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In Vitro effect of Bone Morphogenetic Protein-2 (BMP-2) and Cigarette Smoke Extract (CSE) on Osteoblastic Mesenchymal Stem Cells: Beneficial Biological Effects of BMP-2 Negated By CSE

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Abstract

Introduction: Clinical and demographic studies have shown that tobacco smoking is a major contributor to non- and delayed-union in fracture healing. The cellular and molecular basis for this is poorly understood, and few studies in human fractures have been undertaken.

Aims: To analyse the *in vitro* biological effects of tobacco smoking at the cellular level within the human fracture microenvironment, with specific regard to mesenchymal stem cell (MSC) proliferation and to ascertain whether the application of bone morphogenetic factor-2 (BMP-2) could be used therapeutically to improve fracture healing.

Methods: Fracture haematomas (n=10) were collected from anaesthetised, non-smoking patients who had sustained a tibial fracture, and who were undergoing surgical operative fixation. The semi-solid material was dissected and explanted into tissue culture flasks. Complete culture media was introduced, and cultures were incubated at 37 °C in a humidified 5% CO₂ environment. Cigarette smoke extract (CSE) was produced and infused into the cell cultures to establish an *in vitro* smoking environment. A control group (n=10) was set-up and left untreated by CSE. Harvested, spindle-shaped adherent cells were characterised by immunocytochemistry. Cell populations were counted via flow cytometry to assess and compare proliferation rates between CSE-treated and untreated cell cultures. BMP-2 concentrations (10 and 100 ng/mL (an additional dose of 500 ng/mL in CSE-treated cells)) were infused into cell cultures to enhance *in vitro* cellular viability, which was analysed by means of the MTT assay.

Results: There was a significant reduction in the rate of proliferation of osteoblastic MSCs in CSE-treated cells after 5 days of culture ($p < 0.05$). At a dose of 100 ng/mL, BMP-2 augmented cellular growth and improved cellular viability in cultures not treated with CSE ($p < 0.0001$). No significant improvement was seen in CSE-treated cell cultures.

Summary: The effect of smoking on bone fracture healing appears to contribute to the inhibition of osteoblast proliferation, which may not be reversible with the therapeutic use of exogenous BMP-2. Moreover, the improvement seen in non-smokers does strengthen the case for smokers to cease using tobacco in the perioperative setting in order that such treatments are rendered more effective.

Introduction

Tobacco smoking has serious consequences in patients who have sustained bone fractures, and is known to be associated with delayed- and non-union of fractures

[1,2]. Orthopaedic surgeons have long recognised the harmful aspects of smoking and its detrimental impact on the dynamics of bone regeneration [2-4]. Indeed, Mosely and Finseth (1977) were the first to report that cigarette smoking had a detrimental effect regarding the

healing of hand wounds, and the serious and adverse effects of smoking on healing tissue have been elucidated by numerous clinical trials [5-10]. A seminal study conducted by Adams *et al.* found that the mean time to union of open tibial fractures was 32 weeks for smokers, compared with 28 weeks for non-smokers, thus drawing attention to the detrimental medical effects and socio-economic consequences associated with smoking [11]. The damaging pathophysiological basis of inhaled tobacco smoke is thought to be mediated by the vasoconstrictive and platelet-activating properties of nicotine, the hypoxia-promoting effects of carbon monoxide and the inhibition of oxidative metabolism by hydrogen cyanide at the osteoblastic cellular level [12-14]. These hypotheses are consistent with recent experimental studies on animals that demonstrate a nicotine-impaired blood flow to the site of injury [15,16]. Thus, smoking is of major concern to the orthopaedic surgeon dealing with fractures, and patients are routinely advised to refrain from smoking perioperatively in order to improve lung function and enhance rehabilitation outcomes [15,17-25].

