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In vitro Degradation and biocompatibility of WE43, ZK60, and AZ91 Biodegradable Magnesium Alloys

C.L. Liu[1,2,a], J. Jiang[2], M. Wang[1], Y.J. Wang[3], Paul K. Chu[2,c],W.J. Huang[1,c],

[1] School of Materials Science and Engineering, Chongqing Institute of Technology, Chongqing 400054, China

[2] Department of Physics and Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, China

[3] School of Applied Science and Technology, Chongqing Institute of Technology, Chongqing 400054, China

[a]liuchenglong@cqit.edu.cn, [b]paul.chu@cityu.edu.hk, [c]huangweijiu@cqut.edu.cn

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Abstract. Successful application of magnesium alloys as degradable load-bearing implants is determined by their biological performance especially degradation and corrosion behavior in the human body. Three magnesium alloys, namely WE43, ZK60, and AZ91 are investigated in this work. The in *vitro* degradation behavior, cytotoxicity, and genotoxicity are evaluated by corrosion tests, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and micronuclei tests, respectively. Immersion tests indicate that the ZK60 alloy has the best corrosion resistance and lowest corrosion rate in Hank's solution, followed by AZ91 alloy and WE43 alloy in that order. The MTT results obtained from the three magnesium alloys after 7 days of immersion indicate good cellular viability. However, excessively high aluminum and magnesium concentrations have a negative influence on the genetic stability.

Introduction

As potential biomaterials in orthopedic implants, magnesium and magnesium alloys offer potential advantages to patients with bone fractures or defects compared to other inert metal implants. The advantages include smaller density, lower elastic modulus, inherent biocompatibility, and biodegradation through corrosion [1]. Up to now, researchers have paid more attention to the Mg-Al, Mg-RE, Mg-Ca, Mg-Zn alloy systems [2-5], and have mainly focused on the *in vitro* and *in vivo* corrosion degradation and biocompatibility. However, a shortcoming about magnesium alloys has been reported that some elements may have adverse effects on the human body, e.g. Al and RE. Aluminum is harmful to neurons and osteoblasts and also associated with dementia and Alzheimer disease [2]. In addition, the concentration of magnesium in the extracellular fluid ranges between 0.7 and 1.05 mmol/L, but too much magnesium may impose side effects mentioned in some studies [6]. Therefore, it is necessary to evaluate the biological safety of the corrosion products obtained from these alloys. There have been some reports on the cytotoxicity of magnesium alloys, e.g. binary Mg alloy [6], Mg-Zn-Mn [1], Mg and Mg4Y [7], and so on. In the aforementioned studies, the cytotoxicity of magnesium alloys has been mainly measured via cell death, inhibition of cell

growth, and cell proliferation. However, data concerning the genotoxicity of the extracted medium obtained from biodegradable magnesium alloys are scare. Gentoxicity is considered as an important factor when evaluating the bioactive and biocompatibility of implanted porperties [8].

In the experiments reported here, three Mg alloys, namely WE43, ZK60, and AZ91, were investigated. Their *in vitro* degradation behavior in the stimulated biological environment is studied here. The effects of magnesium and other metallic ions on the proliferation of Chinese hamster ovary (CHO) cell are monitored. Furthermore, in order to evaluate the influence on the genetic stabilization, their genotoxicity is determined by the micronuclei test (MNT).

Experimental details

Three magnesium alloys, Mg-Al-Zn (AZ91), Mg-Zn-Zr (ZK60), and Mg-RE (WE43), were studied. For the *in vitro* corrosion tests, cylindrical samples 15.0 mm in diameter and 4.0 mm long were machined from the alloys. The samples used in the immersion test were prepared by the standard technique of grinding with SiC abrasive paper and polishing with an Al_2O_3 suspension solution, followed by ultrasonic cleaning in acetone, absolute ethanol, and distilled water, respectively.

The immersion test was carried out at 37±1 ^oC Hank's solution. After different immersion periods, the samples were removed from the solution, rinsed and dried in argon. The morphology was observed by optical microscopy at time points of 6, 72 and 168 h. The corrosion morphology of AZ91 sample after 72 h immersion was characterized by scanning electron microscopy (SEM) using a JEOL-JSM-5600LV. The ion concentrations in each sample were measured three times after different immersion periods using a SHIMADZU atomic absorption flame emission spectrophotometer (AA6501S) at an excitation wavelength of 285.2 nm.

