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Letter

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Influence of various sterilization methods on hardness, grain size and corrosion rate of a Mg6Ag-alloy

Abstract: Sterilization is a necessary step for all implant materials. Different methods can influence the materials properties. Especially important for magnesium as degradable materials is the determination of the corrosion properties. In this study the influence of 70% ethanol, glutaraldehyde, autoclaving, dry heat, UV-, gamma- and electron beam-irradiation on mechanical and corrosion parameters were analyzed. As mechanical parameters hardness and grain size were determined. The corrosion rate under physiological conditions, weight of the corrosion layer and corrosion morphology was determined. It could be demonstrated that irradiation treatments and 70% ethanol are suitable methods, as they decrease the corrosion rate. Heat-introducing methods (autoclaving and dry heat) acted as incomplete ageing treatments on this alloy and therefore increased the corrosion rate. Furthermore, osmolality showed a better correlation to the actual corrosion rate than the pH. Therefore an optimum ratio between alloying system, implant and sterilization method has to be established, depending on the intended application.

Keywords: heat treatment; irradiation sterilization; liquid chemical sterilization; magnesium alloy; physiological conditions.

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Introduction

Magnesium alloys specially designed for biomedical applications have been intensively studied [1, 2] and the first clinical applications have been approved [3]. However, different applications need various shapes and processing steps: e.g., the use for sutures involves casting and two concomitant extrusion steps, which have a huge impact on microstructure and mechanical properties [4, 5]. It is widely accepted that any applied processing step has an influence on the material properties and due to the structure-property relationship also on the corrosion rate [6, 7]. Especially for medical applications there are some further important requirements for the final implants before implantation like cleaning, sterilization and packaging, which have not yet been widely considered or studied. A variety of sterilization methods for medical products is available and could be applied: (1) liquid chemical sterilization [8], (2) moist heat [9], (3) dry heat [10], (4) radiation [11], (5) ethylene oxide [12] and (6) formaldehyde [13]. Due to the susceptibility of magnesium and its alloys to water all sterilization methods including liquids should be critically analyzed. Moreover, heat and radiation can also induce alterations in the material, which is an exclusion criterion for many polymers [14].

The choice of an appropriate sterilization method furthermore has to be adapted for feasibility and clinical acceptance. To the author's knowledge, there are only three recent studies concerning the influence of sterilization on magnesium alloys, two from which are describing the influence on the mechanical properties [7, 15] and the other one determined changes in surface free energy and the influence on cyto- and hemocompatibility [16]. None of these studies took into account that a possible induced material alteration could also influence the corrosion rate of the material. The aim of our study therefore was to analyze the effect of various sterilization techniques (70% ethanol, glutaraldehyde, autoclaving, dry heat, UV-, gamma- and electron beam-irradiation) on the hardness as a mechanical

characteristic value and on the corrosion rate under physiological conditions. The chosen alloy is a silver-containing magnesium alloy (Mg6Ag, 6 wt% silver), which was developed especially for biomedical applications [17]. Moreover the size effect was taken into account, as present implant applications deal mainly with small dimensions like screws or stents. Therefore small discs were analyzed.

Materials and methods

Material production

High purity magnesium (99.99%, Xinxiang Jiuli Magnesium Co. Ltd, Henan, China) and silver (99.99%, ESG Edelmetall-Handel GmbH & Co. KG, Rheinstetten, Germany) were used to prepare the Mg6Ag ingots by melting in a steel crucible at 750°C with protection of Argon+2% SF_6. The melt was stirred at 200 rpm for 25 min. Afterwards the temperature was decreased to 730°C, the melt was poured into a preheated mild steel mould and held for 15 min at 680°C. The solidification process was carried out by chilling the mild steel mould in flowing cooling water at a rate of 100 cm/min. After removing the upper and lower parts with shrinkage and debris solution treatment (T4) was done in a resistance furnace (Linn Elektro Therm, AK 40. 06, Bad Frankenhausen, Germany) at 430°C for 16 h. Rods with a diameter of 12 mm were produced by hot extrusion (Strangpresszentrum Berlin, Berlin, Germany). The ingots were heated up to 285°C before hot extrusion was carried out. The temperature of the extrusion die was 300°C. The extrusion ratio and stamp advance rate during extrusion was set to 108 and 0.7 mm/s respectively. Afterwards the rods were again T4-treated (16 h at 430°C). The resulting rods were machined to 10 mm diameter and samples with a height of 1.5 mm were produced by cutting-off. Surface finishing was performed by lapping (Henschel KG, Munich, Germany). The samples were delivered as received to the different sterilization methods without further surface treatment.

