AGEs induce caspase-mediated apoptosis of rat BMSCs via TNFα production and oxidative stress

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Abstract
Diabetic humans and animals exhibit lower bone mass and healing, resulting from diminished bone formation. We have recently reported that type 1 diabetic rats have fewer bone marrow osteoprogenitor cells, and since the formation of advanced glycation end products (AGEs) in bone increases in diabetes, we explored possible mechanisms involved in AGE-induced apoptosis of rat bone marrow stromal cells (BMSCs). BMSCs isolated from 4-month-old rats were exposed to 10–400 \( \mu \)g/ml AGE–BSA for 16 h and apoptosis was quantified with PI/annexin V staining and flow cytometry. Signaling mechanisms were evaluated by preincubating the cells with appropriate inhibitors. The formation of reactive oxygen species (ROS) was quantified by flow cytometric analysis of DCFDA fluorescence and the expression of genes by RT-PCR analysis. AGE–BSA at a concentration of 400 \( \mu \)g/ml increased the apoptosis of BMSCs two- to threefold, an effect completely blocked by a pan-caspase inhibitor. BSA or high concentrations of glucose had no effect. AGE–BSA-induced BMSC apoptosis was attenuated by a p38 inhibitor but not by an NF-κB inhibitor. Treatment with AGE–BSA induced the expression of several pro-apoptotic ligands and receptors, most notably tumor necrosis factor \( \alpha \) (TNF\( \alpha \)), TRAIL, lymphotoxin alpha, CD40, and TNFR2. Furthermore, AGE–BSA-induced apoptosis was completely blocked by pirfenidone, an inhibitor of TNF\( \alpha \) production/secretion. Finally, AGE–BSA increased the production of ROS in BMSCs, and its apoptogenic effect was blocked by the antioxidant N-acetylcysteine (N-acetyl-L-cysteine). Thus, AGE–BSA increases the apoptosis of rat BMSCs via the activation of caspases, involving TNF\( \alpha \) production/secretion, p38 MAPK signaling, and oxidative stress. We propose that increased protein glycation, such as that occurring under hyperglycemia, causes the apoptosis of BMSCs, which might significantly contribute to the development of osteopenia in diabetic animals.

Key Words
- diabetes
- apoptosis
- bone marrow stromal cells
- osteopenia

Introduction
Osteoporosis is a common complication of diabetes mellitus (DM), and patients with either T1DM or T2DM experience a higher incidence of fractures (Schwartz 2003, Yamagishi et al. 2005). Numerous rat and mouse studies have confirmed that T1DM results in reduced bone mass (Goodman & Hori 1984, Hamada et al. 2007). The common denominator of all these studies (human and animal) is that DM is associated with a reduction in bone formation rate as part of the low-turnover osteopenia that develops (e.g. Goodman & Hori 1984).
and Krakauer et al. (1995)). In addition to generalized osteopenia, bone healing in response to surgical interventions (e.g. femoral fractures (Funk et al. 2000)), craniotherapy (Santana et al. 2003), or distraction osteogenesis (Thrailkill et al. 2005)) is diminished in diabetic animals.

In recent years, substantial attention has been paid to the role that advanced glycation end products (AGEs) and oxidative stress (OxS) might play in the pathophysiology of DM. AGEs are formed as a result of non-enzymatic reactions between carbohydrates and various proteins or lipids (Valcourt et al. 2007). In several animal models of DM, increased AGE concentrations have been found to be associated with various diabetic complications such as nephropathy, retinopathy, neuropathy, and impaired dermal healing (Méndez et al. 2010). AGEs accumulate in bone tissue during DM (Katayama et al. 1996, Saito et al. 2006) and may participate in the pathogenesis of DM-related osteoporosis. In support of this possibility, AGEs are capable of affecting bone cells directly since cultured osteoblastic cells (Cortizo et al. 2003, Santana et al. 2003) as well as bone tissue in vivo (Santana et al. 2003) express receptor for AGEs (RAGE).

