Analysis of metallic traces from biodegradation of endomedullary AZ31 alloy temporary implants in rat organs after long implantation times

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Abstract

AZ31 alloy has been tested as a biodegradable material in the form of endomedullary implants in female Wistar rat femurs. In order to evaluate the accumulation of potentially toxic elements from biodegradation of the implant, magnesium (Mg), aluminium (Al), zinc (Zn), manganese (Mn) and fluorine (F) levels have been measured in different organs such as kidneys, liver, lungs, spleen and brain. Several factors that may influence accumulation have been taken into account: how long the implant has been in place, whether or not the bone is fractured, and the presence of a MgF_2 protective coating on the implant. The main conclusions and the clinical relevance of the study have been that AZ31 endomedullary implants have a degradation rate of about 60 % after 13 months,

which is fully compatible with fracture consolidation. Neither bone fracture nor MgF_2 coating seems to influence the accumulation of trace elements in the studied organs. Aluminium is the only alloying element in this study that requires special attention. The increase in Al recovered from the sampled organs represents 3.95 % of the amount contained in the AZ31 implant. Al accumulates in a statistically significant way in all the organs except the brain. All of this suggests that in long term tests AZ31 may be a suitable material for osteosynthesis.

Introduction

Orthopaedic and cardiovascular medicine are generating a growing demand for medical devices (Manuel and Hort, 2011). In temporary orthopaedic and trauma surgery implants, magnesium (Mg) and its alloys exhibit better load-bearing mechanical properties than other biodegradable materials like polymers and ceramics (Blaker *et al.*, 2005; Rezwan *et al.*, 2006). They also have very similar elastic modulus and density values to bone (Staiger *et al.*, 2006), thus avoiding the occurrence of stress shielding. Mg and its alloys are furthermore of great interest because they do not need to be removed after fulfilling their purpose, being resorbable materials (Witte *et al.*, 2008). Their biodegradation process gives rise to corrosion products (ion release) that may be deposited on the implant surface or around the implant site, and may also emigrate and be recovered in urine, blood, plasma and in different organs far away from the implant. According to the current state of the art, the decomposition or corrosion products of a magnesium implant do not generally pose a health hazard. Only a burst release of these products, which could overburden the organism's regulating mechanisms and therefore lead to local physiologically detrimental conditions, is to be considered a problem (Seitz *et al.*, 2014).

This research considers the commercial alloy AZ31, whose mechanical properties and corrosion resistance are superior to pure Mg (Carboneras *et al.*, 2011a; Carboneras *et al.*, 2010), but whose biodegradation process gives rise not only to Mg corrosion products but also to those of zinc (Zn), aluminium (Al) and manganese (Mn). The latter, which is not included as an alloying element, may be considered a contaminant that originates in the manufacturing process (Alvarez-Lopez *et al.*, 2010). It is essential that all the released traces do not reach toxic levels (Feyerabend *et al.*, 2006; Harper and Eley, 2002; Valentini *et al.*, 2007), an aspect that is not often studied in Mg and its alloys (Yuen and Ip, 2010). The most controversial element incorporated in the AZ31 alloy is Al. For some researchers, Al is suspected of playing a major role during the development of

Alzheimer's disease (Bulat *et al.*, 2008; Seitz *et al*., 2014), Parkinson's and dementia (Erasmus *et al.*, 1995), is potentially neurotoxic in humans and laboratory animals (El-Demerdash, 2004) or produces reactive oxygen species (ROS) and free radicals (Nehru and Anand, 2005; Ranjbar *et al.*, 2008). Thus it would be desirable to know where and in what amounts the released ions accumulate.

In order to approximate clinical practice, we fractured a left femoral diaphysis in each rat and then fitted it with an AZ31 implant. In other *in vivo* studies, Mg material was transcortically inserted (*Castellani et al*., 2011; Fischerauer *et al*., 2013; Kraus *et al*., 2012) but not in an endomedullary way, that is, as for repair of a real fracture.

Both long term *in vivo* studies and the quantification of metallic trace accumulation in organs are scarce in the consulted literature (Schilling *et al.*, 2013). Most of them consisted of the study of histological and pathological changes (Dziuba *et al.*, 2013; He *et al.*, 2009). The time factor for *in vivo* experimentation with Mg and its alloys does not usually go beyond 3 to 6 months (Kraus *et al.*, 2012; Witte *et al.*, 2006). In the present research it was decided to analyse metallic traces in rat organs, far away from the implant, originating in the biodegradation process of AZ31 alloy for a longer implantation time (13 months).

On the other hand, promising results from previous *in vitro* studies carried out by this research group using AZ31 alloy with MgF_2 coating (Carboneras *et al.*, 2010; Carboneras *et al.*, 2011b; Lozano *et al.*, 2013) led us to also consider the influence of this coating in the present work. This research line seeks to evaluate metallic traces such as Mg, Al, Zn, Mn and fluorine (F) in different rat organs, such as the liver, kidneys, spleen, lungs and brain, resulting from the biodegradation process of AZ31 alloy intramedullary pins. It also studies the influence of different factors on the accumulation of these traces: how long the implant has been in place, whether or not the bone is previously fractured, and the presence of a protective coating on the implant.

Materials and methods

The implants

The AZ31 alloy was received from Magnesium Elektron Ltd. in the form of a rolled 3 mm thick sheet in O-temper condition (annealed at 345°C). The chemical composition of the AZ31 alloy was determined by wave length dispersion X-ray fluorescence (WDXRF) to be: 3.37 ± 0.09 wt.% Al, 0.78 ± 0.04 wt.% Zn, 0.22 ± 0.01 wt.% Mn (balance Mg). AZ31 was machined to form cylinders of 20 mm in length and 1 mm in diameter, with an average weight of 28.0 ± 0.3 mg. The cylindrical specimens were rounded at both ends in order to eliminate sharp edges, to facilitate their insertion in the laboratory animals, and to avoid edge effects on the degradation rate. To enhance the corrosion resistance of AZ31 alloy, half of the pins were subjected to a chemical conversion treatment applied by immersion of the AZ31 substrates in 48 wt.% HF solution at room temperature for 24 hours (Alvarez-Lopez *et al.*, 2010; Chiu *et al.*, 2007), followed by rinsing with deionised water and drying in a stream of warm air. This treatment gave rise to the MgF_2 protective layer.

All samples were gamma-ray sterilised.