Alloy composition

The actual overall alloy composition was analyzed via ICP-OES (Spectroflame spark analyzer, Spectro, Kleve, Germany) after dissolution of the samples in concentrated nitric acid (1:32,000). The amount of silver was determined by X-ray fluorescence analysis (Bruker AXS GmbH, Karlsruhe, Germany).

Sterilization methods

Several sterilization methods were applied. For each method 15 samples were used. The denomination for each method is given in brackets and will be used throughout the manuscript.

1. 70% Ethanol (EtOH): samples were immersed in 15 mL Falcon tubes (Greiner Bio-One, Frickenhausen, Germany) containing 3 mL of 70% ethanol (Merck Millipore, Darmstadt, Germany and sonicated in an ultrasonic bath for 20 min. Afterwards the samples were transferred to 24-well plates (Greiner Bio-One, Frickenhausen, Germany) under sterile conditions and air dried for 30 min.
2. Glutaraldehyde (GA): a 2% glutaraldehyde solution was prepared by diluting from a 25% stock solution (Sigma-Aldrich Chemie, Taufkirchen, Germany) in distilled water. Samples were immersed in 15 mL Falcon tubes in 3 mL solution and left under the fume hood for 3 h. The transfer was the same as for ethanol.
3. Autoclaving (AC): samples were individually shrink-wrapped in sterilization bags (SteriClin, Vereinigte Papierwarenfabriken GmbH, Feuchtwangen, Germany) and exposed to an autoclave run (121°C, 2 bar pressure, 20 min).
4. Dry heat sterilization (DH): samples were sterilized in borosilicate glass test tubes (Duran Group, Mainz, Germany) capped with aluminium foil in a preheated oven (Memmert, Schwabach, Germany) at 200°C for 2.5 h.
5. UV-irradiation (UV): samples were exposed to UV light (wavelength: 253.7 nm) in 12-well plates (Greiner Bio-One, Frickenhausen, Germany) in a biological safety cabinet (Airstream, Esco Technologies, Hatboro, PA, USA). Exposure was performed for 30 min on each side of the sample.
6. Gamma-irradiation (GI): samples were individually shrink-wrapped in sterilization bags and sterilized at a dosage of 27.6 kGy (BBF Sterilisationsservice, Kersen, Germany).
7. E-beam-irradiation (EBI): samples were individually shrink-wrapped in sterilization bags and sterilized by a 10 MeV e-beam at a dosage of 27.2 kGy (Herotron, Bitterfeld, Germany)
8. Control (C): unsterile samples as received without any further treatment

Prior to the sterilization the samples were weighed (initial weight, W_i) using a high precision scale (Sartorius, Goettingen, Germany). After the sterilization the weight was determined again (sterilization weight, W_s) under sterile conditions. The weight change (ΔW_s) induced by the sterilization methods was calculated by the following formula:

$$\Delta W_s = W_i - W_s \quad (1)$$

Determination of hardness

After sterilization Vicker's hardness (HV 5) was measured using a micro-hardness tester (Emcotest Prüfmaschinen, Kuchl, Austria) according to [18] with a load factor of 49.07 N. Before testing the samples were grinded with a 2500 abrasive paper for some seconds. The hardness was measured on 5 samples per sterilization method at 5 different positions, taking care of the minimum distance to other indentations and the sample's edge. Optical evaluation of the indents was performed by the instrument and the average values for each sample were used for calculations.

