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Cytotoxicity of zinc-containing bioactive glasses in contact with human osteoblasts

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Abstract

Bioactive glasses such as Hench's 45S5 have applications to tissue engineering and bone repair: the insertion of zinc has been proposed to improve their bone-bonding ability and to slacken their dissolution in extracellular body fluids. In view of a potential clinical application, we have investigated whether zinc-containing 45S5 (HZ) glasses might be cytotoxic for human MG-63 osteoblasts. In our experimental conditions, after 24 h of incubation HZ glasses released significant amounts of Zn^{2+} and induced in MG-63 cells release of lactate dehydrogenase (index of cytotoxicity) and the following indexes of oxidative stress: (i) accumulation of intracellular malonyldialdehyde, (ii) increased activity of pentose phosphate pathway, (iii) increased expression of heme oxygenase-1, (iv) increased activity of Cu,Zn-superoxide dismutase, (v) decreased level of intracellular thiols. These effects were inversely related to the zinc content of glass powders, were mimicked by $ZnCl_2$ solutions and were prevented by either metal chelators (EDTA, NTA) or the antioxidant ascorbate, suggesting that Zn^{2+} released fastly from HZ glasses can cause MG-63 cell damage via an oxidative stress. This work highlights the importance of designing Zn-containing bioactive glasses without cytotoxic effects and gives supplementary information about the prooxidant role of zinc in living systems.

Keywords: Bioactive glasses; Zinc; Cytotoxicity; Oxidative stress; Ion release; Surface area

1. Introduction

Some ceramics, such as Bioglass®, sintered hydroxyapatite and glass-ceramic A-W, spontaneously integrate with living bone by promptly forming a carbonated hydroxyapatite layer on their surfaces. They are listed among bioactive materials and are routinely used in dental and orthopaedic applications as bone substitutes [1] and to coat metallic implants, thus facilitating cell attachment and growth as well as matrix production at the implant surface [2]. The concept of bioactive material was first introduced in 1971, with the synthesis of Bioglass® 45S5 by L.L. Hench: this material is composed of SiO_2, CaO, P_2O_5 and Na_2O [3]. Since then, numerous glasses and glass ceramics with similar compositions have been extensively studied for clinical applications [4].

Bioglass® 45S5 has been shown to bond within days to bone as well as to soft tissues, and to stimulate

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osteogenesis in *in vivo* models [5]. The mechanisms whereby this occurs remain still unknown [6]. However, compared with human cortical bone, bioglasses have lower fracture toughness and higher elastic moduli. Therefore, it is desirable to develop bone-bonding materials with improved mechanical properties [1]. The recent advances in sol-gel synthetic techniques offer new ways of controlling the structure, surface area and glass composition, leading to new materials of tailored applications. For instance, the partial replacement of sodium with zinc in bioactive glasses has been proposed to stimulate bone cells proliferation and differentiation and improve bone-bonding ability of bioglass [7].

Zinc is fundamental for cell growth, development and differentiation, but the biochemical mechanisms involved are not completely known [8]. Zinc is a cofactor for many enzymes, stimulates protein synthesis and is essential for DNA replication [9]. Zinc deficiency is associated with skeletal growth retardation and alterations in bone tissue calcification [10]: zinc is thought to activate bone formation [11] and inhibit bone resorption [10]. For these reasons zinc-containing cements are widely used in dentistry [12]. Zinc also prolongs chemical durability of glass, by retarding its dissolution and reaction in aqueous solutions (such as extracellular body fluids), and improves its mechanical properties [13].

New potential bioactive glasses called HZ have been recently formulated, possessing a composition similar to that of Hench's 45S5, but supplemented with increasing amounts of zinc (5–20%, w/w, as ZnO) [14]. In view of a potential clinical application of HZ glasses, we investigated whether they could stimulate cultured human osteoblast cells to proliferate: in preliminary experiments HZ glasses did not increase the cell proliferation rate, but rather their presence in the culture medium was associated with reduced cell viability, in a dose- and time-dependent way. Starting from this observation, aim of our work has been to clarify whether HZ glasses actually exert toxic effects on human osteoblast cells, and whether zinc, which is released by HZ glasses in the cell culture medium, might play a role in such cytotoxicity.

2. Materials and methods

2.1. Glass powders

Bioglass® 45S5 and three different types of zinc-doped 45S5 (HZ5, HZ10, HZ20) were prepared, as previously described [13]: their chemical composition is detailed in Table 1. Briefly, about 100 g of batch were prepared by mixing reagent grade Na_2CO_3, $CaCO_3$, $Na_3PO_4{\cdot}12H_2O$, SiO_2 (and in HZ also $ZnCO_3$) raw materials in a sealed polyethylene bottle for 1 h. Premixed batches were put into a 50 ml platinum crucible and melted in an electric oven at 1400–1550 °C for 2 h. The melt was quickly cooled between two graphite sheets to avoid undesired crystallization phenomena.

Table 1
Composition of glasses (%, w/w)

Sample	ZnO (%, w/w)	SiO_2 (%, w/w)	Na_2O (%, w/w)	CaO (%, w/w)	P_2O_5 (%, w/w)
45S5 (H)	–	44.75	24.43	24.50	5.93
HZ5	4.94	42.54	23.27	23.40	5.64
HZ10	10.05	40.44	22.08	22.02	5.39
HZ20	20.13	37.14	18.75	18.86	4.64

2.2. Cells and reagents

MG-63 human osteoblasts cells (provided by Istituto Zooprofilattico Sperimentale "B. Ubertini", Brescia, Italy) were cultured up to confluence in 35–150 mm-diameter Petri dishes with MEM (Minimum Essential Medium Eagle with Earl's salts, Sigma Aldrich, Milano, Italy) supplemented with 2% fetal bovine serum (FBS), penicillin, streptomycin and L-glutamine in a humidified atmosphere containing 5% CO_2 at 37 °C. The confluent cells were incubated for 24 h in the absence or presence of glasses and other reagents, before the assays, as described in the following paragraphs.