The three magnesium alloys (WE34, ZK60 and AZ91) after sterilization by ethylene oxide (ETO) were used in the immersion test. These alloys were immersed into 37±1 ^oC Hank's solution for 7 days. Afterwards, the extracted media without particles were used to conduct the MTT Assay. A 96 well-plate and CHO were used. 1 x 10^4 cells were seeded in each well. The cell were seeded in a 7-day extraction production and complete culture medium (1:9, 1:3 and 1:1 in volume) in the 96 well-plate. Two different time points (i.e. 24 h and 48 h) were used to measure the metabolic rate of the CHO. 10 µl of 5 mg/ml MTT solution was added and after incubation for 48 h, 100 µl of 10% SDS with 0.01 M HCl was added to dissolve the formed crystals. The absorbance was recorded at 570 nm with a reference wavelength of 640 nm.

The CHO cells were seeded on the samples at a concentration of 25,000 cm^{-2} in the extracted production medium for 2 days. Afterwards, the samples were rinsed with PBS, fixed with 2% paraformaldehyde, and immunofluorescently stained for the cytoskeleton protein f-actin with phalloidin-fluorescein isothiocyanate (Sigma). The nuclei were counterstained with hoechst33342. Pictures were taken with a digital camera (Carl Zeiss Axioplan 2).

The extracted medium from WE43, ZK60 and AZ91 after immersion in Hank's solution for 7 days was used. The cytokineses block MN technique developed by Fenech and Morley was employed to evaluate the formation of MN [9]. The cells which were treated with the solution for 2 days were dissociated by trypsinization and then seeded on 3.5 cm petri dishes. The medium was then replaced by one containing 2.5 µg/ml cytochalasin B from dreschslera dematioidea (CB, Sigma Cat no. C6762). The Chinese Hamster Ovary (CHO) were incubated for additional 24 hours, rinsed with PBS, and fixed in methanol/acetic acid [9:1(v/v)] for 20 min. The air-dried cells were stained with 10 µg/ml acridine orange (AO, Sigma Cat no. 158550) and observed under by fluorescent microscopy (Carl Zeiss Axioplan 2). The MN in the binucleated cells were scored and classified

according to standard criteria [10]. The yield of MN, Y_{MN}, was calculated as the ratio of the number of MN to the number of binucleated cells. At least 500 binucleated cells were scored in each sample for the MN measurement in this experiment. The statistical significance was determined using Student's paired *t* test. A *p* value of <0.05 was considered to represent a significant difference between values.

Results and Discussion

The corrosion morphology is monitored by optical microscopy and the results are shown in Fig. 1. After 6 h immersion in Hank's solution, obvious corrosion attack is apparent. With increasing immersion time, more corrosion holes are formed with deposition of salts including O, Mg, Ca, P, etc., as confirmed by the EDS results displayed in Fig. 2. The observation is similar to previously reported ones [11,12]. With longer immersion time, the AZ91 and WE43 alloy samples are corroded more severely, especially after 7 days immersion. The diameter of the pitting holes on the AZ91 sample increases rapidly to approximately 10 μm. On the contrary, the pitting holes on the WE43 sample do not enlarge, but they may suffer uniform attack. In the AZ91 sample, the small volume fraction of the β phase mainly serves as a galvanic cathode and accelerates the corrosion process of the α –matrix [13]. When the surface film is broken down, the corrosive solution attacks the surface and the α–matrix and new galvanic corrosions are established self-accelerating further dissolution of the α–matrix. It may be the reason why AZ91 suffers severe corrosion attack. The rare earth elements in the WE43 sample may accelerate the kinetics of the formation of MgH_2 during immersion, which gradually decomposes to form $Mg(OH)_2$ in the solution [14]. This process can inhibit hydration of the surface film and acts as a barrier against further corrosion. Moreover, the standard potentials of most rare earth elements are very close to that of magnesium [15], thus resulting in decrease in galvanic corrosion. Compared to the foregoing two alloys, the ZK60 alloy shows slower corrosion rate and the surface film remains almost intact after 7 days of exposure illustrating that further corrosion is effectively impeded. The variations in the corroded morphologies can be further confirmed by measuring the amount of released magnesium ions as shown in Fig. 3. The concentration of magnesium ions for ZK60 is about 430.45±4.8 μg/l after 7 days of exposure, which lower relative to the WE43 samples throughout the immersion period, based on the relative content of Mg in three alloys. The ZK60 alloy is mainly composed of 5% Zn, 0.5% Zr, and Mg. The alloying element, Zn, in the ZK60 alloy can decrease the impurities to very low levels through the precipitation effect, and the homogeneity of ZK60 alloy can be improved [16]. The higher purity means decreased micro cathode sites which are also found in pure magnesium. In addition, the greater Zn content in the ZK60 alloy than in the other two alloys may also provide better protection. Zn participates as phosphate forming the surface film on the alloys during immersion [3].