Cigarette smoke consists of volatile acids and complex compounds of particulate matter. Approximately 500 different gases are released during combustion, including nitrogen, carbon monoxide, carbon dioxide, ammonia, hydrogen cyanide and benzene. In addition to these gasses, there are roughly 3500 chemicals produced and, aside from nicotine, include anatabine and anabasine [26,27]. The tar produced when the particulate matter loses water contains the carcinogens associated with cigarette smoking [28]. Nicotine is regarded as the addictive component, and is known to increase platelet aggregation, decrease microvascular prostacyclin levels and inhibit the physiological function of fibroblasts, red blood cells and macrophages [29]. The pharmacokinetics of the drug entails acting upon nicotinic acetylcholine receptors on certain cells. Moreover, the inhaled circulating carbon monoxide has a greater affinity for haemoglobin binding than oxygen, which reduces oxygen tension in tissues [30,31].

Bone Morphogenetic Proteins (BMPs) are pleiotropic morphogens that are capable of regulating growth, differentiation and apoptosis of osteoblasts, chondroblasts, neural cells and epithelial cells [32,33]. The production of BMPs involves various extracellular matrix (ECM) factors, osteoprogenitors, mesenchymal stem cells (MSCs), osteoblasts and chondrocytes. Bone morphogenetic proteins are essential in fracture healing because they elicit a series of events relevant to chondro-osteogenesis.

This typically involves chemotaxis, mesenchymal and osteoprogenitor cell proliferation and differentiation, angiogenesis and ECM synthesis [34-41].

There is a structural and functional similarity amongst BMPs, although each subtype has a unique role within fracture healing. The BMPs also have an ability to stimulate the synthesis and secretion of other bone repair factors such as insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) [42]. All BMPs, except for BMP-1, are members of the transforming growth factor- β (TGF- β) superfamily. BMP-1 is thus classified as a metalloproteinase that is capable of cleaving the BMP-2 antagonist chordin, and is involved in the direction of bone and cartilage formation *in vivo*. It is for this reason that the protein was first thought to behave like other BMPs, and thus was initially characterised as such [43-46].

In the early stages of a bone fracture, a haematoma is formed at the site of injury, which contains an array of progenitor cells and acute-phase proteins; these help to orchestrate the healing process [47]. Mesenchymal stem cells (MSCs), multipotent in nature, are found within the haematoma itself and surrounding soft tissues produce key cytokines that are released *in situ* and systemically [48,49]. These cytokines are instrumental in inducing a beneficial inflammatory response, and the subsequent formation of granulation tissue at the fracture site. Chemokines, for example, which are vital in attracting neutrophils and other leukocytes to the wound area, are known to be secreted by MSCs [29,50,51]. The precise patho-biological mechanisms resulting from tobacco smoking on the healing process in bones and soft tissues, however, remain largely unknown. The aims of this study, therefore, were to investigate the cellular damage that may occur due to smoking, and to ascertain the possible efficacy of BMP-2 in improving fracture healing rates in patients who smoke.

Methods

Patients and sample collection

Approximately 5.0 mL of the fracture haematoma was aspirated from the medullary area of closed tibial fractures of non-smoking, anaesthetised trauma patients ($n=10$) during surgical operative reduction and fixation within 24 hours of hospital admission. This type of fracture was selected due to there being a surplus amount of haematoma material available, which is ordinarily cleansed from the injury site during reparative surgery. The mean age of the patient group was 43.67 years (range: 23-84), and consisted of 7 males and 3 females. Written informed consent was

obtained from each participant and ethical approval was granted from the Local Research Ethics Committee prior to the commencement of this investigation.

