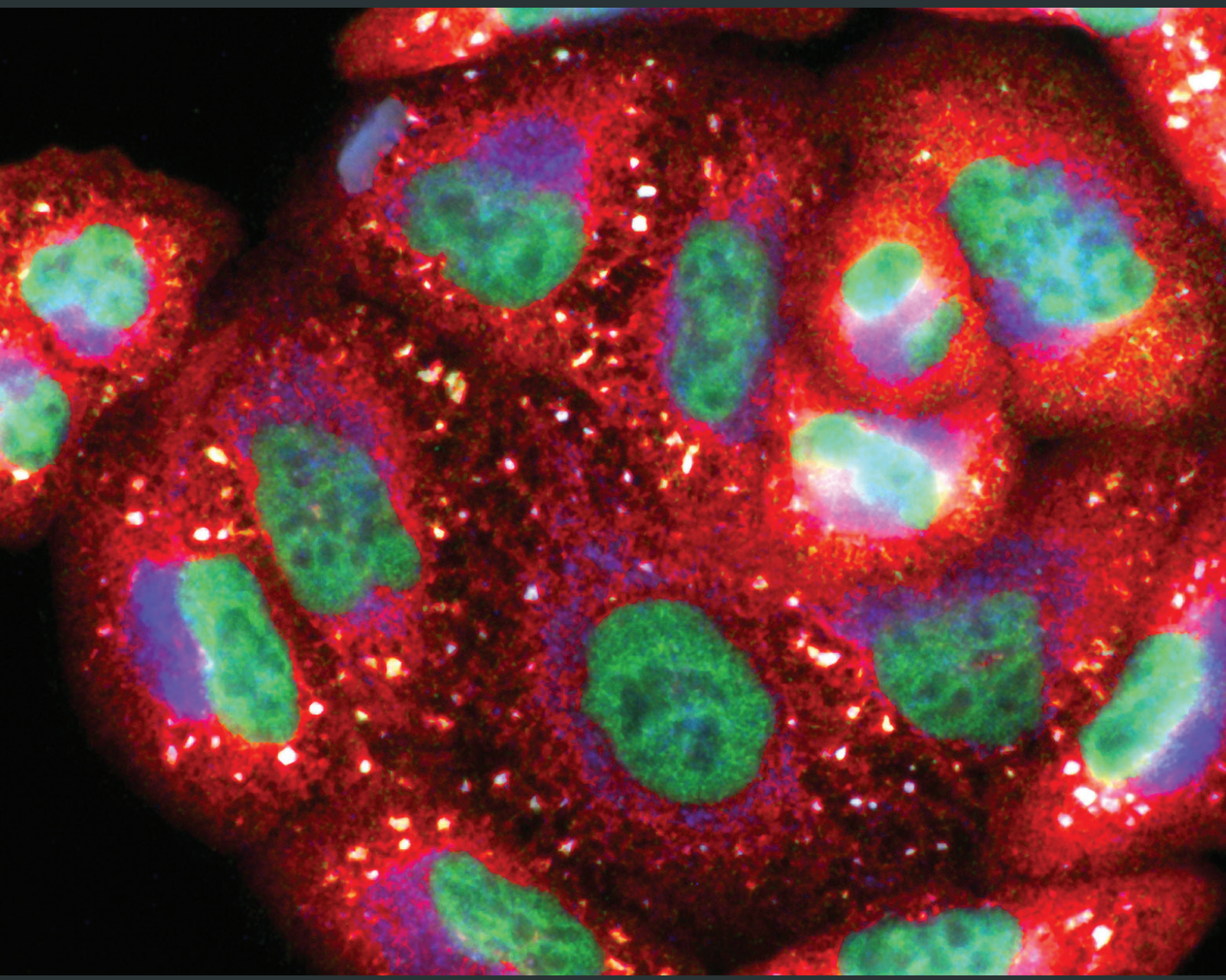
Oxidative Medicine and Cellular Longevity

Magnesium and Other Biometals in Oxidative Medicine and Redox Biology

Lead Guest Editor: Martin Kolisek
Guest Editors: Rhian Touyz, Andrea Romani, and Mario Barbagallo



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Editorial Board

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Editorial

Magnesium and Other Biometals in Oxidative Medicine and Redox Biology

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Magnesium (Mg) and also other biometals (BM) play an essential role in regulating a plethora of biological processes such as (1) metabolism of biomacromolecules, (2) cell division and proliferation, (3) normal mitochondrial homeostasis and energy production, (4) normal redox homeostasis, (5) antioxidant signaling, and (6) crosstalk between major signal cascades. However, exact molecular mechanisms underlying the action of Mg or other BM in these processes are often only partially understood. This may relate, in part, to lack of sensitive and specific tools to assess Mg and BM in biological systems and to relative lack of emphasis of Mg and BM in clinical medicine.

Diabetes mellitus type 2 (DT2) might serve as an example. Hypomagnesaemia has been identified in 9 to 40% of DT2 patients in "Mg-focused" clinical trials. However, Mg status in DT2 patients is rarely determined as routine clinical practice.

Another example highlighting the importance of Mg homeostasis in disease etiology is Parkinson disease (PD). It is not yet clear whether chronic intracellular Mg deficiency causes the disease itself but it is obvious that insufficient dietary intake of Mg or its wasting (GIT, kidney) worsens PD symptoms and accelerates its progression.

Manganese (Mn) is an essential trace element involved in many physiological processes supporting growth and development, and also neuronal functions. On the other hand, pathological accumulation of Mn in the brain has a detrimental, toxic effect on neurons. Dopaminergic neurons in the *substantia nigra* are especially sensitive to Mn toxicity; thus, accumulated Mn may cause manganism, a disease condition with etiology almost identical with PD.

Even though copper (Cu) plays a role in multiple vital enzymatic reactions and physiological processes, it is notoriously known for its essential role in redox homeostasis in cells and consequently tissues and organs of the body. For instance, decreased levels of protein-bound Cu may lead to iron (Fe) accumulation in the brain, thus increased oxidative stress (OS) that is hallmarking prevalence and progression of neurodegenerative and psychiatric diseases. The two pathological conditions resulting directly from the perturbed transport of Cu in the body are Menkes disease (negatively affected is the intestinal P-type ATPase ATP7A transporting Cu^+) and Wilson's disease (negatively affected is the P-type ATPase ATP7B transporting Cu^+ that is localized within trans-Golgi network of hepatocytes and brain cells).

BM not only are important for global biochemistry and physiology of the body, but also have been popular in the field of implantology. For example, low toxicity, durability (when in alloys), and biodegradability made out of Mg a "super-component" of materials that are used for manufacturing of the latest generation of stents or other biodegradable implants. The field of implantology and implant material engineering is progressing rapidly, and it is likely that the success of Mg will be followed by other BM soon.

Processes maintaining normal mitochondrial homeostasis (MH) are essential for life and involve reactive oxygen species (ROS). Excess bioavailability of ROS (oxidative

stress) contribute to cell dysfunction, injury, and mitophagy/autophagy. At the levels of cells and also the whole body, MH deterioration leads to senescence and death. Certain organs (especially those metabolically highly active, e.g., brain, heart, muscles, and liver) are more prone to deterioration of MH than others. Thus, natural ageing may be paralleled with premature ageing of particular organs that often demonstrates as progressive degenerative disease. The factors behind premature ageing of any organ might be encoded genetically, or they have epigenetic, or environmental background, or a combination of all. Mild OS (e.g., Akt-mediated mitochondrial OS) triggers mitophagy. Excessive, strong OS leads to death of the cells. Disbalanced homeostasis of redox-active BM such as Cu, Fe, Mn, Zn, and Mg might have deleterious effects on MH. Therefore, parameters defining status of the homeostasis of aforementioned BM should be routinely considered by the clinicians to project correctly an integrative clinical image of the patient that is necessary to adjust the most appropriate therapy.

Similar to PD, Alzheimer's disease (AD) has been linked to excessive OS, disturbed BM homeostasis, and disturbed MH. I.-M. Balmus et al. in their work assessed (1) levels of Mn, Mg, and Fe, (2) activities of superoxide dismutase and glutathione peroxidase, and (3) concentration of malondialdehyde (lipid peroxidation marker) in blood sera of healthy probands, sera of patients with mild cognitive impairment (MCI), and blood sera of patients with diagnosed AD. These authors found increased lipid peroxidation, low antioxidant defense, low Mg and Fe concentrations, and high Mn levels in MCI and AD patients, in a gradual manner. Outcomes of this study clearly demonstrate aberrant BM homeostasis with OS in MCI and AD. Moreover, these data may help to develop a predictive protocol that could complement AD biomarkers that are already being tested in large clinical trials.

Both, 3-hydroxyanthranilic acid (3-HANA) and 3-hydroxykynurenine (3-HK) are intermediates in the metabolism of tryptophan. 3-HANA was initially considered neurotoxic but later identified as having a neuroprotective effect with therapeutic potential in neuroinflamatory disorders such as AD. On the other hand, elevated levels of 3-HK are having clear neurotoxic effects linked to pathologies of AD and early stage Huntington disease (HD). D. Ramírez-Ortega et al. studied the effect of 3-HANA and 3-HK on Cu toxicity in primocultures of rat astrocytes. These authors identified both kynurenines (1) to potentiate the Cu cytotoxicity in ROS-independent manner and (2) to potentiate the effect of Cu on the decrease of glutathione (GSH) levels. Kynurenine pathway (KP) plays an important role in regulation of OS and inflammation, and in pathologies of major neurodegenerative disorders. Therefore, work of group around D. Ramírez-Ortega et al. urges for further examination of the crosstalk between metabolites of KP and homeostasis of Cu (and perhaps also homeostasis of other BM).

I. Pilchova et al. discuss in their review involvement of Mg regulation of cellular and mitochondrial functions focusing their attention primarily on energy metabolism, mitochondrial calcium (Ca^{2+}) handling, and apoptosis. This work provides an up-to-date topic and emphasizes the importance of mitochondrial Mg homeostasis (MMH) beyond mitochondria and that aberrant MMH may have detrimental effects on cell. At several occasions, the importance of mitochondria-endoplasmic reticulum (ER) crosstalk, in respect to Mg homeostasis and essential intracellular processes, is being accentuated.

The central role of BM in the maintenance of oxidative balance within the frame of metabolic and neurodegenerative disorders is discussed by M. Pokusa and A. K. Trancikova. The review highlights the intersection between etiopathologies of neurodegeneration and of metabolic disorders. It also features ROS and disturbed BM homeostasis as being causative (and perhaps also consecutive) hallmarks of the aforementioned disease conditions.

As previously mentioned, Mg is also a focus of implantology and biomaterial engineering due to its low toxicity and biodegradability. Z. Liu et al. in their work highlight unique properties of Mg and microbicide effect of silver (Ag; Ag nanoparticles generate ROS in living biological systems). By controlling the microstructure and increasing the Ag content, authors obtained Mg-Ag alloys with good antibacterial properties in harsh and dynamic conditions and with almost equivalent cytocompatibility to human primary osteoblasts as pure Mg.

Papers in this special issue highlight new exciting data, comment, and synthesise the newest knowledge on Mg and other BM in oxidative medicine and redox biology. We hope that this special issue will attract broad readership in the field spanning from neurodegenerative to metabolic disorders and implantology. We would like to express our thanks to all the authors, reviewers, and the editorial team for the great support in making this special issue a reality.

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Research Article

3-Hydroxykynurenine and 3-Hydroxyanthranilic Acid Enhance the Toxicity Induced by Copper in Rat Astrocyte Culture

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Copper is an integral component of various enzymes, necessary for mitochondrial respiration and other biological functions. Excess copper is related with neurodegenerative diseases as Alzheimer and is able to modify cellular redox environment, influencing its functions, signaling, and catabolic pathways. Tryptophan degradation through kynurenine pathway produces some metabolites with redox properties as 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HANA). The imbalance in their production is related with some neuropathologies, where the common factors are oxidative stress, inflammation, and cell death. This study evaluated the effect of these kynurenines on the copper toxicity in astrocyte cultures. It assessed the $CuSO_4$ effect, alone and in combination with 3-HK or 3-HANA on MTT reduction, ROS production, mitochondrial membrane potential (MMP), GHS levels, and cell viability in primary cultured astrocytes. Also, the chelating copper effect of 3-HK and 3-HANA was evaluated. The results showed that CuSO4 decreased MTT reduction, MMP, and GSH levels while ROS production and cell death are increasing. Coincubation with 3-HK and 3-HANA enhances the toxic effect of copper in all the markers tested except in ROS production, which was abolished by these kynurenines. Data suggest that 3-HK and 3-HANA increased copper toxicity in an independent manner to ROS production.

1. Introduction

Metals have a vital participation in some cellular processes as enzyme cofactors, as structural and antioxidant components, and also as part of metabolism. For this reason, their balance in cell environment is important and their unbalance causes damage in elemental cell structures as lipids and DNA; therefore, metals excess can be toxic mainly by oxidative stress production [1–3]. Copper is the third most abundant essential transition metal that is naturally found in human liver [4] and the most abundant in the brain [5]; this metal works as structural and functional part in various systems. In CNS, copper can act as cofactor of some antioxidant enzymes like + copper/zinc-dependent superoxide dismutase (SOD-1) and has big importance in respiration mitochondrial respiration as part of cytochrome c oxidase structure [6, 7]. In addition, this metal participates in the neurotransmitter biosynthesis (noradrenaline) and can be stored in ceruloplasmin [8–10]. Copper can be transported into the brain through copper transporter 1 (Ctr1) and toward inner of brain cells by the ATPase copper transporter (ATP7A) and the divalent metal transporter (DMT1) [11–15]. It has been reported that astrocytes have a great influence in cerebral copper homeostasis and they can store big amounts of this metal

due to specific characteristics as vast amounts of DMT1, ferritin, metallothionein, and antioxidants as glutathione (GSH) [13, 16, 17]. However, alterations in copper metabolism have been related with neurodegenerative diseases as Alzheimer's (AD), Parkinson's (PD), Menkes (MD), and Wilson's (WD) diseases, triggering an oxidative stress state in cell environment, resulting in disturbance of energy metabolism and reactive oxygen species (ROS) production [18–21]. ROS can also stimulate endogenous pathways that can be modulated by redox environment as the kynurenine pathway (KP).

KP is the main route of tryptophan (Trp) catabolism. Trp is an essential amino acid, which can be metabolized through different pathways to form important substances as serotonin and melatonin, but more than 95% is degraded through KP [22], whose main aim is NAD^+ production, an electron carrier and cofactor in some redox reactions [23]. KP is present in the liver, kidney, and brain of various mammals such as mice, rats, guinea pigs, rabbits, monkeys, and humans [22, 24, 25]. Along the pathway, different metabolites with neuroactive activity and/or redox properties are produced. KP is highly regulated by redox status of the cellular environment, but its metabolites can also modify this environment due to their redox properties [26]. The alteration in KP metabolite levels has been associated with aging and several neurodegenerative diseases as Huntington, Parkinson, and Alzheimer [27]. Specifically, 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HANA) have been studied by various research groups, which describe controversial results. The metabolite 3-HK is found at nanomolar concentrations in CNS in normal conditions, but its levels are modified in neurodegenerative diseases. In fact, the amount increases as much as three times in Huntington's disease [28, 29]. It has been reported that 3-HK is able to induce cell death through apoptosis in brain regions as well as in cell cultures and *in vivo* experiments (with DNA fragmentation and chromatin condensation) [30–33]. In addition, it has been shown that 3-HK generates oxidative stress besides that it triggers protein aggregates in human lens and finally cataract formation because of its interaction with metals [33–35]. On the other hand, there are reports where it was observed that 3-HK (0–100 μM) works as an antioxidant. In this context, 3-HK and 3-HANA were able to decrease lipid peroxidation and GSH oxidation in brain cortex homogenates [36]; in *Aldrichina grahami* homogenates, 3-HK was able to trap superoxide [37]. In other reports, it has been demonstrated that 3-HK can capture hydroxyl and peroxyl radicals [36, 38]. Recently, it was shown that 3-HK can have chelating properties with metals as ferrous and also can scavenge OH• and $ONOO^-$ in chemical combinatory assays [39].

Moreover, 3-HANA has also ambiguous characteristics which cause toxicity in neuronal cultures and can produce protein damage due to its interaction with metals and with the ability to generate hydroxyl radicals through Fenton's reaction. Besides, it has been reported that 3-HANA can have uncoupling effect in oxidative phosphorylation and is able to decrease oxygen consumption-activating astrocytes and neuron death [33, 34, 40–42]. Nevertheless, 3-HANA is described as scavenger of OH• and $ONOO^-$ in chemical combinatory assays and can act as a chelator of ferrous ion [36, 39]. In addition, 3-HANA can be an inflammatory and neuroprotector molecule since it induces hemeoxygenase-1 and suppresses cytokine and chemokine production stimulated by IL-1/IFN-γ and toll-like receptor (TLR) ligands leading to neuroprotection [43].

Due to the fact that 3-HK and 3-HANA influence the redox environment and knowing that copper can be toxic to the cell, the aim of this work was to determinate the effect of the coincubation of copper with these two kynurenine metabolites, in the toxicity induced by this metal.

2. Materials and Methods

3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HANA), copper sulfate ($CuSO_4$), thiazolyl blue tetrazolium bromide, 2′,7′-diclorodihidrofluoresceine diacetate (DCF-DA), and propidium iodide (PI) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). All other chemicals were of the highest commercially available purity and obtained from known commercial suppliers. Solutions were prepared using deionized water obtained from a Milli-RQ (Millipore) purifier system.

2.1. Copper Chelation Assays. Chelation capacity of both KP metabolites was assessed according to previous report [44], where different concentrations of 3-HK and 3-HANA (0-1 mM) were tested. Briefly, a solution of chelator (50 μl of 3-HK or 3-HANA in different concentrations) was mixed with $CuSO_4$ (50 μl) in HEPES buffer (50 μl). After 2 minutes, 50 μl of hematoxylin or DMSO (blank) was added and mixed for 3 minutes. Then, the absorbance was measured during 4 min. The wavelength used was different for each pH tested. Three different pH (5.5, 6.8, and 7.5) and 2 different buffers were tested (sodium acetate buffer pH 5.5 and HEPES buffer pH 6.8 and 7.5), considering previous reports where it was demonstrated that copper accumulation, as in the pathologies, can change pH environment [45, 46].

2.2. Primary Astrocyte Cultures. Rat-cultured cortical astrocytes were obtained from the brains of 3 days postnatal Wistar rats (PND). Cells were seeded in Roux flasks at a 9×10^6 cells/ml density. The cells were maintained in DMEM supplemented with FBS at 10% under incubation at 37°C with CO_2 (5%), until the cells were again seeded in 24-well plates to be used. Over 95% of the cells were immunoreactive for glial fibrillary acidic protein, an astrocyte-specific marker [39].

2.3. MTT Reduction Assay. According to previous reports [39, 47, 48], cellular function was evaluated by MTT reduction assay. This assay is employed as a functional status test through the formation of formazan salts by the action of dehydrogenases in viable cells [39]. Briefly, astrocytes (100,000 per well) were treated with different copper concentrations (0–500 μM), to stablish the toxic copper concentration. Then, $CuSO_4$ (350 μM) was coincubated with 3-HK

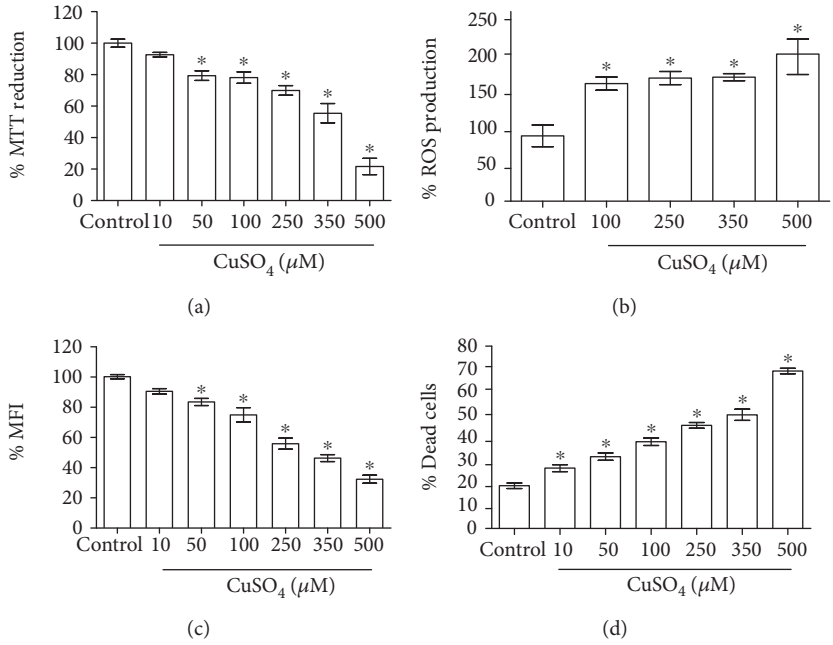

Figure 1: Effect of $CuSO_4$ on cellular function (a), ROS production (b), MMP (c), and cell viability (d) in astrocytes. After incubation for 24 h with copper, MTT, DCFC-DA, JC-1, and iodide propidium were added to each well, respectively. Data are presented as mean values + SEM of 8 independent experiments from 4 different cultures. $^*p < 0.05$ versus control (one-way ANOVA followed by Tukey's test).

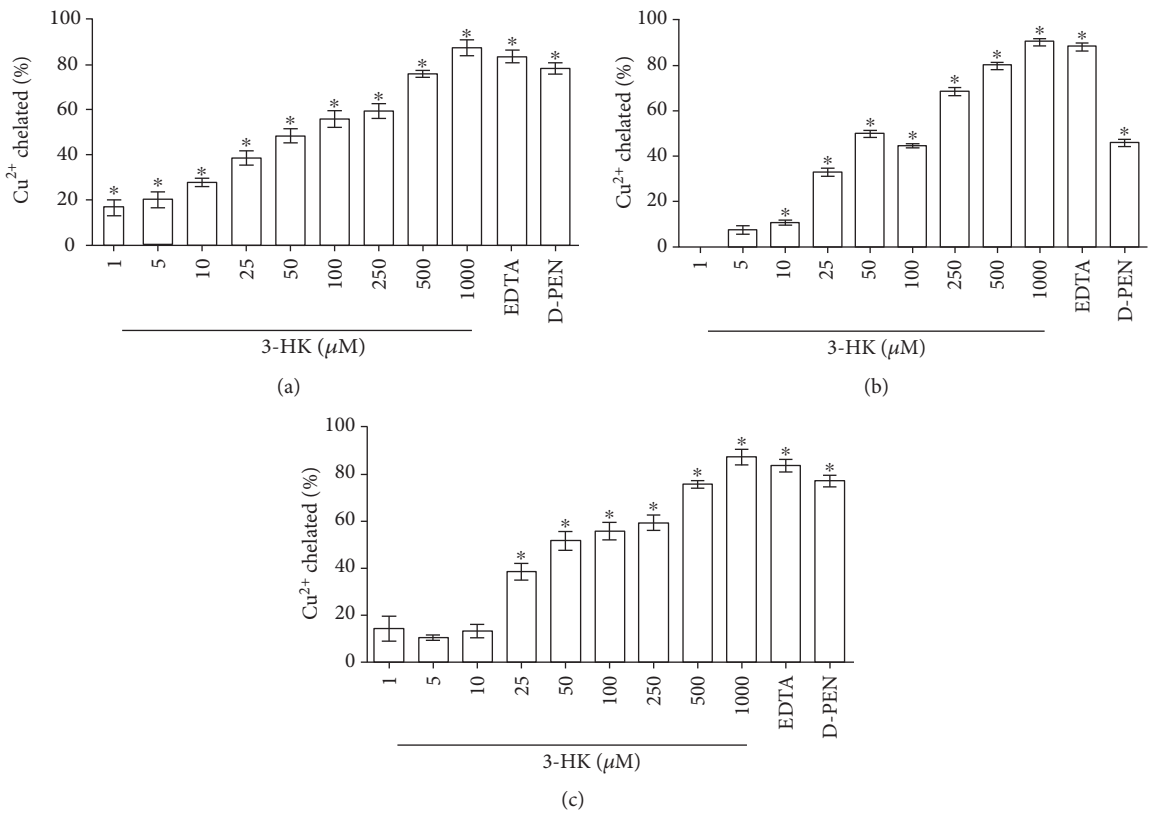

Figure 2: Copper chelation capacity of 3-HK. Different conditions of pH were tested: pH 5.5 (a), pH 6.8 (b), and pH 7.5 (c). Data are presented as mean values + SEM of 8 independent experiments for each concentration. $^*p < 0.001$ versus control (one-way ANOVA followed by Tukey's test).

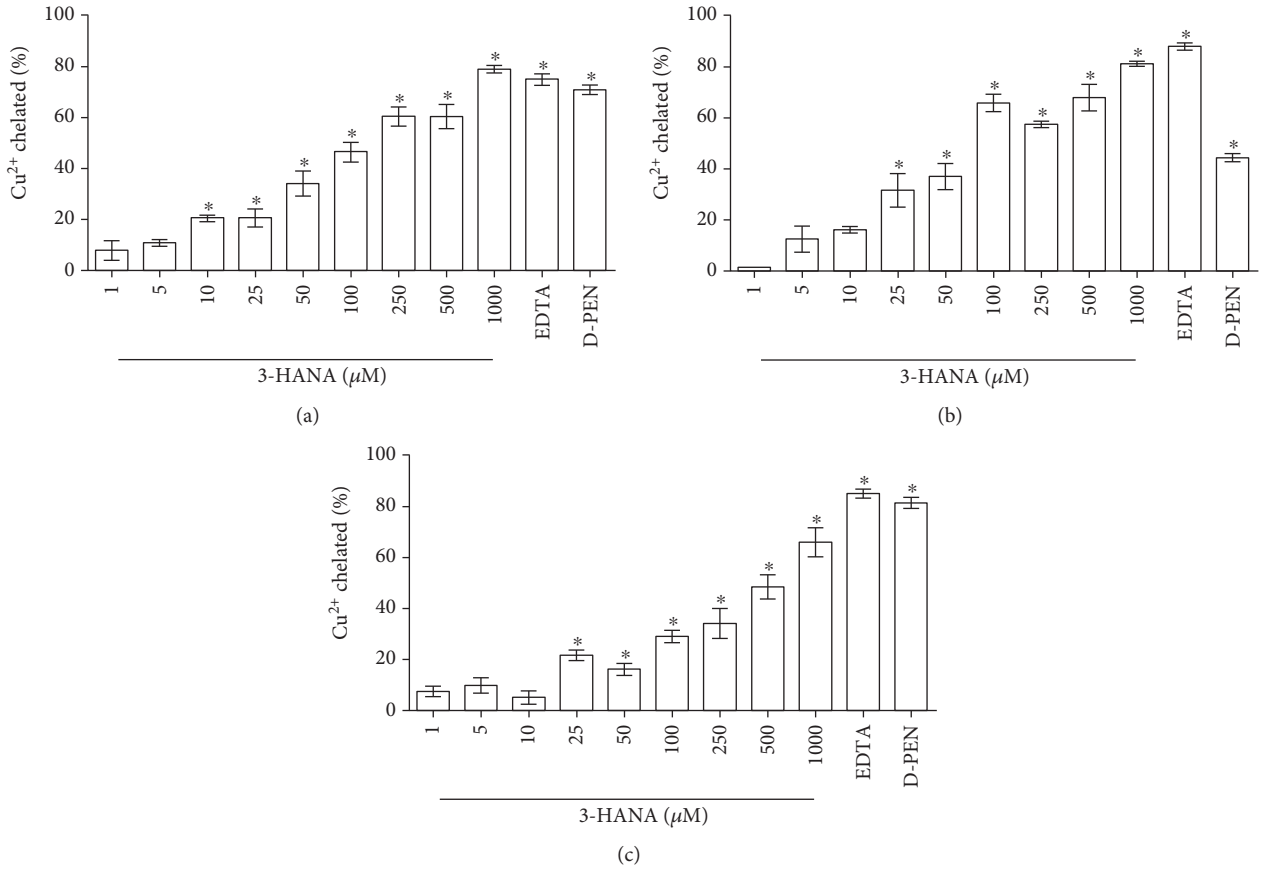

Figure 3: Copper chelation capacity of 3-HANA. Different conditions of pH were tested. pH 5.5 (a), pH 6.8 (b), and pH 7.5 (c). Data are presented as mean values + SEM of 8 independent experiments for each concentration. $^{*}p < 0.001$ versus control (one-way ANOVA followed by Tukey's test).

and 3-HANA (100 μM) in DMEM medium for 24 h at 37°C. After treatment, the medium was removed and 500 μl MTT (1 mg/ml in DMEM medium) was added to each well. MTT was incubated for 3 h at 37°C, then medium was removed, and acid isopropanol was added to dissolve the blue formazan salts. Quantification of resulting blue formazan salts was done at a wavelength of 570 nm in a plate reader (EON, BioTek). The results were expressed as the percentage of MTT reduction versus control values.