Surgery

The animals used in the experiments were 45 female Wistar rats of three months of age and approximately 200 g body weight (bw). The animals were treated in accordance with European Union Guidelines for Ethical Care of Animals (86/609CEE) and Spanish Regulation (RD 1201/2005).

The scheme of Fig. 1 shows the experimental procedure followed in the *in vivo* tests. The animals were classified into two main groups: 9 control rats without implant insertion and without surgery; and 36 rats with an AZ31 alloy implant. Half of the second group (18 rats) were subjected to a femur fracture of their hind left leg by a unicortical cut with a Gillies saw followed by manual breakage of the other cortical, and immediately afterwards the implant was inserted inside the bone

as an intramedullary pin for fracture reduction. The other half (18 rats) were not subjected to any fracture, but the AZ31 alloy implant was inserted by identifying the femoral condyles and drilling an orifice of 1.1 mm in diameter through the intercondylar notch to the central marrow channel. Half of the inserted implants were previously subjected to HF treatment and the other half remained untreated.

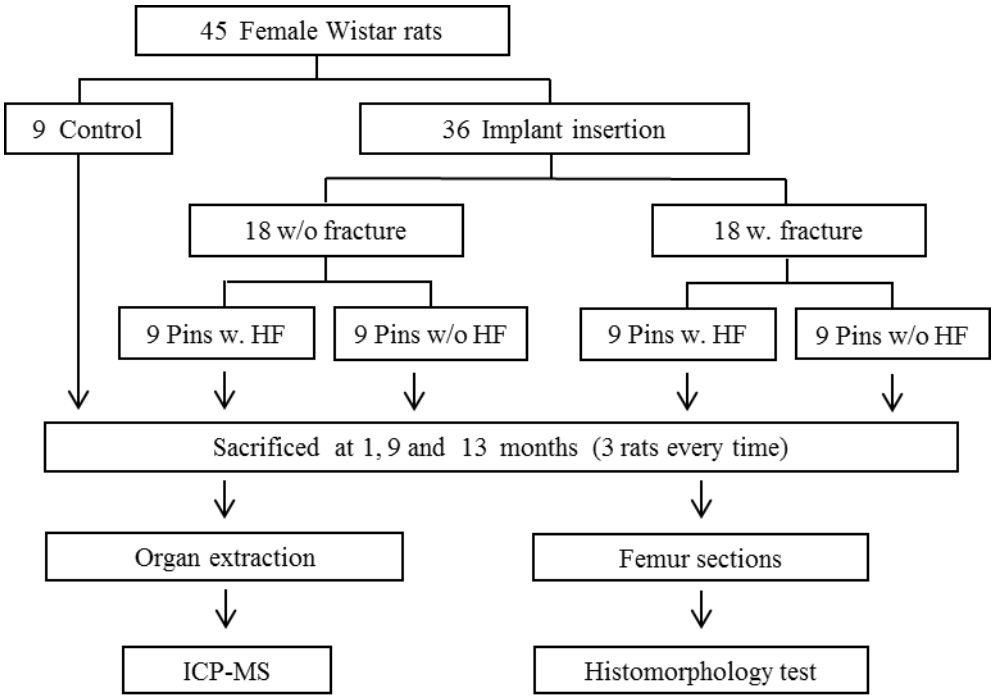


Fig. 1: Experimental procedure diagram

Following the operation, each rat received a prophylactic dose of analgesics and antibiotics: 5 mg Enrofloxacin (BAYTRIL®, 10 mg/kg) and Meloxicam (METACAM®, 2 mg/kg), both in a single dose via subcutaneous injection. All the animals were kept in standard animal house conditions. Food and water were available *ad lib*.

Upon completion of the scheduled monitoring times of 1, 9 and 13 months, the rats were euthanized by intraperitoneal injection of 0.4 mg sodium pentobarbital (Dolethal®) diluted in serum to proceed to extraction of the organs and their subsequent chemical analysis by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The femurs with implants were also extracted in order to carry out

histomorphometric analysis and biodegradation evolution of the AZ31 alloy implants with implantation time.

Sample preparation from the retrieved organs

Several organs were retrieved: kidneys, livers, lungs, spleens and brains. Operations were performed on a clean bench using only zirconia scissors and plastic tweezers in order to avoid any contamination with metals. After removal, the organs were frozen first to -20 °C and then to -80 °C and freeze-dried for 72 hours with a Benchtop 6K freeze dryer (VirTis, SP Scientific, New York, USA).

Dry organ average weights (in g) were: liver (5.48 ± 1.23), lungs (0.66 ± 0.17), brain (0.38 ± 0.08), kidneys (0.66 ± 0.11) and spleen (0.27 ± 0.07). The samples were subsequently homogenised and powdered with a glass ball tube drive mill (Ika Ultra-Turrax, Staufen, Germany). A 0.2 g aliquot of each sample was digested in a 50 mL mix (40 mL distilled water, 8 mL HNO_3 and 2 mL H_2O_2) and then placed in closed high-pressure HPV 80 type vessels and slowly heated to 200 °C in an Ethos Sel 1600 URM Microwave (Milestone, Connecticut, USA) and kept at that temperature for 10 minutes.

ICP-MS analysis

To identify the different types of metallic traces lodged in the rat organs, each solution was analysed with an ELAN 6000 PE Sciex ICP mass spectrometer (Perkin-Elmer, Massachusetts, USA). The use of Inductively Coupled Plasma Mass Spectrometry (ICP-MS), which combines the exceptional characteristics of ICP for the atomisation and ionisation of the injected sample with the sensitivity and selectivity of MS, has been widely used in the determination of elements at trace and ultra-trace levels in different materials (Coedo *et al.*, 1996; Okazaki and Goth, 2008; Okazaki *et al.*, 2004; Rubio *et al.*, 2008; Sarmiento-Gonzalez *et al.*, 2009).

Analyses were performed for the following metallic elements: Mg, Al, Zn and Mn. The latter was evaluated because of intermetallic contamination in the form of Al_6Mn from the manufacturing

process (Alvarez-Lopez *et al.*, 2010). The detection limit calculated on the basis of the standard deviation of ten successive measurements of the blank solution, using the 3σ criterion, was 0.2 ppm. Control tissues were obtained from the rats not subjected to any kind of implant, but also living in the same conditions and the same experimental time as the rats with implants.

Technical precision was monitored with NIST Bovine liver standard (SRM 1577b) for Mg, Al, Zn and Mn quantification. The results were normalised in relation to sample weight.