Fracture haematoma: The extraction of progenitor cells

The semi-solid material was removed from the fluid components in the specimen and gently washed in phosphate buffered saline (PBS) (Sigma, St. Louis). Small dissected pieces of the material were explanted into sterile 25 cm² tissue culture flasks (Sarstedt, Nümbrecht) and briefly allowed to solidify in filtered air before the addition of 4.0 mL of Iscove's Modified Dulbecco's Media (IMDM) (Sigma, St. Louis), containing 10% foetal calf serum (FCS) (Sigma, St. Louis), 1% penicillin and streptomycin (Sigma, St. Louis) and 500 mL of 200 mMol L-glutamine (Sigma, St. Louis). Primary cultures were incubated at 37°C in a humidified, 5% CO₂ incubator and left, undisturbed, for 7 days. After this period, the flask substrata were washed gently in PBS to remove debris and non-viable cells and the culture media was renewed thereafter every 3-5 days. On reaching flask confluence (approximately 23 days), the cells were harvested with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis), and subdivided into further passages. After six passages of culture, cells were stored in the vapour phase of liquid nitrogen (N₂), at a temperature of approximately -160°C, for later analysis.

Immunohistochemical characterisation of MSCs

Adherent cells were harvested and characterised using immunofluorescent microscopy and flow cytometry (Coulter, California). Monoclonal antibodies (MAbs) against cell surface cluster of differentiation (CD) antigens CD29, CD44 (Caltag, Carlsbad), CD73 (eBioscience, San Diego), CD105 (Immunostep, Salamanca) and CD166 (Medical and Biological Laboratories Co., Naka-Ku Nagoya), conjugated with fluorescein isothiocyanate (FITC), were used as positive controls and the haematopoietic stem cell marker (HSCM) CD34 (Novocastra, Newcastle Upon Tyne) was used as a negative control on all cell samples in both assays. Commercially available MSCs (Japan Cell Bank, Shinjuku) were used as a comparative positive control.

Characterisation of MSCs

Immunocytochemistry: After absolute counting using flow cytometry, approximately 5×10^4 cells/mL were transferred onto individual glass slides by using a cytopsin (Thermo Shandon, Runcorn). The resulting cell clusters were fixed in 0.3% methanol at -20°C for 1 minute

before being briefly washed in PBS, and were blocked with 1% bovine serum albumin (BSA) (Sigma, St. Louis) to prevent non-specific binding of antibody to cell surfaces. Primary MAbs (mouse, anti-human) against CD29, CD34, CD44, CD73, CD105 and CD166 were added onto cells on individual slides, and left to incubate at temperature (RT) for 1 hour (h) before washing again in PBS. A polyclonal secondary antibody (rabbit, anti-mouse) (Dako, Carpinteria) was then applied to the cells and incubated for 1h at room RT in the dark. Cells were finally washed in PBS before applying a glass cover slip and analysed on a fluorescent microscope (Nikon, Livingston).

Immunophenotyping: Harvested fibroblast-like cells, that were spindle-shaped in their morphology, were quantitated by flow cytometry and washed with PBS, before blocking with BSA. Approximately 5×10^5 cells were diluted in 900 mL of Isoton II diluent (Beckman Coulter, High Wycombe); 10 mL of primary MAb (specific for CD29, CD44, CD73, CD105 and CD166; CD34 was used as a negative marker), conjugated with FITC, was then added to the solution and left to incubate at RT in the dark for 30 minutes. Samples were then processed on a flow cytometer to quantify the percentage of MSC-positive cells within the cell population for each marker.

In vitro effects of a smoking environment on fracture cells

The Cigarette Smoke Extract (CSE) was produced based on a validated pump system as reported by Bernhard *et al.* [52]. In order to prepare the extract, four cigarettes were 'smoked' through 30 mL of IMDM using a 50 mL syringe. Each cigarette was smoked for 10 puffs, each puff drawing 35 mL of smoke every 30 seconds, which burnt approximately 75% of the cigarette. The volume of smoke generated, therefore, was 350 mL. Each millilitre of CSE contained 0.133 (4/30) of a cigarette's smoke-derived constituents. The resultant CSE was sterilised by filtering through a 0.2 mm syringe filter and the pH was adjusted to 7.4, pipetted into 1.0 mL aliquots, and frozen at -20°C. The brand of cigarettes used were Marlboro Reds Class A, and rated as 10 mg tar, 0.8 mg of nicotine and 10.0 mg of carbon monoxide.