2.4. ROS Production Determination. ROS were evaluated through DCF-DA oxidation [49]. Astrocytes (100,000 per well) were treated with different copper concentrations (0–500 μM), and then copper (350 μM) was coincubated with 3-HK and 3-HANA (100 μM) in DMEM medium for 24 h at 37°C. After that, medium was removed, and cells were washed with saline solution and were added with 75 μl of trypsin. Cells were recollected, and 100 μl of DCF-DA (75 μM) was added to the tubes and reincubated for 20 min at 37°C in darkness. After incubation, ROS formation was quantified by flow cytometry at 488 nm excitation and 532 emission considering 10,000 total events in FlowJo programm. Data are presented as percentage of ROS production versus control.

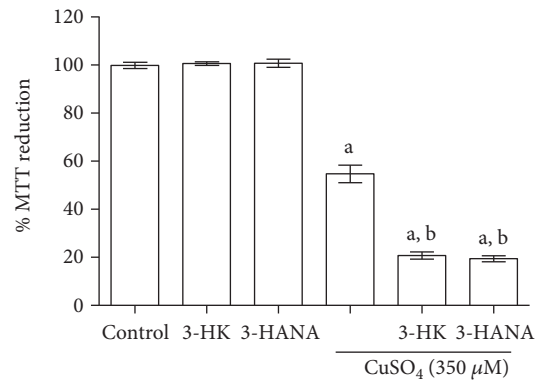

Figure 4: Effect of 3-HK and 3-HANA in the cellular dysfunction induced by $CuSO_4$ (350 μM). After 24 h of incubation with 3-HK or 3-HANA (100 μM) + copper, MTT was added to each well and formazan salt was measured. Data are presented as mean values + SEM of 6 independent experiments from 3 different cultures. $^{a}p < 0.001$ versus control and $^{b}p < 0.001$ versus $CuSO_4$ (one-way ANOVA followed by Tukey's test).

2.5. Mitochondrial Membrane Potential (MMP) Assay. Mitochondrial membrane potential is a marker of healthy cells, and JC-1, a lipophilic cation, is used to evaluate because it is

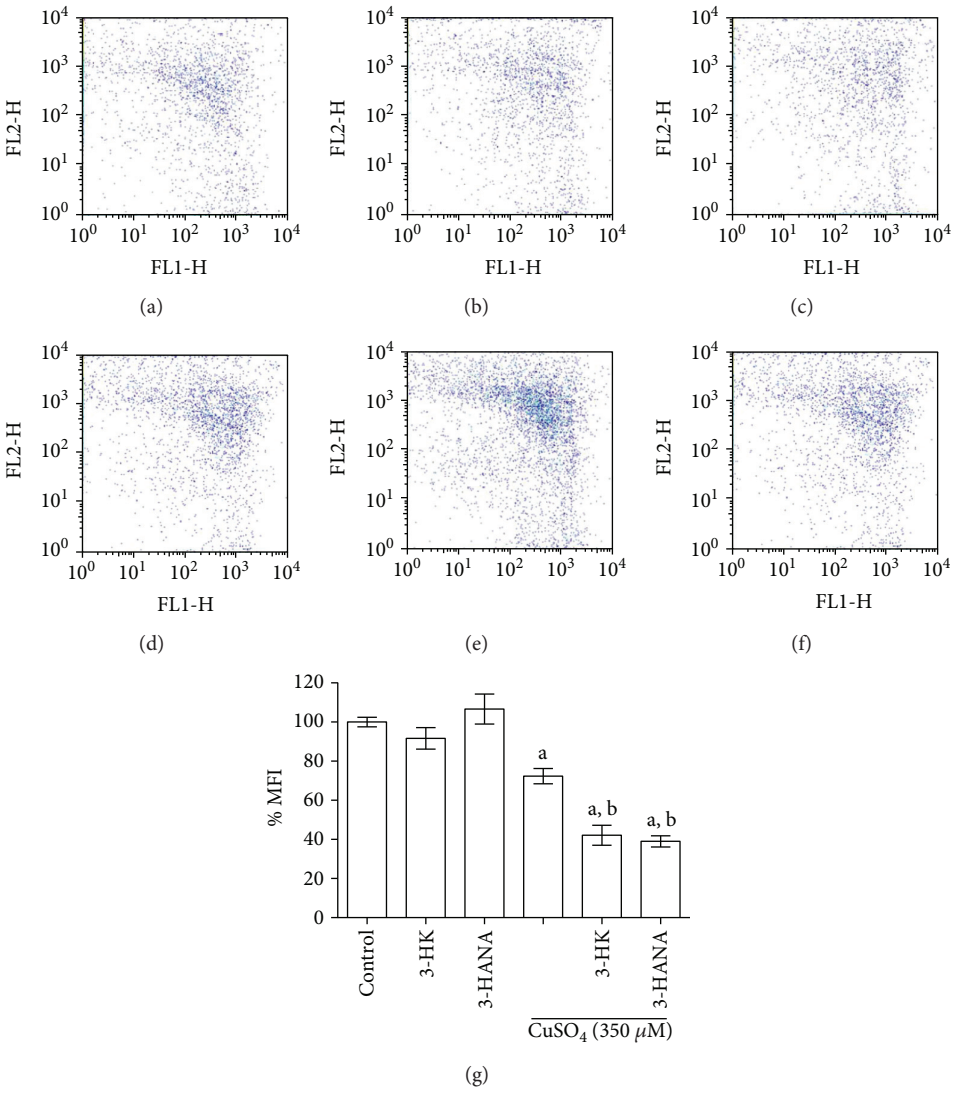

Figure 5: Effect of coincubation of $CuSO_4$ (350 μM) with 3-HK (100 μM) and 3-HANA (100 μM) on MMP in astrocytes. The MMP was measured using JC-1 orange-red fluorescence. Changes in MMP were evaluated by flow cytometry. Representative dot plots of MMP are showed in (a) control, (b) 3-HK, (c) 3-HANA, (d) copper, (e) copper + 3-HK, and (f) copper + 3-HANA. Percentage of mean fluorescence intensity (MFI) is present in (g). Data are presented as mean values + SEM of 6 independent experiments from 3 different cultures. $^a p < 0.001$ versus control and $^b p < 0.001$ versus $CuSO_4$ (one-way ANOVA followed by Tukey's test).

selective to changes in mitochondrial membrane potential and can form red fluorescence aggregates (FL-1 channel, emission length 525 nm) with high MMP, whereas when MMP is low, JC-1 is in its monomeric form (FL-2 channel, emission length at 590 nm) and displays a green fluorescence [50, 51]. After treatments, medium was removed, cells were washed with saline solution, and then 75 μl of trypsin was added to each well. Cells were recollected and centrifuged at 2000 rpm for 10 min. Medium was discarded, and mitochondrial membrane potential was evaluated through the label of cells with 3 μM of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1) for 15 min at 37°C in darkness. Then, cells were washed with buffer assay two times. After washing, cells were resuspended and analyzed by flow cytometry. 10,000 events were assessed. Data are expressed as mean fluorescence intensity (MFI) in FL-2 channel and the percentage of cells that decreased MMP [39].

2.6. GSH Determination. GSH concentration was measured with a glutathione detection assay kit (Abcam 65322). Briefly, astrocytes were incubated with copper (350 μM), 3-HK and 3-HANA (100 μM), and with combinations of both in DMEM medium for 24 h at 37°C. After that, medium was removed, cells were washed with saline solution, and then 75 μl trypsin was added to each well. Cells were recollected (100,000 cells) and centrifuged at 2000 rpm for 10 min. Medium was discarded, cells were washed with cold PBS, resuspended in cell lysis buffer, homogenized, and centrifuged 10 min at 4°C, and supernatant was collected. The cells were deproteinized with perchloric acid and potassium

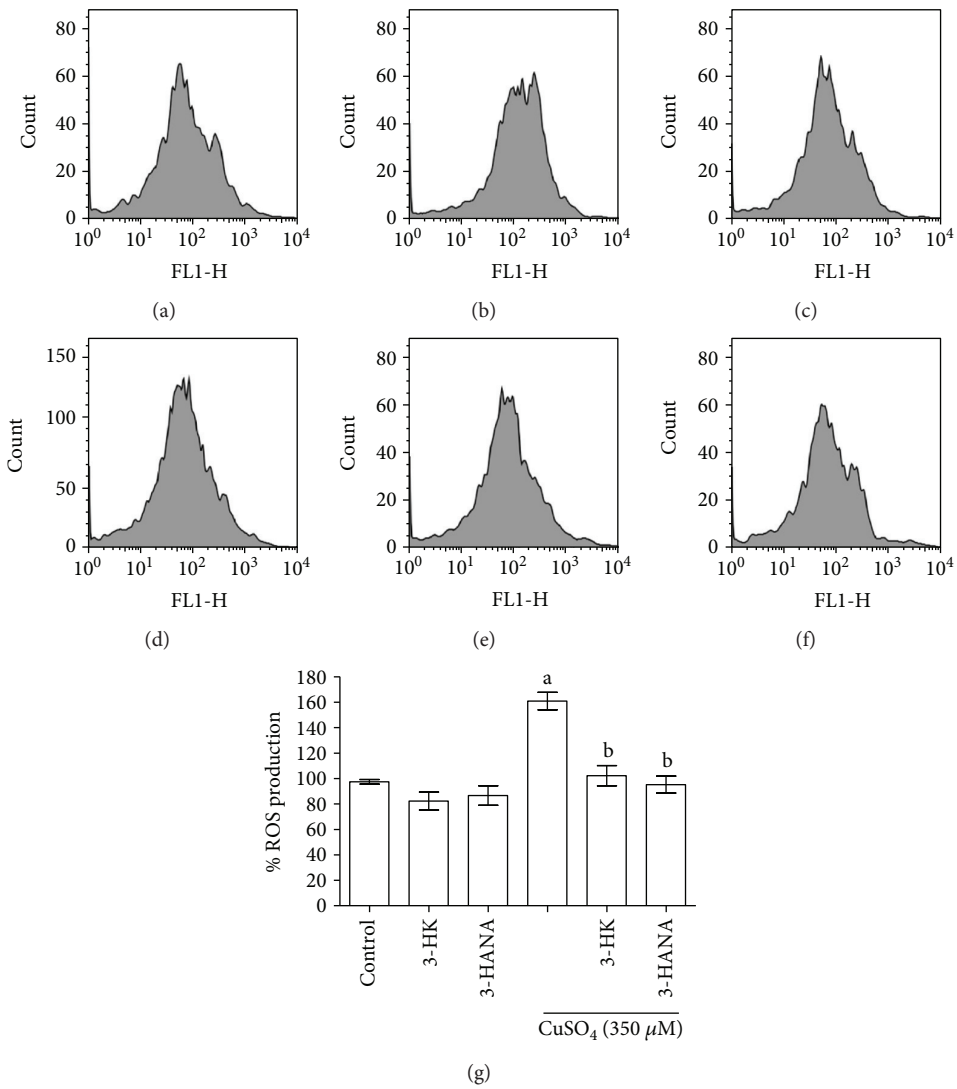

Figure 6: Effect of 3-HK (100 μM) and 3-HANA (100 μM) on ROS production induced by copper. After 24 h of incubation with the kynurenines and copper, DCF-DA was added to all treatments to determinate ROS. Representative dot plots of MMP are showed in (a) control, (b) 3-HK, (c) 3-HANA, (d) copper, (e) copper + 3-HK, and (f) copper + 3-HANA. Percentage of ROS production is showed in (g). Data are presented as mean + SEM of 6 independent experiments from 3 different cultures. $^{a}p < 0.01$ versus control and $^{b}p < 0.001$ versus $CuSO_4$ (one-way ANOVA followed by Tukey's test).

hydroxide. Once deproteinized, samples were ready to use in the GSH determination assay according to the kit's instructions. Briefly, standard curve was prepared from 0.1 μg/μl of GSH and dilutions were done in lysis buffer. 50 μl of standard and 100 μl of each sample were added to each well, and then 2 μl of GST reagent and 2 μl of monochlorobimane (MCB) were added. The plate was mixed, and fluorescence in samples was immediately measured in a plate reader at 360 nm excitation and 460 nm emission in a kinetic mode, every 3 minutes for 1 hour at 37°C. The results were expressed as the percentage change in glutathione levels in treated versus untreated control samples.

2.7. Viability Test Assay. Cellular death was assessed with propidium iodide according to Magana-Maldonado et al. [52]. Astrocytes were incubated with the combinations of $CuSO_4$ (350 μM) with 3-HK or 3-HANA (100 μM) in DMEM medium for 24 h at 37°C. Then, medium was removed, cells were washed with saline solution, and trypsin (75 μl) was added to each well. Cells were recollected and centrifuged at 2000 rpm for 10 min. Medium was discarded, and propidium iodide (PI) was added (5 μg/ml) and incubated for 15 min in darkness. After incubation with PI, samples were analyzed by flow cytometry, and a total of 10,000 events were assessed. PI fluorescence was determinated with a FACSCalibur instrument, and data collection was performed using unstained cells and positive controls for single color. The results were expressed as cells death percentage.

2.8. Data Analysis. The results were expressed as mean values ± SEM. All data were analyzed by one-way analysis of variance and Tukey's post hoc test using the Prism software (GraphPad, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Concentration-Response Effects of Copper Toxicity in Astrocyte Cultures. To evaluate copper toxicity, astrocytes were incubated during 24 h with different copper concentrations (0–500 μM) in DMEM medium. Astrocytes showed decrease on MTT reduction in a concentration-dependent manner in all used concentrations, being the major effect (80%) with 500 μM of copper (Figure 1(a)). After it was proven that copper had effect on cellular function and knowing that the dehydrogenases are responsible of MTT reduction, we evaluated how mitochondrial function was affected and if ROS were implied in copper toxicity on astrocytes. Copper reduced MMP in a concentration-dependent manner (Figure 1(c)) and increased ROS production around 50% versus control; however, this effect was not concentration dependent (Figure 1(b)). After the toxicity pattern was observed, we evaluated cell death through propidium iodide (PI), which is capable of binding and labeling DNA. After incubation during 24 h with different concentrations of copper, the cell death increased significantly since the lower concentration (10 uM) was tested and this effect was concentration dependent (Figure 1(d)).

3.2. Copper-Chelating Ability of 3-HK and 3-HANA. With the purpose of testing if copper had an interaction with 3-HK or 3-HANA, we assessed the chelation capacity of 3-HK and 3-HANA for copper. The assay was carried out at three different pH (5.5, 6.8, and 7.5) since we knew that copper is able to modify the pH. Figure 2 shows the 3-HK ability to form a chelating complex with copper in all pH tested. This 3-HK ability to catch copper was more efficient in acid pH being that the IC50 is at pH 5.5 = 56.223 ± 10.322 μM, IC50 at pH 6.8 = 74.731 ± 9.3231 μM, and IC50 at pH 7.5 = 74.232 ± 16.769 μM.

On the other hand, 3-HANA had chelating capacity for copper too, three different conditions were tested, and in all of them, 3-HANA was able to catch copper (Figure 3). At pH 6.8, the most efficient ability to chelate copper with an IC50 = 112.491 ± 7.212 μM took place, following at pH 5.5 with an IC50 = 146.637 ± 4.922 μM and the pH where it was observed less effect was at pH 7.5 with at IC50 = 559.497 ± 31.422 μM.

3.3. Effect of 3-HK and 3-HANA in the Cellular Dysfunction Induced by Copper in Astrocytes. After copper toxicity was evaluated, we determined the kynurenine effect in the presence of this metal (350 μM). Figure 4 shows that copper decreases cellular function (around 50% versus control), evaluated by MTT reduction assay, and the coincubation with the kynurenines enhances this effect (around 80% versus control). The kynurenines alone do not have effect on MTT reduction.

3.4. Kynurenines Enhance the Reduction of Mitochondrial Membrane Potential Induced by Copper on Astrocytes. The next step was to determine whether the effect on MTT reduction could be related with mitochondrial membrane potential alterations. Representative figures are shown in Figures 5(a), 5(b), 5(c), 5(d), 5(e), and 5(f). 3-HK and 3-HANA (100 μM) alone do not induce effect on this parameter compared with the control group. However, copper (350 μM) is able to reduce around 40% the MMP, while the coincubation of copper with both kynurenines reduced around 60% the MMP versus control.

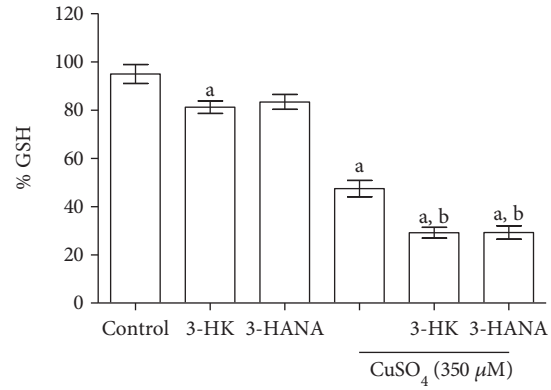

Figure 7: Effect of 3-HK and 3-HANA and their coincubation with copper in GSH levels on astrocytes. GSH levels were determinated after 24 h of incubation with the treatments. Data are presented as mean values + SEM of 8 independent experiments for each treatment. $^{a}p < 0.05$ versus control and $^{b}p < 0.001$ versus $CuSO_4$ (one-way ANOVA followed by Tukey's test).

3.5. 3-HK and 3-HANA Reduce ROS Production Induced by Copper. Considering redox properties of 3-HK and 3-HANA, the next experiment was to know if the potentiation in the copper toxicity induced by the kynurenines was through ROS production. Copper (350 μM) induces around 60% ROS production, and coincubation with 3-HK and 3-HANA abolished this effect. Incubation of 3-HK and 3-HANA alone did not have effect in ROS production (Figure 6). Representative pictures are shown in Figures 6(a), 6(b), 6(c), and 6(d).

3.6. GSH Depletion Is Involved in Toxicity Pattern Induced by the Coincubation of Copper and Kynurenines. As we know, both copper and kynurenines can interact with GSH, and then we evaluated the levels of this endogenous antioxidant that it is in high concentration in astrocytes. 3-HK decreased GSH levels around 20%, while copper decreased them around 55%; 3-HANA did not have effect in this parameter. However, the coincubation of copper with these kynurenines decreased GSH levels around 70% versus control, in both cases (Figure 7).

3.7. Effect of Copper Coincubation with Kynurenines on Cell Viability. Figure 8 shows the effect of copper and kynurenines on cell viability. Representative pictures are shown in Figures 8(a), 8(b), 8(c), 8(d), 8(e), and 8(f). 3-HK and 3-HANA alone did not have effect on cell death, while copper was able to increase the percentage of dead cells around 45%. Coincubation of copper with kynurenines enhanced the number of dead cells (Figure 8(g)). Figure 9 shows representative bright field micrographs of the different treatments. 3-HK and 3-HANA did not show difference compared with control. However, in copper alone (Figure 9(d)) and the

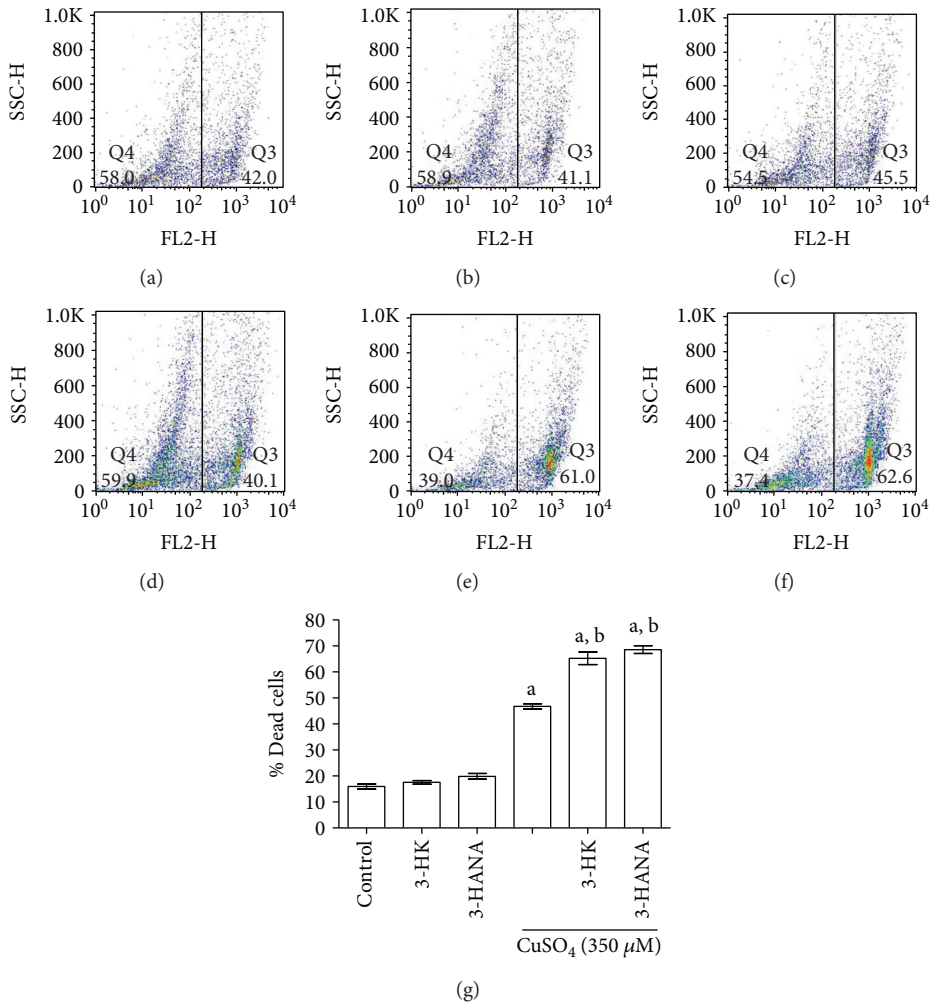

Figure 8: Effect of 3-HK and 3-HANA on cell death induced by copper. The propidium iodide (PI) flow cytometry assay was used for the evaluation of cell viability. Representative dot plots of IP are showed in (a) control, (b) 3-HK, (c) 3-HANA, d) copper, (e) copper + 3-HK, and (f) copper + 3-HANA. Percentage of dead cell is showed in g. Data are presented as mean values + SEM of 6 independent experiments from 3 different cultures. $^{a}p < 0.001$ versus control and $^{b}p < 0.001$ versus $CuSO_4$ (one-way ANOVA followed by Tukey's test).

copper combination with 3-HK (Figure 9(e)) and 3-HANA (Figure 9(f)), a considerable number of dead cells compared with control can be seen.

4. Discussion

3-HK and 3-HANA are metabolites of tryptophan catabolism, which possess redox properties and have been associated with neurodegenerative diseases as HD and AD. These kynurenines are produced through KP, which is highly regulated by redox environment. In this context, copper is an integral part of many important enzymes involved in cellular metabolism; however, its dyshomeostasis can generate oxidative stress and it has been related with some neurodegenerative diseases in which also KP metabolites are involved [53, 54]. In the present work, we evaluated the effect of 3-HK and 3-HANA in the copper toxicity on astrocytes. We performed the experiments in astrocytes since in the brain, these cells are thought to play a key role in copper homeostasis; in fact, it has been proposed that astrocytes can normally accumulate this metal which will be used by themselves or routed to neurons [53], and the second reason to use astrocytes is that 3-HK and 3-HANA cannot be enzymatically degraded in these cells. First, we demonstrate that copper had toxic effects on astrocytes as some previous reports showed [55–57]. Copper was able to decrease cell functionality and MMP and increase ROS production; these factors may be closely related and be dependent on each other. Despite the fact that copper has important functions as cofactor of antioxidant enzymes as SOD 1, copper is also cytotoxic considering that it can participate in ROS production through Fenton's reaction and to displace other elemental metals [58, 59]. Besides, mitochondrial alterations and changes in redox environment induced by copper can lead to decrease cell viability as was observed in this work.

On the other hand, it has been shown that 3-HK and 3-HANA are able to scavenge hydroxyl radical and peroxynitrite in chemical combinatory assays and also are able to chelate some metals such as iron [39]. Keeping in mind this background, we explore if 3-HK and 3-HANA would have

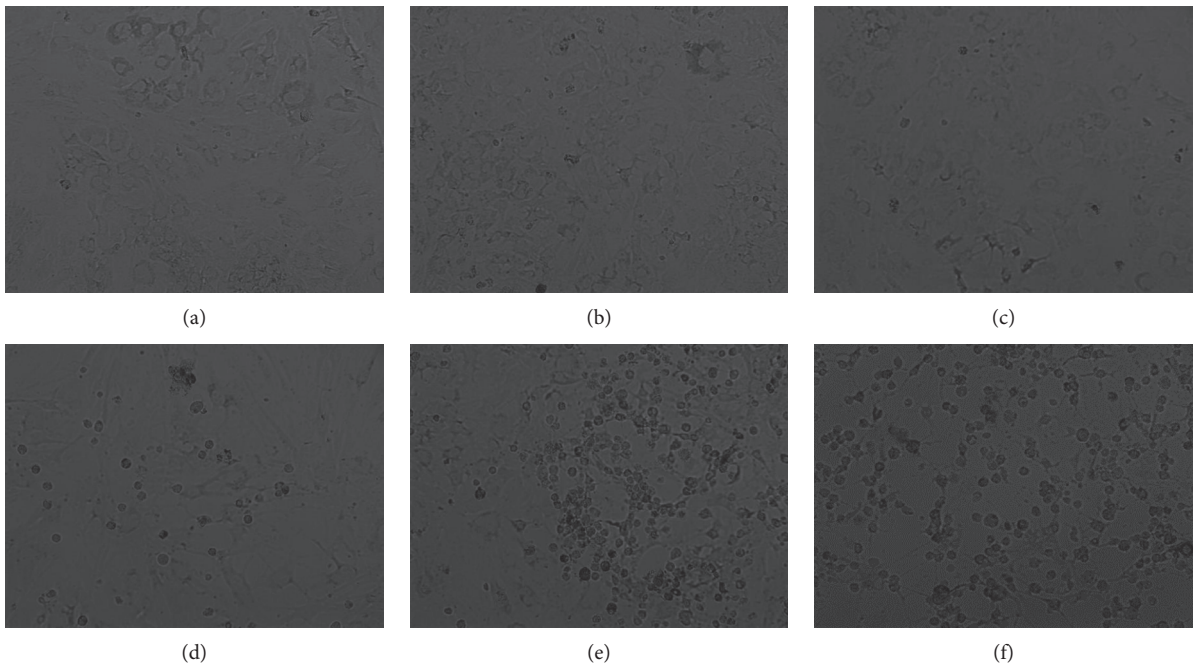

Figure 9: Effect of kynurenines on cell death induced by copper in astrocytes. Representative phase-contrast micrographs showing the effect of coincubation of 3-HK or 3-HANA with copper. (a) Control, (b) 3-HK, (c) 3-HANA, (d) $CuSO_4$, (e) $CuSO_4$ + 3-HK and (f) $CuSO_4$ + 3-HANA.

copper-chelating capability. Copper-chelating probes were placed at three different pH (5.5, 6.8, and 7.5) by two reasons: (1) in the method that we are using to determinate copper chelation, the affinity of hematoxylin for cupric ions is decreased when the pH is also reduced, and mainly (2) because it has been described in previous reports that copper accumulation, as in some pathologies, can change environment pH and this changes can influence the chelating capacity of various molecules [44–46]. Our data show that both kynurenine metabolites were able to chelate copper under different conditions of pH; this results may be due to the nature of 3-HK and 3-HANA since it has been reported that these metabolites are good electron donors in electrochemical experiments [60].