The extracted media from WE43, ZK60, and AZ91 after 7 days immersion in Hank's solution are selected to further investigate the cytotoxicity by examining both the viability and morphology of CHO cells. The extracted media is serially diluted to 50%, 25%, and 10% concentrations by addition of the control medium. Fig. 4 shows the cell viability cultured in different extracted media from the three alloys after 24 and 48 h. The extracted media of WE43, ZK60 and AZ91 alloys cause slightly reduced cell viability in comparison with the negative control for approximately 10%. It can be seen that the cell viability is negatively influenced by the higher extract concentration from the three magnesium alloys, but there appears to be no significant difference among them. The CHO cell morphologies are shown in Fig. 5 after culturing in the extracted media for 48 h. All the results obtained from the WE43, ZK60, and AZ91 extracted media inidcate healthy cell morphology with a flattened spindle shape, which is similar to that in the control medium.

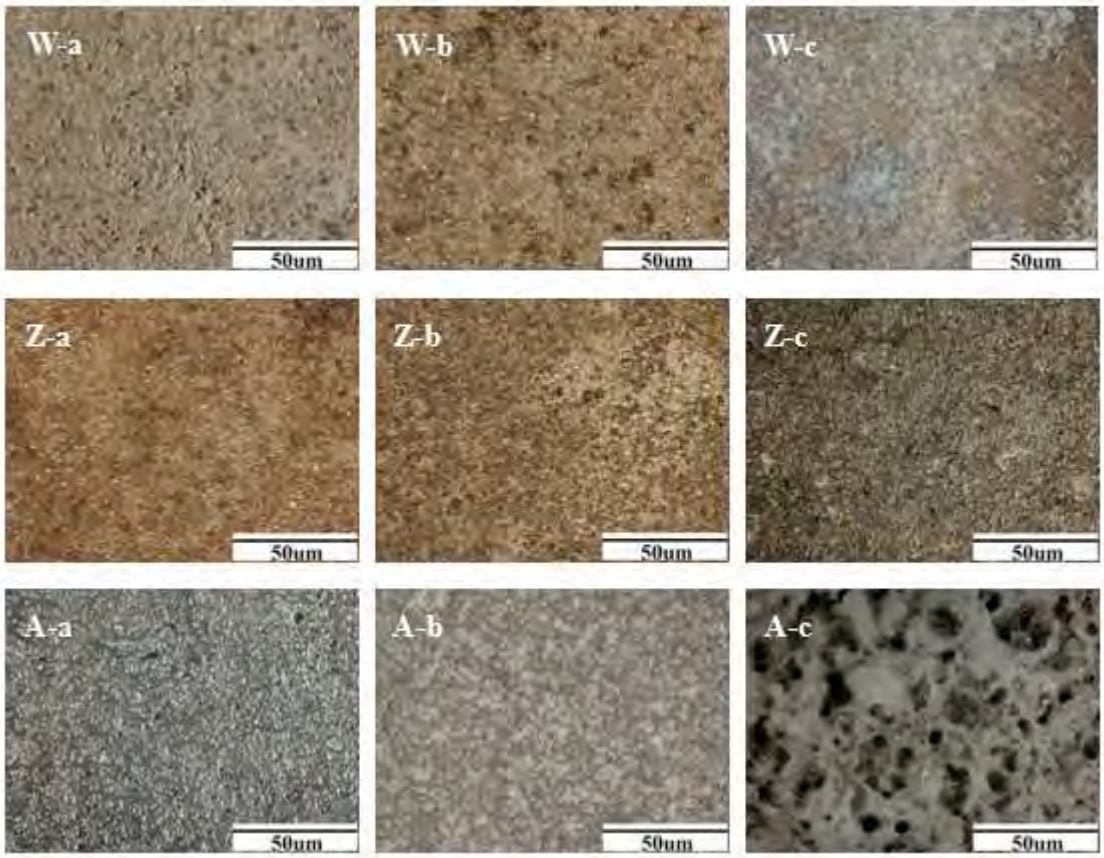

Fig. 1. Optical micrographs of WE43, ZK60, and AZ91 alloys in Hank's solution after different immersion periods: (a) 6 h; (b) 3 days; (c) 7 days. W, Z and A corresponds to WE43, ZK60 and AZ91, respectively.

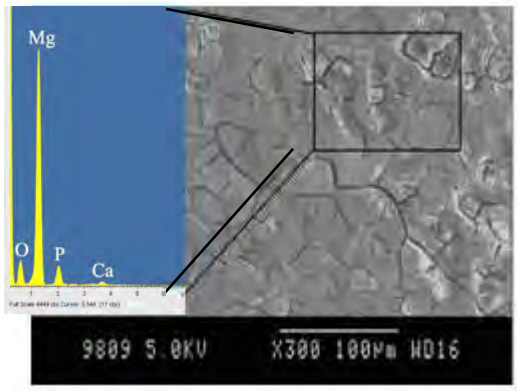

Fig. 2. SEM corrosion morphology of AZ91 sample after 3 days exposure in Hank's solution and EDS spectra obtained from selected regions denoted by rectangle.

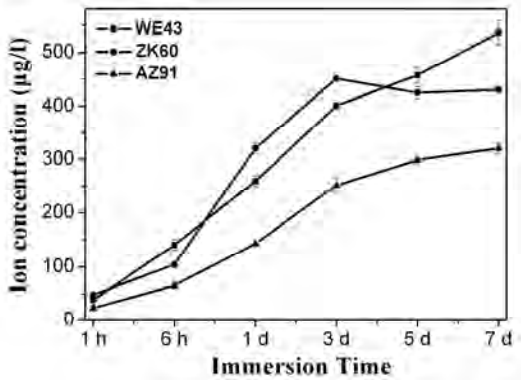

