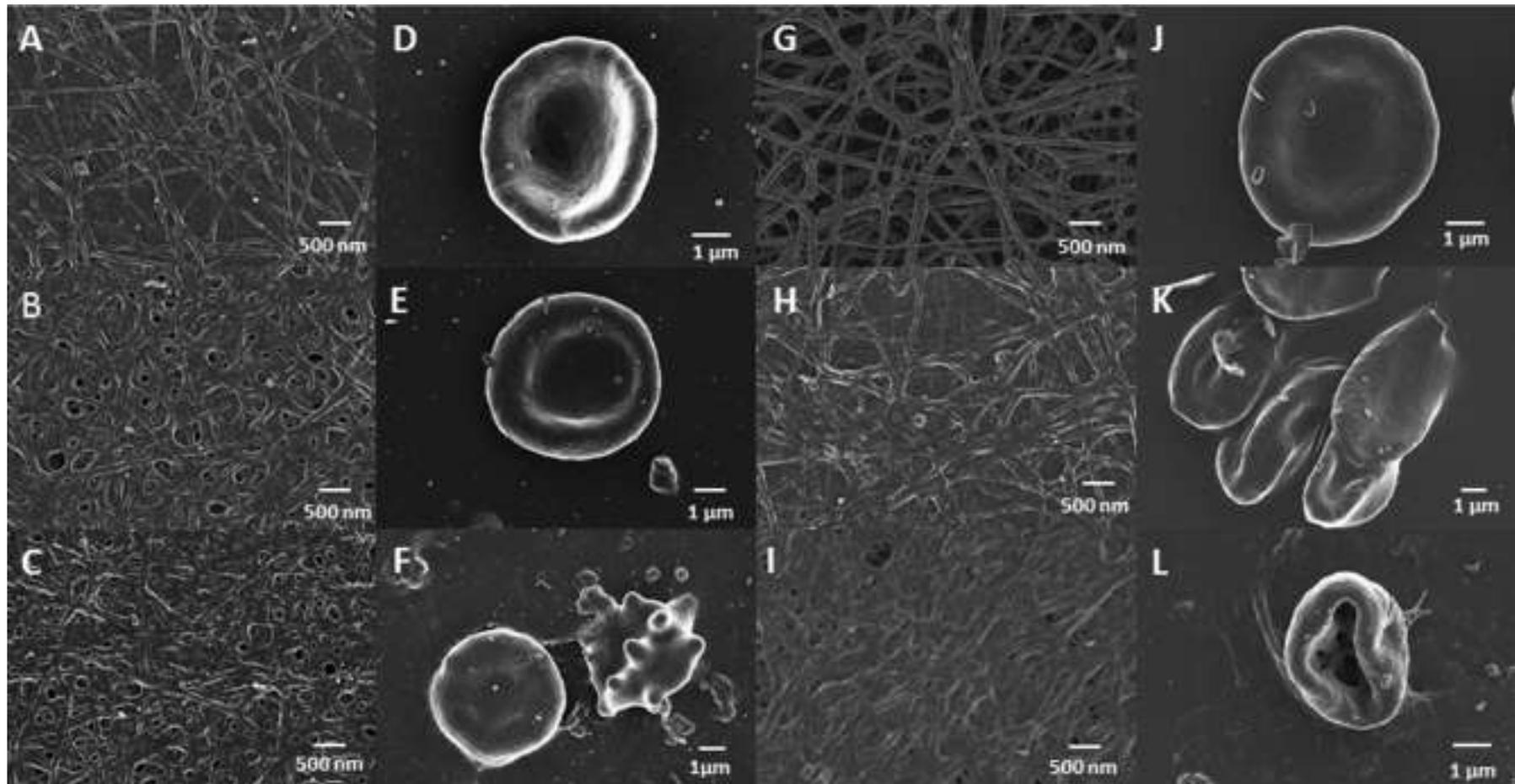


Figure 3. Scanning electron microscopy of subcutaneous implants into mice: A–C week 1 Fibrin (A-control, B- polystyrene microspheres, C- kafirin microparticles). D–F week 1 Whole blood (D-control, E- polystyrene microspheres, F- kafirin microparticles). G–I week 8 Fibrin (G-control, H- polystyrene microspheres, I- kafirin microparticles). J–L week 8 Whole blood (J-control, K- polystyrene microspheres, L- kafirin microparticles).