OxS (i.e. exaggerated, unbalanced production of reactive oxygen species (ROS)) has been implicated in many of the pathologies associated with diabetes: nephropathy (Figueroa-Romero et al. 2008), nephropathy (Forbes et al. 2008), and DM-related loss of bone mass (Hamada et al. 2007, Manolagas & Almeida 2007). OxS can cause death in many cell types (Maiese et al. 2007) including osteoblasts (Chen et al. 2005, Fatokun et al. 2006). Thus, AGE formation and OxS are clearly major potential mechanisms for the development of diabetes-related osteopenia, possibly via the apoptosis of osteoblasts or their precursors.

Bone marrow includes hematopoietic and stromal compartments and osteoblast precursors reside within the latter (Owen 1985, Friedenstein 1990). These precursors (osteogenic colony-forming units (CFU-Os)) can be induced to differentiate into functional osteoblasts in specific in vivo conditions such as in diffusion chambers and under the kidney capsule (Friedenstein et al. 1966, Ashton et al. 1980) or in vitro, where in the presence of appropriate culture conditions (such as dexamethasone (DEX), organic phosphate, and ascorbate), these cells express bone-associated markers and form bone-like nodules (Maniatiopoulos et al. 1988, Malaval et al. 1994).

The number of CFU-Os has been shown to decline in conditions characterized by reduced bone formation rate, such as in aged (Bergman et al. 1996), ovariecetomized (Tabuchi et al. 1986), and unloaded (Keila et al. 1994) animals. On the other hand, treatments that increase bone formation such as systemic administration of PTH or PGE2 increase the number of CFU-Os (Nishida et al. 1994, Weinreb et al. 1997).

We have recently provided evidence that T1DM in rats results in fewer osteoprogenitors in bone marrow, possibly due to their apoptosis (Weinberg E, Maymon T, Moses O & Weinreb M, 2013, unpublished observations), and the purpose of this study was to use in vitro assays to test possible mechanisms whereby AGEs induce the apoptosis of rat bone marrow stromal cells (BMSCs), among which osteoprogenitors reside.

**Materials and methods**

All the experiments carried out in this study were approved by the Animal Use Ethics Committee of Faculty of Medicine, Tel-Aviv University. Bone marrow was harvested from male 10-week-old Sprague Dawley rats and was used to generate stromal cell cultures.

**Materials**

All chemicals and reagents used for tissue culture were obtained from Biological Industries (Beit Haemek, Israel), unless otherwise stated. DEX, d-glucose, 2',7'-dichlorofluorescin diacetate (DCFDA), and the antioxidant N-acetyl-L-cysteine (N-acetylcysteine (NAC)) were purchased from Sigma–Aldrich, Pirfenidone, the tumor necrosis factor (TNF) synthesis inhibitor, was purchased from Santa Cruz Biotechnologies. The rats were killed with CO2, and bone marrow was extracted from both the femurs and cells were seeded in 75 cm2 tissue-culture flasks in a medium composed of minimum essential medium-alpha containing 5.5 mmol/l d-glucose. This medium was supplemented with 13 v/v

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FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 12.5 IU/ml nystatin (basic medium), and 10 nM DEX was added to the medium in some of the experiments. Cultures were washed with PBS after 24 h to remove non-adherent cells and were cultured for 6 days in 5% CO₂ at 37 °C in the same medium, which was changed after 3 days.

**Exposure of rat BMSCs to AGE–BSA**

After 7 days, primary cells were collected using 0.25 w/v trypsin/0.05 w/v EDTA, pelleted by centrifugation (150 g (RCF); 7 min at room temperature), counted in a hemocytometer, and reseeded (passage 1) in 60 mm tissue-culture plates, 2.5 × 10⁵ cells/plate for an additional 48-h attachment period. In this study, all the experiments were carried out in passage 1 cells in triplicates. Subsequently, the cells were incubated with 10–400 µg/ml AGE–BSA in a medium containing 2 v/v FCS. Control treatment consisted of incubation of cells in 2 v/v FCS alone, 2 v/v FCS with BSA, or 2 v/v FCS with an elevated concentration of D-glucose (50 mmol/l) or a mannitol equivalent (5.5 mmol/l D-glucose + 44.5 mmol/l mannitol).