Bernhard's validated volumetric equations are based on the assumptions that a human generates 350 mL of smoke with each cigarette and has a blood volume of 6L. A person smoking 20 cigarettes per day will have the equivalent of 1 cigarette's toxins in each 300 mL (6000/20) of blood. Each mL of blood, therefore, contains 0.0033 (1/300) of 1 cigarette's smoke-derived constituents. By this

calculation, CSE can be considered to contain 40 times (0.133/0.0033) the amount of smoke-derived constituents that would be expected to be contained in the blood of a smoker who smokes 20 cigarettes per day. A 2.5% (1/40) solution of CSE in the IMDM would, therefore, equate to 20 cigarettes per day. CSE-treated cell cultures ($n=10$) were counted at baseline and again after 5 days of infusion and compared with the untreated group ($n=10$). Micrographs of the CSE-treated cultures were ascertained to assess the morphological changes associated with a 2.5% CSE concentration *In vitro*.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay: *In Vitro* effect of BMP-2 on Cell Viability Enhancement

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole, is reduced to purple formazan in the mitochondria of living cells. The solubilisation solution, dimethyl sulfoxide (DMSO) (an acidified ethanol solution), is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this solution can be quantified by measuring at a wavelength of 570 nm by a spectrophotometer. The deeper the purple colour, the greater the mitochondrial presence and increased cellular activity, proliferation and viability. This assay was used to analyse the viability of cells in culture. It is particularly suitable where a growth factor or drug is incorporated into the culture media. Samples were analysed in 96-well plates, and read on a spectrophotometer for absorbance determination. The MTT assay was appropriate to determine the effects of a BMP-2 intervention on the haematoma-derived MSCs.

Cell preparation - MTT assay

The haematoma-derived cells were retrieved from the vapour phase of the liquid nitrogen vessel. Cells were all harvested from non-smoking patients' fracture haematomas ($n=10$) and were allowed to expand in culture until flask-confluence, which occurred in all samples after 5-10 days in the 5th passage. After the application of trypsin and re-suspending in 1.0 mL of complete media, cells were combined with an isoton II solution containing a known quantity of fluorescent beads (Coulter, California) and counted using a flow cytometer so that cell suspension concentrations could be determined prior to plating-out in a 96 well plate. Cells were plated-out at an inoculum of 5.0×10^3 cells per well. Three groups were created in duplicate (20 wells per group) and incubated for 72h with concentrations of BMP-2 at either 10 ng/mL or 100 ng/mL being added in respective groups. A control group was set

up that did not contain any of the BMP-2 growth factor. Each well was made up to 250 μ L using complete media; surrounding empty wells were filled with media only to prevent dehydration. Plates were covered and incubated at 37°C in a humidified incubator for 72 h.

MTT preparation

10 mg of MTT was dissolved in 1.0 mL of distilled water (dH_2O). A final concentration of 1 mg/mL of MTT in IMDM was aliquoted. 50 μ L of the MTT solution was added to each well after 72 h. The reaction was allowed to continue for 4 h in the incubator after adding the MTT solution. After 4 h, the MTT reagent was pipetted from the plate and safely discarded. 100 μ L of DMSO was then added to each well in order to dissolve the purple formazan product within the mitochondria of the adherent haematoma cells. Plates were then read at 570 nm using a microplate reader.

Statistical analysis

All data acquired was analysed using the Student's *t*-test (non-parametric) or ANOVA (parametric), as appropriate, to obtain confidence intervals; *p* values of 0.05 or less were regarded as statistically significant.

Results

Cell culture

Cells were photographed at different stages of culture using a light microscope fitted with a digital camera (Nikon, Livingston). The morphological features of the adherent cells in culture were observed as spindle-shaped and fibroblast-like structures that formed colonies became confluent in the tissue culture flasks within a few days. There was rapid expansion of cells from the initial primary culture, with minimal decline in the rate of proliferation through subsequent passages (Figure 1).