According to redox and chelating properties of 3-HK and 3-HANA, we decided to evaluate their effect on the copper toxicity. Both kynurenines were able to abolish ROS production induced by copper; however, the toxic effect on mitochondrial and cellular function was enhanced by the coincubation of copper with both kynurenines. These effects may be due to the fact that both 3-HK and 3-HANA are able to affect respiratory control (oxygen consumption in states 2 and 3 of mitochondrial respiration) [61] in addition to copper toxicity in mitochondria. Both sceneries affect ATP production and can lead to cell death as can be observed in Figure 9. In this context, a previous report showed that 3-HK and 3-HANA produced cellular damage but in an independent way of ROS production, and actually, the oxidative stress parameters evaluated were even below of basal levels [39]. Moreover, the toxic copper effect enhanced by the kynurenines can be due to the fact that these kynurenines can be oxidized in the presence of copper as copper is reduced, promoting cross-linking in important proteins [34]. Besides, autoxidation of these hydroxykynurenines can form compounds as xanthommatin radical, p-quinone, and 4,6-dihydroxyquinolinequinonecarboxylic acid (DHQCA) which are reactive [62] and can interfere with the mitochondrial function and subsequently provoke cell toxicity. Other parameter evaluated was the effect of copper and kynurenines on GSH, which is in high amounts in astrocytes [17]. GSH is responsible to form complexes with copper being copper a natural pool in astrocytes; this complex is required for the incorporation of copper into metallothionein and for SOD activation. In the case of copper was overmuch, GSH would be the first antioxidant to catch it and avoid a triggering of oxidative effects [63–65]. Our data show that copper (350 μM) decreased GSH levels and this effect was also enhanced by 3-HK and 3-HANA. This could be explained by the fact that in astrocytes, 3-HK can suffer a deamination and this could occur slowly at physiological pH or by the action of kynurenine aminotransferase (KAT), forming 3-hydroxykynurenine glucoside (3-OHKG) in a nonoxidative way, which in turn can form adducts with GSH [66–68]. With the knowledge that KAT is the most abundant KP enzyme in astrocytes, it is not hard to think that 3-HK deamination is taking place and 3-OHKG can form adducts with the large amounts of GSH in this cell type, decreasing GSH available to catch copper and allowing that free copper causes the greater damage observed. Although that process has not been described with 3-HANA, it has been shown that this metabolite is also able to decrease GSH levels in some kind of cells [69]. It is important to take into consideration that 3-HK and 3-HANA could be good ROS scavengers and chelating

agents; however, their interaction with other cellular components could increase the cell vulnerability to damage toward other agents, as in this case, to copper.

5. Conclusion

This research provides important evidence about how two endogen KP metabolites can intensify the cellular damage induced by copper. It is relevant, because in some neurodegenerative diseases, they are found as common factors, the alteration in copper concentrations or in other metals, as well as KP metabolite alterations. The challenge for the future research would be to know the precise modulation of KP metabolites by metals and try to identify therapeutic targets in diseases where these components are present.

Conflicts of Interest

The authors report no conflicts of interest.

Authors' Contributions

The authors alone are responsible for the content and writing of the paper.

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Research Article

Preliminary Data on the Interaction between Some Biometals and Oxidative Stress Status in Mild Cognitive Impairment and Alzheimer's Disease Patients

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Increased interest regarding the biometal mechanisms of action and the pathways in which they have regulatory roles was lately observed. Particularly, it was shown that biometal homeostasis dysregulation may lead to neurodegeneration including Alzheimer's disease, Parkinson disease, or prion protein disease, since important molecular signaling mechanisms in brain functions implicate both oxidative stress and redox active biometals. Oxidative stress could be a result of a breakdown in metal-ion homeostasis which leads to abnormal metal protein chelation. In our previous work, we reported a strong correlation between Alzheimer's disease and oxidative stress. Consequently, the aim of the present work was to evaluate some of the biometals' levels (magnesium, manganese, and iron), the specific activity of some antioxidant enzymes (superoxide dismutase and glutathione peroxidase), and a common lipid peroxidation marker (malondialdehyde concentration), in mild cognitive impairment ($n = 15$) and Alzheimer's disease ($n = 15$) patients, compared to age-matched healthy subjects ($n = 15$). We found increased lipid peroxidation effects, low antioxidant defense, low magnesium and iron concentrations, and high manganese levels in mild cognitive impairment and Alzheimer's disease patients, in a gradual manner. These data could be relevant for future association studies regarding the prediction of Alzheimer's disease development risk or circling through stages by analyzing both active redox metals, oxidative stress markers, and the correlations in between.

1. Introduction

It is now generally accepted that several biometals (BM) such as iron, copper, zinc, manganese, and magnesium are vital in the complex cellular activities and regulation [1–5]. Lately, there is an increased interest regarding the BM mechanisms of action due to their potential to lead to several pathway degeneration when homeostatically impaired. In this way, neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson disease, and prion protein disease are shown to be closely related to several BM levels.

AD is a complex disorder involving both behavioral and molecular distress. In this way, it is now accepted that AD is a multifactorial disease in which several important components have been described: the discrete biochemical changes which firstly occur triggering cellular modifications such as amyloid accumulation and neurofibrillary tangles formation; the histological typical features accompanied by synaptic disruption and neuronal loss; and last but not the least the visible symptoms of the behavioral component—memory loss, cognitive decline, and related comorbidities (affective distress and somatic disorders such as chronic pain or anemia) [6–9]. Also, considering these findings, it is now

accepted that mild cognitive impairment (MCI) is a disorder providing a major risk factor for AD [10]. Moreover, while some overlapping traits between MCI and the early stages of AD in considering the characteristic mild cognitive decline were suggested, it was shown that AD cognitive abilities gradually decline, but MCI patients' cognitive state remains stable for years [10].

Oxidative stress is also a major component of AD pathology. The common knowledge on aging now includes a biochemical theory that partly explains the cellular decline due to oxidative/antioxidant process imbalance occurrence at a cellular level as we age. Together with that, the thorough description of brain biochemical mechanisms which revealed its high oxygen resources needs and its special membrane lipid-rich structure leads to the conclusion that brain tissue is extremely susceptible to oxidative stress. In this context, it was demonstrated that oxidative stress plays important roles in AD pathology, both at its first molecular changes and also during its development up to its final stages.

Also, oxidative stress may also be a result of a breakdown in metal-ion homeostasis which leads to abnormal metal protein chelation. Extensive evidence points to an important implication of several both toxic and redox metal ions which can contribute to DNA and protein damage causing oxidative stress and molecular damage (as reviewed by [11]) by being involved in cycles of electron transfer reactions from and to the substrates which make them extremely important in redox and metal homeostasis, both of which are tightly related [12]. Therefore, any ionic metal unbalance occurring at the cellular or peripheral level is reflected in abnormal redox homeostasis followed by excessive reactive oxygen species (ROS) production, oxidative stress, and their further effects [13].

Furthermore, the critical role of copper, iron, and other trace redox-active transition metals was shown recently to be implicated in the pathogenesis of AD [14, 15]. While our group previously suggested a strong link between oxidative stress and Alzheimer's disease [16, 17], we aimed to assess the possible cause/effect relationship between BM abnormal levels dynamics and the increased oxidative damage occurring in AD pathology. Moreover, we demonstrated a progressive pattern of oxidative markers change during both mild cognitive impairment and Alzheimer's disease patients analysis which could also be linked to a progressive BM level pathological tendency.

Consequently, the aim of the present work was to evaluate some relevant BM levels (magnesium, manganese, and iron), the specific activity of some antioxidant enzymes (superoxide dismutase SOD and glutathione peroxidase (GPx)), and malondialdehyde (MDA) levels as a marker of lipid peroxidation, in MCI and AD patients, compared with age-matched healthy subjects.

2. Materials and Methods

2.1. Patient Recruitment. In this case-control study, we collected blood samples from 30 patients with AD (15 patients) and MCI (15 patients). The control group was consisted from healthy age- and sex-matched participants ($n = 15$). All the participants were recruited from the "Socola" Regional Institute of Psychiatry (Iasi, Romania) based on ethical agreement from the Regional Institute of Psychiatry Board Committee. Also, the cognitive status of the participants was assessed using standard Mini-Mental State Examination (MMSE) [18] and Alzheimer's Disease Assessment Scale-Cognitive (ADAS-Cog) [19]. All the AD patients underwent psychiatric diagnosis fulfilling NINCDS ADRDA criteria [20, 21]. Also, MCI diagnosis followed Petersen et al. criteria (memory impairment accompanied by general cognitive and functional abilities preservation) [22]. The study was conducted according to Helsinki Declaration and national and European regulations on Biomedical Research. All the patients or their families were given and signed a written informed consent for their contribution in this study. Several exclusion criteria were provided for patients' recruitment such as antioxidant supplementation and acute or severe comorbidities.

2.2. Sample Preparation. Blood samples have been collected before breakfast and allowed to clot. After centrifugation (3000 rpm, 15 minutes, 4°C), blood sera were separated, aliquoted, and stored at −22°C until analysis. For BM quantification, the stored aliquots were processed according to the following protocol: 1 ml of sample was digested with 3 ml nitric acid 65% and 2 ml of hydrogen peroxide in decontaminated TFM pressure vessels that were inserted in Speedwave MWS-2 produced by Berghof. The digestion program for samples was in steps as follows: 145°C for 5 min, 190°C for 10 min, and 100°C for 10 min [23]. After the microwave digestion, the samples were transferred in 25 ml decontaminated flasks and filled up to volume with ultrapure water. No special preparation except for the biochemical analysis kit brochures' mentions were needed for the biochemical analysis protocols.

2.3. Biometals Separation and Quantification. High-purity and producer-certified-quality reagents were used for the element separation and measurement. Ultrapure water filtered by LaboStar™3/7 TWF (Siemens) purification system from double-distilled water was used for decontamination, sample preparation, and reagent dilution. High-purity nitric acid 65% (Merck, Germany) and hydrogen peroxide EMSURE 30% stabilized for higher storage temperature (Merck, Germany) were used in the metal digestion process from the biological samples. The standard stock solutions for AAS used in the calibration method were certified by Merck, Germany. All the necessary solution used in calibration and quantification of the metals were manganese (1000 mg/l), iron (1000 mg/l), and magnesium (1000 mg/l).

Atomic absorption spectrometer with a high-resolution continuum source equipped with graphite furnace and platform, ContrAA 600 from Analitik Jena, Germany, was used for all the element measurements. Matrix sample modifiers diluted from certified solutions of $Pd/Mg(NO_3)_2$ (Merck Germany) were necessary. Blind samples for testing any possible contamination from laboratory and reagents were prepared. Standard solutions were used for

Table 1: Demographics, functional description, and oxidative marker assessment of the study groups.

	Control, $n = 15$	MCI, $n = 15$	AD, $n = 15$
Age (means ± SEM, years)	62.5 ± 3.4	63.2 ± 4.2	65.8 ± 3.9
Sex (% F/% M)	46.6% F/53.3% M	33.3% F/66.6% M	40% F/60% M
MMSE score (means ± SEM)	26 ± 0.5	22.2 ± 0.3	18.5 ± 0.3
SOD (U/ml)	0.22 ± 0.008	0.18 ± 0.001***	0.17 ± 0.003***
GPx (U/ml)	0.142 ± 0.001	0.049 ± 0.002***	0.045 ± 0.001***
MDA (nmol/l)	9008 ± 0.14	13.141 ± 0.36***	18.158 ± 0.26***

Results are presented as mean ± SEM; ***$p < 0.001$, as compared to the control group.

quality-control sample preparation consisting in various successive concentrations.

2.4. Biochemical Analysis. Biochemical analysis included SOD, GPx, and MDA quantification. SOD enzymatic activity was determined as enzymatic reaction inhibition rate using a spectrophotometric SOD Assay Kit (Sigma, Germany) according to the manufacturer's instructions. The method is based on the WST (water-soluble tetrazolium) salt reaction with superoxide anion producing a water-soluble formazan dye. Therefore, indirect measurement of SOD activity is obtained by analyzing the linear correlation with the rates of xanthine oxidase catalyzed O_2 reduction which is inhibited by SOD. Following this measurement, the results were normalized by total sera protein concentration and transformed in SOD activity units. GPx activity was determined using GPx Cellular Activity Assay Kit CGP-1 (Sigma, Germany). This kit also uses an indirect determination method based on the oxidation of glutathione to oxidized glutathione coupled with the inverse reaction in the presence of GPx, glutathione reductase, and NADPH as an enzymatic cofactor. The method is therefore based on the NAPDH concentration decrease measurement in the reaction media, correspondent to the GPx activity during which NADPH is oxidized to $NADP^+$. MDA levels were assessed using thiobarbituric acid-reactive substances (TBARs) determination method. Trichoracetic acid (50%, 0.5 ml) and thiobarbituric acid (0.73%, 0.55 ml) were mixed with blood sera (0.1 ml) and vortexed. Afterwards, a 20-minute incubation at 100°C (boiling water bath) and a 10-minute centrifugation (3000 rpm) were performed. The supernatants were read at 532 nm and the absorbances were read against MDA standard curve (the results were expressed as nmol MDA/ml blood serum) [24, 25].

2.5. Statistical Analysis. Firstly, the Shapiro-Wilk normality test for the data sets was performed to study the samples' distribution. One-way ANOVA followed by the Tukey HSD test was performed to demonstrate the significant variance of each metal concentration in blood serum between studied groups. All the statistical analyses were carried out by using OriginPro v.9.3 (2016) software created by OriginLab Corporation, USA. The results were reported as means ± standard error of the means. F values for which $p < 0.05$ were regarded as statistically significant. Pearson's correlation coefficient and regression analysis were used to evaluate the connection between antioxidant defense, lipid peroxidation, and BM serum levels for which Minitab 17 (Minitab Inc., 2013) application was used.

3. Results

In the present study, we measured several important BM levels, in the peripheral blood of MCI and AD patients, compared with age- and sex-matched controls. Chemical analysis of the subjects' blood serum revealed significant differences regarding the magnesium, manganese, and iron levels in the MCI and AD patients' sera.

Data on patients and oxidative stress markers have been previously described [26] but are summarized in Table 1.

We observed an iron levels decrease in the AD patients sera (140.43 ± 16.02 μg/dl) as compared with the healthy sex- and age-matched controls (192.45 ± 42.51 μg/dl). Also, interestingly, we firstly observed a slight increase of the iron levels in the MCI patients (242.47 ± 18.06 μg/dl) followed by the significant decrease in AD patients (Figure 1). Overall one-way ANOVA showed a significant variation between the groups: C – MCI – AD [$F(2, 30) = 3.82$; $p = 0.033$]. Post hoc comparisons using the Tukey test indicated that the mean score for the AD group ($M = 140.43$, SD = 57.77) was significantly different than the MCI group ($M = 242.47$; SD = 57.12).

Regarding the manganese levels, we observed a statistically significant increase of manganese levels in AD patients' sera (3.32 ± 0.07 μg/dl, $p < 0.001$) and MCI patients' sera (3.10 ± 0.09 μg/dl, $p < 0.05$) as compared to the healthy sex- and age-matched controls' sera (2.68 ± 0.16 μg/dl) (overall ANOVA: $F(2, 32) = 8.93$, $p = 0.0008$) (Figure 1). Post hoc analysis revealed that the mean score for the MCI and AD groups were significantly different from the control group regarding the manganese levels and similarly regarding the magnesium levels.

Contrarily, we observed that AD patients' (994.08 ± 69.04 μg/dl, $p < 0.01$) and MCI patients' (1051.40 ± 65.43 μg/dl, $p < 0.05$) magnesium levels tend to decrease as compared to those of healthy controls (1316.46 ± 60.27 μg/dl) (Figure 1). Also, overall one-way ANOVA analysis revealed a significant pattern of magnesium level decrease [$F(2, 35) = 4.73$; $p = 0.015$] for the experiment groups, as compared with the healthy sex- and age- matched controls.

Regarding the BM levels trending, several direct moderate and low correlations (post hoc Pearson's correlation)

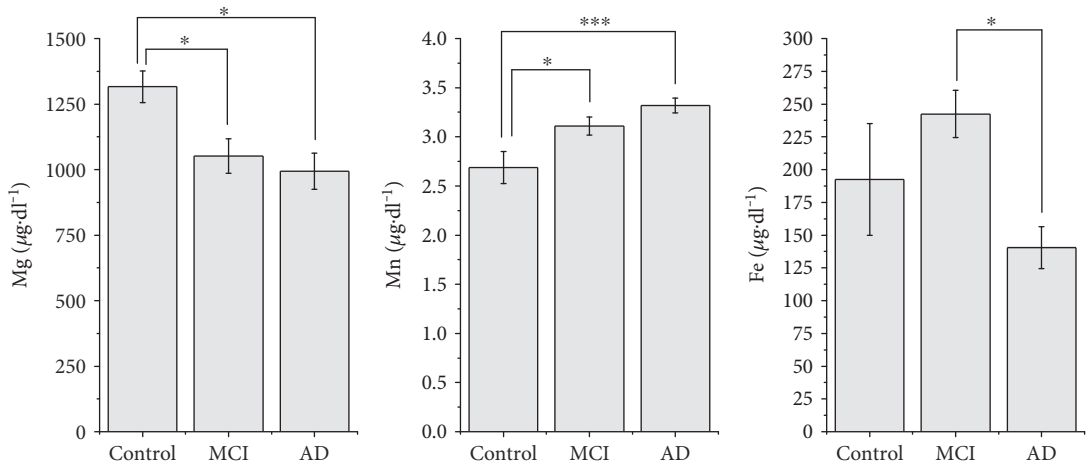

Figure 1: Magnesium, manganese, and iron concentrations in blood sera (presented as mean ± SEM) in studied groups ($^{*}p < 0.05$ and $^{***}p < 0.001$, Tukey HSD test).

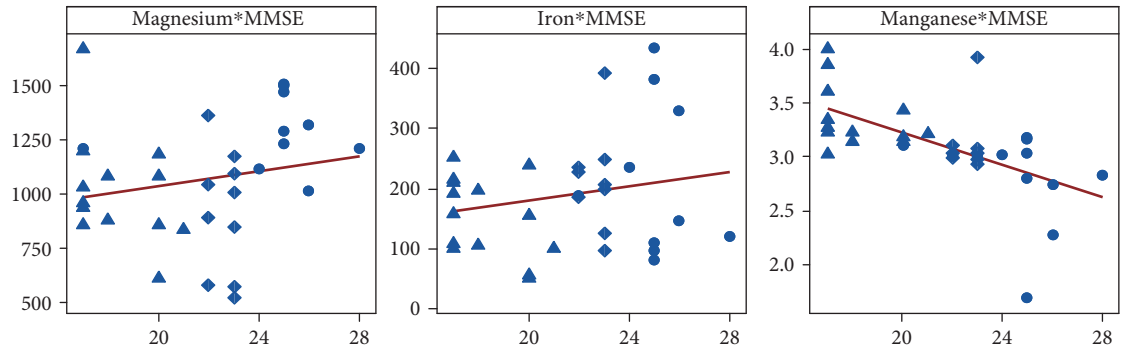

Figure 2: Correlation between MMSE scores and BM levels in control (●), MCI (♦), and AD (▲) patients (explanation in text).

have been observed. In this way, while analysing iron against manganese trending, a moderate negative correlation has been observed (Fe-Mn: $r = -0.431$, $p = 0.012$). Weak negative correlation was obtained while analysing magnesium versus manganese trending (Mg-Mn: $r = -0.27$, $p = 0.12$), and no statistical correlation was found for iron versus magnesium comparison. However, while comparing BM levels with specific psychiatric scales applied to the patients, we observed a strong negative correlation between MMSE score and manganese levels ($r = -0.585$, $p < 0.001$) (Figure 2).

Interesting results were obtained in post hoc analysis (Pearson correlation) of biometal concentrations versus oxidative stress status correlations. In this way, we obtained significant statistical correlations for two of the oxidative stress markers as compared with manganese concentrations (Mn-GPx: $r = -0.564$, $p < 0.001$; Mn-MDA: $r = 0.561$, $p < 0.001$). For magnesium levels, moderate statistical correlations were obtained for Mg-GPx ($r = 0.509$ $p = 0.002$) and Mg-MDA ($r = -0.383$, $p < 0.05$) comparisons. No statistical significant correlations were found during post hoc analysis of serum iron levels and oxidative stress markers.

4. Discussion

The present work aimed to evaluate manganese, magnesium, and iron levels, the specific activity of some antioxidant enzymes (SOD and GPx), and MDA levels as a marker of lipid peroxidation therefore cellular damage, in MCI and AD patients, compared to age-matched healthy subjects. We found increased lipid peroxidation effects, low antioxidant defense, low magnesium and iron concentrations, and high manganese levels in MCI and AD patients.

Previous research suggested some contradictory results concerning the BM serum levels in neurodegenerative disorders. Recent studies reported various tendencies in BM level dynamics in demented patients [16, 26, 27]. In this way, Barbagallo et al. [28] showed decreased magnesium ion levels in AD patients' serum. Also, Cilliler et al. [29] reported a correlation between low AD group magnesium levels and MMSE score. Similarly, manganese level analysis in the recent studies revealed controverted results. The most recent meta-analysis on the matter, Du et al. [30], reports controversial dynamics of manganese levels presenting

several studies which show a significant decrease of this parameter in demented patients' sera, while in other studies, no difference or significant increase were obtained. We also found statistical correlations between MMSE scores versus manganese levels and ADAS-COG versus magnesium and manganese levels (data not shown). Regarding the iron levels, the previous studies suggest no significant change in demented patients [31], while the more recent Paglia et al. [32] study reported a progressive decrease of this parameter in AD, MCI patients, subjective memory complaint, and healthy participants. In our study, we report similar tendencies of the discussed BM in MCI and AD patients, as compared to age- and sex-matched control group and also significant statistical correlations for all the studied biometals versus lipid peroxidation marker, moderate statistical correlations for antioxidant enzymes versus magnesium levels, and no statistical significant correlation between oxidative stress markers and iron levels. However, an interesting iron level pattern of progression was observed. Previous studies show high specific antioxidant enzymes activities [33–35] and a compensatory activity in the body. Also, several studies reported decreased nonenzymatic antioxidant factor levels and high iron, aluminum, and mercury concentrations in demented patients [36]. Furthermore, clinical trials showed increased oxidative stress markers' levels in patients with MCI as well [37–39].

Similar iron pattern of variation was reported by Paglia et al. [32] who showed that the iron level increase (in individuals showing subjective memory complaint) is followed by abrupt concentration decrease in MCI and AD patients. These findings, together with the study of Smith et al. [40] which show increased iron levels in AD and MCI brains forming iron deposits could suggest that anemia may be a common condition found in AD patients. This idea was also suggested by Hare et al. [41] who reported low iron plasma levels in correlation with low-bound transferrin levels. Moreover, our findings could be explained by the importance of iron in oxidative stress modulation. While high levels of iron were observed in demented patients' brain and high oxidative stress is a common trait for AD, the low-iron serum levels could suggest that intense brain redox activity is resulting in excess iron concentrations in that tissue. This may be the reason why an increased iron-modulated immune response could be observed in the brain tissue during initial accumulation of beta-amyloid plaques [42]. However, while our previous results show increased serum oxidative stress levels, it seems that this variation is not dependent on iron activity.

Although controversial results were obtained in similar studies which showed that manganese levels in demented patients are decreased, many reports demonstrate that manganese could contribute to beta-amyloid plaque formation [43] and therefore an increase of this parameter would be possible both in brain tissue and also in blood serum. In this way, Tong et al. reported a plasma Aβ peptides concentration increase in correlation with high serum manganese levels. Although manganese was shown to significantly increase hippocampal glutathione peroxidase (GPx) activity [44] and manganese-dependent SOD (SOD2) [45, 46], it seems that an inverse correlation may be observed in blood sera of demented patients regarding GPx activity and total SOD activity. However, Dobson and Aschner [47] extensively discuss the oxidative stress induction potential of excess manganese. Many redox activity molecular studies on effects of manganese accumulation led to mitochondrial oxidative stress pathway description. Similar to other toxicants, it seems that excessive manganese could lead mitochondrial electron transport chain perturbance, which eventually causes additional ROS, cellular oxidative stress, and furtherly apoptosis. Also, in the redox reactivity series of metals, manganese occupies a leading position; therefore, it possesses the highest potential to generate ROS. In this way, excessive manganese intake may lead to mitochondrial dysfunction and oxidative stress alongside neuronal and astrocytic apoptosis. This aspect could be observed in the strong positive correlation between manganese and MDA levels which reflects the effects of excessive ROS production through molecular level causing lipid peroxidation and cellular damage.

The role of magnesium deficiency in AD pathogenesis has been extensively discussed [48]. Moreover, Durlach [49] brings several more arguments on magnesium mechanisms of modulation in glutamatergic transmission and hippocampal activity. Also, in an AD mouse model, Li et al. [50] points to imminent recovery of cognitive deficits and synaptic loss following magnesium administration. Xu et al. [51] reported that magnesium deficiency in rats may lead to increased free radical oxygen species, also introducing the magnesium implication in inflammation and oxidative lesions. In this way, the correlation between magnesium and the two antioxidant enzymes (GPx and SOD) is entitled since the high rate of ROS production in the presence of low antioxidant activity could be a result of magnesium depletion.

5. Conclusions

Our data show progressive increase of manganese levels in demented patients, as compared with healthy controls and progressive decrease of magnesium levels in the groups. A slight increase in iron serum levels was observed in MCI patients followed by an abrupt level drop in AD patients. We observed several strong correlations between cognitive status and biometals serum levels. Also, several correlations between the studied biometal sera levels and main oxidative stress markers were observed. Positive correlations were found in manganese versus MDA, magnesium versus GPx, and magnesium versus SOD analysis and negative correlations in manganese versus SOD and manganese versus GPx analysis. Still, no correlations were found between serum iron levels and serum oxidative stress markers. These data could be relevant for future association studies regarding the prediction of AD development risk or cycling through stages by analyzing both active redox metals and oxidative stress markers, and the correlations in between.

Conflicts of Interest

The authors have no conflict of interest to disclose.

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Review Article

The Involvement of Mg^{2+} in Regulation of Cellular and Mitochondrial Functions

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Mg^{2+} is an essential mineral with pleotropic impacts on cellular physiology and functions. It acts as a cofactor of several important enzymes, as a regulator of ion channels such as voltage-dependent Ca^{2+} channels and K^+ channels and on Ca^{2+}-binding proteins. In general, Mg^{2+} is considered as the main intracellular antagonist of Ca^{2+}, which is an essential secondary messenger initiating or regulating a great number of cellular functions. This review examines the effects of Mg^{2+} on mitochondrial functions with a particular focus on energy metabolism, mitochondrial Ca^{2+} handling, and apoptosis.

1. Impact of Mg^{2+} on Cellular Functions and Intracellular Mg^{2+} Dynamics

Mg^{2+} is an essential mineral with pleotropic impacts on cellular physiology and functions [1, 2]. It acts as a cofactor of several important enzymes, especially those requiring ATP in order to be fully functional, such as the various protein kinases, proteins involved in nucleic acid metabolism, or ATPases involved in the transport of various ions [1, 2]. In addition, Mg^{2+} alters the electrophysiological properties of ion channels such as voltage-dependent Ca^{2+} channels and K^+ channels [3]. The voltage-dependent block of N-methyl-D-aspartate receptor by Mg^{2+} [4, 5] represents an important phenomenon in the neurosciences. Finally, Mg^{2+} can affect the binding affinity of Ca^{2+} to specific Ca^{2+}-binding proteins, such as calmodulin [6], S100 [7], troponin C [8], and parvalbumin [9, 10]. The effects of Mg^{2+} on Ca^{2+}-handling proteins are responsible for the significant modification of intracellular Ca^{2+} dynamics and signalling [11]. In general, Mg^{2+} is considered as the main intracellular antagonist of Ca^{2+}, which is an essential secondary messenger initiating or regulating a great number of cellular functions in various cells [12].