**Detection of apoptosis**

After 16 h of exposure to AGE–BSA or controls, the cells were collected, pelleted by centrifugation, and stained with annexin V–FITC and PI, according to the manufacturer’s protocol (eBioscience, San Diego, CA, USA). Briefly, the cells were washed with PBS and stained with 5 µl of annexin V–FITC and 10 µl of PI in 1 × binding buffer for 15 min at room temperature in the dark. Live/apoptotic cells were counted using a flow cytometer (FACSort; BD Biosciences, Franklin Lakes, NJ, USA). The total number of apoptotic cells was derived by adding the number of early apoptotic (annexin V-positive, PI-negative) to that of late apoptotic (annexin V-positive and PI-positive) cells. Annexin V-negative, PI-positive cells were considered to be necrotic cells. In some experiments, the cells were preincubated for 1 h with one of the aforementioned pharmacological inhibitors before exposure to AGE–BSA.

**Detection of intracellular ROS**

The intracellular production of ROS was measured using DCFDA, a fluorogenic dye that is used for the measurement of ROS within the cell. Control and AGE–BSA-treated (16 h) BMSCs were collected, pelleted by centrifugation, and resuspended in PBS containing 10 µM DCFDA for 30 min in the dark. The cells were washed with PBS and analyzed using a flow cytometer with excitation at 495 nm and emission at 529 nm. The percentage of stained cells was determined for each sample after subtracting the respective fluorescence of unstained cells.

**Determination of gene expression**

The expression of apoptosis-related genes was examined after the exposure of BMSCs for 16 h to AGE–BSA or control (2 v/v FCS or BSA). Total RNA was isolated using the Perfect Pure RNA Cultured Cell Kit (5 Prime, Gaithersburg, MD, USA) according to the manufacturer’s instructions. The quality and quantity of the RNA were estimated using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was converted to cDNA using the High Capacity cDNA RT Kit (Applied Biosystems). RNAs from three replicates for each treatment were pooled and a custom TaqMan Array 96-Well FAST Plate (Applied Biosystems) containing 92 probes to key apoptosis-associated genes and four probes to endogenous control genes was used. Fold changes of gene expression relative to the β-glucuronidase gene (Gusb) as an endogenous control (relative quantification (RQ) values) were determined according to the 2^(-ΔΔCt) method (Livak & Schmittgen 2001).

**Statistical analysis**

The cells were exposed to the various treatments in triplicates and experiments were repeated three to five times. To combine data obtained from several experiments, the apoptosis of control cells (treated with 2 v/v FCS) was converted to 100% for each experiment. Means and s.d.s were calculated for each treatment and analyzed with one-way ANOVA followed by non-paired t-tests.

**Results**

The exposure of rat BMSCs to AGE–BSA for 16 h significantly increased the number of cells in early and late apoptosis stages (the lower right and upper right quadrants of the PI/annexin V scatter plot (Fig. 1A and B)). While 200 µg/ml AGE–BSA increased the apoptosis of BMSCs significantly (by about 50%), 400 µg/ml had a more pronounced effect (approximately twofold) and this concentration was used for the remainder of the study. These concentrations of AGE–BSA are within the range used in many other studies (e.g. Nah et al. (2007), Shen et al. (2010), Okazaki et al. (2012) and Shi et al. (2013)).
Mechanisms of AGE–BSA-induced BMSC apoptosis

E WEINBERG and others

BSA alone did not increase the apoptosis of BMSCs. A similar exposure to a high-glucose medium or a high-mannitol medium had no effect on the number of apoptotic cells (Fig. 1C). The fraction of necrotic cells (the upper left quadrant of the scatter plot) was typically around 2.5% in these experiments and was not altered by exposure to AGE–BSA or BSA (data not shown).

Apoptosis usually results from the activation of caspases by the offending factor. To assess the involvement of caspases in AGE–BSA-induced cell death of BMSCs, we preincubated the cells with zD-DCB, a pan-caspase inhibitor (Harada & Sugimoto 1998, Allen et al. 2003, Zeldich et al. 2007), and found that it completely and dose dependently abolished AGE–BSA-induced apoptosis (Fig. 2), indicating that this effect of AGE–BSA is entirely caspase dependent.