Fig. 3. Variations of magnesium ion concentrations during immersion in Hank's solution from the WE43, ZK60, and AZ91 alloys,

The aforementioned results disclose that the CHO cells show normal gorwth and reduced viability in the three magnesium alloys extraction media compared to the control. In the cytotoxicity tests, particles in the extracted medium are excluded. Therefore, the ions, Mg, Al and Zn ions for AZ91, Mg, Zn and Zr ions for ZK60, and Mg, Y and RE ions for WE43 in the extracted medium may be the main factor influencing the *in vitro* cytotoxicity evaluation. The results by atomic absorption flame emission spectrophotometry indicate that all the extracted media are composed of mainly magnesium with small amounts of other ions. The Mg ion concentrations in the WE43, ZK60 and AZ91 extracted media are 536.4±10.8 μg/l, 430.5±4.8 μg/l, and 320.2±4.5 μg/l after 7 days

exposure, respectively. The concentrations of other ions, such as Al, Zr, Y, etc, are lower than 0.1 μg/l. Therefore, it was reasonable to believe the existence of Mg ions in the extracted medium is a dominant factor in the three magnesium alloy cytotoxicity test. The results of cell viability for three magnesium alloys are similar to that of Mg-1Al, Mg-1Zr and Mg-1Y [6]. Toxicity effect has been reported for the L-929 cellular response to Al^{3+} at 4.18 mM/l, Y^{3+} at 10^{-4} M/l, and Zr ions at 10^{-3} M/l, respectively [17,18]. Therefore the lower ion concentrations in the extracted medium after 7 days immersion render no significant cytotoxicity to the CHO cell line and cause no significant difference in the cell viability as well as growth morphology.