Recent progress in the field of Mg^{2+} transporter research has led to the identification of plasma membrane Mg^{2+} transporter SLC41A1 [13, 14], mitochondrial Mg^{2+} efflux system SLC41A3 [15], mitochondrial Mg^{2+} influx channel Mrs2 [16], and a mitochondrial Mg^{2+} exporter [17]. Substantial progress has also been achieved with respect to the regulation of whole body Mg^{2+} homeostasis [18]. These discoveries have shed new light on the importance of Mg^{2+} in cellular physiology including mitochondrial functions. Mitochondria have been demonstrated to be capable of both the accumulation of Mg^{2+} and the release of Mg^{2+} [19, 20]. Thus, mitochondria represent an important intracellular Mg^{2+} store. Significant amount of intracellular Mg^{2+} has also been shown to be localised within the lumen of the endoplasmic/sarcoplasmic reticulum (ER/SR) [21]. However, unlike mitochondria, the molecular mechanisms of Mg^{2+} transport through the ER membrane are not yet clear. Since impact of Mg^{2+} on cellular functions was summarised in recent reviews [1–3], we will deal, in this review, with the effects of Mg^{2+} on mitochondrial functions with a particular focus on energy metabolism, mitochondrial Ca^{2+} handling, and apoptosis (Figure 1).

2. Impact of Mg^{2+} on Energy (Oxidative) Metabolism

The oxidation of coenzymes (reduced in glycolysis, reaction catalysed by pyruvate dehydrogenase complex, β oxidation, and Krebs cycle) in the mitochondrial respiratory chain and

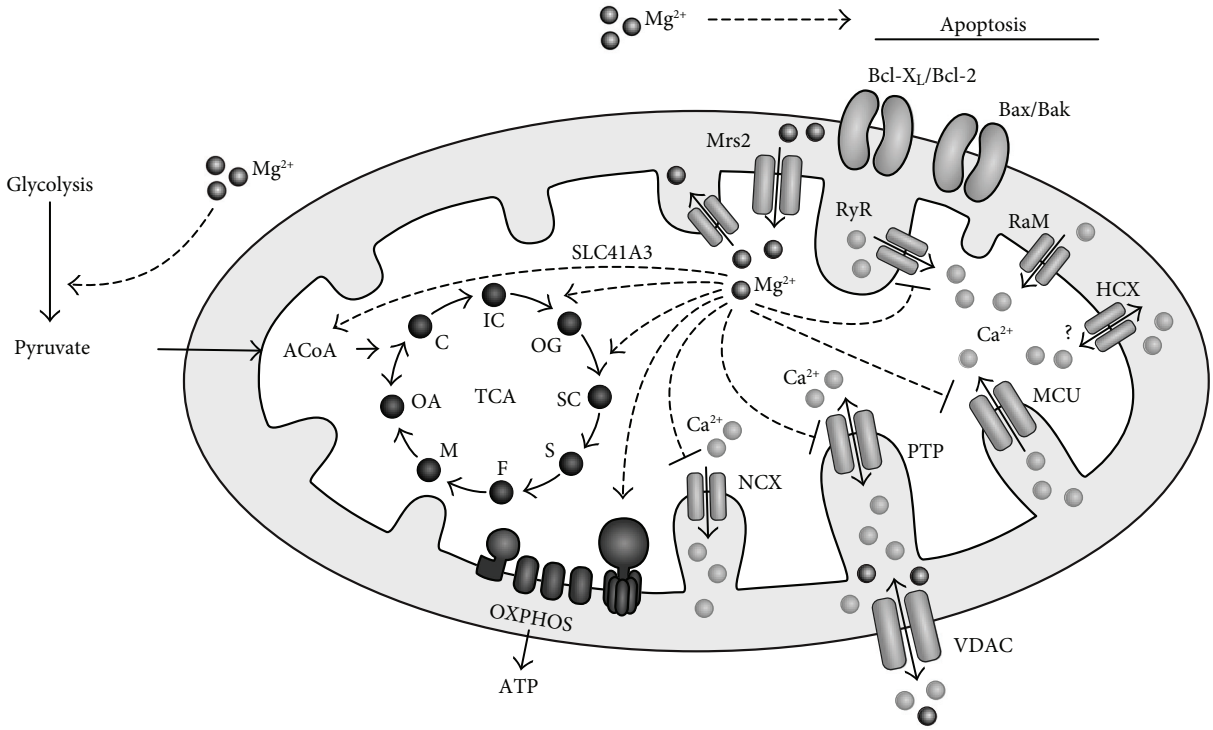

Figure 1: Regulation of mitochondrial functions by Mg^{2+}. Mitochondrial Mg^{2+} activates (————>) three dehydrogenases in the mitochondrial matrix: pyruvate dehydrogenase (conversion of mitochondrial pyruvate to acetyl coenzyme A), isocitrate dehydrogenase (conversion of isocitrate to 2-oxoglutarate), and 2-oxoglutarate dehydrogenase (conversion of 2-oxoglutarate to succinyl coenzyme A). In addition, mitochondrial Mg^{2+} activates F_0/F_1-ATP synthase, which is the terminal complex of mitochondrial oxidative phosphorylation (OXPHOS). This regulatory activity contributes to mitochondrial energy metabolism. Mitochondrial Mg^{2+} inhibits (————|) Ca^{2+} transporters localised in the inner mitochondrial membrane: Ca^{2+}-dependent permeability transition pore (PTP) opening that results in the release of a variety of compounds from mitochondria including Ca^{2+}, mitochondrial Ca^{2+} uniporter (MCU), mitochondrial ryanodine receptor (RyR), and mitochondrial Na^+/Ca^{2+} exchanger (NCX). This regulatory activity contributes to both intracellular and mitochondrial Ca^{2+} homeostasis. Cytoplasmic Mg^{2+} regulates mitochondrial Bax/Bak-dependent apoptosis, which is regulated by proteins of the Bcl-2 family such as Bcl-X_L, Bcl-2. TCA: tricarboxylic acid cycle/Krebs cycle, ACoA: acetyl coenzyme A, C: citrate, IC: isocitrate, OG: 2-oxoglutarate, SC: succinyl coenzyme A, S: succinate, F: fumarate, M: malate, OA: oxaloacetate, RaM: rapid mode of mitochondrial Ca^{2+} uptake, HCX: mitochondrial H^+/Ca^{2+} exchanger, SLC41A3: mitochondrial Mg^{2+} efflux system, Mrs2: mitochondrial Mg^{2+} influx channel, VDAC: voltage dependent anion channel.

the consequent mitochondrial oxidative phosphorylation represent the major pathway of intracellular energy production in the form of ATP for all mammalian cells, except for erythrocytes. A small fraction of ATP is produced in the cytoplasm by the oxidation of glucose in the glycolysis pathway. Many of the glycolytic enzymes (hexokinase, phosphofructokinase, phosphoglycerate kinase, and pyruvate kinase) have previously been shown to be sensitive to Mg^{2+}. The most important effect is attributable to the $MgATP_2$ complex, which is a cofactor for these enzymes, whereas other chelation forms are inactive or inhibitory [22].

The study of the impact of Mg^{2+} on the enzymes of energy metabolism in mitochondria began several decades ago [23, 24]. The earlier approach, which was focused on the description of the Mg^{2+} effect on isolated mitochondrial enzymes [25, 26], has subsequently been substituted by studies focused on the effect of Mg^{2+} on energy metabolism in isolated vital mitochondria [27–29] or vital cells [30, 31]. Some results obtained by the kinetic analysis of isolated enzymes have also been further analysed in more details by mathematical methods [32, 33]. Mg^{2+} has been documented to enhance the activity of three important mitochondrial dehydrogenases involved in energy metabolism. Whereas activities of isocitrate dehydrogenase (IDH) and 2-oxoglutarate dehydrogenase complex (OGDH) are stimulated directly by the Mg^{2+}-isocitrate complex [25] and free Mg^{2+} [34], respectively, the activity of pyruvate dehydrogenase complex (PDH) is stimulated indirectly via the stimulatory effect of Mg^{2+} on pyruvate dehydrogenase phosphatase, which dephosphorylates and thus activates the pyruvate decarboxylase of PDH [35]. OGDH is the rate-limiting enzyme of the Krebs cycle and acts as an important mitochondrial redox sensor [36, 37]. The results obtained by the complex investigation of the impact of Mg^{2+} on ATP synthesis, the mitochondrial transmembrane potential, and respiration indicate that OGDH is the main step of oxidative phosphorylation modulated by Mg^{2+} when 2-oxoglutarate is the oxidisable substrate; with succinate, the ATP synthase is the Mg^{2+}-sensitive step [29]. Indeed, Mg^{2+} has been shown to be the activator of ATP synthesis by mitochondrial F_0/F_1-ATPase [38, 39].

Taken together, the data suggest that Mg^{2+} has significant impact on the metabolic state, which is mediated by its stimulatory effect on the above-mentioned mitochondrial enzymes. However, the mitochondrial metabolic state seems, in turn, to affect the Mg^{2+} concentration of both the matrix [40] and the cytoplasm [41]. Finally, the effect of Mg^{2+} on energy metabolism partially interferes with the stimulatory effect of Ca^{2+} on energy metabolism and mitochondrial Ca^{2+} transport that are particularly important in excitable cells such as neurones [42, 43] and muscle cells [44]. Increase of extramitochondrial concentration of Mg^{2+} that was not associated with increase of Mg^{2+} concentration in mitochondrial matrix led in the presence of Ca^{2+} to the attenuation of state 3 respiration and stimulation of state 4 respiration [45]. This effect was attributed to the Mg^{2+}-dependent inhibition of mitochondrial Ca^{2+} uptake (see further) that resulted in decrease of matrix Ca^{2+} concentration [45].

3. Involvement of Mg^{2+} in Regulation of Mitochondrial Ca^{2+} Transport

Mitochondria are important players in intracellular Ca^{2+} homeostasis and signalling [46, 47]. In response to specific signals, mitochondria are capable of both the active accumulation of intracellular Ca^{2+} and the release of Ca^{2+} from mitochondria via different Ca^{2+} transport mechanisms localised on mitochondrial membranes (Figure 1). Thus, they are considered as rapid-uptake slow-release buffers of cytosolic Ca^{2+} [48, 49]. In addition to cell signalling, mitochondrial Ca^{2+} plays an important role with respect to metabolism and cell survival [50, 51]. Several molecular mechanisms control mitochondrial Ca^{2+} transport [52].

The transport of Ca^{2+} through the outer mitochondrial membrane (OMM) is mediated via voltage-dependent anion channel (VDAC) that can be modulated in various ways [52], but little is known about the effect of Mg^{2+} on VDAC-dependent Ca^{2+} transport. An early study had shown that Mg^{2+} did not alter single channel activity but modified single current amplitudes in the lower conductance channel [53].

Active mitochondrial Ca^{2+} uptake is mediated by a specific transporter, namely the mitochondrial Ca^{2+} uniporter (MCU), which transfers Ca^{2+} through the inner mitochondrial membrane (IMM) at the expense of the proton gradient generated by the mitochondrial respiratory chain. The rate of uptake has been described to be proportional to the mitochondrial transmembrane potential [54], but, recently, the exponential dependence of the relative Ca^{2+} transport velocity on the mitochondrial transmembrane potential has received greater support [55, 56]. Another physiologically important question is associated with the low affinity of MCU for Ca^{2+} (apparent K_d 20–30 μM at 1 mM Mg^{2+}) [57]. The discrepancy between the low Ca^{2+} affinity of the MCU observed in vitro and the high efficiency observed in vivo has been explained on the basis of the microheterogeneity of cytoplasmic Ca^{2+} rising during stimulation. The microdomains of high intracellular Ca^{2+} concentration (10–20 μM) have been suggested to be transiently formed in regions of close proximity to mitochondria and Ca^{2+} channels of the ER or of the plasma membrane [58]. MCU-mediated Ca^{2+} transport in isolated heart, kidney, and liver mitochondria is inhibited in the presence of 1.5 mM Mg^{2+} by approximately 50% in the heart and kidney and by 20% in the liver [59]. Similarly, the inwardly rectifying mitochondrial Ca^{2+} current displaying sensitivity to ruthenium red and selectivity to divalent cations, similar to that of MCU, is reduced by 0.5 mM of cytoplasmic Mg^{2+} concentration to 41% of its conductance in Mg^{2+}-free solutions [60]. Moreover, mitochondrial Mg^{2+} loading has been shown to suppress MCU Ca^{2+}-uptake rates [61]. The data of experimental studies were used for mathematical modelling of MCU-mediated Ca^{2+} transport suggesting a mixed-type inhibition mechanism for Mg^{2+} inhibition of the MCU function [62]. On the contrary, Mg^{2+} increased the rate of the active and ruthenium-red-sensitive accumulation of Ca^{2+} by isolated rat heart mitochondria [63]. The discrepancy has been attributed to the concentration of Ca^{2+} used for measurements. In the last-mentioned study [63], Ca^{2+} uptake was measured at 25 μM Ca^{2+}, thus at a concentration that in the absence of Mg^{2+} is enough to open the permeability transition pore (PTP). Although the rate of Ca^{2+} transport mediated by MCU is inhibited by Mg^{2+}, the net accumulation of Ca^{2+} in mitochondria was increased because of the Mg^{2+}-mediated prevention of Ca^{2+} leakage from mitochondria via PTP.

Some controversial findings have been reported to be related to the mitochondrial accumulation of Ca^{2+} through IMM via the mitochondrial ryanodine receptor (mRyR). Western blot analysis, immunogold electron microscopy, and the high-affinity binding of [^{3}H]-ryanodine indicate that a low level of mRyR is localised within IMM [64]. Similarly to MCU, mRyR is inhibited by low concentrations of ruthenium red (1–5 μM) and by Mg^{2+} [64]. However, the IMM localisation of RyRs by immunogold labelling has not been confirmed by another group [65]. Results obtained in our laboratory also argue against the significant physiological importance of mitochondrial Ca^{2+} uptake via mRyR, since only energised rat heart mitochondria are able to accumulate substantial amounts of Ca^{2+} and the accumulation is prevented by the submicromolar concentration of ruthenium red [63]. Finally, the group of Sheu [66] has suggested that, upon Ca^{2+} overload in the matrix, mRyR might be responsible for mitochondrial Ca^{2+} efflux, thus preventing the activation of PTP (see below).

Recent study documented that Mg^{2+} does not affect the rapid mode of mitochondrial Ca^{2+} uptake [67] that represents another mechanism of Ca^{2+} transport through the IMM distinct from MCU [68].

The main route of mitochondrial Ca^{2+} release has previously been demonstrated to depend on the Ca^{2+}-induced release of Ca^{2+} from mitochondria (mCICR). The mechanism of mCICR involves the transitory opening of the PTP operating in a low conductance mode. Therefore, Ca^{2+} fluxes from mitochondria are a direct consequence of the mitochondrial depolarisation spike (mDPS) caused by PTP opening [69]. In vitro, both mDPS and mCICR can propagate from one mitochondrion to another, generating travelling depolarisation and Ca^{2+} waves. Mitochondria therefore appear to be excitable organelles capable of

generating and conveying electrical and Ca^{2+} signals. In living cells, mDPS/mCICR is triggered by IP_3-induced Ca^{2+} mobilisation leading to amplification of the Ca^{2+} signals primarily emitted from the ER [69]. As documented in our laboratory, the opening of PTP in the low conductance mode depends significantly on the Mg^{2+} concentration [63]. This is in agreement with the previous study that documented the inhibitory effect of divalent cations including Mg^{2+} on Ca^{2+}-dependent opening of PTP [70].

Two additional antiporters are suggested to play an important role with respect to mitochondrial Ca^{2+} release/efflux [51, 57]. In nonexcitable tissues (liver, kidney), such an antiport, appear to be predominantly an H^+/Ca^{2+} exchanger, whereas in excitable tissues (heart, brain), it appears to be primarily a Na^+/Ca^{2+} exchanger [71, 72]. The molecule responsible for the Na^+/Ca^{2+} exchange was identified in 2010 [73]. A possible molecular candidate for the H^+/Ca^{2+} exchange (Letm1) was reported in 2009 [74], although this proposal is still controversial [75, 76]. As suggested by Takeuchi and coworkers [51], further analysis is necessary to determine whether Letm1 is, indeed, the H^+/Ca^{2+} exchanger mediating Ca^{2+} extrusion from mitochondria. The transport activity of the Na^+/Ca^{2+} exchanger is inhibited by Mg^{2+} at concentration 2.5 mM [77], whereas Mg^{2+} does not inhibit the Ca^{2+} flux mediated by the H^+/Ca^{2+} exchanger Letm1, even at ~300-fold excess [75].

4. Mg^{2+} and Mitochondrial Apoptosis

Mitochondria play an important role in the process of the intrinsic pathway of apoptosis [78, 79]. They are both targets of proteins of the Bcl-2 family that are essential regulators of intrinsic apoptosis pathway initiation [79, 80], and the residence of proteins playing a crucial role in the execution of intrinsic apoptosis (cytochrome c, Smac/Diablo, apoptosis-inducing factor, and endonuclease G) [81]. In some cells, the extrinsic (receptor) pathway of apoptosis is connected to the intrinsic pathway via receptor-initiated cleavage of Bid protein, which is also a member of the Bcl-2 family, and the consequent translocation of truncated Bid (tBid) to the mitochondria [79, 81].

In contrast to the well-established role of Ca^{2+} in apoptosis [82], the role of Mg^{2+} has been largely ignored. Several in vitro studies have suggested the stimulatory role of Mg^{2+} in both the extrinsic and intrinsic pathways of apoptosis. Changes in cytosolic Mg^{2+} concentration have been observed in the glycodeoxycholate-induced apoptosis of hepatocytes [83], during the proanthocyanidin/doxorubicin-induced apoptosis in K562/DOX cells [84] and in the Fas ligand-induced apoptosis of B lymphocytes [85]. The elevation of intracellular Mg^{2+} observed in early phase of apoptosis has been explained by Mg^{2+} being necessary to stimulate the activity of Ca^{2+}/Mg^{2+}-dependent endonucleases, which are the executors of apoptosis. Patel et al. [83] have shown that the incubation of cells in Mg^{2+}-free medium prevents the rise in intracellular Mg^{2+} and reduces nuclear DNA fragmentation. On the contrary, Chien and coworkers [85] have documented that an increase in cytosolic free Mg^{2+} is independent of the extracellular Mg^{2+} concentration and the source of the elevated intracellular Mg^{2+} has been suggested to be in the mitochondria. This suggestion is supported by the discovery of mitochondrial Mg^{2+} efflux and influx transporters [15, 16] and by experiments revealing the efflux of Mg^{2+} from mitochondria with preserved integrity (i. e., high transmembrane potential, no swelling) as the response to the apoptotic compound, gliotoxin [86]. Finally, the upregulation of Mrs2 has been shown to be responsible for the inhibition of the adriamycin-induced apoptosis of a gastric cancer cell line, probably by suppressing Bax-induced cytochrome c release from the mitochondria [87]. On the other hand, recent studies have documented both the elevation of mitochondrial [88] and the decrease of cytoplasmic [89] Mg^{2+} concentrations in some models of the induction of apoptosis.

Previous studies have also documented the impact of Mg^{2+} on cytochrome c release from mitochondria, an event that is followed by apoptosome formation and further progression of mitochondrial apoptosis [79]. Although a promoting effect of Mg^{2+} has been suggested, the impact of Mg^{2+} on cytochrome c release seems to depend on the mechanism of OMM permeability increase. The release of both Bax- [90] and tBid-induced cytochrome c [91] has been shown to be independent of the PTP pore but to be highly stimulated by Mg^{2+}. On the contrary, Noxa-induced cytochrome c release is inhibited by Mg^{2+}; this can be explained by the ability of Mg^{2+} to inhibit PTP [92], since PTP opening can result in the release of a variety of compounds from the mitochondria including that of cytochrome c leading to apoptosis [81].

5. Conclusions

Mitochondrial dysfunction has been implicated in the mechanisms of several serious human pathologies including metabolic [93, 94], cardiovascular [95], and neurodegenerative [96, 97] diseases. As we have discussed above, Mg^{2+} affects mitochondrial functions that have an important impact on cell survival. Recent work on Mrs2 knockdown HeLa cells has unambiguously revealed that the disruption of mitochondrial Mg^{2+} homeostasis has a dramatic impact on a cellular energy status and cell vulnerability [31]. Moreover, mitochondrial extruder SLC41A3 has been shown to be involved in the regulation of the whole-body Mg^{2+} balance [98]. These findings argue for more systematic research in the field of Mg^{2+} and mitochondria. Since mitochondria display significant cell and tissue heterogeneity [49, 99], the impact of mitochondrial Mg^{2+} on cellular physiology can also be anticipated to be cell- and tissue-type-dependent. Experiments on a variety of cell types will be important. In addition, the impact of Mg^{2+} on apoptosis initiation and execution in various cells has to be investigated in more detail. With respect to apoptosis, the cell-type specificity and the cause-consequence relations between apoptosis initiation and changes in the intracellular or mitochondrial concentration of Mg^{2+} are still unclear. Moreover, recent studies strongly point to the importance of ER-mitochondria interactions with respect to mitochondrial functions, Ca^{2+} homeostasis, and dynamics [100, 101]. Since the ER

transport of Mg^{2+} is not as clear yet, the study of the transport of Mg^{2+} through the ER membrane and the possible impact of the luminal Mg^{2+} concentration on ER-mitochondria crosstalk and on mitochondrial Mg^{2+} transport and functions will be crucial. Finally, other processes are localised in the mitochondria, which are also considered as the main site of the intracellular production of reactive oxygen species. The effect of Mg^{2+} on these processes has not been discussed in this review, but some interest should be focused on this direction in the future.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

The Central Role of Biometals Maintains Oxidative Balance in the Context of Metabolic and Neurodegenerative Disorders

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Traditionally, oxidative stress as a biological aspect is defined as an imbalance between the free radical generation and antioxidant capacity of living systems. The intracellular imbalance of ions, disturbance in membrane dynamics, hypoxic conditions, and dysregulation of gene expression are all molecular pathogenic mechanisms closely associated with oxidative stress and underpin systemic changes in the body. These also include aspects such as chronic immune system activation, the impairment of cellular structure renewal, and alterations in the character of the endocrine secretion of diverse tissues. All of these mentioned features are crucial for the correct function of the various tissue types in the body. In the present review, we summarize current knowledge about the common roots of metabolic and neurodegenerative disorders induced by oxidative stress. We discuss these common roots with regard to the way that (1) the respective metal ions are involved in the maintenance of oxidative balance and (2) the metabolic and signaling disturbances of the most important biometals, such as Mg^{2+}, Zn^{2+}, Se^{2+}, Fe^{2+}, or Cu^{2+}, can be considered as the central connection point between the pathogenesis of both types of disorders and oxidative stress.

1. Introduction

1.1. Contemporary Concept of Oxidative Stress. The traditional concept of oxidative stress is based on an imbalance between the production of free radicals, namely, reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the antioxidant capacity of the organism. The normal function and survival of eukaryotic organisms is fully dependent on oxygen and energy metabolism. Differences in the oxygen demands of the various tissues follow their special metabolic requirements. Oxidative damage is elevated in proportion to higher oxygen consumption under diverse pathological metabolic conditions [1]. Damaging effects of this phenomenon can be observed in intracellular metabolism and also in the structural features of cells [2]. Free radicals, the active compounds in oxidative damage, are defined as molecules with unpaired electrons in their outermost orbit. A typical oxidizing substance involved in the production of free radicals in living systems is oxygen [3].

Mitochondria together with several other eukaryotic cellular compartments such as plasma membrane [4, 5], cytosol [6, 7], peroxisomes, lysosomes [8], and endoplasmic reticulum (ER) [9, 10] significantly participate in ROS production and its consequent utilization [11]. In mitochondria during aerobic metabolism, the reduction of excessive electronegative oxygen atoms leads to the formation of reactive intermediates such as superoxide that can easily be converted to various forms of ROS. These include the superoxide anion radical, hydroxyl radicals, and the nonradical hydrogen peroxide. In this process, complex I (NADH-ubiquinone oxidoreductase) and complex III (ubiquinol-cytochrome c oxidoreductase) of the respiratory chain are the two main locations of ROS production [12, 13]. In addition, the ER, because of the activity of cytochrome P450-dependent oxygenases, [9, 10] and cytosolic xanthine oxidase provide another source of ROS [6, 7]. Peroxisomes show a very interesting example of high ROS production. They play a crucial role in many metabolic processes including fatty acid oxidation, metabolism of amino acids, and synthesis of lipid

compounds [14], and most enzymes catalyzing these processes produce ROS during their activity [15]. Several studies indicate that a high proportion of peroxide (20–60%) is generated inside peroxisomes and diffuses through the peroxisomal membrane protein 2 (Pxmp2) channel to the surrounding medium [16]. Moreover, in 1972, Boveris and colleagues showed that, in rat liver, the major proportion of peroxide (about 35%) is generated by peroxisomal oxidases [17].

ROS, especially superoxide anion, can be generated nonenzymatically, when the oxygen directly accepts a single electron by the reduced coenzymes, prosthetic groups (e.g., flavins or iron-sulfur clusters), or previously reduced xenobiotic [13, 18].

Other reactive species, such as RNS, are also produced in eukaryotic organisms by cell metabolism [19]. Nitric oxide (NO) is generated by the cytosolic nitric oxide synthases (NOS), which convert L-arginine into L-citrulline and NO [20, 21]. To date, three different isoforms of NOS have been identified, depending on their cellular localization and cellular function. The activity of neuronal NOS (nNOS) and endothelial NOS (eNOS) is regulated by transient interaction with Ca^{2+}/calmodulin [18, 22]. Inducible NOS (iNOS) is not regulated by Ca^{2+}, but its activity is induced by infection, inflammation, or trauma [18, 22].

Both ROS and RNS play dual roles in cell metabolism. On one hand, at the physiological level, both ROS and RNS play important and beneficial roles in various cellular processes. For example, ROS are involved in growth, apoptosis, and gene transcription, and at the system level, they support complex functions, such as the regulation of blood pressure, cognitive function, or immune response [6, 23]. RNS contribute to the regulation of apoptotic and necrotic cell death [6], and at the systemic level, RNS also contribute to blood vessel modulation [24], proliferation, and relaxation of vascular smooth muscle cells, leukocyte adhesion, angiogenesis, and thrombosis [25]. On the other hand, their overproduction in cells and the resulting accumulation of oxidative damage lead to lipid peroxidation, oxidative modification of structural proteins, protein misfolding and aggregation, and DNA mutation as a result of RNA/DNA oxidation [26] and additionally to chronic diseases such as neurodegeneration, cancer, diabetes, cardiovascular disease, stroke, and chronic inflammation [27, 28].

Thus, the cellular concentration of ROS and RNS clearly determines the alteration between their beneficial and harmful effects. However, the exact concentration of specific ROS and RNS at which this shift in function occurs remains unknown. Several authors have suggested that this phenomenon depends on the particular cell type, cellular compartment, time, source of their production, and, of course, the type of ROS and RNS generated [6, 29].

Tissue defense against oxidative damage is based on the antioxidant capacity of exogenous antioxidant molecules such as ascorbate and vitamin E. In addition, endogenic molecules, such as glutathione (GSH), catalase, and the superoxide dismutases (SOD), provide the main antioxidant capacity of living eukaryotic cells. In general, a tight relationship exists between the activity levels of these enzymes and the concentration of various biometals, usually serving as cofactors of these enzymes. For example, copper (Cu^{2+}) and zinc (Zn^{2+}) ions, in particular, have a great impact on the activity of cytoplasmic SOD, whereas manganese (Mn^{2+}) is a metal essential to the function of the mitochondrial type of this enzyme (mSOD) [30, 31]. Iron ions (Fe^{3+}) are an integral component of catalase [32]. Almost no antioxidant enzymatic action can be managed without a specific ion equilibrium. Aberrations in the plasma concentrations of magnesium (Mg^{2+}), Cu^{2+}, Zn^{2+}, and selenium (Se^{2+}) ions are observed together with oxidative stress markers in clinical studies of metabolic or neurodegenerative disorders [33, 34].