Human lung epithelial (A549) and murine alveolar macrophage (MH-S) cells, provided by Istituto Zooprofilattico Sperimentale "Bruno Ubertini" (Brescia, Italy), and N11 mouse glial cells (a gift from Dr. Marco Righi, CNR Institute of Neuroscience, Section of Cellular and Molecular Pharmacology, Milano, Italy) were cultured up to confluence in 100 mm diameter Petri dishes with HAM's F12, RPMI, or DMEM, respectively, plus 10% FBS.

The heme-albumin complex, called methemalbumin (MHA), was prepared by dissolving 13 mg hemin in 2.5 ml of 0.1 M NaOH, containing 12 mg of TRIS base: 5 ml of a 2% (w/v) solution of bovine serum albumin were added and the final pH value and hemin concentration were adjusted to 7.5 and 2.5 mM, respectively. The solution was filtered through a 0.2 μm pore filter [15]. Electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA). The protein contents of cell monolayers and cell lysates were assessed with the BCA kit from Pierce (Rockford, IL). Plasticware was from Falcon (Becton Dickinson, Franklin Lakes, NJ). Unless otherwise specified, other reagents were purchased from Sigma Aldrich (Milano, Italy).

2.3. Lactate dehydrogenase (LDH) leakage

After a 24 h incubation with bioactive glasses and/or other substances, the extracellular medium was withdrawn and centrifuged at 12,000 × *g* for 30 min to remove glass powders and cellular debris. Cells were washed with phosphate-buffered saline solution (PBS), detached with trypsin/EDTA (0.05/0.02%, v/v), resuspended in 1 ml of PBS and sonicated on ice with two 10 s bursts. LDH activity was measured in the extracellular medium and in the cell lysate, as previously described [16,17], to check the cytotoxic effect of glass powders. Both intracellular and extracellular enzyme activity were expressed as μmol NADH oxidized/min/dish, then extracellular LDH activity was calculated as a percentage of the total (intracellular + extracellular) LDH activity in the dish.

2.4. Measurement of malonyldialdehyde (MDA)

After a 24 h incubation with bioactive glasses and/or other substances, cells were washed with PBS, detached with trypsin/EDTA, and resuspended in 1 ml of PBS. Lipid peroxidation was spectrophotometrically detected, measuring the intracellular level of MDA, the end product derived from the breakdown of polyunsaturated fatty acids and related esters, with the lipid peroxidation assay kit (Oxford Biomedical Research, Oxford, MI): the reaction of *N*-methyl-2-phenylindole with MDA in the presence of hydrochloric acid produces a stable chromophore with absorbance maximum at 586 nm [18]. Intracellular MDA was expressed as pmol/mg cellular protein.

2.5. Measurement of pentose phosphate pathway (PPP) activity

After a 24 h incubation with bioactive glasses and/or other substances, cells were washed with PBS, detached with trypsin/EDTA, and resuspended (5×10^5 cells/ml) in Hepes buffer (in mM: 145 NaCl, 5 KCl, 1 $MgSO_4$, 10 Hepes sodium salt, 10 glucose, 1 $CaCl_2$, pH 7.4 at 37 °C) containing 2 μCi of [1-^{14}C]glucose or [6-^{14}C]glucose (58 and 55 mCi/mmol, respectively; Dupont-New England Nuclear, Boston, MA). Cell suspensions were incubated for 1 h at 37 °C, using a closed experimental system to trap the $^{14}CO_2$ developed from [^{14}C]glucose [19]. The metabolic flux through the PPP was measured as previously described [19], by subtracting the amount of CO_2 developed in 1 h from [6-^{14}C]glucose (via tricarboxylic acid cycle) from the CO_2 released in the same time period from [1-^{14}C]glucose (via either tricarboxylic acid cycle or PPP).

2.6. Measurement of glutathione (GSH), glutathione disulfide (GSSG) and total thiols

After a 24 h incubation with bioactive glasses and/or other substances, intracellular and extracellular GSH and GSSG were spectrophotometrically determined with the glutathione reductase-Ellman reagent recycling assay [17,20], and total thiols with the Ellman reagent [17,21], as previously described.

2.7. Western blot analysis of heme oxygenase expression

After a 24 h incubation with bioactive glasses and/or other substances, cells were directly solubilized in a lysis buffer (25 mM Hepes, 135 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM EGTA, 1 mM $ZnCl_2$, 50 mM NaF, 20% glycerol, 10% β-mercaptoethanol, 0.002% bromophenol blue, pH 6.8), supplemented with a protease inhibitor cocktail set III (100 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 80 mM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, 1 mM pepstatin; Calbiochem-Novabiochem Corporation, San Diego, CA). Aliquots containing 30 μg of proteins were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (12% polyacrylamide) and probed with a rabbit antibody anti-human heme oxygenase (diluted 1:1000 in PBS–BSA 1%, D.B.A. Italia s.r.l., Milano, Italy) or with anti-mouse human actin, clone AC-40 (diluted 1:500 in PBS–BSA 1%, Sigma Aldrich). After 1 h of incubation, the membrane was washed with PBS–Tween 0.1% and subjected for 1 h to a peroxidase-conjugated anti-rabbit (sheep, Amersham International, Bucks, UK) or anti-mouse (sheep, Amersham International, Bucks, UK) antibody, diluted 1:1000 in PBS–Tween 0.1% with Blocker Non-Fat Dry Milk 5% (Biorad Laboratories, Hercules, CA). The membrane was washed again with PBS–Tween 0.1% and proteins were visualized by a Western blotting detection system, the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Molecular weight standards were used in all gels.

2.8. Surface area evaluation

To evaluate the changes in surface area of the glasses after immersion in fluids, they were soaked in the amount of 150 mg for 100 ml of solution. Specific surface area of the glass powders was evaluated by adsorption of an inert gas (N_2) at the temperature of liquid nitrogen (77 K). The adsorption isotherm was recorded using an automatic gas-volumetric apparatus ASAP 2010 (Micromeritics,

Norcross, GA), and data were analyzed with the BET (Brunauer, Emmet and Teller) model for specific surface area determination [22].