To monitor the expression of apoptosis-related genes following exposure to AGE–BSA, we used a custom RT-PCR array that measures the expression of 92 such genes. Among the genes that were mostly upregulated by AGE–BSA, we found several genes whose product is a potent inducer of apoptosis, such as Lta (lymphotoxin alpha = TNFβ; Etemadi et al. 2013), Tnfsf10 (TRAIL; Falschlehner et al. 2007), and Tnf (Table 1). In addition, the expression of other ligands (CD40), receptors (Tnfsf1b (TNFR2)), and intracellular adaptor proteins (Traf1) involved in apoptotic signaling were upregulated. In parallel, some anti-apoptotic genes (e.g. Bcl2l1 (Bcl-X), Birc3 (c-IAP2), and Cflar (C-FLIP)) were also upregulated.

Since the expression of Tnf gene was greatly increased after exposure to AGE–BSA, we decided to test the possibility that the production of TNFα by BMSCs subsequent to exposure to AGE–BSA is involved in the resulting apoptosis. Indeed, preincubation of the cells with pirfenidone, which inhibits the production and secretion of TNFα (Nakazato et al. 2002, Grattendick et al. 2008), completely and dose dependently abolished AGE–BSA-induced apoptosis of rat BMSCs (Fig. 3A). This finding indicated that the production of TNFα by BMSCs subsequent to exposure to AGE–BSA plays a crucial role in the ensuing apoptosis.

The apoptosis of various cell types caused by AGEs involves the activation of the p38 MAPK pathway (Shen et al. 2010, Shi et al. 2013); thus, we sought to test the involvement of this pathway in the apoptosis of BMSCs.

Figure 1
(A) Representative scatter plots of PI (Y-axis) and annexin V (X-axis) staining of BMSCs treated for 16 h with a control (low glucose, 5.5 mmol/l) medium (Cont), a medium containing AGE–BSA at a concentration of 400 µg/ml (AGE–BSA 400), or a medium containing 400 µg/ml BSA (BSA 400). (B) AGE–BSA dose response: mean ± s.d. of apoptotic cells (as percentage of control cells) after treatment for 16 h with a control (low glucose, 5.5 mmol/l) medium (G 5.5), a high (50 mmol/l) glucose medium (G 50), a mannitol substitute (5.5 mmol/l glucose + 44.5 mmol/l mannitol) medium (G 5.5 + M 44.5), a medium containing AGE–BSA at a concentration of 400 µg/ml (AGE–BSA 400), or a medium containing 400 µg/ml BSA (BSA 400). **P<0.01 vs control (G 5.5) cells. (C) Mean ± s.d. of apoptotic cells after treatment for 16 h with a control (low glucose, 5.5 mmol/l) medium (G 5.5), a high (50 mmol/l) glucose medium (G 50), a mannitol substitute (5.5 mmol/l glucose + 44.5 mmol/l mannitol) medium (G 5.5 + M 44.5), a medium containing AGE–BSA at a concentration of 400 µg/ml (AGE–BSA 400), or a medium containing 400 µg/ml BSA (BSA 400). **P<0.01 vs control (G 5.5) cells.

Figure 2
Mean ± s.d. of apoptotic cells (as percentage of control cells) after treatment with 400 µg/ml AGE–BSA and increasing concentrations of zD-DCB, a pan-caspase inhibitor. ***P<0.001 vs control (low glucose) cells.
While SB203580, a well-accepted p38 MAPK inhibitor, was toxic to BMSCs at the lower concentrations of FCS used (data not shown), LY2228820 (a water-soluble p38 inhibitor) was not. The preincubation of BMSCs with increasing concentrations of LY2228820 (Ishitsuka et al. 2008, Tate et al. 2013) gradually attenuated, but did not completely abolish the apoptosis induced by AGE–BSA (Fig. 3B). These data indicated that p38 is involved in AGE–BSA-induced BMSC apoptosis, but that other signal transduction pathways participate too. Importantly, AGE–BSA-induced cell death does not seem to involve the activation of NF-κB, since the pretreatment of BMSCs with 10–20 μM SN50, a cell-permeable NF-κB inhibitory peptide, did not diminish apoptosis following exposure to AGE–BSA (data not shown). Since JNK is often linked to apoptotic signaling, we tried to evaluate its participation in AGE–BSA-induced apoptosis; however, the exposure of BMSCs to 5–20 μM SP600125, a specific JNK inhibitor, was toxic to the cells.