Fluorine analysis

The analysis of fluorine was not possible by ICP-MS because its ionisation potential is higher than that of argon. For this reason, the analysis was performed using an Orion 9409BN fluoride ion-selective electrode coupled to an Orion model 720A+ multimeter (Thermo Fisher Scientific, Massachusetts, USA). The analysis was carried out in the solutions obtained from acid digestion of the different organs. According to Inkielewicz and Krechniak (2003), a volume of Total Ionic Strength Adjustment Buffer (TISAB IV) was added to each sample to avoid fluoride complexation by iron (Fe) and Mg ions. TISAB IV maintains the pH under 8, protecting from interferences given by Fe and Mg concentrations no higher than 100 ppm. Since the Fe and Mg concentrations analysed by ICP-MS in liver samples were as high as 360 ppm and 550 ppm, respectively, the samples were diluted 10 times in milli-Q water before analysis. The calibration curve was performed with NaF as standard, with the same TISAB IV conditions used in the analysis of the organ samples.

Histomorphometric analysis

In order to test the evolution of biodegradation by histomorphometric analysis, the rat femurs of the rats, were extracted and fixed in 10 % buffered pH 7 formaldehyde and dehydrated in grading hydroxyethylmetacrylate resin concentrations, as mentioned in Donath and Breuner's method (Donath and Breuner, 1982). The femurs were cut into blocks and then embedded in Technovit® resin. The cutting and grinding of hard tissues was performed with an EXAKT sawing machine and

grinding equipment (Leica Microsystems, Wetzlar, Germany). Five cross sections of about 50 µm thickness were obtained from each femur.

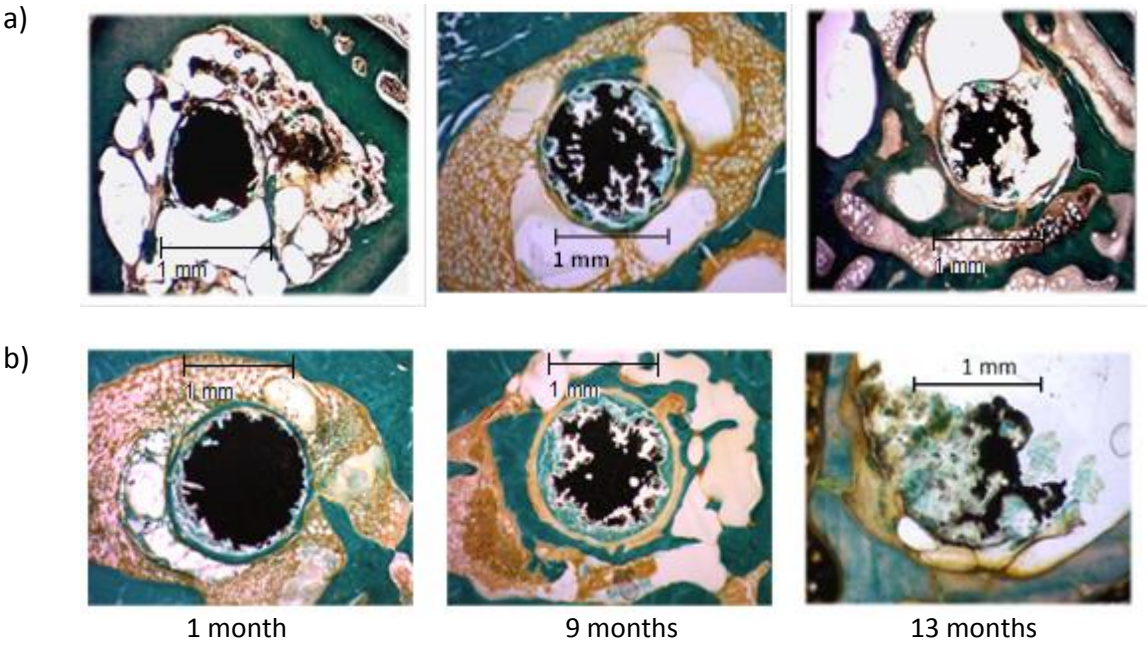
Cross sections were stained with Masson trichrome makes it possible to appreciate the uncalcified osteoid tissue (orange), the calcified mature bone tissue (green) and the cell nuclei (dark red). The stained cross sections of femurs with implants were studied with an optical microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany). The images were transferred to MIP 4 software (Micron Image Processing Software, Digital Image Systems, Barcelona, Spain) to carry out the histomorphometric analysis. This software identifies the initial area occupied by the implant which is easily recognised by the circle of new tissue around it (Fig. 2a and b), and highlights the remaining AZ31 alloy implant by colour density so the loss of implant in each section can be calculated.

Statistics

The data obtained in the determination of metallic traces in the various organs was summarised as mean ± standard deviation (S.D.). T-tests or Mann-Whitney tests were used to compare two independent groups. ANOVA for repeated measures and pairwise comparison with Bonferroni correction were used to compare means at different time-points. ρ-values ≤ 0.05 were considered as significant. Statistical analysis was performed with Statgraphics Online.

Results

Histomorphometric analysis was used to evaluate the biodegradation process of the implants inside the bone. Fig. 2 shows the representative cross-section histomorphometric images at different magnifications of the partially resorbed untreated AZ31 implants in fractured femurs (a) and HF treated pins (b) stained with Masson trichrome after 1, 9 and 13 months from insertion, and (c) the percentage of implant resorption with implantation time for untreated and HF treated pins, respectively.



c)

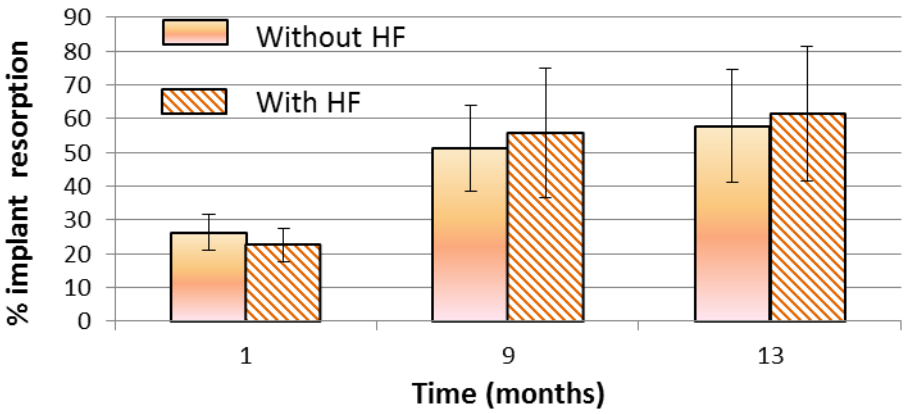


Fig. 2: Cross-section histomorphometric images of a) untreated AZ31 and b) HF treated AZ31 pins, stained with Masson trichrome after 1, 9 and 13 months from insertion (left to right). Central black area is the implant and green area is the cortical bone. c) Percentage of implant resorption for untreated and treated AZ31 pins with time.