2.9. Zinc release from HZ bioglasses

To assess the amount of Zn^{2+} ions released by HZ bioactive glasses as a function of time, leaching tests were run on zinc-containing glasses after incubation in MEM medium used for osteoblasts cultures. HZ particles of size <26 μm were suspended in MEM at a 50 μg/ml concentration and checked for Zn leaching at different soaking times. The amount of zinc released in solution was determined using an inductively coupled plasma (ICP) spectrometer (Perkin Elmer Optima 4200 DV; Perkin Elmer, Shelton, CT), equipped with ultrasonic nebuliser for trace elements.

2.10. Measurement of Cu,Zn-superoxide dismutase (SOD) activity

After a 24 h incubation with bioactive glasses and/or other substances, the superoxide dismutase activity was measured with the superoxide dismutase ELISA Kit (R&D Systems, Minneapolis, MN), using the xanthine/xanthine oxidase/nitroblue tetrazolium (NBT) system to generate and quantitate superoxide disproportionation, which prevents superoxide-mediated reduction of NBT and lowers the yield of blue NBT-diformazan [23]. Briefly, cells were detached by gentle trypsinization and centrifugated for 10 min at 250 × *g*, then the pellet was suspended in 800 μl of Cell Lysis Solution (R&D Systems). After a 5 min centrifugation at 13,000 × *g*, the supernatant was assayed for total SOD and Cu,Zn-SOD activity, using a Lambda 3 spectrophotometer (Perkin Elmer, Shelton, CT) set at 550 nm. To evaluate the contribution of Cu,Zn-SOD activity to the total activity, Mn-SOD and Fe-SOD were inactivated by adding 500 μl of chloroform/ethanol (37.5/62.5, v/v) to the supernatant before the test [24]. SOD activity was expressed as units per milligram of proteins.

2.11. Statistical analysis

All data concerning cellular tests in text and figures are provided as means ± S.E.M. The results were analyzed by a one-way analysis of variance and Tukey's test: $p < 0.05$ was considered significant.

3. Results

To evaluate the best experimental conditions of incubation of MG-63 cells with bioactive glasses, we first determined the potential cytotoxicity of different concentrations of the materials after a 24 h incubation. The results of such experiments are shown in Fig. 1. In the presence of zinc-free H glass (10–50 μg/ml), MG-63 cells did not show a significant increase of LDH activity in their extracellular medium, that is a sensitive index of cytotoxicity [25]. A concentration of 50 μg/ml zinc-doped HZ glasses was needed to induce a significant leakage of LDH in the culture medium, which progressively increased with the zinc content in the glasses (Fig. 1). According to these results, we decided to test all materials at the concentration of 50 μg/ml, corresponding to 10 μg/cm^2 of cell monolayer, in order to put in evidence the condition in which the glasses exhibit cytotoxicity. Since the cytotoxic effect appeared to be related to the percent content of zinc, and our bioactive glasses are known to release this metal in solution, we measured the amount of Zn^{2+} ions released by HZ bioactive glasses in MEM as a function of time (Fig. 2). Under the same experimental conditions of cytotoxicity tests, both the concentration of Zn^{2+} ions in solution and its increase with time correlated with the amount of zinc in the bulk of particles. After 3 h of soaking in MEM, the concentration of Zn^{2+} in solution was half the maximum zinc concentration that can be obtained by complete glass dissolution (Fig. 2); after 24 h, the

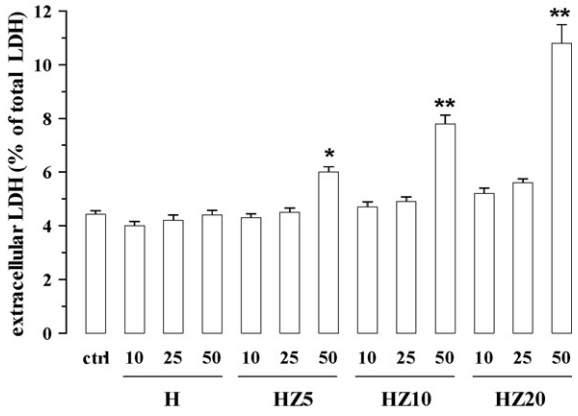

Fig. 1. Effect of different concentrations of glass powders on LDH release in the supernatant of MG-63 cells. Cells were incubated for 24 h in the absence (ctrl) or presence of one of the following bioglasses: 45S5 (H), 45S5 containing 5% (w/w) ZnO (HZ5), 45S5 containing 10% (w/w) ZnO (HZ10), 45S5 containing 20% (w/w) ZnO (HZ20), under the concentrations of 10, 25 and 50 μg/ml (corresponding to 2, 5 and 10 μg/cm^2 of cell monolayer, respectively). After this incubation the LDH activity was measured in the extracellular medium and in the cell lysate, as described in Section 2. Extracellular LDH activity was calculated as percentage of total (intracellular + extracellular) LDH activity in the dish. Measurements ($n = 3$) were performed in triplicate, and data are presented as means ± S.E.M. vs. control (ctrl): *$p < 0.002$; **$p < 0.0001$.

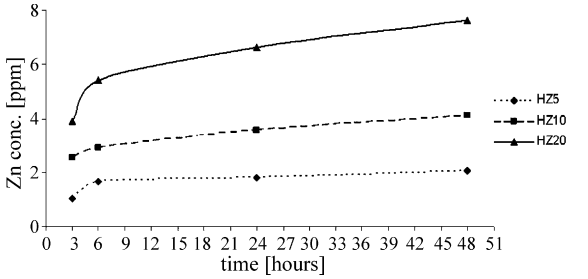

Fig. 2. Zinc release in MEM. Particles of zinc-doped glasses (HZ5, HZ10, HZ20) having a size <26 μm were suspended in MEM at a 50 μg/ml concentration and checked for Zn^{2+} release at different soaking times (3, 24, and 48 h). The concentration of zinc was determined as described under Section 2. 2 ppm = 30.5 μM, 4 ppm = 61 μM, 8 ppm = 122 μM. Data are presented as means ± 0.02 ($n = 4$). In each determination the final value was obtained after subtracting the amount of zinc in MEM alone, used as a blank (0.05 ± 0.05 ppm, 0.7 ± 0.7 μM, $n = 4$).