Microstructural analysis

To perform the analysis of microstructure samples were embedded in Demotec 30 (Demotec Demel e.K., Niddau, Germany), grinded for 3 min at 145 rpm with 2500 grinding paper, and polished for 60 min

at 150 rpm with waterfree OPS-solution (Struers, Willich, Germany) under nitrogen atmosphere. Afterwards the samples were shortly etched in a picric acid solution (35 mL H_2O, 6.5 g acetic acid and 5 g picric acid in 140 mL ethanol; all solutions VWR International, Darmstadt, Germany [19]). Optical light polarization microscopy was performed on a light microscope with 1000× magnification (PM, 020-520-008 DM/LM, Leica GmbH, Wetzlar, Germany). Grain sizes (mean square grain size) were determined by the line-intercept method from the microstructure micrographs using AnalySIS software (Olympus Soft Imaging Solutions, Muenster, Germany).

Determination of corrosion rate, pH and osmolality

Before and after sample sterilization the weight of the samples was determined as described above (W_i and W_s, respectively). The initial pH (SENTRON ARGUS X pH-meter, Fisher Scientific GMBH, Schwerte, Germany) and osmolality (Osmomat 030, Gonotec, Berlin, Germany) for the immersion medium (Dulbecco's modified eagle medium; DMEM Glutamax, Life Technologies, Darmstadt, Germany with 10% fetal bovine serum; FBS, PAA laboratories, Linz, Austria) was recorded.

Fifteen samples of each sterilization methods were immersed in agarose-coated 12 well plates in 3 mL corrosion medium (In average 0.07 g sample/mL corrosion medium) for 72 h under cell culture conditions (37°C, 20% O_2, 5% CO_2, 95% rH). At the end of each experiment the pH-value and osmolality of the supernatant was measured. To determine the change of these values induced by incubation, three wells were filled only with medium without samples were used as controls.

After immersion in the corrosion medium, samples were rinsed with distilled water and dried in a vacuum oven (Salvis Lab, Rotkreuz, Switzerland) at 37°C overnight. Afterwards the weight of the samples in dry state (W_{ds}) was determined.

Subsequently the formed corrosion products were removed by treating the corroded disk with Chromic acid (180 g/L in distilled water, VWR international, Darmstadt, Germany) for 20 min at room temperature. Afterwards they were thoroughly rinsed in distilled water and 100% ethanol. The samples were again dried overnight and the weight after removal of corrosion products (W_{rcp}) was determined. The weight of the corrosion layer (W_{cl}) was calculated using the equation:

$$W_{cl} = W_{ds} - W_{rcp} \quad (2)$$

The corrosion rate was calculated in mm/year using the equation:

$$CR = 8.76 \times 10^4 \, \Delta g / A \cdot t \cdot \rho \quad (3)$$

where Δg is the weight change in grams ($W_i - W_{rcp}$), A is the surface area of the sample in cm^2, t is the immersion time in hours, and ρ is the density in g/cm^3.

Statistical analysis

Statistics were performed using the SigmaStat package (Systat software GmbH, Erkrath, Germany). Standard analyses comparing more than two conditions were done by using one way analysis of variance (ANOVA) on ranks with Dunn's multiple comparison post hoc test.

Results

Alloy composition and impurities

The real alloy composition was quite close to the nominal composition, it contained a slightly higher amount than denominated by the nominal composition (6.3 wt% Ag). The impurity level was also determined and in an acceptable range (Iron (Fe): 0.00212, copper (Cu): >0.001, nickel (Ni): 0.001, silicon (Si): 0.00132, beryllium (Be): 0.00004, all in wt%).