Characterisation of MSCs by immunocytochemistry

Adherent, fibroblast-like cells were isolated and characterised as MSCs by immunofluorescence and immunophenotyping (cells were found to be strongly positive (>95%) for CD29, CD44, CD105 and CD166, which are qualitative and quantitative techniques, respectively). Human MSCs, sometimes referred to as marrow stromal cells, are capable of becoming differentiated into osteoblasts, chondrocytes, adipocytes, myoblasts and, possibly, neuronal-like cells [47] and, therefore, are essential components of fracture healing.

Figure 1: Micrograph (a) shows a primary culture explanted from a fracture haematoma sample at day 7 (representative sample). The spindle-shaped progenitor cells are forming colonies around a section of haematoma. Micrograph (b) shows proliferating cells reaching confluence within 7 days of subculture into the 1st passage, and was subsequently seeded-out into new cultures ($\times 100$ magnification).

The markers used to characterise the cells are not truly specific for MSCs, although it is agreed in the literature that stromal cells do not express the antigens to haematopoietic CD markers CD11, CD14, CD34 or CD45. They do, however, express CD29, CD44, CD71, CD73, CD90, CD105, CD166, Stro-1 and intracellular adhesion molecule-1 (ICAM-1) [53]. The following images (Figure 2) were observed using a fluorescent microscope after labelling the extracted cells with known MSC surface antigen markers, conjugated with FITC. The HSCM CD 34 was used as a negative control, and cells did not fluoresce when this antibody was used. Cells from the same patient and passage were used in each case as a representative sample.

Figure 2: Fluorescent microscope slides showing immunocytochemical labelling of cells isolated from haematoma cells using MABs against CD29, CD44, CD105, CD166 (MSC surface antigens). The presence of the FITC fluorescence is highly suggestive of an MSC population within cultures (representative sample).

Immunophenotyping

Flow cytometry was used to quantify the amount of positive cells in the haematoma samples (Figure 3). Cells were again labelled with MABs specific to MSC surface antigens conjugated with FITC. Flow cytometry was employed to detect labelled cells and ascertain the amount of positive cells in a given cell population derived from the fracture haematoma. The following histograms are taken from a representative patient sample in the 5th passage.

Figure 3: MSCs derived from the fracture haematomas were strongly positive for CD29 (A), 44 (B), 105 (C) and 166 (D) (each $> 90\%$) on flow cytometry.

In Vitro Effects of Smoking on Fracture MSCs

Cell counting using flow cytometry showed that CSE-treated and non-CSE-treated cells proliferated rapidly *in vitro*, as shown in figure 4. CSE-treated cells, however, were significantly inhibited *in vitro* cell culture, compared with non-CSE-treated cells ($p < 0.05$).

Figure 4: Cell proliferation comparison in MSCs extracted from fracture haematomas using flow cytometry. CSE-treated and untreated after 5 days' of *in vitro* culture. Values are normalised means, and correlated to percentage changes (%Δ) from baseline MSC inoculum. Error bars represent standard error from the mean ($\pm$ SEM) and statistical analysis performed using Student's *t*-test.

In Vitro Effect of BMP-2 on Cell Viability Enhancement

Figure 5 shows the effects of adding BMP-2 (10 and 100 ng/mL) on MSC viability and proliferation *in vitro*, when compared with cells that did not receive the reagent (control). The results show that BMP-2 has a highly significant effect on the growth of viable MSCs *in vitro* that could possibly accelerate fracture healing in patients, if given exogenously.

Figure 5: *In vitro* effects on fracture MSC development after 72 h with 10 and 100 ng/mL of added exogenous BMP-2, and the control group (BMP-2; 0 ng/mL), using the MTT assay. All values are means, with error bars representing standard error of the mean ($\pm$ SEM); all groups $n=10$. Statistical analysis performed using ANOVA (Analysis Of Variance).