HZ5 glass was completely dissolved, whereas longer reaction times (up to 48 h) were necessary for HZ10 and HZ20. In fact, the final concentrations detected after 48 h were 2.1 ± 0.1 ppm (32.1 ± 1.5 μM), 4.1 ± 0.2 ppm (62.7 ± 3.0 μM) and 7.6 ± 0.2 ppm (116.3 ± 3.0 μM), for HZ5, HZ10 and HZ20, respectively. These values are very similar to the theoretical concentrations expected after a complete dissolution: 2 ppm (30.5 μM), 4 ppm (61 μM), 8 ppm (122 μM) for HZ5, HZ10 and HZ20, respectively.

The Zn content of bioactive glasses influenced their dissolution: the increase of surface area of HZ particles suspended in MEM was greatly inhibited in comparison with the zinc-free H glass (Table 2). Results recalled those obtained for the same materials in different fluids, such as TRIS solutions [22], and were similar for the glasses having the lowest (HZ5) and the highest (HZ20) zinc content.

To clarify the role played by released Zn^{2+} ions in HZ glasses cytotoxicity, the leakage of LDH was investigated in further experiments, wherein bioactive glasses were incubated together with either a metal chelator (EDTA, NTA) or an antioxidant molecule (ascorbate). In the presence of each of these compounds, the HZ-elicited increase of extracellular LDH was completely inhibited (Fig. 3).

Table 2
Specific surface area of H, HZ5 and HZ20 glasses after increasing reaction times in MEM

Time (h)	Specific surface area ($m^2\ g^{-1}$)		
	H	HZ5	HZ20
0	2	0.8	2.6
24	23	9	5
168	133	35	18

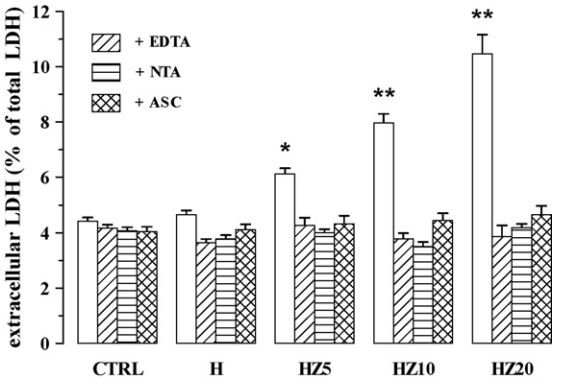

Fig. 3. Effect of glass powders on LDH release in the supernatant of MG-63 cells. Cells were incubated for 24 h in the absence (CTRL) or presence of ethylenediaminetetraacetate (EDTA, 1 mM), nitrilotriacetate (NTA, 1 mM), ascorbate (ASC, 100 μM) and/or one of the following bioglasses (50 μg/ml): 45S5 (H), 45S5 containing 5% (w/w) ZnO (HZ5), 45S5 containing 10% (w/w) ZnO (HZ10), 45S5 containing 20% (w/w) ZnO (HZ20). After this incubation the LDH activity was measured in the extracellular medium and in the cell lysate, as described in Section 2. Extracellular LDH activity was calculated as percentage of total (intracellular + extracellular) LDH activity in the dish. Measurements ($n = 4$) were performed in triplicate, and data are presented as means ± S.E.M. vs. control (CTRL): *$p < 0.002$; **$p < 0.0001$.

After a 24 h incubation, HZ glasses induced a significant rise in the intracellular content of MDA, a product of the peroxidation of cell membrane lipids [18]. This effect increased with zinc content, was completely blocked by EDTA and NTA, and was strongly inhibited by ascorbate (Fig. 4). Instead, zinc-free H glass did not cause a significant effect on intracellular MDA levels (Fig. 4).

A 24 h incubation of MG-63 cells with HZ glasses induced a significant decrease in the intracellular thiols, which was prevented when HZ glasses were incubated together with either EDTA or ascorbate (Fig. 5A). GSH is considered to be the major thiol redox buffer in the cell. GSH effectively scavenges free radicals and other reactive oxygen species (ROS) both directly and indirectly (through enzymes such as glutathione peroxidase, which catalyzes the GSH-dependent reduction of H_2O_2 and other peroxides). The more specific glutathione reductase-Ellman reagent recycling assay showed, as expected, that GSH contributed to the vast majority of thiols content in MG-63 cells (Fig. 5B): HZ glasses induced a significant decrease in intracellular GSH, which could be partly attributable to GSH oxidation to intracellular GSSG, and partly to GSH efflux out of the cells (Fig. 5B). GSH efflux is frequently observed in association with apoptotic damage [26].

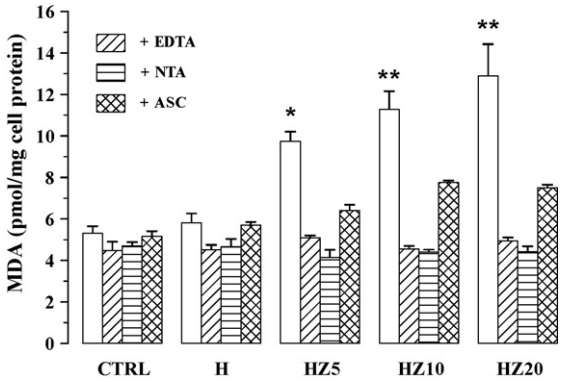

Fig. 4. Effect of glass powders on the intracellular level of MDA in MG-63 cells. Cells were incubated for 24 h in the absence (CTRL) or presence of ethylenediaminetetraacetate (EDTA, 1 mM), nitrilotriacetate (NTA, 1 mM), ascorbate (ASC, 100 μM) and/or one of the following bioglasses (50 μg/ml): 45S5 (H), 45S5 containing 5% (w/w) ZnO (HZ5), 45S5 containing 10% (w/w) ZnO (HZ10), 45S5 containing 20% (w/w) ZnO (HZ20). After this incubation time MDA was determined as described under Section 2. Measurements ($n = 3$) were performed in triplicate, and data are presented as means ± S.E.M. vs. control (CTRL): *$p < 0.005$; **$p < 0.0001$.