When the antioxidant capacity of these molecular instruments is insufficient to bring the free radicals back to the basic nonreactive state, organic molecules such as DNA, RNA, lipids, and enzymes are the main targets of oxidative events mediated by the ROS [35]. The intracellular machinery, which secures redox balance, is similar to other cellular systems and is able to adapt. Signaling pathways, which regulate the strength of antioxidant capacity, act mainly through the activation of the antioxidant response element (ARE) in the promoter regions of genes by means of NF-E2-related factor 2 (Nrf2). The binding of ROS-sensing basic leucine zipper (bZIP) transcription factor Nrf2 to the promoter ARE region (Nrf2/ARE) results in the upregulation of the expression of a wide range of antioxidant enzymes [36]. In addition to ROS or RNS, prolonged hyperglycemia (increased level of sugar) with proinflammatory advanced glycation end (AGE) products can also lead to the activation of the Nrf2/ARE signaling pathway [37].

Because events involved in the maintenance of the oxidative balance can be observed in most cell types in the body, the defects in these processes are of major importance in the development of systemic changes in the inflammatory response, energy metabolism, membrane dynamics, or tissue regeneration. All these activities are the basis for the pathogenesis of all types of metabolic and neurodegenerative disorder. The main objective of this review is to focus on the common features shared by these two distinctly different disorders in the way that the cellular oxygen balance is preserved. Emphasis is placed on the central physiological role of metal ions relevant to pathogenesis of both types of disorders. The assessment of available information concerning the oxidative background of both metabolic and neurodegenerative disorders might contribute to the identification of oxidative stress as one of the main causes responsible for the metabolic roots of neurodegenerative disorders.

2. Oxidative Stress and Metabolic Disorders

Randomly produced ROS not only have harmful effects but also exhibit a physiological role in the innate immune response after the respiratory burst of immune cells. Several chronic diseases, including metabolic disorders, may be partly caused by the constant activation of the immune system, which might further result in tissue and also systemic oxidative stress. The reduction of oxygen to hydrogen peroxide or peroxynitrite, which takes place in immune cells, for

example, neutrophils during the respiratory burst, is based on the activity of NADPH oxidase (NOX) and iNOS [38, 39]. Higher local expression of ROS-producing NOX is tightly bound to the elevated levels of hypoxia-inducible factor 1 (HIF-1) and factors responsible for angiogenesis (vascular endothelial growth factor (VEGF)). These proteins are the key players in processes improving tissue oxygenation ability during hypoxic or other pathological conditions that lead to an energy deficit attributable to substrate oxidation [40]. Oxidation-sensing factor, HIF-1α, and VEGF are often discussed in the context of obesity, because cellular hypoxia has been observed in the adipose tissue of obese individuals [41]. During hypoxia, ROS production is rapidly elevated. The precise mechanisms are still not known, but oxygen deprivation and its impact on the mitochondrial electron transport chain have to be taken into consideration. ROS and also RNS themselves are similarly responsible for the activation of broad ranges of proinflammatory factors such as nuclear factor-κB (nuclear factor κ-light-chain-enhancer of activated B cells; NF-κB), activator protein-1 (transcription factor; AP-1), cellular tumor antigen p53, and protein C-est-1 (ETS proto-oncogene 1, Ets-1; transcription factor) together with proliferatory and hypoxia sensing factors VEGF and HIF [42]. Moreover, proinflammatory NF-κB itself can stimulate HIF-1α basal expression by binding to the HIF gene promotor region [43, 44], possibly serving as an explanation of this inflammation and hypoxia-sensing switching point. As is summarized in the scheme in Figure 1, the upregulation of HIF-1α in hypoxic adipose tissue under obesity conditions is, however, positively correlated with the exacerbation of insulin resistance and glucose intolerance [45]. This negative effect of HIF-1α on glucose tolerance is mediated by the attenuation of adipogenic factors such as peroxisome proliferator-activated receptor γ (PPARγ), glucose transporter type 4 (GLUT4), and pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1) and is associated with the metabolic deprivation of adipocytes together with fatty acid accumulation [46]. The disruption of HIF-1α in adipocytes of a transgenic mouse model has been shown to improve the metabolic function of these adipocytes and to ameliorate insulin resistance [47].

All of this evidence strengthens ideas regarding the cooperative effects of hypoxia and inflammation in the pathophysiology of metabolic disorders. However, a rough collection of data obtained in recent years indicates that proinflammatory cytokines secreted by immune-competent cells and adipocytes might in turn trigger the development of insulin resistance. On the other hand, anti-inflammatory medication may reverse this process [37]. The infiltration of macrophages into adipose tissue is tightly bound with an excessive accumulation of fat in obesity and with the secretion of proinflammatory cytokines, such as plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor α (TNF-α), and interleukin6 (IL-6). Proinflammatory cytokines can also originate in white adipocytes, which have self-endocrine potential. In white adipose tissue, such an enhanced secretory capacity for proinflammatory cytokines creates an even stronger background for the development of low-grade inflammation and related oxidative stress [48].

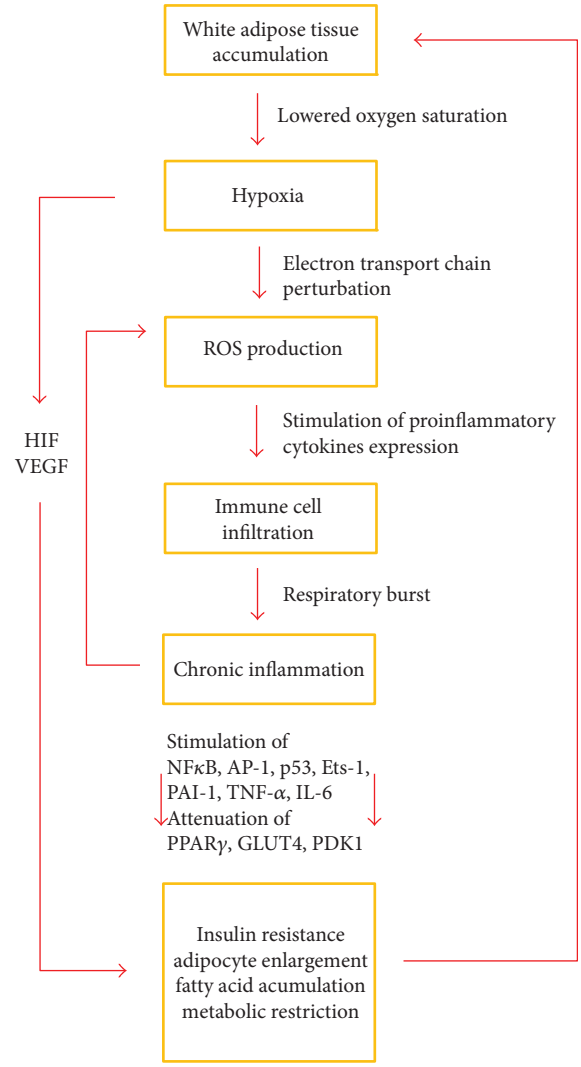

Figure 1: The progression of pathological changes in adipose tissue after initial fat accumulation with a focus on participation of hypoxic condition. Lowered oxygen saturation in enlarged adipose tissue leads to hypoxic conditions. Mitochondrial dysfunction in hypoxic tissue causes alterations in the electron transport chain and thus an increase in generated ROS, which are critical for the further activation of immune cells and the development of chronic inflammation. The activation of relevant genes leads to the pathogenesis of metabolic disorders and creates the vicious cycles further empowering the pathological changes. The interconnections between the pathological processes leading to the final metabolic disorders are marked in boxes. Red arrows indicate the flow of changes and the vicious cycles between each pathological stage, together with the causative effect and the ablation of gene expression between specific grades of pathogenesis.

Adipokines, which are endocrine-active substances originating from white adipocytes, are critical factors contributing to the regulation of free radical formation. Leptin, an adipokine, whose secretion capacity is dependent on total adipose tissue mass, is well known for its restrictive effect on food intake. Because of these anorexic effects, leptin has been considered as a potential therapeutic agent for obesity. Contrary to its beneficial effects, a higher leptin concentration

in blood (hyperleptinemia) is well known to elevate the level of oxidative stress by the stimulation of mitochondrial and peroxisomal oxidation of fatty acids. Stimulation of fatty acid utilization can be understood as beneficial in obesity, but the pro-oxidative stimulation of mitochondrial and peroxisomal metabolism is a critical factor in ROS generation. An increase in mitochondrial metabolism during hyperleptinemia is also observed in immune cells. This effect has been suggested to be related to the proliferation and activation of monocytes infiltrating the adipose tissue [49]. Leptin, by promoting pro-oxidative events, increases the phagocytic activity of macrophages and also induces the synthesis of proinflammatory substances such as IL-6 and C-reactive protein (CRP) [50]. In addition, adiponectin, another adipokine secreted by adipocytes, shifts macrophages towards the anti-inflammatory phenotype. However, proinflammatory cytokines such as TNF-α and IL-6 inhibit its synthesis [51]. This suggests that, in contrast to leptin, adiponectin acts as an anti-inflammatory agent. Study of the 3T3-L1 adipose cell line has shown that one of the stimulatory effects of adiponectin secretion is mediated by both insulin and amino acids [52]. Another study of animal models has proposed that adiponectin allows insulin action by its stimulatory effects on glucose uptake through the activation of the AMP-activated protein kinase (AMPK) [53]. In agreement with these observations, low adiponectin levels in plasma are associated with insulin resistance, as has been seen in obese patients [54]. On the other hand, insulin resistance is positively correlated with the blood level of a third type of adipokine called resistin, which is similar to leptin being associated with the stimulation of the secretion of proinflammatory molecules [55, 56]. The physiological effects of resistin have been suggested to be associated with glucose storage, as it has been observed to be elevated during long-term physical exercise [57]. Moreover, the ablation of the resistin gene in mice stimulates the regulation of gluconeogenic enzymes [58]. Low-grade inflammation of excessive white adipose tissue mass may therefore result in the chronic pathologic upregulation of resistin plasma levels, accompanied with the development of glucose intolerance. In agreement with these data, insulin resistance and hyperglycemia in a rat metabolic toxicity model treated with hyperglycemia-causing agent hydrochlorothiazide (HCTZ) and a high-fat diet have been associated with higher levels of malondialdehyde (oxidative stress marker, ROS-induced metabolite of polyunsaturated lipids) [59].

Insulin resistance, as the main pathogenic factor of type II diabetes mellitus (T2DM), has a dramatic impact on energy substrate distribution, accompanied by the modification of mitochondrial function. A study by Anderson and colleagues has clearly shown that the mitochondria of obese and insulin-resistant rodents and humans produce elevated levels of ROS when compared with those of their lean counterparts [60]. A higher intake of energy substrates by obese individuals enhances the proton gradient of the inner mitochondrial membrane with a higher probability of electron leakage from the terminal respiratory chain. This uncontrolled disruption of electron potential is usually associated with ROS generation [61]. In cases of developed insulin resistance, a lower glucose intake, and the starving of the cells, ROS has a negative effect on the ATP concentration and thus leads to the upregulation of the burning of fatty acids by beta-oxidation. The ATP production by β-oxidation is associated with the same effect on transmembrane potential magnification. Whether the enhanced generation of ROS is the cause of insulin sensitivity impairment or vice versa, the effect of higher nutrient intake is still not fully understood [62]. The effectiveness of antioxidant therapy in obesity and T2DM is the subject of intensive discussion [62, 63].

Altered mitochondrial function, which is behind the onset of insulin resistance and the onset of metabolic syndrome, might be based on mitochondrial dysfunction related to mitochondrial cytopathies [64]. The term "mitochondrial dysfunction" can be considered as general pathophysiological alterations that result in diminished antioxidant defense through ROS production, reduced oxidative phosphorylation, and decreased ATP production [65]. A decreased level of the elimination of damaged mitochondria may be the result of alterations in mitochondrial fission and fusion processes and the inhibition of mitophagy [66]. On the contrary, the stimulation of mitochondrial biogenesis improves metabolic status and is considered to be protective against the development of T2DM. Genes, such as PPARγ, peroxisome proliferator-activated receptor γ coactivator 1 (transcriptional cofactor; PGC1-α), nuclear respiratory factors 1 and 2 (NRF1 and 2), and mitochondrial uncoupling protein 1 (UCP1), which are involved in the regulation of mitochondrial biogenesis, are upregulated not only by the stimuli of physical activity and myogenesis but also by dietary restriction and low temperature [67]. Quantitative real-time polymerase chain reaction (PCR) carried out in the 3T3-L1 adipose cell line has revealed that the supplementation of growth medium by balanced deep-sea water with higher concentrations of various ions such as Ca^{2+} and Mg^{2+} leads to an increase in the expression of genes involved in mitochondrial biogenesis in preadipocytes, such as PGC1-α, NRF1, and mitochondrial transcription factor A (TFAM) [68].

3. Biometals in Metabolic Disorders

The precise role of biometals in metabolic modulation has not been fully uncovered, but the available data support the hypothesis of a strong relationship between these trace elements and essential hypertension, endothelial dysfunction, insulin resistance, oxidative stress, and the atheroinflammatory state. The pathological activation of the immune system might be a consequence of disturbed ion homeostasis. The activation of immune cells leads to an intracellular increase of their Ca^{2+} concentration as a potential consequence of ion misbalance, especially that of biometals [69]. As summarized in Table 1, numerous clinical studies emphasize the increase/depletion or disturbances of biometal ratios in the pathophysiology of metabolic disorders [33, 70, 71]. Bioactive metals such as Mg^{2+}, Zn^{2+}, Se^{2+}, Cu^{2+}, and Mn^{2+} are collectively considered as antioxidant trace elements [70, 72]. They act as cofactors for antioxidant metalloenzymes [72, 73]: Cu^{2+} and Zn^{2+} have been identified as cofactors of

Table 1: Association of changes in reviewed biometal levels with the development of metabolism defects.

Biometal	Type of change +/−	Observed effects	Citation
Mg	+	↓ intracellular Ca^{2+}	[82, 83, 94]
		↑ oxidative glucose breakdown by stimulation of PDH activity	[82, 83]
	−	Positive correlation with obesity	[74, 75]
		↑ oxidative stress markers	[76, 77]
		Hyperglycemia, insulin resistance	[59]
		↑ proinflammatory cytokines	[92, 93]
		↓ function of ATP-dependent ion pumps	[88, 191, 192, 194]
Zn	+	↓ glycemia	[103]
	−	↑ immune system reactivity	[99, 100]
		↓ lowered antioxidant capacity via downregulation of Nrf2	[36]
Cu/Mn	+	↑increased risk of T2DM poor glycemic control	[33, 102]
Se	−	Found in prediabetic patients together with lowered Zn^{2+} and Mg^{2+}	[33]

PDH: pyruvate dehydrogenase; Nrf2: NF-E2-related factor 2.

cytoplasmic superoxide dismutase (Cu-Zn-SOD) [31] and Mn^{2+} as a cofactor of mitochondrial SOD (Mn-SOD) [30].

3.1. Magnesium. In general, Mg^{2+} has one of the most important roles in the regulation of metabolism. A negative correlation of insulin resistance and hyperglycemia with Mg^{2+} plasma concentration has been observed in rats after their administration with the hyperglycemia-causing agent HCTZ [59]. Obese subjects frequently exhibit metabolic disorders together with elevated inflammatory markers, such as the C-reactive protein (CRP) and alanine transferase (ALT), markers associated with an increased risk of cardiovascular disease and liver damage. Several studies have described the depletion of magnesium concentrations in the blood of obese individuals [74, 75], and this effect seems to be correlated with a greater degree of oxidative stress [76]. Decreased Mg^{2+} levels, together with an increased concentration of malondialdehyde (an oxidative stress marker), have been found in blood samples, in particular, in younger insulin-resistant patients in comparison with healthy controls [77]. Moreover, the status of glucose and triglyceride metabolism has been significantly improved in magnesium-supplemented pregnant women suffering from gestational diabetes mellitus [78]. Oral supplementation by magnesium chloride to obese women reduces plasma ALT levels, together with a tendency towards a reduction in CRP levels [79]. Furthermore, magnesium as a cofactor plays a role in glutathione (GSH) production by gamma-glutamyl transpeptidase [80]. Therapeutical treatment by magnesium sulphate in another study has been shown to lead to the increased activity of superoxide dismutase and catalase [81].

As a cofactor of pyruvate dehydrogenase phosphatase (PDP), Mg^{2+} also facilitates the dephosphorylation of pyruvate dehydrogenase (PDH) and, thus, its activation. PDH is the rate-limiting enzyme guiding the intermediate metabolites from anaerobic glucose breakdown to oxidative metabolic pathways [82]. Because of these specific roles, Mg^{2+} can speculatively be considered as an ion of the aerobic/anaerobic switch of glucose degradation. Furthermore, according to observations of Kelley and colleagues, PDH activity is below the physiological level in the skeletal muscle of patients with T2DM [83]. From another point of view, diabetic patients are well known to have problems with the maintenance of ion homeostasis, because of the high prevalence of nephropathy within these patients. Hypothetically, this mechanism might be a link between a lower Mg^{2+} concentration and a lower retention capacity of DM kidneys, particularly in the case of nephropathic comorbidity [84]. Under healthy conditions, insulin activates the reuptake of Mg^{2+} by the activation of transient receptor potential melastatin type 6 (TRPM6) channel [85]. However, in the absence of insulin signaling, such as during insulin resistance, Mg^{2+} reuptake may be also impaired. Even more interesting is the observation that oxidative stress reduces TRPM6 activity [86].

The inhibition of the erythrocyte ions of the Na^+/K^+ and Ca^{2+} ATPases in hibernated black bears is further evidence of ion channel regulation by oxidative stress or hypomagnesemia [87]. During hibernation, these bears suffer from a higher degree of oxidative stress, since a higher level of oxidative stress marker malondialdehyde has been detected in their blood. Actually, the activity of both these ion pumps is regulated by Mg^{2+}-dependent phosphorylation. In general, the presence of Mg^{2+} or of some other divalent cations (Mn^{2+}, Co^{2+}, and Fe^{2+}) is essential for ATP hydrolysis at significant rates and is crucial for the action of all ATPases, including the Ca^{2+} pump. An explanation for this effect of Mg^{2+} is its participation in the formation of a complex with ATP, thereby facilitating the hydrolysis of ATP to fuel the Ca^{2+} pump (Figure 2). By this mechanism, Mg^{2+} represents the natural antagonist of calcium effects in living systems, by mediating the excretion of this ion through the cytoplasmic membrane [54, 88].

The concentration of Ca^{2+} should be tightly regulated under all conditions, mainly in the regulation of the immune response. A strong association of the hyperactivity of immune cells, Ca^{2+} concentration, and Mg^{2+} deficiency can be found in the literature [89, 90]. Rats fed on an Mg-deficient diet for eight days show a significant increase in intracellular Ca^{2+} concentrations after the administration of platelet-activating factor, compared with controls [91]. According to another group of authors, a short-term deficiency of

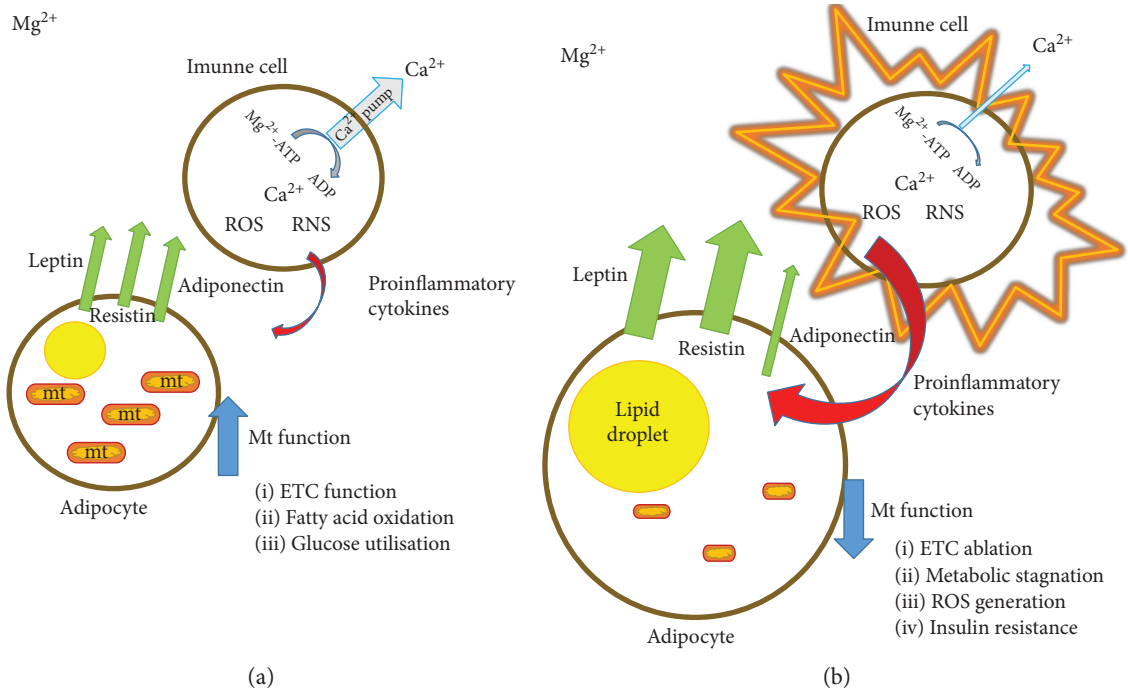

Figure 2: Schematic illustration demonstrating the relationship between adipocytes and immune cells in white adipose tissue with an accent on magnesium regulatory functions. (a) Normal magnesium levels preserve the standard physiological activity of the ATP-dependent Ca^{2+} pump. A low intracellular concentration of calcium keeps immune cells in an inactive state. (b) A decrease in the magnesium concentration reduces the excretion rate of calcium ions from cells via the Mg^{2+}-dependent Ca^{2+} pump. Activated immune cells in adipose tissue secrete proinflammatory cytokines with inhibitory effects on adipocyte metabolism. Genes responsible for mitochondrial biogenesis (PPARγ, PGC1-α, NRF1–2, and UCP) are attenuated under proinflammatory conditions, leading to the attenuation of mitochondrial functions. Higher expression of leptin is connected to an increase in fat tissue mass caused by metabolic stagnation. Upregulation of resistin is complementary to the stimulated expression of proinflammatory cytokines. Adiponectin secretion is attenuated by proinflammatory cytokines resulting in the aggravation of glucose tolerance and the development of insulin resistance.

Mg^{2+} (21 days) in rats leads to an increase of a broad variety of cytokines such as IL-1α, IL-1β, IL-2, IL-6, and TNF-α [92]. Furthermore, a study performed by Bussiere and colleagues has demonstrated the upregulation of a wide variety of genes associated with the immune response of neutrophils in Mg-deficient rats compared with that in control rats. The authors have also identified genes involved in apoptosis, coding heat shock proteins, cytoskeletal proteins, and proteins implicated as stress response regulators and effectors and enzymes implicated in thromboxane synthesis. These genes have been named by the authors as a genes implicated in the immunoinflammatory process of Mg^{2+} deficiency [93]. On the contrary, studies focused on a higher magnesium concentration as a result of its addition to growth media have identified several significant effects of the elevated concentration of this biometal in immune cells. In isolated human leukocytes, magnesium-supplemented growth media lead to a decrease in the intracellular Ca^{2+} concentration and, furthermore, to a significant decrease in immune response activation by chemotactic factor FMLP (N-formylmethionyl-leucyl-phenylalanine) [94]. In addition, leukocyte activity reduction, in response to magnesium supplementation in growth medium followed by Ca^{2+} reduction in cell models, can be explained by the regulatory mechanism of Mg^{2+} on Ca^{2+} ATPase [95].

3.2. Zinc and Other Biometals in Metabolic Disorders. Metabolic disorders, such as essential hypertension and T2DM, are also associated with disturbances of other metal ion concentrations. Zinc, among the other biometals, is the second most prevalent trace element in the human body. In contrast to iron, zinc is a redox-inactive biometal and serves as an important component of more than 2700 enzymes including hydrolases, transferases, oxidoreductases, ligases, isomerases, and lysates [96]. This large number of enzymatic activities modulated by zinc explains the requirement for zinc in DNA, RNA, protein, and lipid synthesis, possibly explaining its major role in the preservation of the stability of the genome, proteome, and other biomolecules [97]. This action includes, but is not limited to, the antioxidant effects of zinc and its participation in DNA repair, the DNA damage response, and the synthesis of molecules (e.g., methionine) necessary for DNA methylation. Furthermore, it contributes to the maintenance of the redox equilibrium through various mechanisms. For example, zinc is an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a cofactor of SOD, and induces the generation of metallothionein, a scavenger of oxidants [98].

Moreover, zinc is crucial for the normal development and function of cell-mediated immunity associated with T cells. The deficiency of this biometal also negatively influences

the secretion of interleukin 1beta (IL-1β) by macrophages [99]. The altered production of cytokines during zinc deficiency can lead to inflammation as evidenced by the induction of IL-1β secretion in zinc-depleted macrophages [100]. In rats, a zinc-depleted diet for 4 weeks causes a significant increase in Mg^{2+} and Fe^{2+} concentration in serum. In particular, the elevation of iron ions in the extracellular space is associated with oxidative stress induction [101]. According to Gouaref and colleagues, serum concentrations of Zn^{2+} are significantly decreased in patients suffering from essential hypertension and T2DM. Several other types of metabolic disorders are, unlike the lowering of zinc, accompanied by higher levels of Cu^{2+} [33]. Both Zn^{2+} deficiency and an excess of Cu^{2+} have been associated with an increased risk of T2DM and cardiovascular diseases. In addition, in hypertensive patients, but not in T2DM patients, the Zn^{2+}/Cu^{2+} ratio is significantly decreased. This suggests that the Zn^{2+}/Cu^{2+} ratio reflects the transition from the hypertension phase to T2DM-associated hypertension [33]. In agreement with these observations, a higher Zn^{2+}/Cu^{2+} ratio has been associated with a reduced risk of poor glycemic control in T2DM patients [102]. Oral administration of zinc together with acetylsalicylic acid to T2DM rats reduces plasma glucose levels and prevents diabetic cardiomyopathy [103]. Positive effects of zinc supplementation have been also investigated in a study of obese mice fed on a high-fat diet (HFD) [104]. HFD significantly decreases the expression of transcription factor Nrf2 in the *aorta tunica media* in a time-dependent manner. Zinc deficiency aggravates the downregulation of this transcription factor, which is associated with the stimulation of antioxidant genes through the ARE in the promoter regions of the relevant genes, as mentioned above [36]. Zn^{2+} supplementation prevents the decrease in aortic *Nrf2* expression induced by HFD [104].

Several authors have also investigated the association of the homeostasis of other ions with metabolic disorders. Yadav and colleagues have observed, in addition to reduced levels of Zn^{2+} and Mg^{2+}, a reduction in Se^{2+} serum levels in insulin-resistant individuals compared with those in healthy controls [105]. Serum concentrations of Se^{2+} together with Zn^{2+} are, according to Gouaref and colleagues, significantly decreased in patients suffering from essential hypertension and T2DM. On the contrary, several types of metabolic disorders are accompanied with higher levels of Cu^{2+} and Mn^{2+} [33].

4. The Role of Oxidative Stress in Neurodegenerative Disorders

The higher susceptibility of the brain to oxidative stress arises from its extraordinary utilization of oxygen. Despite the brain sharing only 2% of body mass, it consumes approximately 20% of the total oxygen production [106]. In particular, neurons and astrocytes, which are the two major cell types in the brain and whose function is fully dependent on oxygen and glucose, consume approximately 10-fold more oxygen compared with other cells [107]. Moreover, neurons are nondividing cells with a long life duration; therefore, they are heavily exposed to the accumulation of oxidative stress. In addition, redox-active metals, which play an active role in ROS production, are abundant in the brain [108]. Despite this fact, neurons do not possess an extra antioxidant capacity or special antioxidant systems.