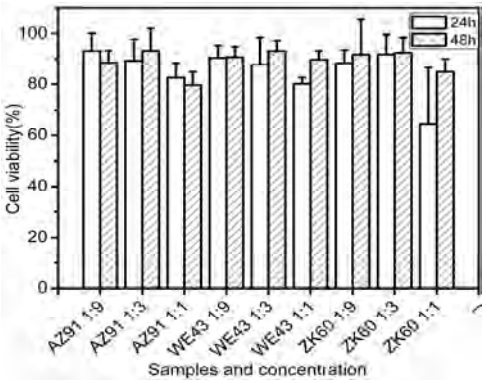

Fig. 4. CHO viability after 24 and 48 h of culture in WE43, ZK60, and AZ91 alloy extracted media after 7 days immersion, respectively, and the signs of 1:9, 1:3 and 1:1 corespond to 10%, 25% and 50% diluted alloy extracted medium by addition of the control medium.

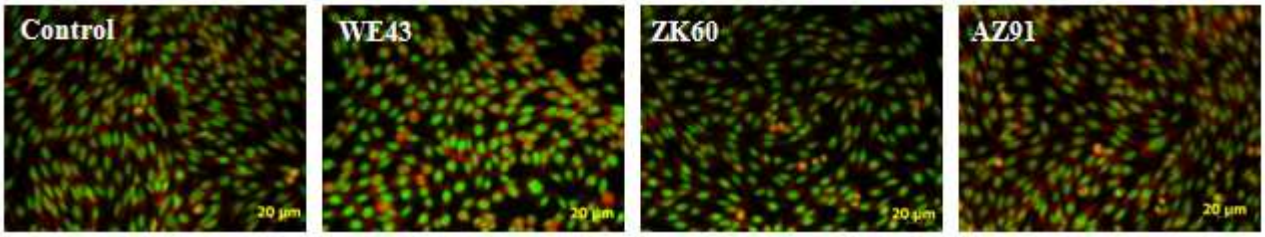

Fig. 5. Optical morphologies of CHO cells cultured in the WE43, ZK60, and AZ91 alloy extraction in Hanks' solution after 7 days immersion and complete culture medium (1:9) for 48 h.

Fig. 6 displays the morphometrical assay by immuneofluorescent staining of the cytoskeleton protein f-actin (phalloidin-FITC, green). The nuclei counterstained with hoechst33342 (blue) show that there are no significant differences in the cell morphology between three magnesium alloys. Therefore, MN studies are performed to evaluate the potential genotoxicity. Fig.7a shows one of the typical CHO MN seeded in the complete medium for 48 h. Some of the CHO become binucleated cells (indicated by white arrows) after further culturing for 12 h in the complete medium containing 2.5 μg/ml CB. In addition, some of binucleated cells produce MN as shown by the red arrow. The cytoplasm is stained red whereas the nuclei and MN are stained green and yellow.

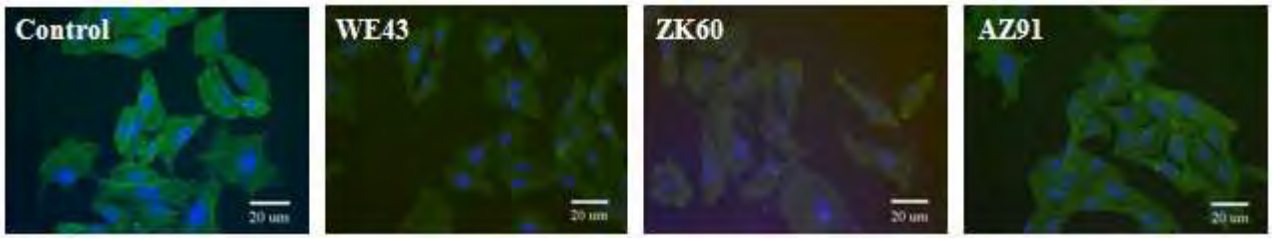

Fig. 6. CHO seed in the control and 50% concentration WE43, ZK60 and AZ91 alloy extracted medium after 7 days immersion for 48 h, stained immunofluorescent for the cytoskeleton protein f-actin (phalloidin-fluorescein Isothiocyanate, green), and the nuclei counterstained with hoechst 33342 (blue).