The AZ31 implant, in black, can be easily identified in the centre of each image, surrounded by a fine film of connective tissue separating the medullary cavity. The cavity is partially occupied by trabeculae bone (green), abundant fat cells with a dark meshed shade and fibres of connective tissue (orange) whereas implant appears in black. The periphery of the image is occupied by mature cortical bone (green) that provides evidence of bone neoformation in the medullary cavity, distinguishing a thick trabecula of cortical bone and bone marrow of an orange coloured dotted

appearance. It is interesting to note how the edges of the implant exhibit pitting corrosion that gives the surface a sawtooth appearance. Biodegradation starts at the outer surface of the pins and progresses towards their centre. The resorbable zones that surround the surface of the implant are being occupied by corrosion products composed of inorganic compounds, Mg oxides and hydroxides, observed in Fig. 2a and b as grey areas. No foreign body cells or inflammatory signs have been noticed in any of the samples.

Tomography images show that new bone is formed around all Mg implants. Even more, in the presence of ongoing corrosion, high levels of bone-device contact are observed after 9 and 13 months in vivo that have been also verified by computed tomography images (Iglesias *et al.*, 2015). After 9 months, mature healing is observed, with full thickness bone around the pins at 9 and 13 months. This bone forming ability is in agreement with the results published by A. Chaya et. al who state that the results of in vivo degradation of Mg devices provide stabilization to facilitate healing, while degrading and stimulating new bone formation (Chaya *et al.*, 2015). A similar behavior was observed by Kraus et al. with WZ21 alloys who state that the bone function does not seem to be harmed, the bone recovered surprisingly quickly after WZ21 pin degradation and kept its integrity for more than 4 weeks showing good osteoconductive properties by enhancing bone accumulation at the pin surface (Kraus et al., 2012).

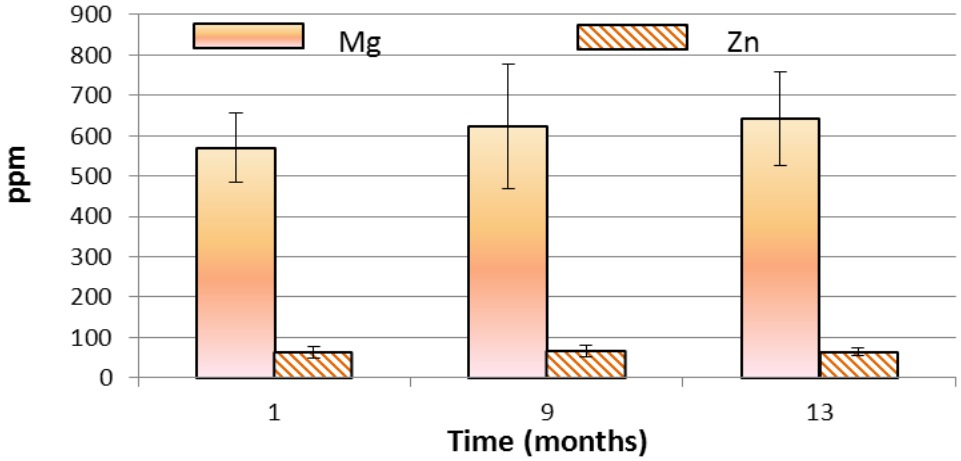
Fig. 2c quantifies the implant resorption percentages for untreated and HF treated pins after 1, 9 and 13 months. There were no statistically significant differences in the resorption between the untreated and HF treated implants. At the beginning of implantation the implant surface is immersed in the physiological medium, rich in Cl and inorganic salts that promote the interaction with the surface and biocorrosion starts on the surface. The cathodic reaction produces not only hydrogen gas but also OH^- ions which induces the precipitation of magnesium oxy-hydroxide compounds. These compounds act as a barrier that prevents the ingress of electrolyte and slow down the corrosion. During the first two months the volume of gas pockets produced around the

AZ31 implant and measured by computed tomography are appreciable as could be seen in a previous work (Iglesias *et al.*, 2015). The gas volume measured is drastically reduced at longer implantation time until the end of experimentation (13 months).

On the other hand, the MgF_2 coating does not exert influence on the degradation process at the long implantation times studied in the work. This result can be explained taking into consideration that the continuous contact of physiological medium with the MgF_2 coating finally gives rise to the degradation of the coating exposing the bare implant to the physiological medium. The electrochemical potential of both, MgF_2 and AZ31 surfaces are very different as could be verified by Scanning Kelvin probe (SKP) in a previous work (Carboneras *et al*., 2010) giving rise to the formation of galvanic couples that accelerate the corrosion on the implant surface.

The influence of implantation time on the accumulation of metallic traces in the complete set of the organs of the rats after 1, 9 and 13 months of implantation time is shown in Figure 3, for Mg and Zn (a), and Mn and Al (b).

a)



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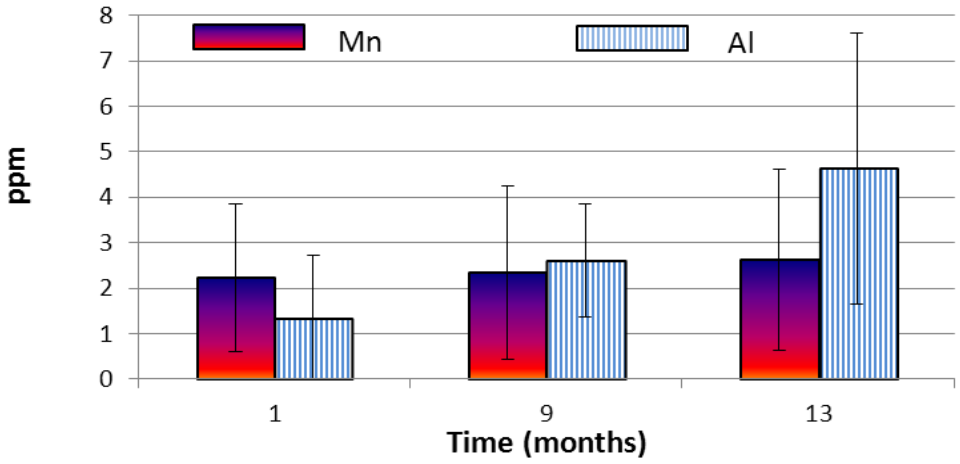

Fig. 3: Metallic traces in the complete set of the organs of the rats after 1, 9 and 13 months of implantation time: a) Mg and Zn; b) Mn and Al.