Influence of sterilization on hardness, microstructure and grain size

Compared to the control (HV 5=52.8±3.8) most of the sterilization methods led to a slight decrease of the hardness values (Figure 1). The highest decrease was observed for dry heat (DH, 47.9±1.9), followed by UV (48.2±5.5), autoclave (49.5±3.2) and glutaraldehyde (49.6±5.3). However, the differences were not significant. The determination of grain sizes revealed that compared to the control (37.2 µm) gamma-irradiation (39.1 µm) and ethanol (40.1 µm) were in a similar range. All other treatments led to an increase of grain size. Glutaraldehyde treated samples showed an intermediate value (47.8 µm); UV and e-beam sterilization led to similar results (51.0 and 52.7 µm, respectively); autoclaving also led to an increase (51.2 µm) and dry heat led to the highest increase (54.6 µm). A good correlation could

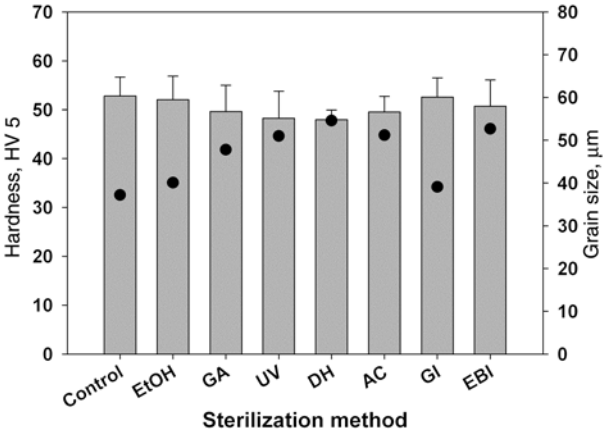

Figure 1: Hardness values (HV 5, vertical bars) and grain size (circles) of the samples after exposure to the different sterilization methods. No significant changes could be observed. A decrease of hardness was in all cases accompanied by an increase of grain size. The denominations are according to the terms described in materials and methods.

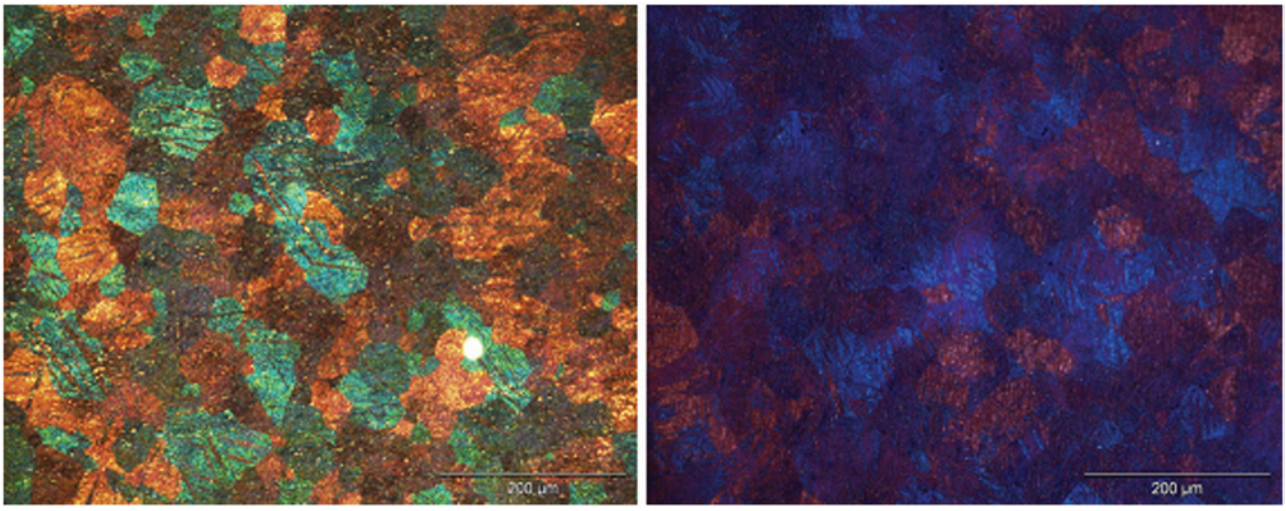

Figure 2: Examples for optical micrographs of the microstructure. Left panel: microstructure after dry heat treatment, right panel: gamma-irradiation. A coarse grain structure can be observed with only a low amount of second phases. Polarized light microscopy, 200× magnification.

be observed between grain size and hardness, increasing grain size led to a decrease of hardness (Figure 2).