GSSG is reduced back to GSH by the NADPH-dependent glutathione reductase [27], which oxidizes NADPH to $NADP^+$ during the reaction. The metabolic flux through PPP is a sensitive index of the cell exposure to oxidizing molecules, since the glucose 6-phosphate dehydrogenase, which catalyzes the first step of the pathway, is activated by a decrease of the NADPH/$NADP^+$ ratio [28,29]. HZ20, the most toxic HZ in the previous experiments, induced a significant activation of PPP, which was prevented when the bioglass was coincubated with either EDTA or ascorbate (Fig. 6). On the other hand, H glass did not exert any significant effect on thiols level, GSH/GSSG ratio and PPP metabolic flux (Figs. 5 and 6).

The expression of heme oxygenase-1 (HO-1) is enhanced by oxidative stress [30] and increases with depletion of intracellular GSH levels [31]. Western blot experiments showed that, after a 24 h incubation with HZ glasses, the expression of HO-1 in MG-63 cells augmented progressively with increasing zinc content of the glass: when zinc was 5% of the glass weight (HZ5), the increase in protein was very low, but it was clearly detectable when zinc was 10% (HZ10) and 20% (HZ20) w/w (Fig. 7). No change in HO-1 expression was detectable when HZs were incubated with cells in the presence of EDTA or ascorbate, or when H was used in place of HZ glasses (Fig. 7). The known HO-1 inducer methemalbumin was used as a positive control inducer of protein overexpression [15].

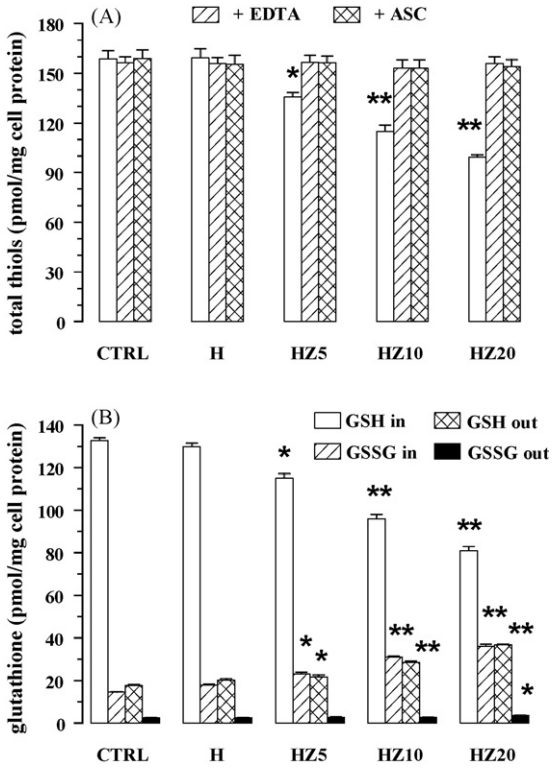

Fig. 5. Effect of glass powders on the intracellular level of total thiols (A) and glutathione (B) in MG-63 cells. (A) Cells were incubated for 24 h in the absence (CTRL) or presence of ethylenediaminetetraacetate (EDTA, 1 mM), ascorbate (ASC, 100 μM) and/or one of the following bioglasses (50 μg/ml): 45S5 (H), 45S5 containing 5% (w/w) ZnO (HZ5), 45S5 containing 10% (w/w) ZnO (HZ10), 45S5 containing 20% (w/w) ZnO (HZ20). After this incubation time intracellular thiols were determined as described under Section 2. (B) Cells were incubated for 24 h in the absence (CTRL) or presence of one of the following bioglasses (50 μg/ml): 45S5 (H), 45S5 containing 5% (w/w) ZnO (HZ5), 45S5 containing 10% (w/w) ZnO (HZ10), 45S5 containing 20% (w/w) ZnO (HZ20). After this incubation time both intracellular (in) and extracellular (out) level of glutathione (GSH) and glutathione disulfide (GSSG) were determined as described under Section 2. Measurements ($n = 3$) were performed in triplicate, and data are presented as means ± S.E.M. vs. corresponding control (CTRL): *$p < 0.01$; **$p < 0.001$.

Zinc, although not involved directly in the enzymatic activity, is required to maintain the protein structure of Cu,Zn-SOD [32]. The effect of Zn on cellular SOD activity is still controversial: a zinc-deficient diet in humans has been observed to occur along with less activity of erythrocyte Cu,Zn-SOD [33], but, paradoxically, in zinc-deficient rats the testicular Cu,Zn-SOD activity was higher than in rats fed with adequate amounts of zinc [34]. We checked the effect of bioglasses on Cu,Zn-SOD activity in MG-63 cells. Basal Cu,Zn-SOD activity in MG-63 cells (1.26 ± 0.05 U/mg cell proteins, $n = 6$) was very similar to that observed

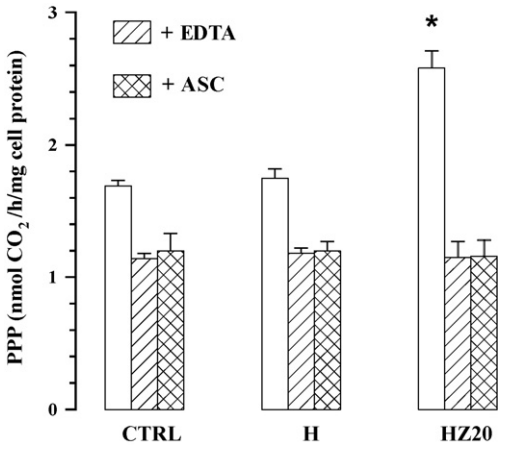

Fig. 6. Effect of glass powders on PPP activity in MG-63 cells. Cells were incubated for 24 h in the absence (CTRL) or presence of 45S5 (H, 50 μg/ml), 45S5 containing 20% (w/w) ZnO (HZ20, 50 μg/ml), ethylenediaminetetraacetate (EDTA, 1 mM) and/or ascorbate (ASC, 100 μM). After this incubation time PPP activity was determined as described under Section 2. Measurements ($n = 3$) were performed in triplicate, and data are presented as means ± S.E.M. vs. control (CTRL): $^*p < 0.01$.