In mammalian cells, RNS, physiological messenger molecules, are normally produced at very low levels. In neurons, NO and RNS are generated by Ca^{2+}-activated nNOS and neuroinflammatory stimuli-activated iNOS [109, 110]. nNOS activity requires the triggering of N-methyl D-aspartate-type glutamate receptors (NMDAR), which promote Ca^{2+} influx into the cells (Figure 3). Furthermore, activated NMDAR also leads to the generation of ROS [111]. In terms of neurodegenerative diseases, amyloid β (Aβ) oligomers or 1-methyl-4-phanel-1,2,3,6-tetrahydropyridine (MPTP) leads to an increased NO production and neurotoxicity via the stimulation of iNOS expression (Figure 3). Moreover, iNOS knockdown or knockout protects cells against the MPTP-induced neurotoxicity in animal models [112].

The selective vulnerability of certain neuronal populations, which are affected in a progressive and irreversible manner, is a common feature of neurodegenerative diseases. These neuronal populations are usually the first that show functional degeneration and cell death during aging and, even more prominently, during neurodegenerative diseases [113]. Several decades ago, observations that decreased levels of GSH and increased levels of lipid peroxidation and protein oxidation are commonly present in the brain tissues of patients with Alzheimer's disease (AD) or Parkinson's disease (PD) patients suggested that ROS/RNS accumulation is involved as a major pathogenic process in age-related and neurodegenerative disorders [19, 28, 114–116]. Despite oxidative stress, which is a common pathological mechanism, the vulnerability of diverse neuronal populations to oxidative damage varies in the different neurodegenerative diseases and within the neuronal population in a certain brain region. For example, the *entorhinal cortex* and the hippocampus CA1 region are the most affected brain regions in AD patients, whereas dopaminergic neurons in the *substantia nigra* represent the most vulnerable neurons in PD brains. Interestingly, dopaminergic neurons in PD brains are affected, whereas the ventral tegmental area (VTA) neurons are not [7]. This phenomenon is most probably attributable to the different degrees of oxidative stress present in the different neuronal populations and to the different expression profiles of the antioxidant systems [7]. For example, hippocampal CA1 neurons, compared with CA3 neurons, express a higher level of ROS-producing genes and, thus, are exposed to a higher level of oxidative stress. In dopaminergic neurons, ROS are generated, on one hand, as a result of dopamine metabolism by monoamine oxidase (MAO) (Figure 3) but, on the other hand, as a result of dopamine auto-oxidation [7, 113].

Taking this into consideration, we have to postulate the existence of additional molecular factors that regulate the selective loss of certain neuronal populations. The "multiple-hit hypothesis", one of the suppositions for the development and progression of neurodegenerative disease, involves the interplay between molecular pathways in a sequential order [117–121]. This means that the neurodegenerative process

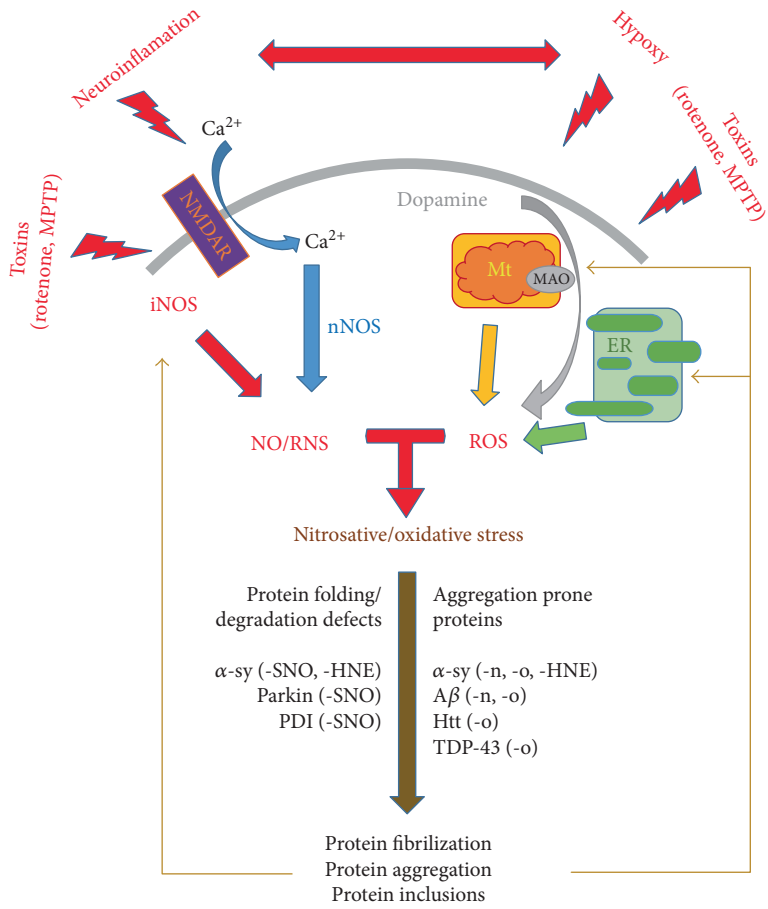

Figure 3: The causative mechanisms of the pathological elevation of nitrosative/oxidative species and their abilities to modify relevant proteins associated with the pathogenesis of neurodegenerative diseases. The activation of iNOS by inflammatory processes and of nNOS by Ca^{2+} influx through NMDAR leads to an increase in intracellular levels of RNS. On the other hand, ROS is generated upon mitochondrial dysfunction, and endoplasmic reticulum (ER) stress is a result of dopamine metabolism by monoamine oxidase (MAO). Hypoxic conditions and toxic compounds, such as rotenone and MPTP, are also considered as carriers of harmful effects to the physiology of the mentioned organelles. However, rotenone and MPTP also exert stimulatory effects on iNOS. Damaged and aggregated proteins create a solid base on which to create a vicious cycle by the strengthening of iNOS activity and by the deepening of mitochondrial and ER dysfunctioning.

is a result of the combined toxic stress from dopamine oxidation or mitochondrial function impairment, together with the failure of neuroprotective mechanisms, including the loss of function of parkin, the failure of antioxidant pathways, or stress-induced autophagic degradation [120]. In the context of energy metabolism, one more aspect should be adequately considered. Oxygen or glucose insufficiency in sensitive brain regions is associated with the overexpression and lowered clearance of Aβ in AD [122, 123]. Hypoxic tissues with reduced mitochondrial function are far more vulnerable to ROS-induced oxidative damage [124].

4.1. Involvement of ROS/RNS in Protein Folding and Aggregation as a Hallmark of Neurodegenerative Diseases. Similarities in pathological mechanisms underlying neurodegenerative diseases result from aberrant protein folding, the consequent aggregation of disease-specific proteins in cells, and the presence of ubiquitinated inclusion bodies. The relationship between protein misfolding and aggregation and excessive ROS/RNS production is well documented, although the exact mechanism of this pathological process is not fully uncovered. For example, ubiquitin E3 ligase (parkin, PARK2) and ubiquitin C-terminal hydrolase 1 (UCH-L1, PARK5) are critical for protein degradation via the ubiquitin-proteasome system (UPS). Mutations in parkin and UCH-L1, which often lead to their functional impairment and, thus, to the impairment of UPS, are both linked to PD [125–127]. Protein levels of UCH-L1 have been found to be downregulated in idiopathic PD and AD brains [128]. Significantly elevated levels of S-nitrosylated parkin (SNO-parkin) have been observed in the postmortem analysis of sporadic PD brains and in PD animal models [129, 130]. Furthermore, the overproduction of NO, for example because of MPTP or rotenone exposure (Figure 3), results in S-nitrosylation and the further oxidation of these proteins [128, 129, 131]. Upon S-nitrosylation, the E3 ligase activity of parkin is transiently increased, followed by its inhibition. The initial increase in E3 ligase activity enhances the ubiquitination of target proteins, the phenomenon observed in Lewy bodies (LB). The consequent inhibition of parkin activity impairs its ubiquitination

activity and thus impairs UPS and protein degradation [110]. In this context, parkin in concert with PINK1 kinase (PARK6, another familiar PD-related gene) plays a role in mitochondria quality control and the subsequent removal of damaged mitochondria. Upon activation, PINK1 phosphorylates and activates parkin resulting in the ubiquitination of proteins of the outer mitochondrial membrane and consequently promotes mitophagy. Similar to the effect of SNO-parkin on UPS, the initial increase in parkin activity promotes mitophagy, whereas further exposure to NO induces the attenuation of mitophagy [132]. Additionally, UCH-L1 undergoes oxidative modification by 4-hydroxy-2-nonenal (HNE) leading to its abnormal function. An abnormal interaction with components of chaperone-mediated autophagy-dependent degradation (Lamp2a, Hsc70, and Hsp90) results in accumulation of chaperone-mediated autophagy substrates, such as α-synuclein (α-syn, PARK1, and PD-related gene), in cells [133].

Protein disulphide isomerase (PDI), a cellular defense protein, with chaperone and isomerase activity, plays a role in protein-folding quality control. PDI is upregulated as a response to ER stress induced by misfolded and aggregated proteins [9, 134]. This chaperone and protective effect of PDI is attenuated upon S-nitrosylation (SNO-PDI). Consistent with this information, increased levels of SNO-PDI have been detected in the brains of patients with PD, AD, and amyotrophic lateral sclerosis (ALS) [135, 136] and in response to iNOS activation in animal models of ALS [137] or in response to mitochondrial toxins, such as rotenone and MPTP, in cellular models [110, 135]. This indicates that, during neurodegenerative processes, proteins related to protein degradation, protein folding, and folding quality control undergo aberrant oxidative or nitrosative modifications, which result in the attenuation of the physiological function of these proteins.

Oxidative/nitrosative modification strongly impacts the structural properties of proteins directly linked with certain neurological disease; this occurs because of the ability of these modified proteins to form fibrillar units and formation of ubiquitin-positive inclusions in cells. For example, Aβ undergoes oxidative and nitrosative modifications that have been demonstrated to induce the formation of Aβ protofibrils and fibrils from monomeric Aβ. Nitrated and oxidized forms of Aβ have also been found in AD senile plaques. These modifications of Aβ have been suggested initially to stabilize the formed Aβ dimers and thus to induce plaque formation [138–140]. Moreover, the modifications of microtubule-associated protein tau (a protein linked with AD pathology) by HNE facilitate hyperphosphorylation and the consequent aggregation of tau and the major conformational changes of this protein, leading to neurofibrillary tangle formation [141–143]. In in vitro models, oxidized fatty acids also have a strong impact on tau polymerization. Transgenic animal models deficient in SOD2 or folate (folic acid, antioxidant) develop oxidative stress followed by tau phosphorylation and aggregation and the formation of amyloid Aβ plagues [144].

The misfolding and aggregation of α-syn represent the basic mechanism of dopaminergic neuronal loss associated with PD. Oxidative and nitrosative posttranslational modifications, including oxidation (o-α-syn), nitration (n-α-syn), and HNE modification (HNE-α-syn), facilitate the generation of protofibrillar structures and the further oligomerization of α-syn, with the highest impact of HNE-α-syn [145]. In in vitro studies of dopaminergic Lund human mesencephalic (LUHMES) neurons, HNE modification enhances α-syn interactions with membranes. HNE-α-syn exposure of differentiated LUHMES neuronal cells initiates intracellular ROS production followed by neuronal death. This can be effectively prevented by treatment with antioxidants [145].

As for previously discussed proteins, huntingtin (Htt; protein related to Huntington's disease (HD)) and TAR DNA-binding protein (TDP-43; protein related to amyotrophic lateral sclerosis (ALS)) undergo oxidative modifications with a similar effect on their conformational changes and protein aggregation [18].

5. Biometals in Neurodegenerative Disorders

The impaired cellular homeostasis of metal ions might initiate neurodegeneration through various mechanisms that have complementary roles in the pathogenesis of the different types of neural degeneration. These pathomechanisms include well-established oxidative stress, which is tightly bound to the incorrect generation of metalloproteins, the activation of microglial cells, and inflammation [146].

5.1. Iron. Iron is an important cofactor of many proteins, with a high redox flexibility, and plays a critical role in processes such as respiration, oxygen transport, nitrogen fixation, DNA synthesis and repair, and neurotransmitter synthesis [147–150]. Redox-active iron is directly linked with an increase in the generation of oxidative stress, together with inhibition of GSH activity with changes in the intracellular reduction potential attributable to GSH oxidation [150, 151]. Chelated reduced forms of iron do not participate in oxidative stress events and have been shown to prevent the degeneration of dopaminergic neurons in transgenic animal models [152]. With respect to neurodegenerative disease, the postmortem analysis of PD brains has revealed, in addition to α-syn and ubiquitin deposits, an increased concentration of iron [150]. Diverse iron distributions within the brain regions have been observed throughout the progression of PD. An explanation of this phenomenon can be found similarly in the differential expressions of iron trafficking and storage factors ferroportin and ferritin in the affected brain parts [151, 153]. Recent in vitro studies have shown that mutant α-syn interacts with metals and that iron (Fe^{2+}, Fe^{3+}) and copper (Cu^{2+}) seem to aggravate the formation of thick α-syn fibrils and induce neuronal toxicity [154, 155]. This has been further confirmed in transgenic animal models and by the in vitro cellular overexpression of human α-syn, with a significant increase in the iron concentration and redistribution of iron from the cytoplasm to the perinuclear region within α-syn-rich inclusions [150]. On the other hand, aggregated α-syn provokes the metal ions (Fe^{2+}, Mn^{2+}) that mediate oxidative stress, thus closing the harmful circle between α-syn aggregation and the generation of

Table 2: Role of reviewed biometals in neurodegenerative diseases.

Biometal	Type of change	Effect	Citation
Fe	Oxidized form (Fe^{3+})	(i) Promotes Aβ and α-syn aggregation (ii) Detected in AD and PD brains	[150, 158, 159, 195]
		↑ intracellular ROS generation	[145, 150, 156–158]
		↑ GSH oxidation	[150, 151]
Cu	Free/unbound	(i) Promotes aggregation of α-syn ↑ oxidative stress	[160, 162, 163]
	Decrease	↑ of Fe levels	[160]
		(i) Detected in substantia nigra of PD brains	[161]
Zn	Free/unbound	(i) Promotes aggregation of Aβ	[162]
	ATP13A2 deficiency	↑ intracellular free Zn^{2+} ↑ ROS production	[161, 167, 168]
Mn	Decrease	(i) LRRK2 G2019S (NOT wt) stays active under ↑ Mn → biological sensor of Mn levels	[171, 178, 179]
	ATP13A2 deficiency	↑ intracellular Mn^{2+} ↑ α-syn-induced toxicity	[167, 180, 181, 196]
	Increase	(i) Indirect hyperphosphorylation of tau (ii) Tau-mediated neuronal death	[183]
Mg	Decrease	(i) High risk factor of PD development (ii) Regulation of Mg homeostasis (iii) Protection against protein aggregation	[184–187]
		(iv) Dopamine generation defects	[184]
	Stabilization/slightly increase	(i) Protection against risk of PD development in animal models and humans	[77, 189]

LRRK2 G2019S: mutation type of leucine-rich repeat kinase 2; ATP13A2: probable cation-transporting ATPase 13A2; GSH: glutathione.

oxidative stress [145, 150, 156, 157]. The chelated form of iron has also been identified in AD-specific Aβ plaques [83]. In the *Drosophila* model, iron chelated by Aβ plaques has been recognized as a primary location of intracellular free radical generation (Table 2), mainly by the Fenton reaction [158]. Rival and colleagues have even provided evidence that the expression of ferritin subunits and treatment with iron chelators are able to relieve Aβ-induced toxicity in the *Drosophila* model [159].

5.2. Copper and Zinc. Copper, as a metalloenzyme cofactor, is essential for normal brain development and function. Disregulation of its homeostasis has been implicated in PD, AD, HD, and ALS. In this context, free unbound copper is involved in oxidative stress and α-syn oligomerization and aggregation [160]. Copper ions display a decrease in their total concentration as reported in the *substantia nigra* in the majority of studies of PD patients [161]. In addition, copper regulates the iron levels in brain by ferroxidase ceruloplasmin activity. In PD patients, elevated levels of iron are accompanied by decreased levels of copper and ceruloplasmin in the brain; on the contrary, elevated levels of free copper result in decreased ferroxidase activity in the cerebrospinal fluid [160]. Copper is able to bind to α-syn and promotes its fibrillation and aggregation [162, 163]. In addition, the α-syn-copper complex alters the redox properties of copper and, thus, induces oxidative stress and even oxidizes several endogenous antioxidants, such as GSH [164]. DJ-1 (PARK7), the PD-linked redox-sensitive chaperone and oxidative stress sensor, inhibits α-syn aggregation and consequently neuronal cell loss [165]. This protein is also prone to bind copper and has a protective effect against copper-induced oxidative stress in cellular models [160]. This has further been supported by the evidence that mice lacking DJ-1 are more sensitive to MTPT exposure [166].

As has been well described, a higher intracellular concentration of ROS is also connected with the release of other biometals from their binding to metalloenzymes (Table 2). This is, in particular, valid for the Zn^{2+} cation whose elevated levels have been found mainly not only in the *substantia nigra* but also in other tissues of PD patients [161]. Recognizing the significant role of zinc, Ramirez and colleagues have demonstrated the higher vulnerability of human-originating neural cells to oxidative stress in cases of ATP13A2 (PARK9) deficiency. ATP13A2 (PARK9) belongs to the lysosomal type 5 P-type ATPase family [167]. Its cationic selectivity is still not determined, and the regulation of the homeostasis of several biometals, including Zn^{2+} and Mn^{2+}, seems to be tightly bound to this ATPase. Furthermore, the chelation of Zn^{2+} ions by a specific Zn^{2+} chelator and the reintroduction of ATP13A2 into the deficient cells lead to a decrease in ROS-mediated toxicity [168].

In the context of AD, copper is another biometal (beside zinc and iron) that has been identified in the amyloid plaques of AD patients [162]. As early as 1999, White and colleagues showed that mice lacking amyloid precursor protein (APP) accumulated copper in the cortex and liver. On the contrary, mice overexpressing APP exhibited a decrease in the copper level in the brain [169]. Based on these data, the authors proposed that the amyloid precursor protein is a

membrane-bound copper transporter [170]. Furthermore, both copper and zinc have the ability to bind to Aβ in vitro, and in neuronal cells, these interactions result in the generation of oxidative stress, Aβ aggregation, and neuronal cell loss. Interestingly, the affinity of copper to Aβ depends on the length of the Aβ species, with a higher affinity to Aβ (1–42) compared with Aβ (1–40). This also corresponds to the ability of Aβ (1–42) to reduce Cu^{2+} to Cu^{3+} and to its effect on the generation of oxidative stress and neurotoxicity [162].

5.3. Manganese. Manganese, an essential cofactor for enzymes including arginase, glutamine synthetase, and SOD2, is critical for normal development and biological functions [171, 172]. Various transporters and the binding of this metal to numerous proteins maintain the homeostatic level of manganese in cells [173, 174]. Excessive exposure to manganese is connected, in addition to manganism, with the development of neurodegenerative diseases [173, 174]. This condition is accompanied with typical neurodegenerative mechanisms including oxidative stress, the disruption of mitochondrial function followed by ATP depletion, protein aggregation, and the attenuation of neurotransmitter synthesis [109]. In this regard, manganese stress in dopaminergic neurons inhibits dopamine synthesis and induces dopamine release from intracellular stores [171]. In vitro studies of neuronal cells have provided evidence that excessive manganese exposure accelerates the expression of α-syn and promotes its fibrillation and aggregation [163]. On the contrary, α-syn overexpression in neuronal cellular models increases the sensitivity to manganese exposure [171, 175, 176].

Several studies have also reported the involvement of manganese in the regulation of leucine-rich repeat kinase 2- (LRRK2-) mediated pathogenesis in PD (Table 2). Mutation G2019S, with enhanced kinase activity, is linked with familiar PD [177]. In vitro studies have suggested that, in addition to Mg^{2+}, Mn^{2+} may act as a cofactor of LRRK2 (PARK8) activity, with a preference for Mg^{2+}. In contrast to wild-type LRRK2, G2019S-mutated LRRK2 shows equal catalytic rates in the presence of both Mg^{2+} and Mn^{2+} [171, 178, 179]. Based on these observations, LRRK2 has been suggested to act as a biological sensor of manganese levels, whereby wild-type LRRK2 reacts to increased manganese levels by a decrease in its kinase activity. On the other hand, PD-linked G2019S remains active, even under elevated levels of manganese, indicating the putative pathological mechanism [178].

Furthermore, at least in yeast and mammalian cells, ATP13A2 has been demonstrated to have protective effects against toxicity mediated by various metals, including Mn, Cd, Ni, and Se [180–182]. Mutations in ATP13A2 have been identified as the cause of Kufor-Rakeb syndrome, a juvenile recessive neurodegenerative disorder [167, 180]. As mentioned above, ATP13A2 is a lysosomal type 5 P-type ATPase [167], with a function in the regulation of biometal homeostasis. The protective role of ATP13A2 against α-syn-induced toxicity has also been confirmed in cellular and in animal models of PD [181].

In terms of AD, a manganese challenge in neuronal cell lines leads to the hyperphosphorylation of tau via extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) activation. This is followed by glycogen synthase kinase-3β (GSK-3β) activation, which in general is associated with tau hyperphosphorylation and tau-mediated neuronal death [183].

5.4. Magnesium. Magnesium, the second most abundant intracellular cation, is critical for many biological processes including cellular energy, gene transcription, cellular growth, survival, and differentiation [184]. In the context of neurodegenerative diseases, magnesium is, in general, considered as a neuroprotective agent. Magnesium homeostasis and the role of the transient potential melastatin 7 (TRPM7) transporter have been implicated in neurodegenerative disease such as PD, AD, and HD [184]. Chronic low intake or deficiency in Mg^{2+} is considered as a high risk factor of PD development (Table 2) and dopaminergic neuron death and, in animal models, leads to a higher susceptibility to MPTP-mediated neurotoxicity [185–187]. On the other hand, supplementation with Mg^{2+} protects or decreases the risk of PD pathogenesis in animal models and in humans [188, 189]. Transgenic zebrafish carrying the mutated form of TRPM7 exhibit defects in dopamine generation or release, with a PD-like phenotype, which is, importantly, partially recovered by the administration of dopamine [184]. The specific mechanism of these outcomes cannot be described precisely with regard to the "crossroad" position of Mg^{2+} among the metabolic pathways or regulatory effects on various ion pumps, cotransporters, and hormone activities [190–192]. Indeed, the increase or stabilization of the intracellular concentration of Mg^{2+} presents one of the impacts of the antidepressant imipramine. In addition, according to one hypothesis, DJ-1 (PARK7) plays a key role in intracellular stabilization of Mg^{2+} by the androgen-receptor-mediated inhibition of the Mg^{2+} efflux effector SLC41A1 [76]. The stabilization or slight increase of intracellular Mg^{2+} concentration is, as mentioned above, connected with the proper or at least improved function of several ion pumps effective in ATP hydrolysis [88]. The mechanism of impact of DJ-1 on Mg^{2+} homeostasis can also be understood as representing one of the numerous connection points of oxidative status and ion balance regulation. DJ-1 is a protein deglycase, which can also act in a chaperone-like manner and regulate the aggregation state of α-syn. The ability of DJ-1 to protect cells against the formation of α-syn fibrils is dependent on the oxidative status. This molecular redox balance sensor has a minimal affinity to α-syn in the unoxidized state, but the oxidation of DJ-1 increases its affinity to α-syn and thus preserves it from toxic fibril formation [193].

6. Conclusions

6.1. Common Features of Metabolic Disorders and Neurodegenerative Diseases. The causative pathogenesis of both metabolic and neurodegenerative diseases involve common mechanisms. The symptomatology of neurodegenerative disorders is the result of pathophysiology occurring not only in nerve cells but also in other cell types. Similarly, the pathophysiology of metabolic disorders does not avoid

neural tissues. Furthermore, extensive recently obtained data suggest a metabolic background to neurodegenerative diseases. Recent evidence has emerged increasingly supporting the hypothesis that AD, one of the most prevalent neurodegenerative diseases, should also be considered as a type 3 DM (T3DM).

The influence of ionic imbalances, in particular, the decrease and/or increase of biometal concentration, and the disturbances in their contents within the various types of peripheral cells or brain regions are considered to play an important role in the development of both types of diseases. The information summarized in this review refer to specific pathomechanisms that take into account the changes in the levels of biometals and their close relationship to inflammatory processes, alterations in energy metabolism, and the generation of oxidative stress. Intracellular reduction in energy supplementation caused by mitochondrial deficiency, hypoxic conditions, or inflammatory changes has been found to have relevant association with both peripheral and neuronal degenerative disorders. Oxidative stress, alternating between the causal factor and the consequence of the disease pathophysiology, should therefore be recognized as a hallmark of the majority of metabolic and neurodegenerative diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Influence of the Microstructure and Silver Content on Degradation, Cytocompatibility, and Antibacterial Properties of Magnesium-Silver Alloys In Vitro

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Implantation is a frequent procedure in orthopedic surgery, particularly in the aging population. However, it possesses the risk of infection and biofilm formation at the surgical site. This can cause unnecessary suffering to patients and burden on the healthcare system. Pure Mg, as a promising metal for biodegradable orthopedic implants, exhibits some antibacterial effects due to the alkaline pH produced during degradation. However, this antibacterial effect may not be sufficient in a dynamic environment, for example, the human body. The aim of this study was to increase the antibacterial properties under harsh and dynamic conditions by alloying silver metal with pure Mg as much as possible. Meanwhile, the Mg-Ag alloys should not show obvious cytotoxicity to human primary osteoblasts. Therefore, we studied the influence of the microstructure and the silver content on the degradation behavior, cytocompatibility, and antibacterial properties of Mg-Ag alloys in vitro. The results indicated that a higher silver content can increase the degradation rate of Mg-Ag alloys. However, the degradation rate could be reduced by eliminating the precipitates in the Mg-Ag alloys via T4 treatment. By controlling the microstructure and increasing the silver content, Mg-Ag alloys obtained good antibacterial properties in harsh and dynamic conditions but had almost equivalent cytocompatibility to human primary osteoblasts as pure Mg.

1. Introduction

The clinical application of biodegradable implant and prosthesis has shown rapid growth to keep with the demands of a rapidly aging population. However, implant-associated orthopedic surgery infections are common postoperative wound infections and can cause biofilm formation on the implants or bones [1, 2]. Biofilms are resistant to antibiotics and can protect bacteria from host immune mechanisms. Once a biofilm has formed, the only treatment is to remove the implant and the diseased tissue [3–5]. Prevention is the preferred method to address the growing problem of implant-associated infections [6, 7].

Pure magnesium (pure Mg) and its alloys, as potential biodegradable implant materials, have the advantage of not requiring removal after bone tissue healing [8]. Therefore, infection caused by a second surgery can be avoided. In vitro, pure Mg exhibited some antibacterial properties due to its alkaline pH [9–11]. In the early stage of degradation, it can create an alkaline environment, which is adverse to the survival and reproduction of bacteria [12, 13]. However, it is not clear whether these changes will occur in vivo, although it was shown that pure Mg induces osteoblasts and suppresses bacteria in a chronically infected rabbit tibial osteomyelitis model [14]. However, the length of time that an effective antibacterial concentration maintained in the local position is not sufficient, which will influence the resistance to infection and will affect osteomyelitis treatment [14, 15]. One cause of these effects is that the degradation rate of pure Mg and magnesium alloys in vivo is lower than that in vitro

[16–18]. In this case, a high pH cannot be maintained, so it sounds unrealistic for pure Mg or its alloys to achieve long-term inhibition to bacteria.