DEX is a critical factor in the induction of osteoblastic differentiation of rat BMSCs (Herbertson & Aubin 1995, Aubin 1999). Since diabetes in vivo increases the apoptosis of mature osteoblasts (Motyl et al. 2012), we hypothesized that the differentiation of cells due to DEX treatment might render them more sensitive to the apoptosis induced by AGE–BSA. BMSCs were treated with 10 nM DEX for 6 days (a regimen that we have repeatedly used to induce osteoblastic differentiation in rat BMSCs (Weinreb et al. 2002, 2004)) and then exposed to 400 μg/ml AGE–BSA for 16 h. Exposure to DEX, by itself, slightly increased the apoptosis of control cells (9 vs 6%); however, whereas AGE–BSA increased the apoptosis of BMSCs not treated with DEX about twofold, it increased apoptosis of DEX-treated BMSCs threefold (Fig. 4).

Finally, we investigated the role of OxS in AGE–BSA-induced BMSC apoptosis. The exposure of cells to AGE–BSA increased their DCFDA fluorescence, indicating an increase in intracellular ROS generation in the cells (Fig. 5A). In the experiments summarized in Fig. 5A, AGE–BSA increased the apoptosis of BMSCs 2.5-fold (not shown). The pretreatment of cells with the antioxidant NAC at 1–2 mM completely and dose dependently inhibited the increase in DCFDA fluorescence induced by AGE–BSA, i.e. prevented the excessive production of ROS within the cells (Fig. 5B). Furthermore, the pretreatment of cells with NAC effectively inhibited apoptosis following exposure to AGE–BSA (Fig. 6).

In summary, AGE–BSA increased the apoptosis of rat BMSCs two- to threefold, an effect that was associated with increased caspase activity, TNF production/secretion, p38 MAPK activation, and OxS. The apoptosis of BMSCs subsequent to increased protein glycation such as that occurring in diabetes might impair osteoblastogenesis and thus contribute significantly to the development of osteopenia in diabetic conditions.

### Table 1

<table>
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<th>Gene symbol</th>
<th>RQ (AGE–BSA)</th>
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<td>C40</td>
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<tr>
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**Figure 3**

(A) Mean ± S.D. of apoptotic cells (as percentage of control cells) after treatment with 400 μg/ml AGE–BSA and increasing concentrations of pirfenidone, an inhibitor of TNFα production/secretion. ***P < 0.01; **P < 0.001 vs control (low glucose) cells. (B) Mean ± S.D. of apoptotic cells (as percentage of control cells) after treatment with 400 μg/ml AGE–BSA and increasing concentrations of LY2228820, a p38 MAPK inhibitor. *P < 0.05; **P < 0.01; ***P < 0.001 vs control (low glucose) cells.
This may add diabetic osteopenia to a growing list of diabetic complications in which TNFα is involved (e.g. Navarro & Mora-Fernández, 2006, Rosser, 2011 and Xu et al., 2013). In addition to TNFα, AGE–BSA increased the mRNA levels of other apoptogenic ligands, such as lymphotoxin alpha (Etemadi et al., 2013) and TRAIL (Falschlehner et al., 2007). AGE–BSA also increased the expression of CD40, which was reported to mediate the release of TNFα in osteoblasts (Die et al., 2012) as well as induce the apoptosis of carcinoma cells via the production of TNFα and TRAIL (Eliopoulos et al., 2000) and that of biliary epithelial cells via the expression of FasL (Afford et al., 2001). Also of interest is the fact that TNFα usually exerts its apoptogenic effect through TNFR1, and we found an increased expression of Tnfrsf1b (TNFR2) after exposure to AGE–BSA. This receptor binds to lymphotoxin alpha, the expression of which was also increased by AGE–BSA in our study, and can also induce the apoptosis of some cells (MacEwan, 2000, Ban et al., 2008). In parallel to the induction of powerful pro-apoptotic genes, some anti-apoptotic genes were upregulated (e.g. Bcl2l1 and Cflar), probably as an attempt to preserve cell viability.