in size than those from control mice but otherwise similar in shape after week 1 (Fig. 3 E) but were more deformed after 8 weeks (Fig. 3K). Those from the kafirin microparticle injected mice were also slightly smaller in size than the controls but were mis-shapen with a number of pointed protuberances (Fig. 3F). This is not seen in control mice and is an indication of a general inflammatory profile and confirms the LM results (Fig. 2). White blood cell differential counts showed no significant differences between treatments (control, polystyrene microspheres, and kafirin microparticles) at week 1 or 8 (Fig. 4). However, when eosinophil and lymphocyte counts were compared between the weeks, the eosinophil count was significantly higher and the lymphocyte count significantly lower at week 8 compared with week 1, for all the treatments. Elevated eosinophil counts of are an indication of non-specific inflammation.²⁴

All the blood results from the mice injected with kafirin microparticles indicate that the kafirin microparticles caused chronic inflammation, abnormal red blood cells and gross fibrin formation. The hypercoagulation induced by the kafirin microparticles may have been due to a general inflammatory response. However, kafirin microparticles have a large surface area and are made from kafirin, which is rich in glutamine²⁵. They are subject to rapid breakdown by digestive proteolytic enzymes (86% within 2 h). Thus, it is probable that the kafirin microparticles were hydrolysed by intra-cellular protease enzymes. As a result, the localised pH would be reduced by proteolysis (pH stat REF) from the normal murine tissue of approx. pH 7 (REF). The glutamine released would then be deamidated, producing glutamic acid. Deamination of zein, producing glutamic acid, has been shown to take place *in vitro* at acid pH.²⁶ Glutamate causes accelerated blood clotting and fibrin formation²⁷ and so may be responsible for the adverse blood response seen in the mice injected with kafirin microparticles.