Silver (Ag) has effective antibacterial properties and has been used to treat burns and chronic wounds for centuries [19]. Silver nanoparticles (AgNPs) and silver ions can bind to proteins, change the membrane of bacteria, interfere with DNA expression, create reactive oxygen species (ROS), and affect thiol group compounds that exist in respiratory enzymes to inhibit respiratory processes [20–22]. The emergence of antibiotic-resistant strains of bacteria has promoted the use of metallic silver to prevent infections of indwelling devices [20]. There are cases of silver applications that focus on the antibacterial properties, for example, wound dressing, bone cement, and megaprosthesis [23–25]. Silver-coated megaprosthesis can release silver ions and reduce the infection rate compared to the group without silver [25]. In addition, metallic silver can stimulate osteogenic differentiation [26].

However, the accumulation of a high amount of silver in the human body can cause argyria or argyrosis, which results from the deposition of significant amounts of insoluble silver precipitates in the dermis of the skin and the cornea or conjunctiva of the eyes [27, 28]. However, no pathological damage to tissues can be observed. The threshold amount of silver that can evoke argyria ranges from 3.8 to 5 g or even 10 g over the whole lifetime of adults [29]. The total body silver concentration that can cause argyria is 1 g for children under 10 years old [30]. Hence, the application of silver in the human body should consider these limitations. In clinical course, the amount of silver coated on megaprosthesis ranges from 0.4 to 1.69 g in adult patients [25]. However, no relevant evidence shows that such a low amount of silver in the human body or chronic silver exposure can cause pathological changes in any tissue or organ [27–29, 31]. Moreover, the loss of cell viability in vitro due to metallic silver or silver compounds is dose dependent [26, 32, 33]. Metallic silver has a lower risk of toxic effects compared with soluble silver compounds [34].

How to avoid or treat orthopedic implant contamination and biofilm formation is a complicated issue [35]. Novel, biodegradable magnesium alloys with antimicrobial properties are desirable considering the surgical contamination and appearance of multiresistance bacteria. Many methods have been studied, for example, coating and surface morphology, to endow permanent implants or even magnesium alloys the function of suppressing bacteria or reducing bacterial adhesion [20, 36–39]. However, maintaining long-term and stable prevention of implant-associated infection remains a problem [40]. In addition, the current methods cannot maintain the long-term antibacterial properties of biodegradable magnesium alloys.

In this study, metallic silver was alloyed with pure Mg so that the silver could be released continuously and react with bacteria if the bacteria attach to the surface of Mg-Ag alloy or the surrounding tissue during the whole degradation period. We planned to reach the best antibacterial properties of Mg-Ag alloys by alloying silver inside as much as possible and obtain good cytocompatibility comparable to those of pure Mg by adjusting their microstructure via thermal-mechanical processing and heat treatment. It is expected that biofilms cannot form on the Mg-Ag alloy and surrounding tissue, even in a harsh environment with a large amount of bacteria and under flow conditions, for example, the human body. By this method, it is hoped that implant-associated and recurrent infections can be prevented successfully when Mg-Ag alloy is used as a bone implant in the future.

2. Material and Methods

2.1. Materials Preparation. Magnesium (99.99 wt%, Xinxiang Jiuli Magnesium Co., Ltd., Xinxiang, China) and silver granules (99.99 wt%, ESG Edelmetall-Handel GmbH & Co. KG, Rheinstetten, Germany) were used for the preparation of Mg-6Ag and Mg-8Ag alloys. Pure magnesium was cut into small pieces and placed in a steel crucible with the corresponding amount (6 wt% or 8 wt%) of silver. The metals were melted at 750°C under a protective atmosphere of 98% Ar (argon) and 2% SF_6 (sulphur hexafluoride) and then stirred for 30 minutes at 200 rpm. After the temperature decreased to 730°C, the melt was transferred to a permanent steel mould (diameter ø = 120 mm) that was coated inside with the mould release agent ALU-STOP LC25 (Büro für angewandte Mineralogie, Dr. Stephan Rudolph, Tönisvorst, Germany). The mould was held for 15 min at 680°C under a protective atmosphere and was then immersed in flowing room temperature water gradually at a speed of 100 cm/min until the melted magnesium alloy solidified. Pure Mg ingots were recast into cylinder shape following the same solidification procedure. The tops and bottoms of the ingots with shrinkage and impurities were removed. The contents of elements in the Mg-Ag alloys were analyzed by X-ray fluorescence (Bruker AXS S4 Explorer, Bruker AXS GmbH, Germany) and with a Spark Analyser (Spectrolab M, Spektro, Germany).

According to the Mg-Ag phase diagram, silver has low solubility of 15 wt% in magnesium at eutectic temperature which is the lowest melting point of a mixture of components [41]. Homogenization treatment and hot extrusion were performed to acquire a homogeneous microstructure and stable mechanical properties. The homogenization treatment was conducted in a resistance furnace (Linn Elektro Therm AK 40. 06, Bad Frankenhausen, Germany) at 430°C for 16 h, followed by quenching in room temperature water. The ingots were then heated up (285°C for Mg-6Ag and 300°C for Mg-8Ag) and processed by hot extrusion (Strangpresszentrum Berlin, Berlin, Germany), for which the extrusion ratio and the advance rate of stamp were 108 and 0.7 mm/s, respectively. The temperature of the container and steel die was 300°C. The extruded rods (ø = 12 mm) were cut into discs (ø = 10 mm and h = 1.5 mm) (Henschel KG, Munich, Germany). T4 heat treatment (solution treatment) of the discs was conducted by placing them in a steel box filled with Ar and holding them in a resistance furnace (Vulcan™ A-550, Dentsply Ceramco, USA) at 450°C for 8 h and before quenching. The discs were ground on sandpaper (2500#, mesh) to remove the oxidation layer caused by heat treatment.

2.2. Microstructure Analysis. Samples were prepared by grinding on sandpaper from 220# to 2500#, followed by polishing using water-free OPS (oxide polishing suspension) on a rubber plate. The samples were etched in picric solution (100 mL ethanol, 20 mL distilled water, 6-7 mL glacial acetic, acid and 12–15 g picric acid (99%), all chemicals from Sigma-Aldrich Chemie, Taufkirchen, Germany) and were observed by polarized light microscopy (Leica 020-520.008 DM/LM, Wetzlar, Germany) and scanning electron microscopy (SEM, TESCAN vega 3 SBU, Brno, Czech Republic). The precipitates in the extruded Mg-Ag alloy were characterized by Bruker X-ray diffraction (XRD).

2.3. Immersion Test. An optimized in vitro test setup was used for the immersion tests [42, 43]. Cell culture medium (CCM), Dulbecco's Modified Eagle's Medium (DMEM), and GlutaMAX +10% FBS (fetal bovine serum, PAA laboratories, Linz, Austria) were used for the immersion test and cell culture. The cell culture conditions were 5.0% CO_2, 20% O_2, 37.0°C, and 97.0% rH (relative humidity). Discs were weighed by a Scaltec SBA32 (Scaltec, Goettingen, Germany) and sterilized ultrasonically in 70% ethanol solution for 30 min. After drying, the discs were transferred to 12-well plates, which were filled with 2 mL CCM in each well, and then incubated in the cell culture conditions for 1 week. The old cell culture medium was replaced by a fresh one after 48 and 120 hours. After the immersion test was carried out for 168 hours, the degradation products were removed by chromic acid (180 g/L in a. dest., Sigma-Aldrich Chemie, Taufkirchen, Germany). The discs were dried in a vacuum box and weighed by the precision electronic balance. The mean degradation rate was calculated using the previously described weight loss method [44].

2.4. Cytotoxicity Test. Human primary osteoblasts were selected for the cytotoxicity evaluation. The human primary osteoblasts came from patients undergoing total hip arthroplasty with local ethical committee agreement. Pure Mg and Mg-Ag discs were sterilized ultrasonically in 70% ethanol solution for 30 min. Extracts of pure Mg and Mg-Ag for the MTT assay were prepared by immersing samples into CCM (0.2 g/mL) for 3 days in the cell culture conditions. The concentration of Mg, calcium (Ca), and Ag in the extracts was measured via inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700x ICP-MS, Waldbronn, Germany). The extracts were further characterized by measuring their pH and osmolality at room temperature using an ArgusX pH meter (Sentron, Roden, Netherlands) and a Gonotec 030-D cryoscopic osmometer (Gonotec, Berlin, Germany), respectively. A 50 μL aliquot of CCM containing 2000 human primary osteoblasts was seeded into each well of 96-well plates. The plates were transferred to the incubator and kept in the cell culture conditions for 24 hours to ensure that the human primary osteoblasts attached to the bottom. The diluted pure Mg and Mg-Ag extracts were prepared by adding CCM at ratios of 1 : 5 and 1 : 10. Then, the old CCM was replaced with fresh CCM (control group), pure Mg and Mg-Ag extracts, and diluted extracts ($n = 6$ for each concentration). After culturing for 3 days, 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide solution (MTT) (Sigma-Aldrich, Steinheim, Germany) was added to each well and incubated for 4 hours. Then, 100 μL SDS (sodium dodecyl sulphate) lysis buffer (Sigma-Aldrich Co., LLC, Steinheim, Germany) was added into the wells and incubated overnight. Finally, the values were measured using an ELISA multiwell plate reader (Tecan, Maennedorf, Switzerland) and the background value was removed.

In the adhesion test, pure Mg and Mg-Ag discs were placed in 12-well plates after the discs incubated in CCM in the cell culture conditions for 24 hours. A total of 10^5 human primary osteoblasts were seeded on the surface of each disc. To ensure that the human primary osteoblasts attached to the surface, the seeded samples were kept in the incubator for 30 min. Then, 12-well plates were slowly filled with 3 mL of fresh CCM and transferred into the incubator and cultured for 3, 6, or 9 days. The CCM was changed every 3 days. The pH and osmolality of the replaced medium were measured. Discs were washed gently in sterilized and distilled water and transferred into wells filled with LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, USA) according to the manufacturer's protocol. After incubation for 20 min, the distribution and viability of human primary osteoblasts on pure Mg and Mg-Ag discs were observed via fluorescent microscope (Nikon Eclipse Ti-S, Tokyo, Japan).

2.5. Biofilm Test and Evaluation. The biofilm test was conducted in a bioreactor system (Figure 1). This dynamic system has a cross-flow condition in the chambers, which ensures that the bacteria go through the surface of the discs. The flow rate of the medium in chamber was 0.3 mL/min. These conditions allow the possibility of initial biofilm formation on the discs [45]. During the running time of 15 hours, the temperature and pH were 37°C and 7.2, respectively. All the parameters mentioned above were controlled by the bioreactor system (BioFlo®/CelliGen® 115 (New Brunswick™), Eppendorf AG, New Brunswick, USA). Reference discs (titanium (Ti)) were always used as an internal control for the test. Pure Mg and Mg-Ag alloys were treated with 25.0 kGy gamma sterilization (BBF Sterilisationsservice GmbH, Kernen, Germany) before the biofilm test [44]. The whole test was performed in a microaerophilic and sterilized environment to ensure bacterial activity. The bacteria culture medium (BCM) consisted of nutrient broth (pH = 7.2), 3 g meat extract, 5 g peptone (Sigma-Aldrich Co., LLC, Steinheim, Germany), and 1 L distilled water. Phosphate-buffered saline (PBS, pH = 7.4) was prepared with 8 g NaCl, 0.2 g KCl, 1.47 g Na_2HPO_4, 0.24 g KH_2PO_4 (Sigma-Aldrich Co. LLC, Steinheim, Germany), and 1 L double-distilled water.

Staphylococcus aureus (*S. aureus*, DSM number 20231) and *Staphylococcus epidermidis* (*S. epidermidis*, DSM number 3269) were used in the biofilm test. These bacteria are commonly found in implant-associated orthopedic infections or osteomyelitis [3, 46–48], although there is contention about which is the most common bacteria isolated from clinical infections, especially implant-associated infections [49–51]. The bacteria were provided by the Leibniz Institute DSMZ-German Collection of Microorganisms and

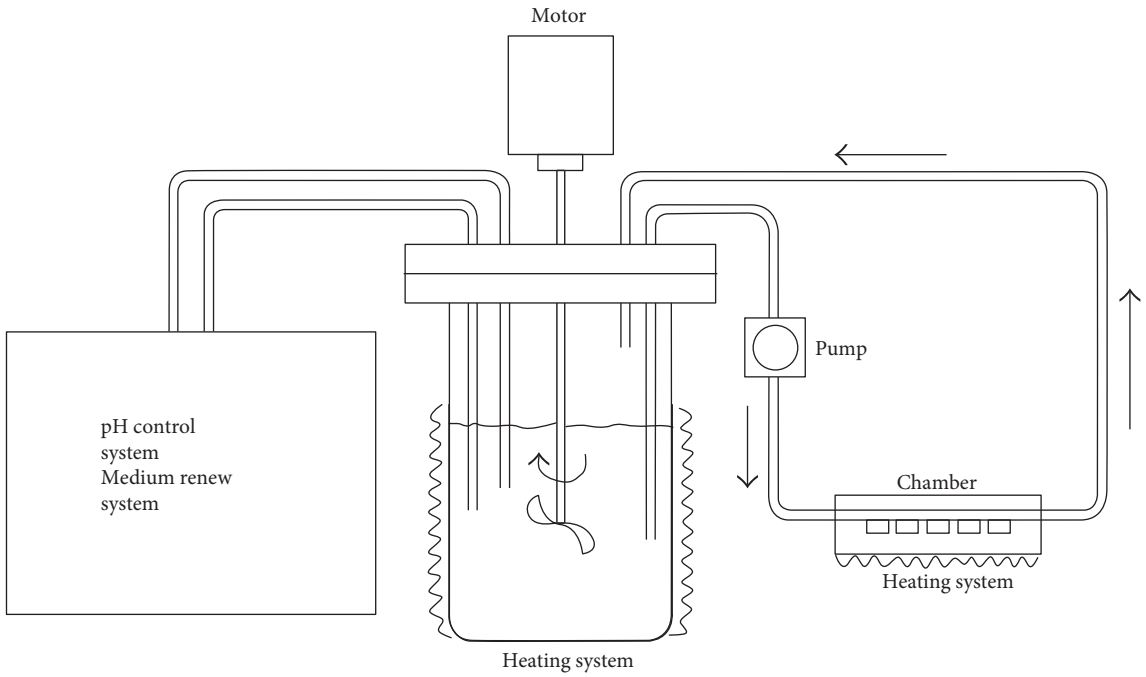

Figure 1: Schematic diagram of the bioreactor system.

Cell Cultures in Germany. The bacteria were cultured overnight and were mixed and imported into the bioreactor system after checking their viability. The density and ratio of the mixed bacteria in medium were 10^6 /mL and 1 : 1, respectively.

After the bioreactor system ran for 15 hours, all of the discs were removed from the chamber and labelled by adding LIVE/DEAD BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific Inc. (Life Technologies), Eugene, USA). Some discs were observed by confocal laser scanning microscopy (CLSM, LSM 710, Carl Zeiss Microscopy GmbH, Jena, Germany). Images of the whole surface and the local details of the discs were taken by CLSM. The other discs ($n = 12$ for each type of sample) were rinsed gently in distilled water, placed in glass bottles with PBS, and transferred to an ultrasonic bath (SONOREX SUPER 10P, BANDELIN electronic GmbH & Co. KG, Berlin, Germany). A plastic scraper was used to remove the bacteria from the surfaces of the discs under sonication. The PBS solutions containing bacteria were diluted, placed on a counting chamber, and counted using a fluorescence microscope (BX51, Olympus Optical Co. (Europa) GmbH, Hamburg, Germany).

2.6. Sample Preparation for SEM and Generation of 3D Images. In adhesion test, the procedures to prepare the SEM samples with human primary osteoblasts were fixation in 2.5% glutaraldehyde solution in buffer (Sigma-Aldrich Co. LLC, Steinheim, Germany) for 2 hours, staining in 1% osmium tetroxide (Sigma-Aldrich Co. LLC, Steinheim, Germany) for 30 min, dehydration for 1 hour using increasing concentrations of 2-propanol (EMSURE®, Darmstadt, Germany) (20%, 40%, 60%, 80%, and 100%), and critical point drying (Leica EM CPD030, Bal-TEC AG, Balzers, Liechtenstein). Then, the samples were placed on an SEM sample holder with N650 Planocarbon (Plano GmbH, Wetzlar, Germany). In biofilm test, three-dimensional (3D) images of the discs were merged using SEM pictures with different tilt angles (0°, 7°, and 15°) before and after removal of the degradation products.

Table 1: Major and trace element composition of the Mg-Ag alloys.

Alloys	Ag wt%	Fe wt%	Cu wt%	Ni wt%	Mg wt%
Mg-6Ag	6.26	0.00205	0.00100	0.00108	Balance
Mg-8Ag	8.51	0.00184	0.00104	0.00106	Balance

2.7. Data Analysis. Statistical analyses were performed by one-way analysis of variance (ANOVA) in Origin 9.0G with the appropriate post hoc comparison test (Tukey's test). A p value <0.05 was considered significant. The graphs present the results as the mean value with the standard deviation (SD) as the error bars.

3. Results

3.1. Metallography and Microstructure. The actual elemental composition of the Mg-Ag alloys is listed (Table 1). Pure Mg has a similar grain size as extruded Mg-6Ag. Extruded Mg-8Ag has finer grains (average grain size (AGS) $= 7.9 \pm 4.5\ \mu$m) than extruded Mg-6Ag (AGS $= 28.2 \pm 13.4\ \mu$m). However, the grain size increased obviously after the T4 treatment and showed a nonuniform trend, but the microstructures of Mg-6Ag and Mg-8Ag became similar after T4 treatment (Figure 2). According to the XRD patterns, the precipitates in the extruded Mg-6Ag and Mg-8Ag are $Mg_{54}Ag_{17}$ (Figure 3(b)). Most of the precipitates are distributed along the grain boundaries of the extruded Mg-Ag alloys, as shown in the SEM images. There is a small amount of precipitate (0.2%) in the extruded Mg-6Ag

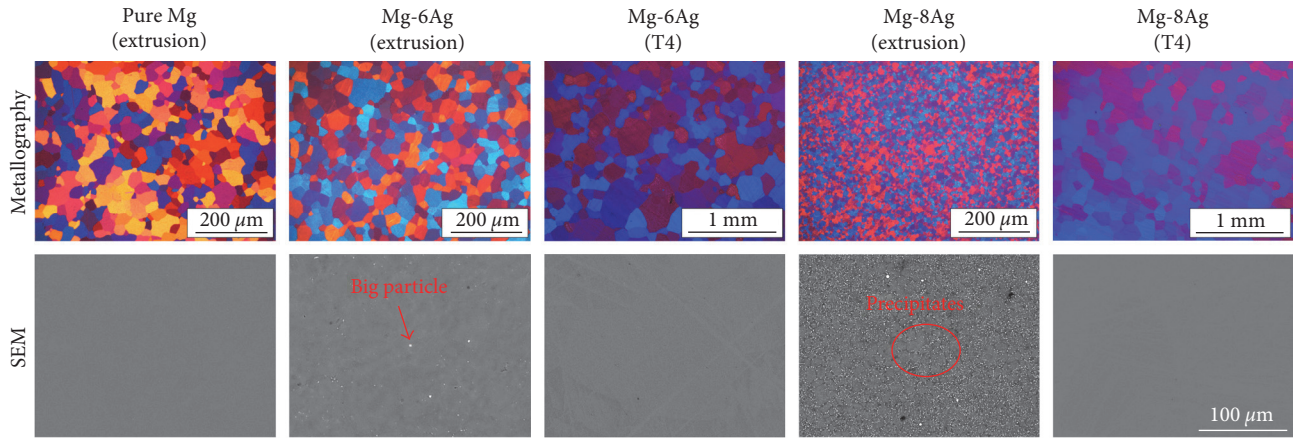

Figure 2: Metallography, distribution, and quantity of secondary phases and precipitates in the extruded Mg-Ag alloys before and after T4 treatment. The grain sizes of extruded pure Mg, extruded Mg-6Ag, T4-treated Mg-6Ag, extruded Mg-8Ag, and T4-treated Mg-8Ag are 29.9 ± 15.5, 28.2 ± 13.4, 154.5 ± 103.4, 7.9 ± 4.5, and 159.9 ± 89.7 μm, respectively. The SEM in BSE (back-scattered electron) mode was used. The ratios of second phases and precipitates in the extruded Mg-6Ag and Mg-8Ag are 0.2% and 2.3%, respectively.

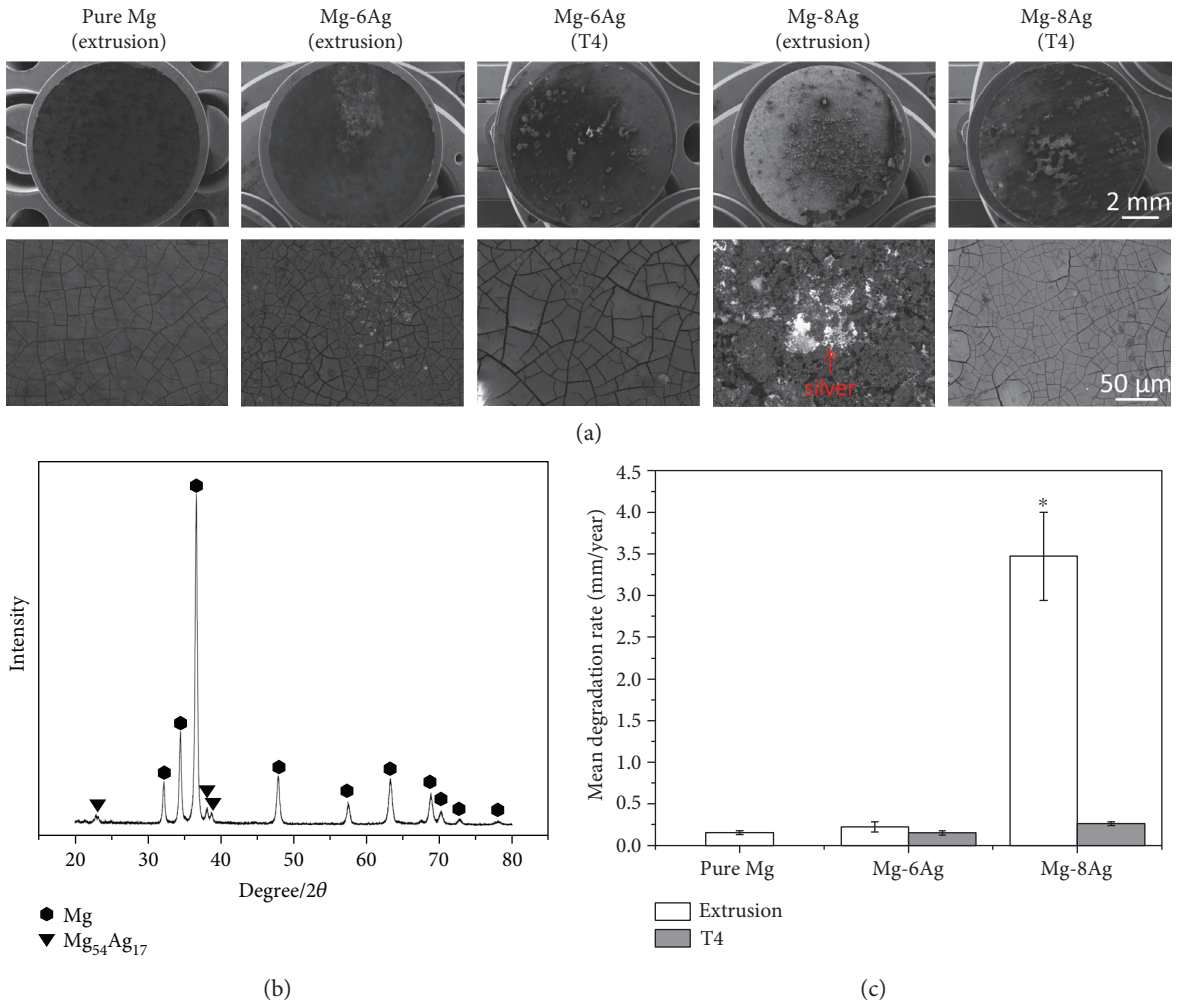

Figure 3: (a) Surface morphology of discs after the immersion test. The images of the local details are in BSE mode. (b) XRD pattern of the extruded Mg-8Ag alloy. (c) The mean degradation rate of pure Mg and Mg-Ag alloys in CCM in the cell culture conditions. The mean degradation rate of the extruded Mg-8Ag is significantly higher than the others. The "*" indicates a significant difference compared with the other values, $p < 0.05$.

Table 2: Increments of pH and osmolality of pure Mg and Mg-Ag alloys compared to those of CCM.

Extracts	pH	Osmol/kg
Pure Mg (extrusion)	0.815	0.107
Mg-6Ag (extrusion)	0.920	0.110
Mg-6Ag (T4)	0.955	0.066
Mg-8Ag (extrusion)	0.895	0.104
Mg-8Ag (T4)	0.965	0.068

and a large amount (2.3%) in the extruded Mg-8Ag. Some larger particles can be found in the extruded Mg-6Ag and Mg-8Ag that are residuals of the secondary phase after the homogenization treatment of the ingots. After T4 treatment, nearly all the secondary phases and precipitates in the extruded Mg-Ag alloys dissolved into the alloys.

3.2. Morphology and Degradation Rate. After the immersion test, the surface condition of the discs can be observed (Figure 3(a)). Some black or white dots, which are degradation products, are present on the surface of the pure Mg and Mg-Ag alloys in low-magnification images. However, the extruded Mg-8Ag disc showed more severe degradation than the other discs. Loose degradation products and accumulated silver are observed on the surface. There is also some accumulated silver on the surface of the extruded Mg-6Ag disc. However, there are only cracks on the pure Mg and T4-treated Mg-Ag discs after the degradation layer is dehydrated.

The addition of silver led to a slight increase in the degradation rate of Mg-6Ag alloy compared to pure Mg (Figure 3(c)). A further increase in silver significantly enhanced the degradation to 3.47 mm/year. This effect could be drastically reduced by T4 treatment, which led to degradation rates of less than 0.5 mm/year. In this case, the degradation rate increased linearly with the amount of silver.

3.3. Cytocompatibility

3.3.1. MTT. The pH and osmolality of the extracts from the pure Mg and extruded Mg-Ag alloys were elevated compared to the CCM (Table 2). More Mg existed in the extracts than in CCM, but the concentration of Ca in the extracts decreased (Table 3). Magnesium/calcium phosphates formed in the degradation layer during degradation [52–55]. After T4 treatment, the osmolality of the Mg-Ag alloy extracts was lower than that of the pure Mg and extruded Mg-Ag extracts. The concentrations of Mg, Ca, and Ag in the primary extracts from extruded Mg-Ag were higher than those from T4-treated Mg-Ag.

In the MTT assay, all primary extracts, including pure Mg extract, showed cytotoxicity compared with CCM because of high pH and osmolality (Figure 4). Their values were below the cytotoxic limit of 75% cell viability. After fivefold dilution, most of the extracts did not show cytotoxicity, except for the extract of the extruded Mg-8Ag, which also did not reach the level of 75% because the silver concentration was still higher than the tolerance

Table 3: Calculated concentrations of elements in the extracts of extruded pure Mg and Mg-Ag alloys.