Figure 6 (below) shows the effects of adding BMP-2 (10, 100 and 500 ng/mL) on MSC viability and proliferation *in vitro*, when compared with cells that did not receive the reagent (control). The results show that BMP-2 has a non-significant effect on the growth of viable MSCs *in vitro* that have been cultured in a smoke-treated environment, despite the addition of 500 ng/mL of BMP-2 growth factor.

Figure 6: BMP-2 Infusion in CSE Environment.

Discussion

The aims of this study were two-fold. The first aim was to focus upon the detrimental biological effects of smoking that often lead to delayed fracture healing, concentrating specifically on the cellular aspects within the fracture microenvironment. The second was to assess whether the addition of BMP-2 would improve cellular growth in an *in vitro* smoking environment. The fracture haematomas were collected from non-smoking patients who had sustained a fracture of the tibia, and who were undergoing operative reduction and fixation within 24 h of injury. Fracture haematomas are known to be a rich source of MSCs, which are regenerated and become differentiated into osteoblasts during the formation of new bone throughout the process of fracture healing by a number of chemical differentiating mediators [47,49]. An important aspect is the capability of osteoblastic stem cells to generate various key molecular growth factors at the site of injury, of which BMP-2 is an important promoter [54]. Our hypothesis in the current study is that stem cells involved in fracture healing are adversely affected by smoking, and we were able to demonstrate this by the utilisation of an *in vitro* smoke-treated model. We also questioned whether a fracture environment could be enhanced with the addition

of exogenous BMP-2 in both smoke-treated and untreated osteoblastic cells by improving cellular viability and growth, and if any differences could be observed between the two groups studied.

After the cells were cultured through 5 passages and quantified, at flask-confluence, *in vitro* proliferation rates were then compared between CSE-treated and untreated cultures to assess cellular proliferation rates. It was found that the cells treated with CSE proliferated more slowly than the untreated cells after 5 days. The significant cellular degeneration and death seen with cultures that were infused with CSE highlight the biological harm caused by tobacco smoke on cells that have an essential involvement in the fracture healing process. This negative impact of tobacco smoke on healing bones presents a serious cause for concern to patients and orthopaedic surgeons. Our *in vitro* results are in agreement with the clinical findings of Adams *et al.* who noted an extended time to union of 4 weeks in a fractured tibia in smokers. The results shown here suggest a toxic effect; likely due to the persistence of tissue-damaging gasses such as carbon monoxide and hydrogen cyanide, and such findings have not been previously reported in *in vitro* human studies [55]. The observation that CSE-treated MSCs proliferate at a slower rate is highly suggestive that certain growth factors synthesised by these cells could also be affected, and that this may be corrected *in vitro* by the introduction of exogenous BMP-2. In addition, possible cytoplasmic growth factor deprivation in the smoke-affected MSCs could hinder the recruitment of further cells and mediators to the fracture site during early bone repair, further inhibiting the reparative process.

The application of soluble growth factors to mesenchymal cell cultures, such as BMP-2, has been the subject of debate largely due to its beneficial use in studies concerning bone tissue engineering [44,56]. As members of the TGF- β superfamily, BMPs are, principally, differentiation factors and regulators of the bone induction cascade in skeletal tissue regeneration [57]. The growth factor BMP-2, which is active in the early stages of fracture healing, can be administered exogenously (as recombinant BMP-2 (rhBMP-2)) to various sites in order to accelerate bone regeneration. It has been shown in one particular *in vivo* study to enhance bone formation in bone gap healing [58]. In another study it was shown to improve osseointegration of implants when covalently bound to biomaterial surfaces [59].