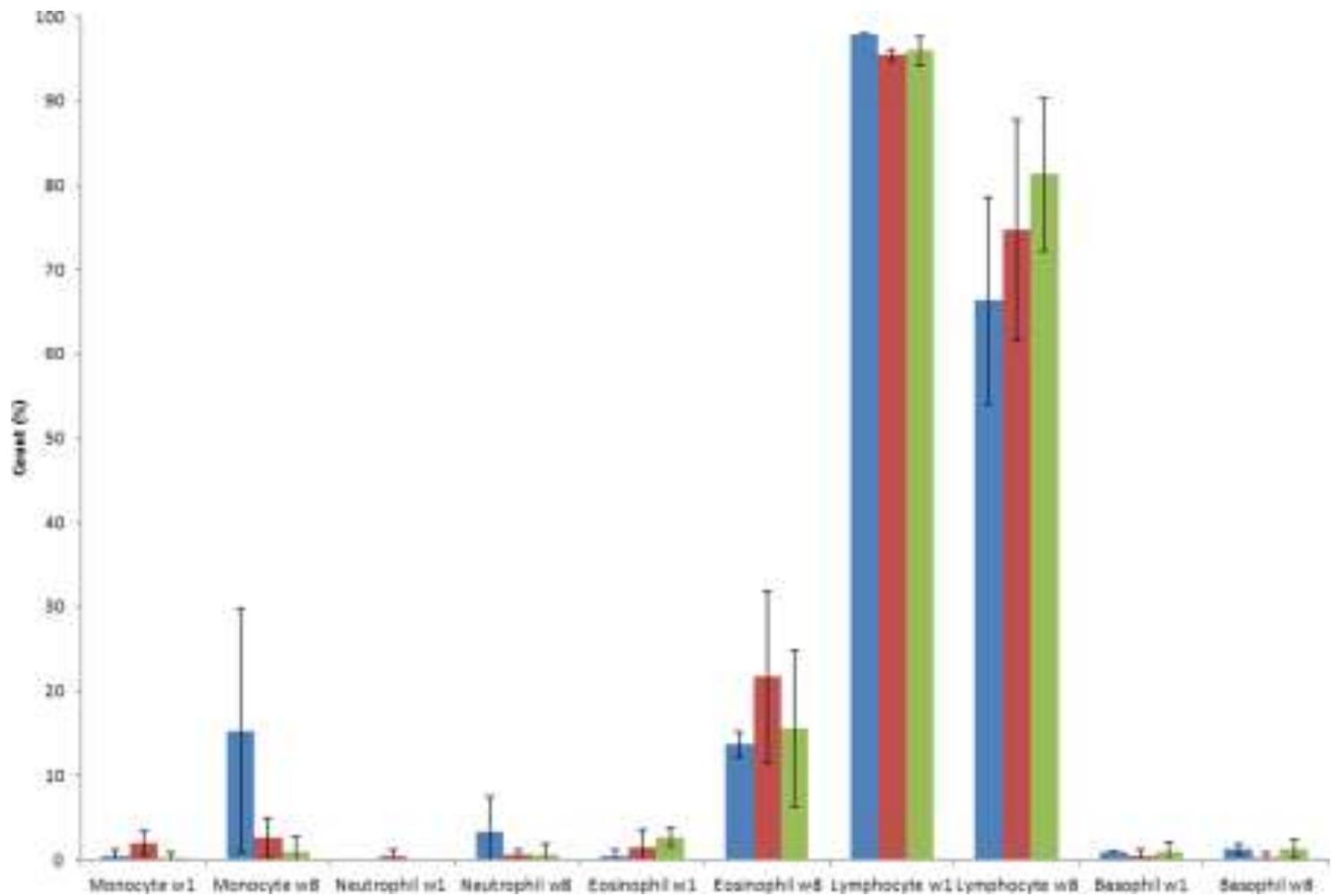


Figure 4. White blood cell counts of mice with subcutaneous implants: week 1 and 8, Blue-control, red-kafirin microparticles, green- polystyrene microspheres particles.

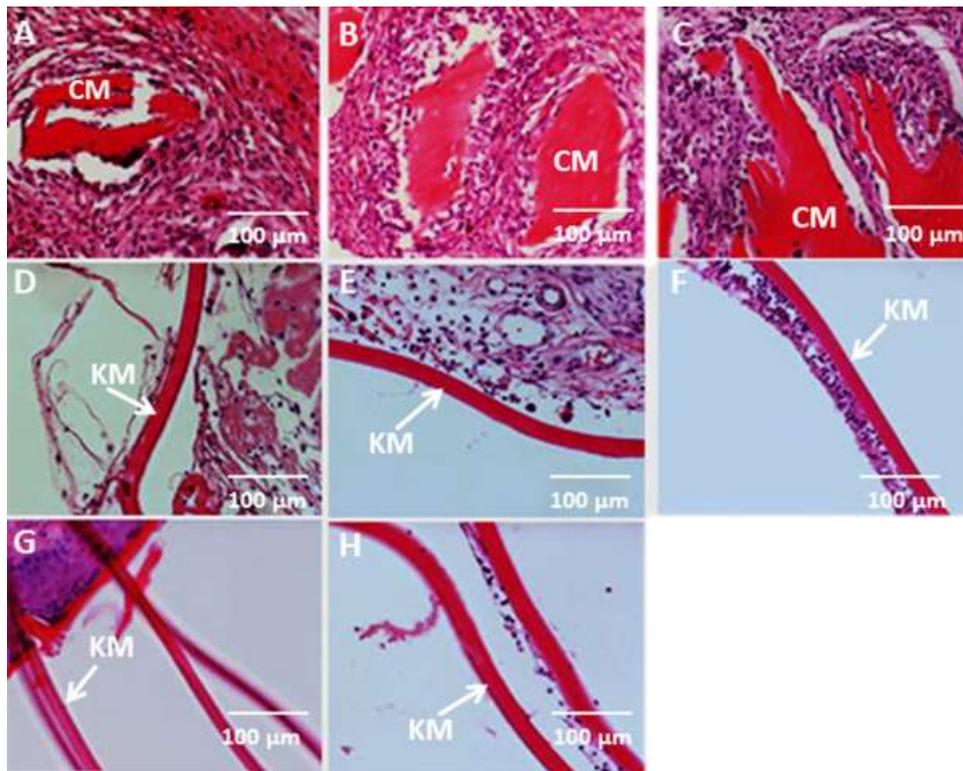
The second part of the study involved the use of films made from kafirin microparticles. The films have a much smaller total surface area than the vacuolated kafirin microparticles. There is evidence that kafirin microparticle films are more resistant to digestive_{proteolytic} enzymes (40.7% digested after 2 h)¹⁶ than kafirin microparticles (86% digested after 2 h)¹⁴ and so may be more inert when used as an implant in a small animal model. Kafirin microparticle films cross-linked with two commonly used cross-linking agents, either glutaraldehyde or sorghum polyphenols were included to investigate any potential adverse effects of kafirin microparticle films when treated with these potentially toxic cross-linking agents. These agents were also included to test whether the cross-linking process would render the films more inert and more resistant to enzymic breakdown than the untreated kafirin microparticle films. Films treated with a model protein active, rhBMP-2, were included primarily to investigate whether there was a synergistic reaction to the combination of kafirin film and rhBMP-2. Rats were used instead of mice, as the film implants were too large for the mice to accommodate. Both murine and rat models have been extensively used to study the safety and efficacy of protein-based subcutaneous implants.