Extracts	Dilution	Concentration (mg/L)		
		Mg	Ca	Ag
Pure Mg (extrusion)	1	1210	27	<0.1
	1/5	258	65.4	<0.1
	1/10	139	70.2	<0.1
Mg-6Ag (extrusion)	1	1280	26	1.2
	1/5	272	65.2	0.24
	1/10	146	70.1	0.12
Mg-6Ag (T4)	1	1010	17	0.31
	1/5	218.8	62.6	0.062
	1/10	119.9	68.3	0.031
Mg-8Ag (extrusion)	1	1150	25	104
	1/5	246	65	20.8
	1/10	133	70	10.4
Mg-8Ag (T4)	1	930	15	0.64
	1/5	202.8	62.2	0.128
	1/10	111.9	68.1	0.064
Cell culture medium (CCM)		20	75	<0.1

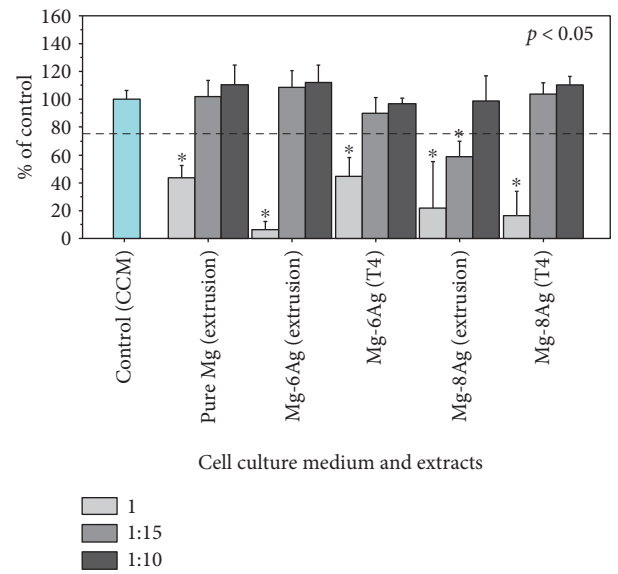

Figure 4: Viability of human primary osteoblasts determined by MTT assay in the primary, 1 : 5, and 1 : 10 extracts. The dotted line marks 75% cell viability, which indicates no potential cytotoxicity [58]. The "*" indicates statistically significant difference at $p < 0.05$ versus the control group (CCM).

of human primary osteoblasts. After fivefold and tenfold dilutions, the extract from T4-treated Mg-8Ag showed good cytocompatibility as did the diluted pure Mg extracts and CCM, although all primary extracts exhibited cytotoxicity to human primary osteoblasts.

3.3.2. Live/Dead Staining and Adhesion Test. The pH and osmolality were measured after preincubation for 24 hours and culturing human primary osteoblasts on the samples

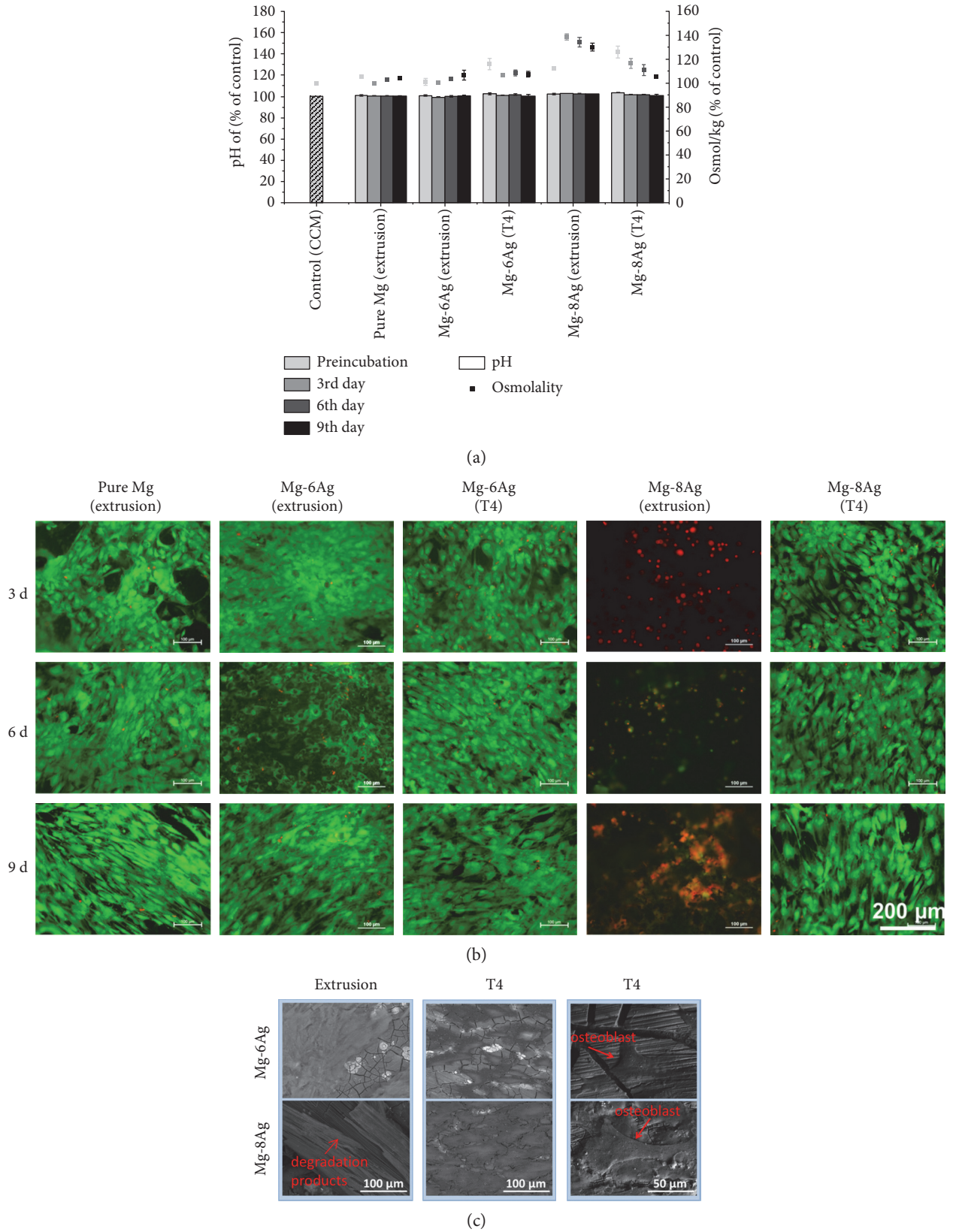

Figure 5: (a) Variation of pH and osmolality during the incubation. (b) Live/dead staining of human primary osteoblasts on pure Mg and Mg-Ag alloys. The clear green parts represent living osteoblasts and the red dots dead osteoblasts. (c) SEM images of the adhesion tests of human primary osteoblasts. The first and second vertical rows show thick cell layers covering the surface after 9 days. The third vertical row shows the status of a single osteoblast attached to the degradation layer after 3 days.

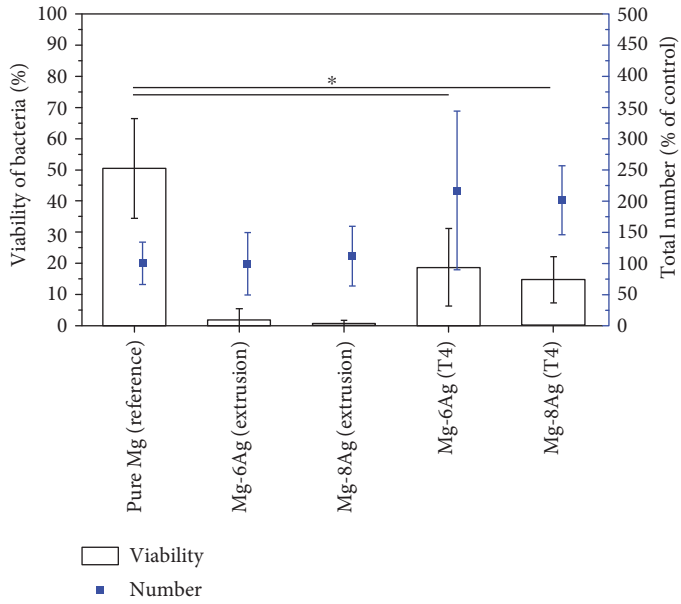

Figure 6: Viability of bacteria and total bacteria on the discs. From left to right—extruded pure Mg, extruded Mg-Ag alloys and T4-treated Mg-Ag alloys after extrusion. Pure Mg is the reference for the total number of bacteria. The "*" means that pure Mg has a statistically significant difference in viability at $p < 0.05$ versus T4-treated Mg-Ag discs.

for 3, 6, and 9 days. The pH of the pure Mg and T4-treated Mg-Ag discs decreased gradually over time (Figure 5(a)). The osmolality of the extract from pure Mg was stable, and the osmolality of the extract from extruded Mg-6Ag increased slightly over time. However, the osmolality of the extract from extruded Mg-8Ag remained at a high level after preincubation, and it had nearly the highest pH and osmolality at all time points. The extracts of the T4-treated Mg-Ag discs had higher osmolality after preincubation, but the osmolality decreased rapidly over time.

The regions with the same cell density were selected for comparison after live/dead staining (Figure 5(b)). The degradation rates of the extruded pure Mg and Mg-6Ag were much lower than those of the extruded Mg-8Ag in CCM in the cell culture conditions according to the pH and osmolality (Figure 5(a)). Human primary osteoblasts can survive and attach to extruded pure Mg, extruded Mg-6Ag, and T4-treated Mg-6Ag discs. After 3 days, on pure Mg and Mg-6Ag, some dead osteoblasts were detected. After 6 days, the number of dead osteoblasts decreased. After 9 days, no differences were detected between the viability of human primary osteoblasts on pure Mg and Mg-6Ag discs. However, the extract from the extruded Mg-8Ag always had the highest average pH and osmolality, which indicates a faster degradation rate. The pH and osmolality near the surface of the extruded Mg-8Ag discs were higher, so many bubbles formed on the surface and a large amount of silver was released. As a result, no human primary osteoblasts attached to and survived on the extruded Mg-8Ag discs. After T4 treatment, the pH and osmolality of Mg-8Ag discs were lower and showed better cytocompatibility than before. Human primary osteoblasts can attach and proliferate on T4-treated Mg-8Ag discs as well as on pure Mg and Mg-6Ag discs, but a slightly higher amount of dead cells was observed in the initial stage (after 3 days). Cell layers and the details of human primary osteoblasts could be observed on all surfaces of the Mg-Ag discs, except for the extruded Mg-8Ag disc, where there are only degradation products (Figure 5(c)).

3.4. Biofilm Test

3.4.1. Viability of Bacteria. The extruded Mg-Ag alloys showed the best antibacterial effect (Figure 6). The viability of bacteria was much lower on the Mg-Ag alloys than on pure Mg. There was a relative high viability of bacteria on pure Mg of 50.35%. However, the viability on T4-treated Mg-6Ag and Mg-8Ag discs was 18.64% and 14.75%, respectively, which was significantly lower than that on pure Mg. In addition, more bacteria were observed on T4-treated Mg-Ag discs than on the extruded Mg-Ag discs.

3.4.2. Biofilm Culture. In the biofilm test, incubation for 15 hours can form an initial biofilm on Ti discs, which were set as the negative control for internal evaluation. A nearly complete young biofilm can be observed on the Ti disc (Figure 7(a)). A large amount of live bacteria on Ti disc could be observed in the high-magnification images (Figure 7(b)). The total amount of bacteria on pure Mg is obviously lower than on Ti disc. Extruded Mg-Ag discs showed local pitting degradation and had a faster degradation rate in BCM with a constant pH (7.2) than in CCM (Figure 7(a)). Many dead bacteria were present on the extruded Mg-Ag discs based on the overview pictures. However, the overview of the biofilm showed no obvious difference between pure Mg and T4-treated Mg-Ag discs. By judging from the high-magnification images (Figure 7(b)), details of live and dead bacteria on the T4-treated Mg-Ag alloys were shown. Most of the bacteria on the surface of the T4-treated Mg-Ag discs were dead compared to the bacteria on pure Mg.

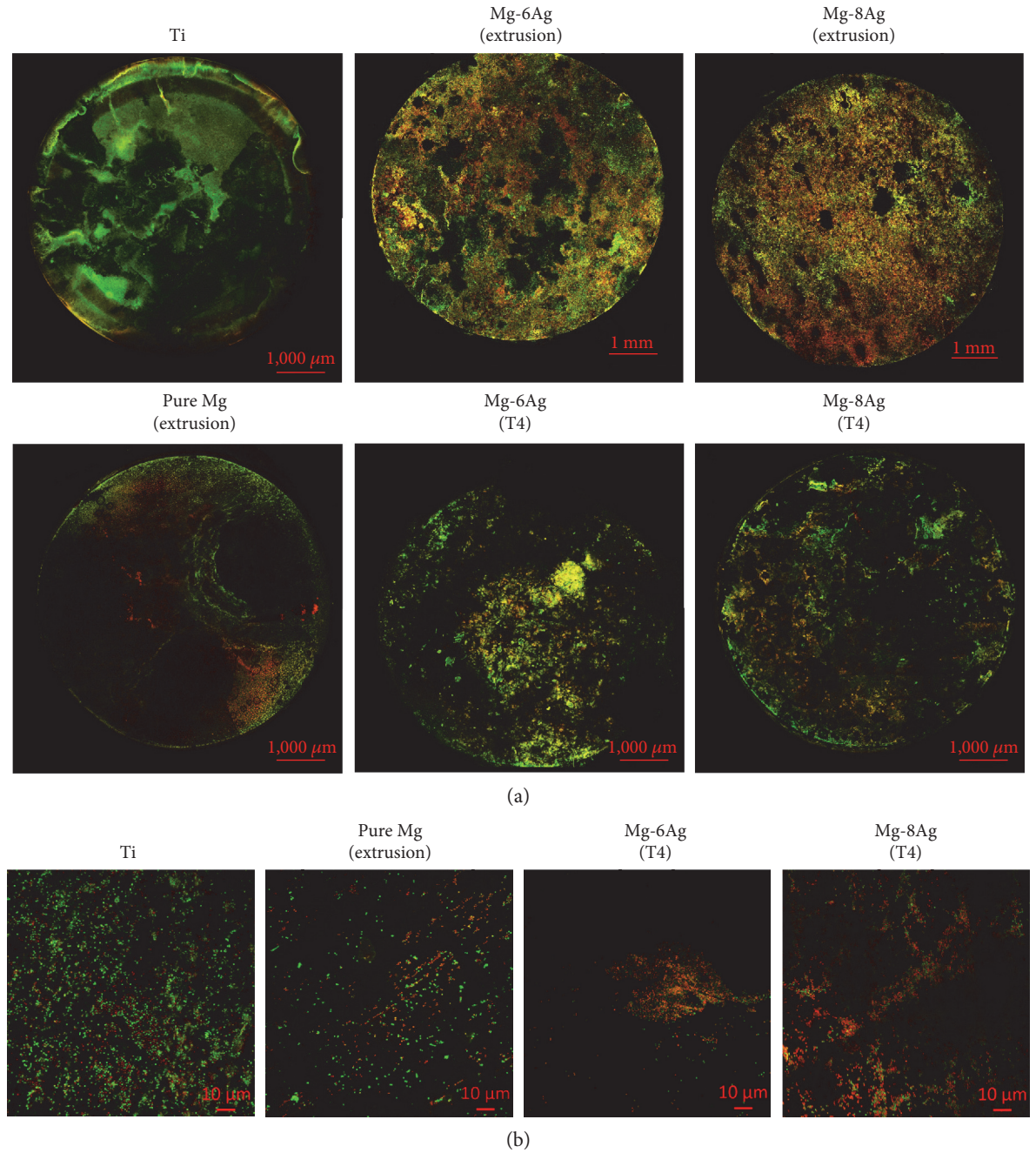

Figure 7: (a) Overview of biofilm formation. (b) Details of bacteria on discs.

3.4.3. Surface Morphology after the Biofilm Test in the Flow System. The 3D images of pure Mg and T4-treated Mg-Ag discs before and after removal of the degradation products are shown (Figure 8). The surfaces of the discs with degradation products appear coarse. It can be observed that many degradation products are present on the surface of the pure Mg and Mg-Ag discs. There are also some needle-like crystals on the T4-treated Mg-6Ag disc and many needle-like crystals on the T4-treated Mg-8Ag disc, so they look very rough in the 3D images. However, after removal of degradation products, the peaks disappeared and many degradation pits were revealed, especially on pure Mg, where a porous surface was exposed. However, only shallow and broad pits were observed on the surface of T4-treated Mg-Ag discs, which indicates a more homogeneous degradation mechanism. The average roughness (Sa) of pure Mg, T4-treated Mg-6Ag, and T4-treated Mg-8Ag are 8.68 ± 0.8, 6.42 ± 0.42, and 8.88 ± 1.92, respectively, and the developed interfacial areas (Sdr) are 37.87 ± 1.44, 19.26 ± 2.72, and 22.39 ± 2.23, respectively. Therefore, the Sa of pure Mg and T4-treated Mg-Ag alloys is on the same level, and the Sdr of the T4-treated Mg-Ag alloys is significantly lower than that of the pure Mg at $p < 0.05$. Therefore, the T4-treated Mg-Ag alloys have less contact area with the medium than pure Mg during degradation.

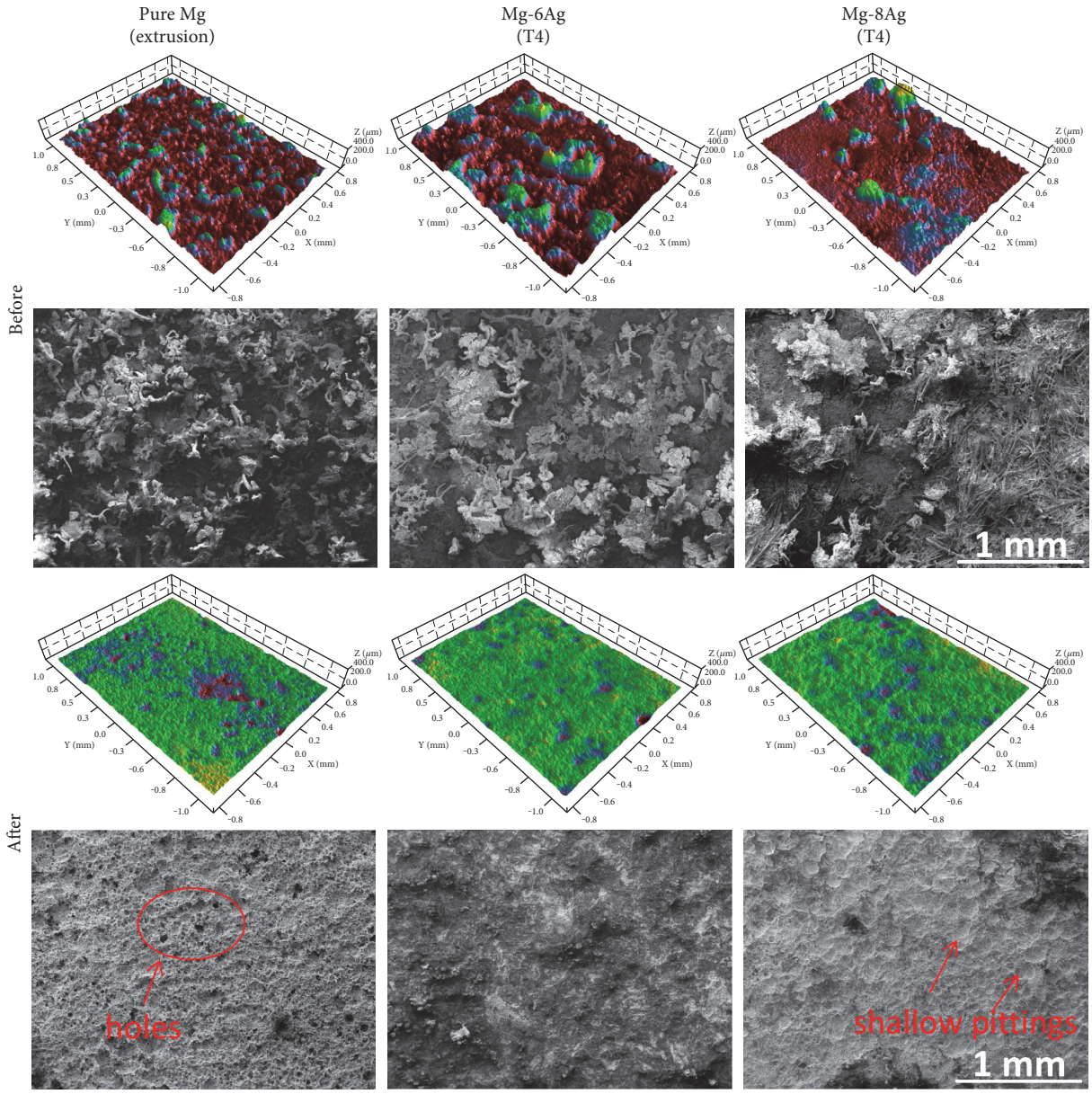

Figure 8: 3D images and surface conditions (SEM) before and after removal of the degradation products. The average roughness (Sa) of pure Mg, Mg-6Ag, and Mg-8Ag are 8.68 ± 0.8, 6.42 ± 0.42, and 8.88 ± 1.92, respectively. The developed interfacial areas (Sdr) are 37.87 ± 1.44, 19.26 ± 2.72, and 22.39 ± 2.23.

4. Discussion

Silver release plays a key role in eliminating multiple bacteria and preventing biofilm formation [23]. The novel point is that the addition of silver to pure Mg significantly improved the antibacterial effect in a dynamic environment because silver was released from the matrix continuously during the whole degradation of Mg-Ag alloy as an orthopedic implant. In a previous study with cast material, the effect of silver addition could already be demonstrated [56]. However, cast magnesium alloys would never be used to produce implants. Therefore, in this study, extruded Mg-Ag alloys were evaluated. Extrusion generally led to a finer microstructure and was associated with lower degradation rates, imbalancing the silver release. Therefore, we evaluated higher silver contents in this study. We found that Mg-Ag alloys possessed much higher degradation rates when the silver content reached in magnesium 8 wt%. The high pH, osmolality, and silver concentration had negative effects on the viability of human primary osteoblasts, although good antibacterial properties were shown in dynamic conditions. T4 heat treatment decreased the degradation of Mg-Ag alloys. As a result, human primary osteoblasts could survive on the surface of T4-treated Mg-Ag alloys. Meanwhile, the Mg-Ag alloys still had a much better antibacterial effect than pure Mg.

Silver has a major influence on the degradation behavior of Mg-Ag alloys. Since it has low solubility in magnesium at ambient temperature [41], more secondary phases

or precipitates exist in Mg-Ag alloys when the silver content is higher [56]. Both the amount and distribution of precipitates can affect the degradation behavior due to the principle of microgalvanic corrosion [12, 54]. The degradation rate increases linearly with increasing quantity of precipitates and the precipitates can cause localized degradation phenomenon [57]. For example, the extruded Mg-8Ag had higher degradation rate than the extruded Mg-6Ag. T4 treatment near the eutectic temperature can eliminate the secondary phases and precipitates. Since the $Mg_{54}Ag_{17}$ precipitates did not have the ability to restrict the migration of grain boundaries at high temperature, the grains were enlarged during T4 treatment. The elimination of precipitates weakened galvanic corrosion, so the degradation rate decreased [12]. For pure Mg and T4-treated Mg-Ag alloys, they had different grain sizes and both of them had no precipitates inside. T4-treated Mg-6Ag had nearly the same degradation rate compared to pure Mg. T4-treated Mg-6Ag had bigger grains than pure Mg, so grain size was not the key factor to influence the degradation rate. T4-treated Mg-6Ag and T4-treated Mg-8Ag had no significant difference of grain size. However, T4-treated Mg-8Ag had higher degradation rate than T4-treated Mg-6Ag to some extent. In this case, solid solution of silver in magnesium influenced the degradation rate when it reached 8 wt%. The pure Mg and T4-treated Mg-Ag alloys had lower degradation rates than the extruded Mg-Ag alloys in CCM. T4-treated Mg-Ag alloys had more homogeneous and flatter degradation surfaces and lower pitting degradation trends than pure Mg in the flow chamber according to the 3D images, even though the low pH (7.2) of BCM had an adverse effect on the stability of the degradation layer [57]. The morphology difference was related to the solution of silver and microstructural changes via T4 treatment. Hence, the existence of precipitates mainly influenced the degradation behavior followed by solution of silver rather than grain size.

The degradation rate of Mg-Ag alloys can influence the pH and osmolality of the medium and silver release, which are closely related to cytocompatibility [43]. A high degradation rate largely leads to increased pH, osmolality, hydrogen generation and silver release. However, the pH will not always increase linearly with the degradation rate because of a "saturation effect". The increment of osmolality is because of Mg ion release instead of Ca and other ions. According to the MTT and adhesion test, high pH, osmolality, and silver contents can cause cytotoxicity to human primary osteoblasts, for example, extruded Mg-8Ag. It is also not easy for cells or bacteria to attach to the surface due to the hydrogen generation, although good antibacterial properties can be obtained. For pure Mg, the cytotoxicity of the primary extract is caused by the high pH and osmolality. Similarly, pure magnesium cannot rely on a high pH to achieve its antibacterial effect, regardless of cytotoxicity, because high osmolality or magnesium ion concentration causes osmotic shock in human cells [58]. Therefore, a high degradation rate is not required of magnesium alloys used as orthopedic implants, and the degradation rate should be controlled to meet the cytotoxicity criteria first.

The biofilm assay was conducted under flowing conditions. The in vitro design of the dynamic system with large numbers of bacteria in the flowing medium represents harsh conditions, although the in vivo conditions normally show clearly lower bacteria concentrations. The flowing conditions and pH control system can exclude the pH effect of the corroding Mg alloys as much as possible. Pure Mg did not show satisfactory antibacterial properties under these conditions. Based on the viability of bacteria on pure Mg discs, there are still considerable numbers of live bacteria that should not be neglected. From the overview of the biofilm and bacteria distribution on the discs, it appears that pure Mg has the potential to form many colonies or a biofilm layer under suitable conditions, although the total amount of bacteria is less than that on the negative control groups [59]. Admittedly, the pH plays an important antibacterial role due to the alkaline environment created during degradation [9–11]. The alkaline environment has adverse effects on bacteria in static conditions. It is unclear whether pure Mg has sufficient antibacterial properties in these conditions in a dynamic environment, such as the human body.

There was a large amount of silver released from the extruded Mg-Ag alloys because of high degradation rate. As a result, they showed good antibacterial properties. However, the viability of bacteria was not as low as expected, for example, 99.9%. One reason is that a large portion of silver flowed away with the medium and another reason is that a large amount of bacteria (10^6/mL) exists in the medium, which indicates harsh conditions. The T4-treated Mg-Ag alloys got better antibacterial properties than pure Mg. T4-treated Mg-8Ag released more silver (twofold) than T4-treated Mg-6Ag, but bacteria viability decreased only by 4 percent due to the flow system of the bioreactor. In this case, less-released silver ions reacted with the bacteria attached to the surface of the discs. According to the decreasing trend of bacteria viability, if we continue to increase the silver content in pure Mg, for example, 15 wt%, more effective antibacterial properties of Mg-Ag alloy could theoretically be achieved in the bioreactor. However, the degradation rate approaches its limit at an acceptable range for orthopedic implants [60].

Silver ions can bind strongly and build complexes with thiols, metallothionein, albumins, and macroglobulins in vivo [29, 61, 62]. The antibacterial properties of Mg-Ag alloys are related to the silver concentration in the infection site, which is determined by the amount of silver in the Mg-Ag alloys and its release rate. If only a small amount of silver was released, the remaining silver was not sufficient to inhibit bacteria. In contrast, if the amount of active silver ions released was greater, the antibacterial properties would be more effective [25], so it is better to alloy as much silver as possible in pure Mg to ensure effective antibacterial properties on the basis of a controllable degradation rate. However, the total silver in the Mg-Ag alloy should not exceed the amount that can cause argyria in the human body.

5. Conclusion

In this study, the relationship between the microstructure, silver content, degradation behavior, cytotoxicity, and antibacterial properties of Mg-Ag alloys was revealed. The microstructure has a strong influence on the degradation

behavior of Mg-Ag alloys. The degradation rate will be high if there are many precipitates in the Mg-Ag alloys. To reach a lower degradation rate, the microstructure was adjusted via solution treatment (T4). As a result, precipitates dissolved into the magnesium matrix and the grains enlarged. T4-treated Mg-Ag alloys showed low degradation rate as pure Mg and more homogeneous degradation than pure Mg. The T4-treated Mg-Ag alloys had no discernible in vitro cytotoxicity to human primary osteoblasts compared with pure Mg. Moreover, the antibacterial properties depend on silver release. By increasing the silver content and controlling the degradation rate, the T4-treated Mg-Ag alloys showed good antibacterial properties in the bioreactor system with flow conditions and abundant bacteria inside.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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