The apoptosis of various cell types caused by AGEs involves the activation of the p38 MAPK pathway (Shen et al., 2010, Shi et al., 2013), and we found that this pathway
intracellular ROS production, in parallel with increased apoptosis. Furthermore, the pretreatment of cells with NAC, an antioxidant, prevented the rise in both ROS production and apoptosis. As has been mentioned earlier, there is a growing recognition that OxS is a major mechanism for many diabetic complications (e.g. Negre-Salvayre et al. (2009)), including osteopenia (Hamada et al. 2007, Mordwinkin et al. 2012). Many of the studies showing that AGEs induce the apoptosis of osteoblastic cells have documented increased ROS generation in the treated cells (Yamagishi et al. 2005, Gangoiti et al. 2008, Schurman et al. 2008). Our data thus show, for the first time, that the apoptosis of BMSCs induced by AGE–BSA depends on intracellular OxS. These data are in agreement with our recent finding that the amount of malondialdehyde, an indicator of lipid peroxidation and hence tissue OxS, is significantly (~35%) elevated in the proximal tibial metaphysis (which comprises mainly bone marrow) of diabetic, compared with normoglycemic, rats (Weinberg E, Maymon T, Moses O, Weinreb M, 2013, unpublished observations). Thus, TNFα and ROS are major players in the apoptogenic effect of AGEs on rat bone marrow cells/osteoprogenitors, as in many of the other diabetic complications (Graves et al. 2006, Negre-Salvayre et al. 2009, Roszer 2011).

There is a widespread agreement that diabetic osteopenia results from diminished bone formation. Since we have recently shown that diabetic rats have fewer osteoprogenitors in their bone marrow (Weinberg E, Maymon T, Moses O, Weinreb M, 2013, unpublished observations), we propose that AGE-induced BMSC apoptosis is a major mechanism whereby hyperglycemia exerts a deleterious effect on the osteoprogenitor pool. Reduction in the number of osteoprogenitors in bone marrow could impair the recruitment of osteoblasts needed for general bone remodeling as well as for bone healing (Funk et al. 2000, Santana et al. 2003, Thrailkill et al. 2005). Since AGEs have also repeatedly been shown to suppress the osteoblastic differentiation of various bone marrow stromal stem cells (Kume et al. 2005, Franke et al. 2011, Okazaki et al. 2012), their impairment of bone formation in diabetic animals may be multifaceted.

In summary, AGE–BSA causes the apoptosis of rat bone marrow stromal stem cells via the induction of TNFα formation, generation of ROS, and activation of caspases. These actions are partly mediated by the activation of the p38 MAPK pathway. The apoptosis of osteoprogenitor cells by AGEs may be an important mechanism for the suppression of bone formation in diabetes.

Figure 6
Mean ± s.o. of apoptotic cells (as percentage of control cells) after treatment with 400 µg/ml AGE–BSA and increasing concentrations of the antioxidant NAC. ***P<0.001 vs control (low glucose) cells.

Our finding that DEX pretreatment increases AGE–BSA-induced BMSC apoptosis can be explained in at least two ways. One explanation is that DEX treatment induces osteoblastic differentiation in BMSCs (evidenced by the acquirement of alkaline phosphatase activity and the production of mineralized extracellular matrix (Rickard et al. 1994, Weinreb et al. 2002, 2004)) and by doing so renders them more sensitive to AGEs-induced apoptosis (Roszer 2011). This notion stems from the observations that diabetes increases the apoptosis of mature osteoblasts in vivo (Liu et al. 2006, Motyl et al. 2012). Another explanation is that DEX can induce the apoptosis of osteoblasts by itself in vitro (Chua et al. 2003, Yun et al. 2009) and also that prolonged glucocorticoid treatment in vivo results in osteoporosis involving the apoptosis of osteoblasts (Migliaccio et al. 2007). Thus, DEX and AGE–BSA could induce synergistic apoptosis in BMSCs. In support of this possibility, DEX slightly increased the apoptosis of control cells, not treated with DEX, in this study. By contrast, one report has shown that DEX inhibits TNFα-induced apoptosis of osteoblasts (Chae et al. 2000), and since DEX-induced apoptosis may use other intracellular pathways (Li et al. 2012), a detailed mechanistic analysis should clarify this issue.

We provide convincing evidence that OxS has a critical role in AGE–BSA-induced BMSC apoptosis. The exposure of BMSCs to AGE–BSA significantly increased their DCFDA fluorescence, attesting to an increased
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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