Implant biocompatibility assessment, rat model

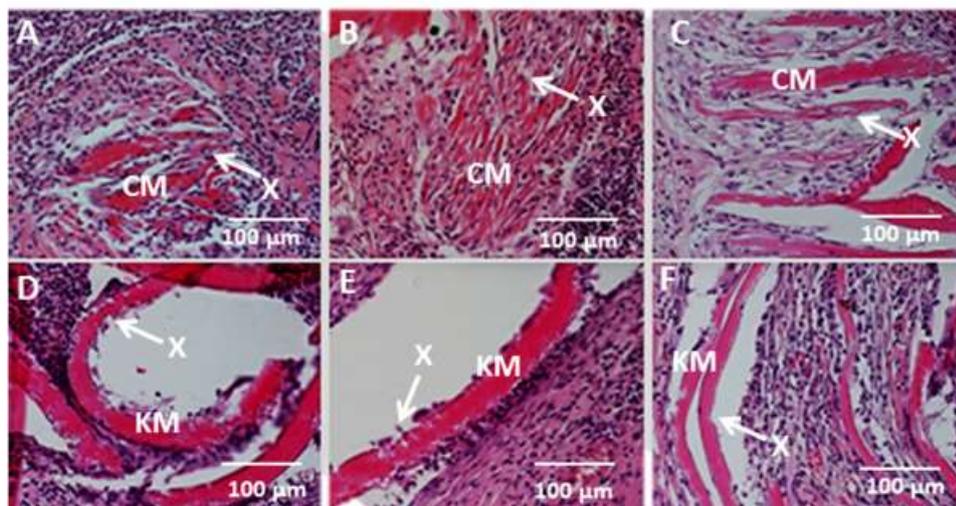
Visible wound healing was in progress by Day 7 post implantation for all treatments and by Day 28 there was complete healing of most of the wounds indicating that the rats reacted normally to the trauma of skin surgery. Skin ulceration was examined as part of the biocompatibility assessment. The areas of ulceration were small and microscopically confirmed at the implantation sites. On the surface of the skin superficial exudative crusts and some areas of full-

thickness epithelial necrosis were seen. Mild inflammation was present in the dermis underneath the slightly ulcerated skin. At Day 7 post implantation, these superficial ulcerations were found at both collagen and kafirin microparticle film implant sites (Table 1A). Implant sites of kafirin microparticle films treated with the cross-linking agents, glutaraldehyde or sorghum polyphenols and rhBMAP-2 showed no more severe responses than the untreated kafirin microparticle films (Table 1A). At Day 28 post implantation, a very low level of ulceration in the epidermis was found in the collagen implantation sites and none at the kafirin microparticle film sites (Table 1B).

Histological examination of tissue at the implant site, Day 7 post implantation, showed small micro-granulomas around all the implants, typical of a foreign body inflammatory reaction (Table 1A, Fig. 5i). The response was much more prominent for the collagen implants compared to the different kafirin microparticle film implants, (Table 1A) and was probably due to the greater surface area of the collagen as opposed to the kafirin microparticle film. The number of inflammatory cells present at the implant site is dependent on the amount of particles available for phagocytosis.²⁸ The collagen implant (Fig. 5 i A) had a larger surface area available for phagocytosis when compared to the kafirin film (Fig. 5 i D), resulting in more cells migrating into the area of inflammation. Multinucleated giant cells were also present, indicative of the presence of poorly soluble, indigestible material in the tissue.²⁹ The smaller macrophages or epithelioid cells fuse (forming the giant cells) to enhance total phagocytic activity. In contrast, the slow rate of the kafirin microparticle film degradation (Fig. 5 i D) and even slower rate of degradation of kafirin microparticle films treated with glutaraldehyde (Fig. 5i G) or sorghum polyphenols (Fig. 5 i H) would result in less particulate material available for phagocytosis,