The data show higher Mg, Al and Mn contents as time increases and the stabilisation of Zn values. This increase over time is not statistically significant except in the case of Al, which continues to increase in the last period (9 to 13 months).

In order to exclude the possibility that the increase in metallic traces with implantation time may be due to other factors not related with the insertion of the AZ31 alloy implant, a comparison was made between the metallic traces analysed in the control rats (without implant but same age) and the rats with implants at 13 months. The results obtained are set out in columns (a) and (b) of Table 1. After a long implantation time, higher metallic trace levels are seen in the rats with AZ31 endomedullary implants than in the control rats, thus confirming that degradation of the implant material takes place and that metallic traces have migrated and accumulated in the different organs.

	With implant (a)		Without implant (b)	
	Mean♦	S.D.♦	Mean♦	S.D.♦
Mg	641.08	115.32	563.60	111.72
Al	Ω 4.64	2.98	Ω 0.81	0.75
Mn	2.62	2.00	2.13	1.98
Zn	65.37	10.38	64.41	11.28

♦=Data in ppm. Ω = Significant difference at 0.95 confidence level.

Table 1: Metal element contents in rats with implant (a), and in control rats [b] without implant, at the longest time (13 months).

Taking into account possible toxicity problems related with Al, together with its statistically significant difference, an exhaustive analysis has been made of its presence in the different organs. Fig. 4 shows the distribution of Al content in each analysed organ from 1 to 13 months of implantation time.

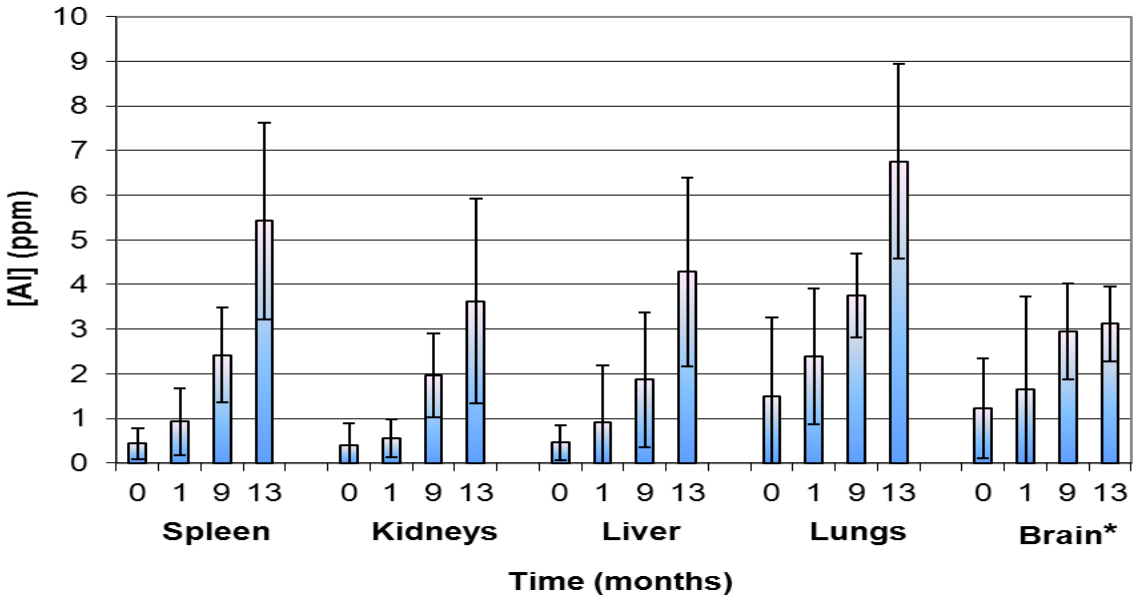

Fig. 4: Aluminium content in organs of rats euthanized after different implantation times. Bars mean S.D. ***** = Brain is the only organ with no significant difference between 0 and 13 months (at 0.95 confidence level).

The Al content has risen in all the rat organs and is seen to accumulate most in the lungs (almost 7 ppm) followed by the spleen (5.4 ppm). In the case of the brain, there seems to be a certain stabilisation after the ninth month. The brain is the only organ that does not show significant differences in Al content (ANOVA > 0.05) between 0 and 13 months (Fig. 4).

Table 2 shows the Al content in different organs for both the control rats and those fitted with implants.

	Without implant	**With implant**	**ANOVA**	**Δ Al (ppm)**	**Dry organ (mg)**	**Δ Al (mg)**	**% pin Al recovered**
	Mean ± S.D	Mean ± S.D	ρ	[a]	[b]	[c]	[d]
Spleen	0.44 ± 0.35	5.42 ± 4.98	**0.012***	4.98	353 ±16	1.76E-03	0.31
Kidney	0.41 ± 0.48	3.62 ± 3.21	**0.028***	3.21	671 ± 15	2.15E-03	0.38
Liver	0.46 ± 0.39	4.27 ± 3.81	**0.017***	3.81	4114 ± 93	15.67E-03	2.77
Lung	1.50 ± 1.76	6.76 ± 5.26	**0.031***	5.26	410 ± 38	2.16E-03	0.38
Brain	1.22 ± 1.12	3.11 ± 1.90	0.331	1.90	321 ± 66	6.10E-04	0.11
Σ organs						22.35E-03	**3.95**

Data in ppm (dry weight). * = significant difference at 0.95 confidence level.

Table 2: Aluminium content in different organs of control rats and rats with implants after 13 months: [a] mean increment, from differences between rats with and without implants, [b] mean weight of dry organs, [c] Al increase (in mg), calculated as [a] x [b] data, [d] recovered Al percentage respect to original whole implant Al mass.