Gentoxicity can be assayed by MNT, because MN can embody DNA damages including base damage, DNA double strand breaks and phophodiester backbone damage [19]. In higher eukaryotes, inactivating an essential gene of a single nonrepaired damage can be sufficient to cause cell death via apoptosis. In addition, if the repair protein fails to repaire DNA damage correctly, genetic instability, gross chromosomal rearrangenments, and accumulation of mutations can result. These events then trigger cell-cycle checkpoints resulting in permanent growth arrest or death of affected cells. If the checkpoints are inactivated by mutations, tumorigenesis ensues. It have been mentioned that magneisum can stabilize the structures of DNA [20]. However, very high concentrations of magnesium and other metal elements especially aluminum may cause genotoxicity which is an important factor when evaluating the biocompatibility of implanted materials. DNA damage can be embodied by the increase in the MN ratio [8]. The MN ratios of CHO treated by the Mg alloy extracted media are shown in Fig. 7b. Differences in the micronulcei ratio are found from cells incubated in the extracted medium. The cellular MN ratios treated by W9:1, Z9:1 and A9:1 are 26.9±0.99, 28.3±1.4 and 26.3±3.7 indicating that W9:1 and Z9:1 corrosion products can induce cellular DNA damage. The celluar MN ratios treated by W1:1, Z1:1 and A1:1 are 42.1±1.28, 38.7±2.4 and 119.1±3.7. The Mg ion concentration is also investigated and this results reveal that the micronuclei ratio and Mg conerntration in the culture medium have a positive correlation. In another words, overly high concentrations of Mg ions lead to a high ratio of DNA damage. The highest MN ratio (119.1±3.7) is detected from the A1:1 sample. The possible reason is irritation by the increased Al ion concentration in the culture medium. The results of MN assay reveal that W43 and ZK60, especially AZ 91 alloys can induce potential genotoxicity. Excessively high concentrations of Mg and other ions especially Al ion released from the Mg alloys should be considered because they are protentially harmful to the human body.

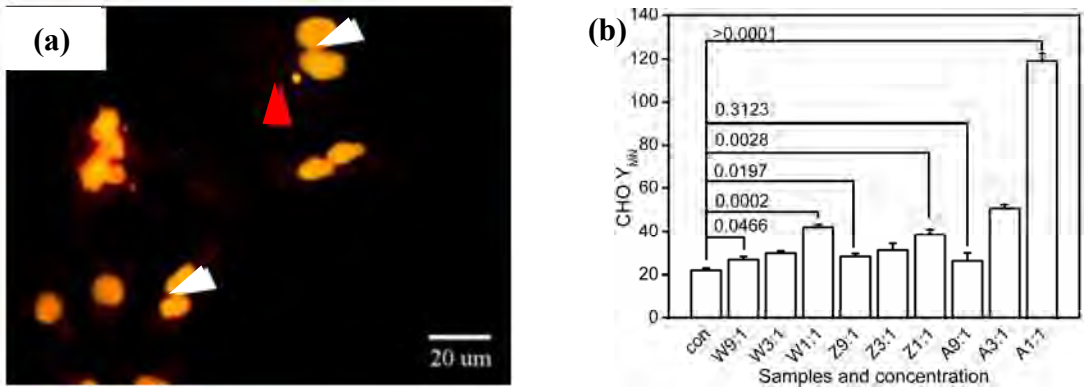

Fig. 7. (a) One of the typical CHO cells binucleated and MN (400 x), indicated by white and red arrows, respectively; (b) MN ratio of CHO cell induced WE43, ZK60, and AZ91 alloy extraction in Hanks' solution after 7 days immersion and complete culture medium (1:9,1:3 and 1:1) . Comparison of Y_{MN} between different samples is made by student's *t*-test.