Influence of the sterilization methods on the corrosion rate

Weight change due to corrosion

Nearly all of the sterilization methods led to an average weight gain in samples, except UV-sterilization, but this was associated by a high standard deviation (Average: −0.28±1.83 mg). The highest weight gain was observed for e-beam sterilization (1.13±1.85 mg), followed by dry heat sterilization (1.04±1.04 mg) and glutaraldehyde treatment (0.63±1.45 mg). Autoclaving led to the most reproducible results, observable in the lowest standard deviation (0.233±0.87 mg). The most inhomogeneous results were obtained with UV and e-beam irradiation (Figure 3). Although the statistical overall comparison with ANOVA on ranks indicated a significant difference in the value distribution ($H=15.7$, $p<0.05$), no significant differences between the treatments could be detected (Dunn's post-hoc test).

Corrosion rate determined by mass loss

The determined corrosion rates were compared against the unsterilized control (ANOVA on ranks, Dunn's post-hoc test, corrosion rate (CR) 0.94±0.47 mm/year). Dry heat dramatically increased the corrosion rate by a factor <6 (CR=6.47±0.94 mm/year; $Q=3.273$, $p<0.05$). Autoclaving also led to an increase of the corrosion rate (CR=1.72±0.32 mm/year), however the increase was not significant. In contrast, all other methods led to a decrease of the corrosion rate, which was significant for ethanol (CR=0.34±0.15 mm/year; $Q=4.43$, $p<0.05$), gamma-irradiation (CR=0.42±0.33 mm/year; $Q=3.299$, $p<0.05$) and electron beam irradiation (CR=0.42±0.36 mm/year; $Q=3.115$, $p<0.05$). Glutaraldehyde treatment showed values comparable to the control. The weight of the corrosion layers showed a highly correlated behavior. Significant differences observed for the corrosion rate were also observed for the weight of the corrosion layer.

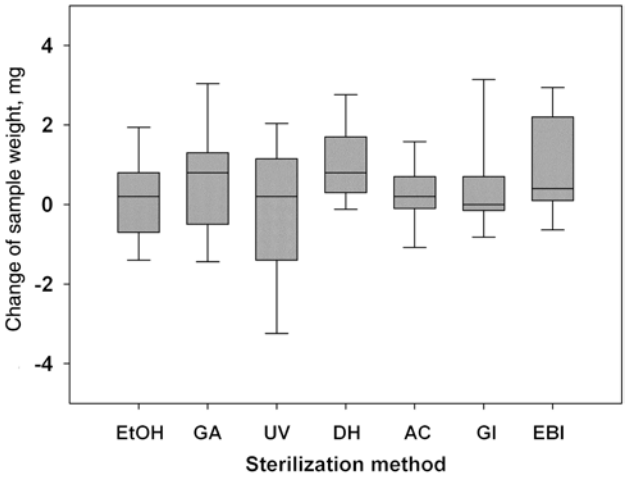

Figure 3: Box- and Whisker plot of the change of sample weight induced by the various sterilization methods. Given are the median and the 25%/75% percentiles. No significant differences could be detected between the groups.

Osmolality and pH

PH and osmolality are discussed as indicators for the corrosion rate. However, in this study both values were measured after 72 h, and at this time point saturation effects already could occur. The pH-value (Figure 4, bars) followed some trend of the corrosion rate: (1) a significant reduction compared to the control for ethanol-treatment ($Q=3.399$, $p<0.05$); and (2) a significant increase measured for dry heat and autoclave ($Q=3.866$ and $Q=3.484$, respectively; $p<0.05$). However, the pH-values compared between these two methods showed no significant differences, despite of the difference in the corrosion rate. The second observation was that although gamma- and e-beam irradiation showed a significantly lower corrosion rate, the pH was in the same range as the control. Compared to the pH, the osmolality (Figure 4, Scatter plot) seems to be a better indicator, as it showed a higher correlation to the corrosion rate. It showed a significant increase in dry heat ($Q=3.141$, $p<0.05$) and autoclaving ($Q=2.939$, $p<0.05$), as well as a significant decrease for gamma-irradiation ($Q=3.217$, $p<0.05$) and e-beam irradiation ($Q=2.918$, $p<0.05$). However, also with this method the lower corrosion rate of the ethanol treatment was not detected.