To quantify the Al percentage recovered from the organ, the following calculation was performed: the mean weight of the implant is 28 mg, of which 3.37 % is Al, so the AZ31 alloy implant contains 0.94 mg Al. Considering that the biodegradation of the implant after 13 months is about 60 %, the released Al is 0.57 mg. If the Al increase (column [a] of Table 2) is normalised considering the weight by organ (column [b] of Table 2) the Al increase in mg is obtained (column [c] = [a] x [b] of Table 2). The last column is the Al percentage recovered from each organ (column [d] of Table 2).

The innovative aspect of the research carried out in this work is that it seeks to approximate a real situation of use of this material in fracture osteosynthesis. Pairwise comparisons were made between rats with and without fracture for each element and organ. As all the ANOVA results were

in excess of 0.05, no significant differences were found (at 95 % confidence) that can be attributed to the fracture factor.

With regard to the last variable analysed in this study, the influence of prior treatment of the AZ31 implants with HF, Fig. 5 compares the F content in the organs of rats euthanized after 13 months.

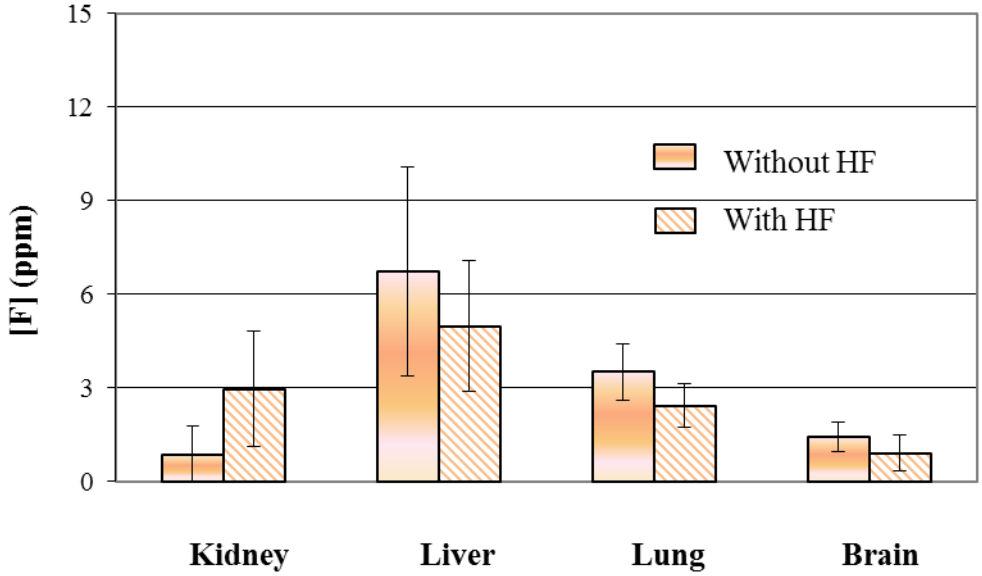

Fig. 5: Fluorine content in organs of rats euthanized after 13 months. Bars mean S.D. No significant difference at 0.95 confidence level between implants treated or not with HF.

It can be seen that the F content in the different organs is independent of the presence of the MgF_2 coating on the Mg implant. The *in vivo* degradation of the implants with and without treatment shows no clear protective effect of the MgF_2 layer.

Discussion

The AZ31 Mg alloy is a suitable material to repair fractures mainly due to its mechanical properties and degradation ability (Carboneras *et al*., 2011a; Seitz *et al*., 2014). The biodegradation process, 60% after 13 months (Fig. 2c), is responsible for the presence of metallic traces accumulated in the organs. In this work the distribution of the metallic traces in different organs has been studied after 1, 9 and 13 months. Yuen (Yuen and Ip, 2010) established the theoretical maximum annual levels

for no-observed-adverse-effect (NOAEL) for some degradable Mg alloy implants. Following Yuen's guidelines, the authors have analysed the metallic release from AZ31 alloy implants from the lowest element content in the alloy, in this case manganese, to the most dangerous and toxic for the body, aluminium.

Considering Mn from the point of view of toxicity, it is considered one of the least toxic metals when consumed orally (Nielsen, 1999; WHO, 1974), but fairly toxic when administered by inhalation (Hathcock, 2004). Workers exposed to atmospheres with extreme concentrations, e.g. in excess of 27 mg/m^3 (Jankovic, 2005) (more than 400 times a normal ambient level) (WHO, 2000) have developed neurotoxicity (manganic madness) with tremors similar to Parkinson's (ATSDR, 2012; Hathcock, 2004).

The maximum tolerable Mn dose has been set theoretically at 7 10^{-3} mg kg^{-1} d^{-1} (Yuen and Ip, 2010). After a year and considering the rat weight (200 g), this means that the maximum theoretical dose is 5.11 10^{-1} mg. On the other hand, the AZ31 alloy (28 mg in our experiments) contents 0.22 % Mn so this means 6.16 10^{-2} mg. The tolerable dose is equivalent to 13.8 times the weight of our implant, since it is only 60% degradated after 13 months (8.3 times if 100 % implant were released in one year). Mn is excreted in faeces after being stored in bile by the liver, so special care must be taken in patients with impaired liver function.

The second element to be considered has been Zn, which is an essential element, so problems are usually related with a deficiency. Only the inhalation of extremely high concentrations can produce illness such as reversible zinc fever (Hildebrand and Hornez, 1998). In accordance with Yuen (Yuen and Ip, 2010), the maximum tolerable Zn dose has been set theoretically at 5.53 10^{-2} mg kg^{-1} d^{-1} or, in our case, 4.04 mg in a year (for a 200 g rat). In our 28 mg implant with a Zn content of 0.78 %, there are 2.18 10^{-1} mg. The tolerable dose would be 30.8 times the weight of the implant material inserted in this study (18.5 times if 100 % implant were released in one year). Despite

using alloys with 6 % of this metal (more than 7 times the Zn content in our AZ31 alloy), Yu (Yu *et al.*, 2013) considered it to be non-toxic, in fact quite the reverse, as it stimulates bone formation.

With regard to Mg, although the literature reports toxic effects (EGVM, 2003; WHO, 2004) or even mortal effects (Birrer *et al.*, 2002; Harper and Eley, 2002) when administered at very high doses, moderate increases in Mg contents do not cause toxicity in cell cultures (Carboneras *et al.*, 2011b; Feyerabend *et al.*, 2010; Lozano *et al.*, 2013; Rezwan *et al.*, 2006; Valentini *et al.*, 2007) or in the human body (Li *et al.*, 2004; Saris *et al.*, 2000; Song, 2007). In other studies, Mg alloys implanted *in vivo* promote bone growth in their vicinity (Li *et al.*, 2008; Witte *et al.*, 2007a; Xu *et al.*, 2007).