in other cell types, such as human lung A549 epithelial cells (1.39 ± 0.13 U/mg cell proteins, $n = 5$), mouse N11 glial cells (1.12 ± 0.08 U/mg cell proteins, $n = 5$) and murine MH-S alveolar macrophages (1.48 ± 0.07 U/mg cell proteins, $n = 5$). In each cell type Cu,Zn-SOD was responsible for 85–90% of total SOD activity (not shown). A 24 h incubation of MG-63 cells with HZ20 elicited a strong increase of Cu,Zn-SOD activity, while

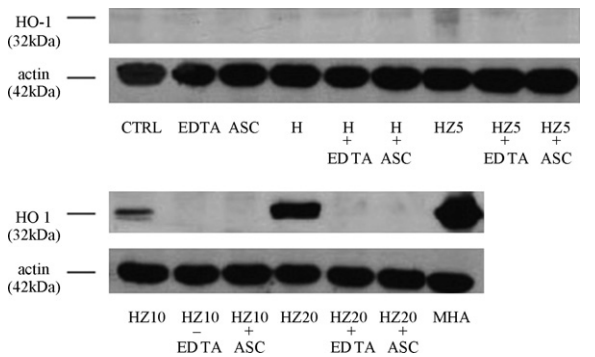

Fig. 7. Western blot detection of human heme oxygenase (HO)-1 protein in MG-63 cells. Cells were incubated for 24 h in the absence (CTRL) or presence of ethylenediaminetetraacetate (EDTA, 1 mM), ascorbate (ASC, 100 μM) and/or one of the following bioglasses (50 μg/ml): 45S5 (H), 45S5 containing 5% (w/w) ZnO (HZ5), 45S5 containing 10% (w/w) ZnO (HZ10), 45S5 containing 20% (w/w) ZnO (HZ20). Incubation of the cells with methemalbumin (MHA), a known inducer of HO-1, was used as positive control. The level of β-actin, the product of an housekeeping gene, was used to check the equal protein loading. The figure is representative of three experiments with similar results.

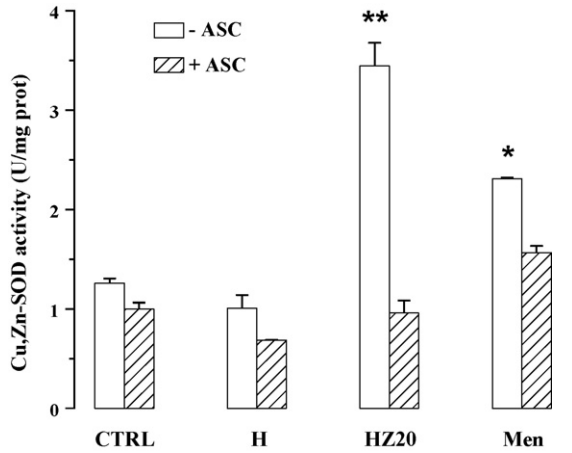

Fig. 8. Effect of glass powders on the Cu,Zn-SOD activity of MG-63 cells. Cells were incubated for 24 h in the absence (−ASC) or presence of ascorbate (+ASC, 100 μM) and in the absence (CTRL) or presence of either H or HZ20 glass (50 μg/ml). Menadione (men, 100 μM) was incubated with the cells for 2 h only, because too toxic after longer time periods; then the medium was discarded and the cells were left to incubate for further 22 h in fresh medium. After incubation, Cu,Zn-SOD activity was measured as described under Section 2. Measurements ($n = 4$) were performed in duplicate, and data are presented as means ± S.E.M. vs. control (CTRL): $^*p < 0.001$; $^{**}p < 0.0001$.

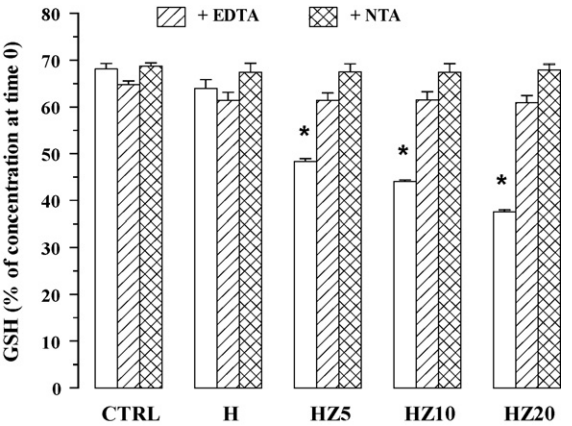

Fig. 9. Effect of glass powders on the concentration of glutathione (GSH) in cell-free culture medium. A 1 mM solution of GSH in MEM was incubated for 3 h at 37 °C, in the absence (CTRL) or presence of ethylenediaminetetraacetate (EDTA, 1 mM), nitrilotriacetate (NTA, 1 mM) and/or one of the following bioglasses (50 μg/ml): 45S5 (H), 45S5 containing 5% (w/w) ZnO (HZ5), 45S5 containing 10% (w/w) ZnO (HZ10), 45S5 containing 20% (w/w) ZnO (HZ20). After this incubation time intracellular GSH was determined as described under Section 2 and expressed as percentage of the GSH detected in the same solution at time 0 (0.97 ± 0.04 mM, $n = 15$). Measurements ($n = 3$) were performed in triplicate, and data are presented as means ± S.E.M. vs. corresponding control (CTRL): $^*p < 0.0001$.

under the same experimental conditions Zn-free H glass did not induce any significant change of enzyme activity compared to control (Fig. 8). The HZ20-induced increase of Cu,Zn-SOD activity was completely prevented by the coincubation with the reducing agent ascorbate and mimicked by the incubation with the oxidizing agent menadione (Fig. 8).