Corrosion morphology

Prior to the removal of corrosion products the corrosion layers showed some differences. With dry heat and autoclaving the material dissolution was already observable.

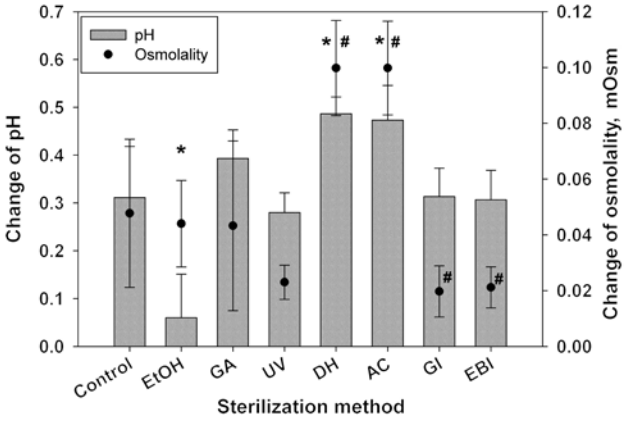

Figure 4: pH (horizontal bars) and osmolality (scatter plot) values measured for the different sterilization methods after 72 h. Given are the mean values±standard deviation. Significant differences (ANOVA on ranks, Dunn's post-hoc test against the control group) are indicated for pH as stars and for osmolality as hashtags.

Black areas, which indicate a highly porous layer, were partly observable after the application of all other sterilization methods, and especially after ethanol treatment. The formation of white corrosion products (which were determined to be presumably nesquehonite ($MgCO_3 \cdot 6\ H_2O$) in other studies) were observed after all treatments except dry heat and autoclaving. After removal of the corrosion products strong pitting corrosion could be observed after dry heat sterilization and autoclaving. The control exhibited a lot of small pits. After treatment with ethanol the amount of pits was reduced, but their size was larger including areas of strong pitting. The glutaraldehyde treatment led to more filiform corrosion morphology mainly in the center of the disc. A similar morphology could be observed after UV-sterilization, but with a more homogeneous distribution. Gamma irradiation led also to filiform corrosion which concentrated in the central region. The e-beam treatment showed a low overall corrosion, but very strong localized attack (Figure 5).

Discussion

The aim of our study was to analyze the influence of various sterilization techniques on the hardness, microstructure and corrosion behavior. The use of a control group without sterilization was performed for the first time, as normally under sterile conditions it is avoided to work with non-sterilized samples. A lot of care was taken that samples which showed bacterial contaminations were excluded from this group. Also to the authors knowledge this is the first study which has analyzed the influence of sterilization methods on the corrosion rate.

As expected the introduction of heat is detrimental to this alloy. This was especially observable for the application of dry heat at 200°C. For as cast material the experimentally determined and optimized ageing treatment (T6) was at 185°C for 8 h [20], leading to an increase in hardness and a slight increase of the corrosion rate. However, in the present study the temperature was higher and applied for shorter time. Moreover, a decrease of hardness was observed. According to the Hall-Petch relationship this indicates an increase of grain size, which was the highest of all treatments. This leads to the assumption that this sterilization treatment acts as an incomplete heat treatment leading only to grain coarsening and not to the intended formation of second phases, which would lead to an increase of hardness. Therefore it has a quite detrimental influence on the chosen alloy, especially regarding the corrosion rate. In contrast, for rare earth containing