(i)



(ii)

Figure 5. (i). Images of hematoxylin–eosin stained sections of subcutaneous implants into rats showing evidence of degradation of implants. Day-7 post implantation. (A). Collagen standard. (B) Collagen + low rhBMP-2 dose. (C) Collagen + high rhBMP-2 dose. (D) Kafirin microparticle film control. (E) kafirin microparticle film + low rhBMP-2 dose. (F) Kafirin microparticle film + high rhBMP-2 dose. (G) Glutaraldehyde-treated kafirin microparticle film. (H) Sorghum polyphenol-treated kafirin microparticle film. Figure 5 (ii) day-28 post implantation. (A) Collagen standard. (B) Collagen + low rhBMP-2 dose. (C) Collagen + high rhBMP-2 dose. (D) Kafirin microparticle film control. (E) Kafirin microparticle film + low rhBMP-2 dose. (F) Kafirin microparticle film + high rhBMP-2 dose. KM- kafirin microparticle film material; NB. glutaraldehyde-treated and sorghum polyphenol-treated kafirin microparticle film implants were only subjected to a 7-day implantation. KM- kafirin microparticle film material; CM-collagen material, X-degraded section of implant.

resulting in less cellular attraction to the area. By Day 28 post implantation, a foreign body reaction was evident at all of the implantation sites (Table 1B, Fig. 5 ii), varying from mild to severe. The only significant difference being between the kafirin microparticle film control implants and the collagen control implant (Table 1B, Fig 5 ii). The gross appearance of the implants Day 28 post implantation showed all the implants were surrounded by tissue capsules. There was some infiltration of blood vessels within the encapsulated collagen implants, with no difference in gross appearance between the collagen control and the rhBMP-2 loaded collagen. More prominent infiltration of blood vessels occurred within the encapsulated kafirin microparticle film implants than with collagen. There was no difference in gross appearance of the implant sites between the control and the rhBMP-2 loaded kafirin microparticle film implants. Macroscopic and histological evaluation showed no abnormal pathology for any of the organs from rats implanted with any of the treatments at both time intervals. Overall, the data showed that kafirin microparticle film implants, including films treated with glutaraldehyde, sorghum polyphenols or rhBMP-2 were non-irritant to the rats, agreeing with studies showing that zein, which is homologous to kafirin,¹¹ has good cell compatibility^{6,30} and has shown no adverse effects when used as a scaffold material in animal studies.¹⁰

Biodegradation of implant material

Lysis of the implant material was used as a measure of implant degradation. Day 7 post implantation, degradation of the collagen implants had started, whilst the kafirin microparticle film implants were all intact (Table 1A, Fig. 5 i). By Day 28 post implantation, most of the collagen implants had been completely degraded (Table 1B, Fig. 5 ii, A-C). In contrast, the kafirin microparticle film implants, although showing signs of degradation, were mainly intact

(Table 1B, Fig. 5 ii, D-F). Collagen implants have been shown to degrade relatively quickly as a result of proteolysis.³¹ In this study, the presence of kafirin microparticle film implants on Day 28 post implantation indicated that the kafirin microparticle films were biodegradable but at a much slower rate than the collagen implants. This was probably due to the low susceptibility of kafirin microparticle films to proteolysis, as described above and the larger surface area of the collagen implants compared to the kafirin microparticle films, regardless of the same implant weights being used.

Evidence of ectopic bone morphogenesis

Radiographs of the rats post-mortem showed no indication of new bone formation for any of the implants by Day 28 post implantation (data not shown). This concurred with histological data for the kafirin microparticle film implants but not for the collagen implants (Table 1 B). By Day 28 post implantation, histological evaluation showed evidence of osteogenesis at the high dose rhBMP-2 collagen implant sites but not at any of the kafirin microparticle film implant sites (Table 1B). Possibly, at the high dose rhBMP-2 collagen implants sites, the beginning of bone morphogenesis was present but it was not sufficiently advanced for calcification to have occurred and thus was not visible on the radiographs. Alkaline phosphatase (ALP) activity was evaluated as a marker of early osteoblast differentiation as it is elevated in the presence of increased osteoblastic activity.³² The high dose rhBMP-2 loaded collagen implants had significantly higher ALP levels than any of the other treatments (Table 2), indicating the presence of some bone morphogenesis and in agreement with the histological results for this treatment.