Taken as a whole, these data suggest that the toxic effect of HZ glasses could be attributed to an oxidative injury induced by Zn^{2+} ions released from glasses during the incubation with the cell cultures. In these experimental conditions, cells were not necessary to the generation of oxidative stress: after a 3 h incubation of GSH in cell-free MEM, the amount of reduced cofactor was decreased by about 30%, a likely consequence of spontaneous autooxidation (Fig. 9). HZ glasses (but not H) elicited during the same time period a further significant decrease of GSH, if compared to the control solution: again, the decrease of GSH was proportional to the zinc content in bioglasses, and it was not observed in the presence of EDTA or NTA (Fig. 9).

In order to clarify the link between the toxic effects of zinc-containing bioactive glasses and the release of Zn^{2+} in solution, we also incubated the MG-63 cells with amounts of Zn^{2+} (added as $ZnCl_2$) similar to those found in MEM after 24 h of incubation with HZ5 and HZ10 glasses. In these experimental conditions, both $ZnCl_2$ concentrations induced a significant increase in extracellular LDH and intracellular MDA and a decrease in intracellular thiols (Fig. 10). Again, the presence of EDTA or ascorbate in the medium blocked these changes (Fig. 10).

4. Discussion

The insertion of metal ions, such as zinc, has been proposed in order to reduce the high solubility of conventional bioactive glasses and increase their osteoconductivity. Zinc-containing bioactive glasses have already shown a higher durability in aqueous solutions than zinc-free ones: an increasing amount of zinc in their composition retards more and more the dissolution pro-

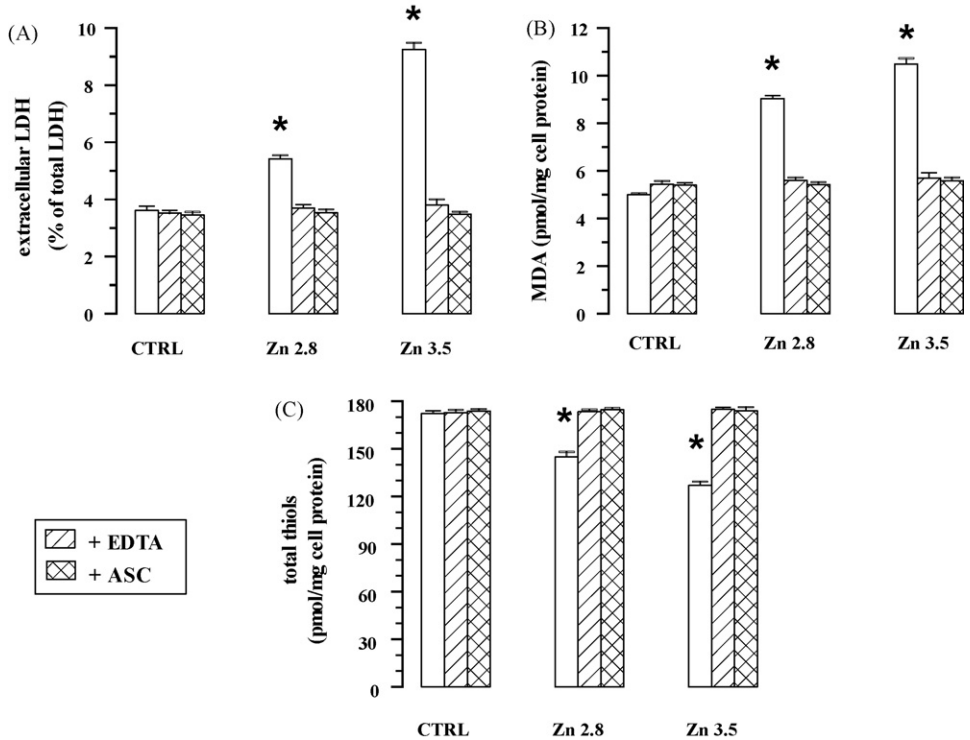

Fig. 10. Effect of $ZnCl_2$ on LDH release, intracellular MDA and intracellular thiols in MG-63 cells. Cells were incubated for 24 h in the absence (CTRL) or presence of 43 μM $ZnCl_2$ (releasing 2.8 ppm of Zn^{2+}: Zn 2.8), 53 μM $ZnCl_2$ (releasing 3.5 ppm of Zn^{2+}: Zn 3.5), ethylenediaminetetraacetate (EDTA, 1 mM) and/or ascorbate (ASC, 100 μM). After this incubation time, extracellular LDH (panel A), intracellular MDA (panel B) and intracellular thiols (panel C) were measured as described under Section 2. Measurements ($n = 3$) were performed in triplicate, and data are presented as means ± S.E.M. vs. control (CTRL): $^*p < 0.001$.

cess. This is evidenced by the slackening of the increase in surface area, a good marker for the advancement of glass dissolution, after soaking of zinc-containing bioactive glasses in different fluids [13,14], including the culture medium MEM that we have used in this experimental work.

In the experimental conditions used for the cytotoxicity tests, ion release experiments have shown that the effect of Zn on glass durability is not sufficient to prevent a complete dissolution of the material. This leads to an increased concentration of Zn^{2+} ions in solution, which, when occurring *in vivo*, might be relevant in eliciting changes of cell functions and metabolism. To clarify this issue, we have incubated human MG-63 osteoblasts with four bioglasses, differing only as to their content of zinc. We have observed that the presence of this metal increases, in a dose-dependent way, the release of LDH in the extracellular medium (an index of cytotoxicity), the accumulation of intracellular MDA (an index of lipoperoxidation), and increased PPP activity and HO-1 expression (two sensitive signals of cell response to oxidative stress). In parallel, the level of intracellular thiols and the GSH/GSSG ratio were decreased, in inverse relationship with the zinc content of glass powders. The HZ20 (but not H) glass induced also an increase of Cu,Zn-SOD activity, which was not relatable to the Zn supplementation per se, but rather to the oxidative stress induced by the Zn-containing bioglass. Indeed, it is known that both Mn- and Cu,Zn-SOD activities increase after cell exposure to an oxidative stress: genes of both isoforms contain binding sites for redox-sensitive transcription factors, such as AP-1 and NF-kB, and metal-binding responsive elements [35].