Turhani *et al.* [56] investigated the supplementation

effect of increasing concentrations of rhBMP-2 *in vitro* on the osteoblastic cell line SaoS-2, which was derived from an osteogenic sarcoma and has osteoblastic phenotypic characteristics. Cell cultures were subjected to various dose incremental concentrations of exogenous rhBMP-2 (10, 100 and 500 ng/mL), which were analysed after 24 h, 48 h and 72 h for cell viability. The group found that there was a dose-dependent significant increase of cellular viability with the addition of rhBMP-2. It was concluded, however, that further investigations were still needed to elucidate the intricate regulatory mechanisms influencing cell responses to exogenous growth factors, particularly BMP-2 [56]. Nevertheless, our study differs from that of Turhani and co-workers, as it describes cells that were derived from consenting hospital patients who had sustained a tibial fracture, as opposed to using a commercially-available cell line, therefore utilising cells that are biologically important in the bone healing process in humans. Indeed, deriving MSCs from fracture haematomas and analysing how smoke-damaged MSCs may result in abnormal healing appears not to have been previously reported.

The addition of exogenous BMP-2 (as a plausible therapeutic strategy to ameliorate fracture repair) in a smoke-treated environment was a characteristic feature of this study. Could it offer a potential osteoinductive clinical treatment for smokers who are displaying symptoms of mal- and delayed- union? Our MTT data on CSE-treated osteoblastic cells suggest that it does not. Cells with a 2.5% CSE infusion showed no significant changes between incremental doses of BMP-2 in smoke-infused environments. This is possibly due to damaged mitochondria, which are located in the lamellipodia of migrating progenitor cells. As the MTT assay relates to the presence of mitochondria to gauge cellular viability (by reduction to purple formazan), a lack of mitochondria would therefore attenuate the reaction. Agonistic improvements attributable to BMP-2, however, were seen in smoke-free MTT cell cultures at a dose of 100 ng/mL, and this concentration had a highly significant stimulatory effect on improving MSC viability ($p < 0.0001$). These findings suggest that it would be advisable for patients to cease smoking for as long as possible following reparative surgery in order for the BMP-2 to be optimally effective, and may be particularly important in individuals who are being considered for BMP-2 as an exogenously prescribed therapeutic intervention. The lack of efficacy of BMP-2 in CSE-treated MSCs, as demonstrated in Figure 6, is more likely due to damaged mitochondria, caused by the cigarette smoke toxins. It intimates that this growth factor, and quite possibly many others, may not be effective in cigarette-

smoke environments, whether *in vitro* or *in vivo*. Patients should be made aware by their clinicians, surgeons and health care professionals that medical interventions may not be optimally beneficial unless they cease smoking for as long as possible, and certainly in cases where medications that are designed to expedite bone healing are indicated. Smoking cessation programmes should be encouraged and recommended for patients who find it difficult to break the habit. In light of these observations, urgent clinical trials should now be carried out using exogenous growth factors, such as BMP-2, in smokers who have sustained a fractured tibia requiring surgical intervention, and, in particular, to ascertain the efficacy of 100 ng/L of BMP-2 on improving fracture healing.

Summary

Tobacco smoking is known to have a deleterious effect on the dynamic processes of both wound and fracture healing; numerous clinical studies involving humans have been shown to demonstrate this detrimental effect. Very few *in vitro* biological investigations, studying smoking and fracture healing, have been carried out in human tissues at the cellular level. Orthopaedic surgeons have recommended that there should be a period of smoking cessation prior to and following surgery. The results from this study suggest that there are detrimental biological factors present in smokers that compromise the efficacy of bone repair and regeneration following a traumatic fracture for a considerable time beyond surgical intervention. A history of cigarette smoking, therefore, should be obtained from individuals so that the risks and complications following a bone fracture may be discussed with them. Both the surgeon and patient should be aware of the deleterious effects of smoking when assessing or planning surgery and that smoking should cease in the perioperative period in order to achieve the best surgical outcomes. In patients who are unable or unwilling to stop smoking, then interventional growth factors, such as BMP-2, may not be optimally effective and probably should not be prescribed.

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Conflict of interest: There are no conflicts of interest.

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