Table 1 Histological scoring for inflammatory and osteogenic response of rat tissue to kafirin microparticle film and collagen loaded with BMP-2 and polyphenol and glutaraldehyde treated KMP film implants by Day 7 and Day 28 post implantation

A. Day 7 post implantation

Carrier material	Treatment	Foreign body reaction	Granulomatous reaction	Ulcerated skin	Lysis [#]	Osteogenesis
Collagen						
	Control	2.3b (0.5)	3.0b (0)	1.0a (0.8)	2.5b (0.6)	0a (0)
	Low rhBMP-2	2.3b (0.6)	3.0b (0)	0.7a (0.6)	2.3b (0.6)	0a (0)
	High rhBMP-2	2.3b (0.5)	3.0b (0)	0.8a (0.5)	2.0b (0.8)	0a (0)
Kafirin Microparticle Film						
	Control	0.3a (0.5)	1.3a (0.5)	1.0a (0.8)	0a (0)	0a (0)
	Low rhBMP-2	0.8a (1.0)	1.8a (1.0)	1.3a (1.0)	0a (0)	0a (0)
	High rhBMP-2	0.3a (0.5)	1.8a (1.0)	0.8a (1.5)	0a (0)	0a (0)
	Glutaraldehyde	0.3a (0.5)	1.0a (0)	0.8a (0.5)	0a (0)	0a (0)
	Polyphenol	0.8a (1.0)	1.8a (1.0)	1.0a (0.8)	0a (0)	0a (0)

B. Day 28 post implantation

Carrier material	Treatment	Foreign body reaction	Granulomatous reaction	Ulcerated skin	Lysis [#]	Osteogenesis
Collagen						
	Control	2.5b (1.0)	2.8a (0.5)	0.3a (0.5)	2.3b (0.5)	0a (0)
	Low rhBMP-2	2.0ab (0)	2.3a (1.0)	0.3a (0.5)	1.5ab (1.5)	0a (0)
	High rhBMP-2	2.5b (0.6)	2.0a (0.8)	0.3a (0.5)	2.3b (0.5)	1.0a (0)
Kafirin Microparticle Film						
	Control	1.5a (0.6)	2.0a (0.8)	0a (0)	1.8ab (1.8)	0a (0)
	Low rhBMP-2	1.8ab (0.5)	2.3a (1.0)	0a (0)	1.3ab (1.0)	0a (0)
	High rhBMP-2	1.8ab (0.5)	2.0a (0.8)	0a (0)	1.0a (0.8)	0a (0)

Results were graded as follows: negative/none (0), mild (1), moderate (2), severe (3).

Total number of implants per treatment =4. RhBMP-2 Dose: Control=0 µg/g, Low=0.107 µg/g; High=2.14 µg/g

[#] Osteolysis was used as measure of degradation of the implant material

Values in a column followed by different letters are significantly different (p<0.05)

Numbers in the brackets are standard deviations, (n=4)

Table 2 Alkaline phosphatase (ALP) activity of kafirin microparticle film or collagen standard implants loaded with rhBMP-2 after 28 days of implantation into rats

Carrier material	rhBMP-2 Dose	ALP concentration (pmol/g)
Collagen	Control	174.3 c (11.5)
	Low	184.3 c (8.6)
	High	244.3 d (7.4)
Kafirin microparticle film	Control	111.0 b (3.8)
	Low	72.4 a (8.2)
	High	118.6 b (8.4)

Values in a column followed by different letters are significantly different ($p < 0.05$)

Numbers in the brackets are standard deviations, (n=4)

RhBMP-2 Dose: Control=0 $\mu\text{g/g}$; Low=0.107 $\mu\text{g/g}$; High=2.14 $\mu\text{g/g}$.

CONCLUSIONS

Kafirin microparticles injected subcutaneously in mice induce chronic inflammation, whereas films made from the same material showed no abnormal inflammatory reactions when implanted subcutaneously in rats. The difference can be attributed to the much larger surface area of the microparticles resulting in more rapid breakdown, releasing toxic products such as glutamate. This study raises awareness that the form of prolamin protein based biomaterials, such as kafirin and zein should be born in mind when assessing the safety of such materials for potential use as implanted scaffolds.

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