Studies on the short-term toxicity of Mg administered intravenously demonstrated that the lethal dose value (LD50) in rats is 174 mg kg^{-1} in females and 206 mg kg^{-1} in males (WHO, 2004). For a 200 g rat, this means between 35-40 mg in a single dose. As Mg is released gradually, and Mg levels in the blood are regulated by the kidneys, the excess is excreted in urine (Reifenrath *et al.*, 2011; Xu et al., 2007). Moreover, the skeleton also sequesters this metal, given that close to 50 % of Mg in the body is stored in the bones (Rezwan *et al.*, 2006).

According to Yuen (Yuen and Ip, 2010), annual insertion of implants with 64-73 g Mg is considered tolerable in humans. That would be equivalent to implants with 180-200 mg Mg in one year in the experimental animal design proposed in this research. Our AZ31 implant contains less than 27 mg Mg, i.e. 12 times less than the safe dose proposed by Yuen (7 times if 100 % implant were released in one year).

In view of the above, it seems reasonable to rule out any toxic effect of the levels of Mg, Mn or Zn used in the implants in this work on the proposed animal model.

With regard to Al, this element is potentially neurotoxic in humans and laboratory animals (El-Demerdash, 2004) and has been shown to accumulate in organs such as the brain, bones, kidneys and blood (Oteiza *et al.*, 1993). Al produces reactive oxygen species (ROS) and free radicals (Nehru and Anand, 2005; Prakash and Rao, 1995; Ranjbar et al., 2008; Strong *et al.*, 1996) that

cause the peroxidation of lipids (LPO), proteins and DNA. Numerous studies have been carried out on rats, establishing a relationship between aluminium and an increase in LPO and inhibition of antioxidant enzymes in the brain, liver and kidneys (Abubakar *et al.*, 2004; Bhalla and Dhawan, 2009; Kaneko *et al.*, 2007; Luo *et al.*, 2007; Mahieu *et al.*, 2009; Mohammadirad and Abdollahi, 2011; Ranjbar *et al.*, 2008; Sanchez-Iglesias *et al.*, 2009; Sharma and Mishra, 2006; Sharma *et al.*, 2007; Stevanovic *et al.*, 2009; Zatta *et al.*, 2002; Zatta *et al.*, 2000). Al is also suspected to play a part in neurodegenerative diseases like Alzheimer's (Bonnefont-Rousselot *et al.*, 2004; Bulat et al., 2008; Gupta *et al.*, 2005; Liao *et al.*, 2006; Sargazi *et al.*, 2006; Yuen and Ip, 2010; Zatta, 2006), Parkinson's and dementia (Erasmus *et al*., 1995), as well as in knowledge and behavioural deficits (Altmann *et al.*, 1999; Sjögren *et al.*, 1997) and hepatotoxicity (Chainy *et al.*, 1996). A link has also been established between LPO in the brain and the appearance of Alzheimer's (Xie and Yokel, 1996).

The total Al content in the human body is estimated to be between 30-50 mg, of which almost half is in the skeleton and 25 % in the lungs (Ganrot, 1986). De Broe (1986) reported different values, finding 40 % of Al in bones, 40% in muscles, and only 1 % in the brain, 1 % in the liver and 12 % in the lungs.

The maximum tolerable Al dose in rats has been set theoretically at 1.64 10^{-2} mg kg^{-1} d^{-1} (Yuen and Ip, 2010). This means 1.19 mg in 200 g of rat after a year. A WDXRF analysis of 28 mg implant yields a level of 3.37 %, i.e., 0.94 mg Al in all the implant. Yuen uses very strict safety limits for Al, always at the theoretical lower limit, prioritising safety. He also adds a safety factor of 10 times in order to absorb possible inter-population variations (the chance that some individuals may be 10 times more sensitive than those considered in the study). If this safety factor was not taken into account, the equivalent would be approximately 21 times that used here (12 times if 100 % implant were released in one year).

Taking into account possible toxicity problems related with Al, together with its statistically significant difference, an exhaustive analysis has been made of its presence in the different organs. Table 2 shows the Al content in different organs for both the control rats and those fitted with implants. The Al content has risen in all the organs of the rats with implants, though the brain is the only organ where these differences are not significant.

This is in agreement with other studies using different types of implants, where the spleen is seen to be the organ in which the greatest amounts of trace ions accumulate (Debroe *et al.*, 1986; Sarmiento-Gonzalez *et al*., 2009; WHO, 1974), probably due to its relationship with the immune system. This mechanism concords with Witte's findings in rabbits (Witte *et al.*, 2007b), where the corrosion products containing Al that remained in the implant area were absorbed by macrophages.

The highest content in absolute terms is found in the lungs, but the increase here has been moderate, as this is where the highest base level was found in the controls. This is in agreement with the findings of other authors (Yu *et al.*, 2013).

With regard to the liver and spleen, the presence of Al may be explained as part of the body's excretion process, limiting Al toxicity in these organs, as pointed out by De Broe (Debroe *et al.*, 1986), whereby "*the sequestration of aluminium in the liver by lysosomes of hepatocytes and Kupffer cells, and in the spleen by macrophages, might explain the absence of proven toxicity for liver or spleen*".

It is not easy for Al to enter the brain, as the blood-brain barrier impedes its passage (Yokel *et al.*, 1999; Yokel and McNamara, 2001; Zatta *et al.*, 1991). At the same time, the rate of elimination of Al from the brain is low (Sanchez-Iglesias *et al.*, 2007; Yokel *et al.*, 1999) and it is suspected that it may build up there over the individual's lifetime (Ganrot, 1986; Markesbery *et al.*, 1984; Priest, 2004).

Average Al values in the brain of healthy humans have been determined in previous studies as being between 1-5 ppm (dry weight) (Bjertness *et al.*, 1996; Hamilton *et al.*, 1973; Markesbery *et*

al., 1984; Tipton and Cook, 1963). Our data, which yields a value of 3.11 ppm (dry weight) in the brain, may be considered to be within the range of normal values, although care must be taken to ensure that the number and size of inserted implants does not exceed the safety limit.

The Al values found in the different studied organs (Table 2) indicate that while a perceptible increase has taken place (column [a] of Table 2), only a tiny amount of the original mass of the implants has been retained in these organs (column [c] of Table 2). A 28 mg implant with 3.37 % Al is equivalent to 0.94 mg Al, and the sum of the recovered Al from the sampled organs represents 3.95 % of the total amount theoretically available (column [d] of Table 2).