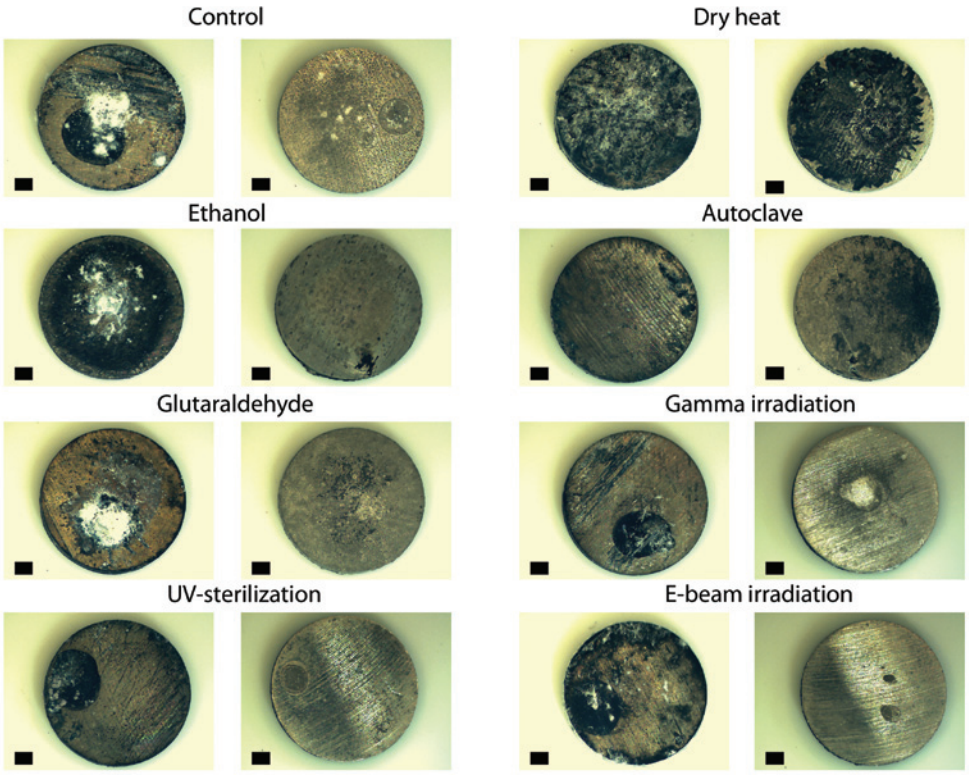

Figure 5: Morphology of the corrosion layers after immersion and drying (left images) and surface and corrosion morphology after removal of the corrosion products (right images). 65× magnification. Scale bar=1 mm.

alloys this temperature is in an appropriate range, but significant changes of the material properties occur after much longer time periods [21, 22]. In the study of Seitz et al. [15] this treatment showed the highest effect on the mechanical properties of LAE442, MgCa0.8 and pure Mg. Liu et al. [16] showed that dry heat increased hydrophilicity of the surface and led to a comparably higher surface free energy. According to the standard [10], the applicable temperature range is between 180 and 200°C, therefore this sterilization method could also be used as targeted heat treatment, when performed under appropriate conditions and validation. Autoclaving as standard method for (permanent) medical instruments seems also not to be applicable to this magnesium alloy, as the corrosion rate was increased by a factor of about 2. Here the combination of moisture and heat was responsible for the changes of grain size, leading to an inhomogeneous surface (Figure 5) and therefore inducing strong pitting corrosion. Taking together the results for silver-containing alloys dry heat and autoclaving cannot be recommended.

The use of liquid chemical sterilization methods showed very interesting results. Interestingly, while ethanol treatment led to grain sizes and hardness similar to the control, the glutaraldehyde treated samples samples showed bigger grains and accordingly a reduced hardness. This unexpected result will be analyzed in more detail in further studies. However, the weight change due to sterilization was higher with GA, indicating a thicker layer on the sample. In the only other study dealing with these sterilization methods, with ethanol the introduction of solely oxygen was observed, whereas with GA a high amount of carbon and small phosphorous contributions were observed, which decreased surface hydrophilicity and increased surface free energy [16]. While the influence of GA on the corrosion rate is negligible, ethanol led to a significant decrease of corrosion rate. The corrosion morphology for both treatments indicated a homogeneous corrosion layer. The pitting observed with ethanol can also be due to material inhomogeneity. The disadvantage of GA is the toxicity of the solution, which makes a lot of cleaning steps necessary afterwards [8]. Here the influence on cellular adhesion would be of high interest. In contrast, the treatment with 70% ethanol is easy to perform in the laboratory and much less time consuming. For cell tests it can be used as a standard method, when irradiation treatments are not available. However, the influence on corrosion was shown for the first time in this study. In the authors

laboratory this is a standard treatment prior to corrosion measurements and cell experiments as sterility under physiological conditions is of utmost importance. It should be mentioned that an increase in surface free energy [16] is favorable for cell adhesion.