After the 24 h incubation time used in these experiments, HZ glasses were almost completely dissolved in MEM. To get further evidence of the likely role played by Zn^{2+} ions in the cytotoxic and redox effects observed in cells incubated with HZ glasses, we incubated MG-63 cells directly with the amounts of Zn^{2+} (added as $ZnCl_2$) found in MEM after incubation with HZ5 and HZ10 glasses. In the same experimental conditions, $ZnCl_2$ produced effects similar to those observed after the incubation with HZ glasses. The toxic effects of both HZs and Zn^{2+} ions were prevented by the presence in the culture medium of either metal chelators, such as EDTA and NTA, or the antioxidant vitamine ascorbate. Taken together, these experimental evidences suggest that, after a 24 h incubation in MEM culture medium, HZ glasses with size <26 μm release a Zn^{2+} concentration that can cause cell damage via an oxidative stress.

After iron, zinc is the most abundant trace metal in human organisms. It is associated with a large number of proteins, including metalloenzymes, structural proteins and transcription factors, and contributes to physiological processes including neurotransmission, hormone secretion, DNA synthesis and gene expression [9]. Yet, the contribution of zinc in many physiological and pathological events still needs to be fully explained. For instance, this metal has been reported to play a dual role in affecting cell death: although zinc seems to be an inhibitor of many forms of apoptosis [36], exposure to excessive concentrations may contribute to neuronal cell death in acute neurological disorders [37] and to apoptosis of human peripheral blood lymphocytes [38]. Zinc has been also implicated in the necrosis of pulmonary artery endothelial cells exposed to *t*-butyl hydroperoxide [9]. Zinc has been hypothesized to exert an antioxidant action through different mechanisms, including sulfhydryl protection against oxidation, induction of metallothionein (a cysteine-rich protein able to scavenge oxidants and bind redox active metals), its presence in the antioxidant enzyme Cu,Zn-superoxide dismutase and its ability to replace redox active metals (copper and iron) from membrane binding sites [39]. For instance, zinc supplementation has been reported to prevent alcoholic liver injury in mice through attenuation of oxidative stress [40]. On the other hand, zinc neurotoxicity produced by either acute exposure to high concentrations or chronic exposure to low concentrations appears to be due mostly to the formation of ROS [41]. Furthermore, excessive zinc intake has been suggested to elevate systemic blood pressure levels in normotensive rats through an oxidative stress [42]. Low amounts of zinc protect retinal pigmented epithelium cells from serum starvation, but too much zinc is toxic to these cells, through the production of free radicals: antioxidants blunt such zinc toxicity [43].

The mechanism by which Zn^{2+} induces cell death and oxidative stress is still unresolved. Zn^{2+} is considered to be redox-inactive under most physiological conditions: unlike iron or copper, Zn^{2+} would not catalyze directly the formation of ROS and, as such, is thought to be relatively non-toxic per se. Several studies point to a critical role for mitochondrial dysfunction in Zn^{2+} toxicity, but the specific cell signaling pathways that contribute to Zn^{2+}-induced injury remain ill-defined [44]. It has been also observed that zinc overload induces and activates NADPH oxidase in cortical neurons and astrocytes in a protein kinase C-dependent manner: thus, NADPH oxidase may contribute to ROS generation in these cells [45]. Interestingly, the inter-

action of zinc with oxygen has been recently shown to generate H_2O_2 and $^{\bullet}OH$ radicals [38], suggesting that Zn^{2+}-induced ROS generation would not necessarily imply mitochondrial dysfunction or NADPH oxidase activation. This is in agreement with the results of our cell-free experiments, where HZ induced a significant decrease of GSH in the absence of cells, an effect that was completely reverted by the metal chelators EDTA and NTA.

Data about the in vivo release of zinc from biomaterials are still few and do not concern bioglasses. After implantation of zinc-containing (0.06–0.6%, w/w) tricalcium phosphate/hydroxyapatite ceramics in the femora of New Zealand White rabbits for 4 weeks the zinc plasma concentration was unchanged, suggesting that zinc was released only in the vicinity of the implants [46]. The intramuscular injection of tricalcium phosphate suspensions containing higher amounts of zinc (6–12%, w/w) induced a significant increase of zinc plasma levels in rats [47]. Macroscopic examination revealed no inflammatory reaction in the injected sites, but the eventual systemic toxicity was not investigated [47]. The same authors found, by in vitro experiments, that when the amount of zinc in these ceramics exceeded the 1.2% (w/w) value the growth rate of mouse osteoblastic MC3T3-E1 cells on their surface steeply decreased [48]: such a cytotoxic effect was related to the release of zinc in the medium, but the mechanisms were not investigated. Our results suggest that also human osteoblasts are sensitive to the cytotoxic effects of another bioactive material, i.e. Bioglass® 45S5, when it contains ≥5% (w/w) zinc. Such a cytotoxicity is a function of the concentration of Zn^{2+} ions released by glass dissolution in the culture medium. Furthermore, we also provide supplementary information on the prooxidant role of zinc in living systems: to our knowledge, this is the first evidence about zinc toxicity in human osteoblastic cells. Once reported only for nervous system, an oxidative damage triggered by zinc has been observed more recently in other cell types, including endothelial [9] and retinal cells [43] and peripheral blood lymphocytes [38]. Furthermore, our data confirm that zinc is not as redox-inert as it has been hypothesized before. The consequence of the interaction of zinc with living tissues is likely to be dependent on cell type, zinc concentration and zinc bioavailability. Future works should be devoted to investigate the best operating conditions of zinc concentration and glass morphology (grains, slabs, etc.) to avoid a too fast release of zinc from the glasses and therefore a cytotoxic effect on osteoblast cells, in view of a practical use of these systems as new biomaterials. In this regard, the release of ascorbate or other antioxidant molecules from a bio-compatible material, such as mesoporous silica [49–51], joined with the HZ glass powders might minimize the cytotoxic effect of a high zinc concentration. It is difficult to state presently how our in vitro model can mimic what may happen in vivo at the interface biomaterial/tissue: we hope that our observation could stimulate further research aimed to assess the potential toxicity of zinc-containing bioactive glasses in *in vivo* models.

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