According to the MTT results, the viability data do not appear to supply adequate information with regard to the genotoxicity of the materials surface, even though potential genotoxicity exists. Firstly, according to our studies, the MTT data are not sensitive enough to reflect the cellular behavior in the tested culture medium. Secondly, when cellular chromosomes are broken by some irritations, there are many mechanisms to repair the lesions. There is the possibility that these lesions fail to be repaired and then most of the cells will die via apoptosis. Then it is possible viability data will show the difference.

Conclusions

The *in vitro* degradation behavior and biocompatibility of WE43, ZK60, and AZ91 Mg alloys have been investigated. The composition and surface films determine the degradation behavior in Hank's solution. ZK60 shows the best corrosion resistance and anti-corrosion stability in Hank's solution, followed by AZ91 alloy and WE43 alloy. The cytotoxicity evaluation using CHO cells indicates that the three magnesium alloys do not induce cytotoxicity, but the ion concentrations and incubation time influence cell viability. Moreover, the concentration of the three alloy extracted media influences the cellular micronuclei ratio. The MN level is almost the same for the same concentration of ZK60 and WE43, but 50% AZ91 extration medium causes the highest ratio of DNA damage. In conclusion, overly high Mg ion and Al ion concentrations in the culture medium contribute to increased cellular DNA damage.

Acknowledgements

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References

[1] F. Witte, J. Fischer, J. Nellesen et al: Biomaterials Vol. 27 (2006), p. 1013.
[2] Z.J. Li, X.N. Gu, Y.F. Zheng: Biomaterials Vol. 29(2008), p. 1329.
[3] E.L. Zhang, D.S. Yin, L.P. Xu et al: Mater. Sci. Eng. C Vol. 29 (2009), p. 987.
[4] Y.W. Song, D.Y. Shan, R.S. Chen et al: Mater. Sci. Eng. C Vol. 29 (2009), p. 1039.
[5] R.C. Zeng, W. Dietzel, F. Witte et al: Adv. Biomater. Vol. 10 (2008), p. B3.
[6] X.N. Gu, Y.F. Zheng, Y. Cheng et al: Biomaterials Vol.30 (2009), p. 484.
[7] J. Fischer, M.H. Prosenc, M. Wolff et al: Acta Biomater. Vol.2009
[8] J. Jiang, K.F. Huo, P.K. Chu: Biomaterials Vol. 29 (2008), p. 544.
[9] M. Fenech, A.A. Morley: Mutat Res-Rev Mutat Res Vol. 161(1986), p. 193.
[10] R.J. Albertini, D. Anderson, G.R. Douglas et al: Mutat. Res.-Rev. Mutat Res Vol. 463(2000), p. 111.
[11] Y.C. Xin, C.L. Liu, P.K. Chu: J. Mater. Res. Vol. 22(2007), p. 2004.
[12] L.P. Xu, E.L. Zhang, D.S. Yin et al, J. Mater. Sci.: Mater. Med. Vol. 19 (2008), p. 1017.
[13] G. Ballerini, U. Bardi, G. Ceraolo: Corr. Sci. Vol. 47 (2005), p. 2173.
[14] G.G. Perrault, in: A.J. Bard (Ed.): Encyclopedia of Electrochemistry of the Elements, Vol VIII, Marcel Dekker, New York, 1978.
[15] R. Arrabal, E. Matykina, F. Viejo: Cor. Sci. Vol. 50 (2008), p. 1744.
[16] G.L. Song, A. Atrens: Adv. Eng. Mater. Vol. 1 (1999), p. 11.
[17] J.C. Wataha, C.T. Hanks, R.G. Craig: J. Biomed. Mater. Res. Vol. 26 (1992), p. 1297.
[18] A. Yamamoto, R. Honma, M. Sumita: Mater. Res. Vol. 39 (1998), p. 331.
[19] J.W.G. Van Putten, H.J.M. Groea, K. Smid et al: Cancer. Res. Vol. 61 (2001), p. 1585.
[20] K.K. Khanna, S.P. Jackson: Nat. Genet. Vol. 27 (2001), p. 247.