The radiation treatments generally seem to be the most suitable sterilization methods for this alloy. Hardness was influenced by UV and e-Beam sterilization due to an increase of grain size, while gamma-irradiation only slightly increased the grain size of the samples compared to the control. UV also showed the highest impact (and standard deviation) on sample weight after sterilization (Figure 3). The corrosion morphology for all three methods is preferable for medical applications due to their homogeneity and the lowest amount of pitting (Figure 5). An exception was the e-beam treatment, where deep local pits were observable, but this can be due to material impurities evoked by extrusion comparable to ethanol, but this should be analyzed in further studies. This implies also that for medical applications the need for highly reproducible test samples is of utmost importance. As it was observed, irradiation treatments can lead to an increase of hydrophobicity and a reduction of surface free energy [16], which should have an effect on cell adhesion. Therefore the study of cell adhesion and survival is part of further studies. An interesting observation in this group was that the pH-value was not correlated to the corrosion rate, whereas the osmolality showed a good correlation (Figure 4). The reason for this behavior is not clear, but indicates a change of hydrogen release due to these sterilization methods compared to the control. Further examinations of the influence of irradiation therefore should be performed, as this is a commonly used method for medical implants capable of high-throughput.

As a further result of this study osmolality shows in most cases a good correlation to the corrosion rate, more likely than measuring the pH-value. Due to the intended use for cell culture application under physiological conditions the buffering capacity of the media is quite high already [23]. As it can be observed in Figure 4 there are some limitations – if the corrosion rate is too high, saturation effects are taking place, which lead to an increase in the weight of the corrosion layer, as a saturated solution is in equilibrium of the chemical processes (Figure 6). For this study an indication can be stated that with a given corrosion rate of about 2 mm/year already an immersion time of 72 h is too long to observe differences between this corrosion rate and higher ones. As a consequence, if osmolality is used as a screening parameter, an increase of 0.1 Osm indicates that the saturation limit is reached.

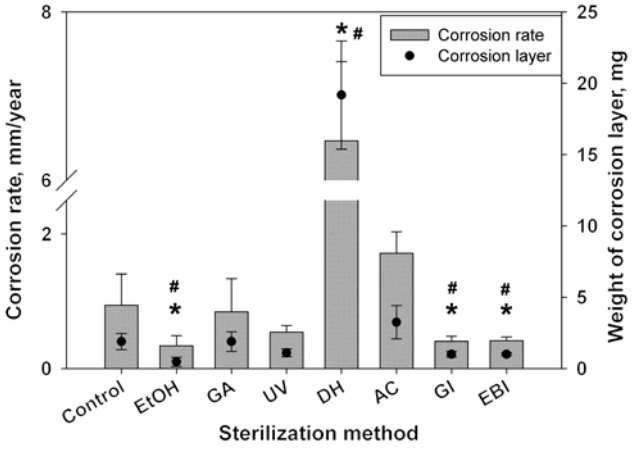

Figure 6: Corrosion rate and weight of the corrosion layer after immersion. A good correlation between the two values is observable. Significant differences ($p<0.05$) are indicated as stars (*) for the corrosion rate and as hashtags (#) for the weight of the corrosion layer.

Conclusions

The determination of mechanical properties alone is not sufficient to elucidate the influence of different sterilization methods. Corrosion rate, mechanism and morphology also have to be considered. Moreover, the influence of all methods has to be determined for each alloying system separately in terms of size effects, alloying system and final implant shape. Therefore an optimum ratio between alloying system, implant and sterilization method has to be established, depending on the intended application.

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