These results agree with existing data from studies of parenteral medication, which is the most similar way to an implant for Al to enter the blood. In healthy adults 99 % of this Al is eliminated through the kidneys (in urine) and to a lesser extent also through the liver (in bile) (Hernandez-Sanchez *et al.*, 2013). In studies of direct injection of Al^{25} in humans, after 24 hours only 0.5 % remained in the blood (Priest *et al.*, 1995; Priest *et al.*, 1991), so its removal is very efficient. This effective functioning of the excretory system, together with the deposition of corrosion products in the vicinity of the implant, are the two main mechanisms that explain the relatively low Al concentration in the studied organs, in line with Witte's remarks (Witte *et al.*, 2007b) that "*only a small portion of ionic aluminium is bound to albumin and is transported out of the blood mainly into the skeleton, liver, kidneys, and brain, and in much smaller amounts into other soft tissues*". Only in cases of diminished renal function (e.g. in elderly persons, patients with damaged kidneys, newborn children) does Al tend to accumulate in the body (Gura, 2010; Klein, 2003).

Time influence

The influence of time on the accumulation of metallic traces in the complete set of organs was studied. Only Al presents a statistically significant increase after 13 months. Great caution is advisable in this respect, since it is not known whether Al would continue to accumulate after longer times not considered in this study. Nevertheless, although all the organs show significant

differences (ANOVA ≤ 0.05) between 0 and 13 months, the brain ($\rho = 0.086$) is an important exception considering that there seems to be a certain stabilisation after the ninth month. Of all the organs analysed, in absolute values Al is seen to accumulate most in the liver, (column [d] of Table 2). This is due to the greater weight of the liver compared to the other organs analysed (column [b] of Table 2). The Al excess in the liver is related to the known excretory function.

It is difficult to compare these results with data published in the literature as this is a pioneering work for such long testing times in animal experimentation. For shorter implantation times, Witte (Witte *et al.*, 2007b), working with rabbits, found little migration of Al particles to tissues close to the implant site, and the gradual disappearance of Al between 3 to 6 months.

Bone fracture influence

The innovative aspect of the research carried out in this work is that it seeks to approximate a real situation of use of this material in fracture synthesis. To evaluate the influence of bone fracture on the ion release, a controlled fracture was performed in some of the experimental animals. The ANOVA results of the comparison between rats with and without bone fracture for each studied metallic element and organ show no significant differences (at 95 % confidence) that could be attributed to the fracture factor.

The relatively slow biodegradation process of the material is compatible with the fracture consolidation process, without loss of mechanical or functional properties.

HF treatment influence

Fluoride is a natural anion within the skeleton and teeth, so the release of fluoride ions into the organism is expected to be harmlessness (Thomann *et al.*, 2010). For example, MgCa alloy cylinders (Thomann *et al.*, 2010) or LA63 scaffolds (Schilling *et al.*, 2013) coated with fluoride have shown good biocompatibility *in vivo*. Thomann supposed that F was partly resorbed and evacuated. Elimination of F takes place via urine, faeces, etc., but the kidneys are the organs that play the most important role in its elimination (Thomann *et al.*, 2010). This is in agreement with our

results, as differences have not been seen in the F content in the organs irrespective of the presence or absence of the MgF_2 coating on the AZ31 alloy implant (Fig. 5).

On the other hand, chronic excessive fluoride intake may result in fluorosis (Yang *et al.*, 2011). Chronic fluorosis is a slow and progressive process causing symptoms related to muscle-skeletal and dental systems. Damage caused by chronic fluorosis has also been reported in many tissues including the kidney, liver and brain (Oncu *et al.*, 2006). For this reason, F accumulation in these organs has also been studied (Fig. 5).

The results obtained with respect to the influence of prior treatment of the AZ31 implants with HF do not reflect a protective effect of the MgF_2 layer on the AZ31 implant surface and no clear difference is seen between the ANOVA for each individual element (Fig. 5).

These results are in disagreement with Witte (Witte *et al.*, 2008), who used a fluoride layer that slowed implant degradation, but agree with the findings of Thomann (Thomann *et al.*, 2010), who reported no decrease in degradation despite the continuing presence of the fluoride layer after six months of implantation. According to Thomann (Thomann *et al.*, 2010) there seems to be barely any degradation of the MgCa0.8 inside the bone medullary cavity. Our biodegradation results are different because both the tested alloy composition (AZ31) and implantation time (13 months) are different to those considered by Thomann. In our studies, biodegradation after 13 months reaches values of around 60 % of the implant, as can be seen in Fig. 2c. The conclusion under this heading is that the addition of a protective MgF_2 layer does not decrease the accumulation levels in organs of the studied traces, in contrast to previous *in vitro* results (Carboneras *et al.*, 2011a; Carboneras *et al.*, 2011b; Lozano *et al.*, 2013), so *in vivo* degradation must depend on other parameters that have not yet been identified. These different behavior shows that an important variable that can control the biodegradation rate is the electrolyte thickness, i.e., the volume of fluid that bathes the surface of the implant (Montoya *et al.*, 2014). This variable can even be more important in the biodegradation than the presence of the MgF_2 coating.

In vivo degradation of Mg implants does not provide evidence of significant toxic levels of metallic trace accumulation in organs that induce serious alterations in their normal function. Nevertheless, it is worth noting the great ability of the organs of lodging metallic traces when blood magnesium levels and the inner organs are unaltered whereas the immediately surrounding tissue can be severely affected by some magnesium alloys (Dziuba *et al.*, 2013).

Conclusions

1) After a long implantation time, insertion of the AZ31 implant as an endomedullary pin and its subsequent biodegradation leads to the statistically significant accumulation of Al traces in all the studied organs.

2) After 13 months, the increase in Al recovered from all the sampled organs represents 3.95% of the amount contained in the AZ31 implant. Al accumulates in a statistically significant way in all the organs except the brain.

3) The presence of a fracture in the bone where the implant is inserted has not led to a significant increase in metallic traces in the different organs compared to the animals without bone fracture. This is the case for all the elements and all the organs.

4) Prior treatment of the implant with HF does not lead to significant differences in the accumulation of metallic traces after long implantation times. The protective effect of the MgF_2 layer found *in vitro* has not been reproduced in this *in vivo* study.

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