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ARTICLE

Biocompatibility of Morphology on Laser-Processed Magnesium Alloy Surfaces

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Abstract: The surface of magnesium alloy was laser-processed, and the laser-etched morphology was determined as grooves by observing the surface morphology of sheep rib bone. The wettability of different morphologies was investigated by contact angle test. Through the cell adhesion test, the effects of different morphologies on cell adhesion, growth and migration were investigated. Results show that the wetting angle of the block-shaped surface is smaller than that of the groove-shaped surface, and block-shaped surface has better hydrophilicity. Compared with the smooth surface, the block-shaped surface has better cell adhesion, and the depressions and bumps are full of cells, suggesting that the micropatterns prepared by the laser processing are conducive to the enhancement of biocompatibility.

Key words: magnesium alloy; laser surface modification; bionic; wettability; cell adhesion

Due to the in-situ degradation ability of magnesium alloy in the human body, its biocompatibility and bone conductivity can actively stimulate the formation of new bones in the human body. Excellent strength, ductility and biological corrosion resistance are important prerequisites for magnesium alloy as the biodegradable implant in orthopedic applications. Magnesium alloy is the bone repair metal material with great development potential[1]. In recent years, with the progress of research, the surface structure has become one of the important factors affecting the combination of implant and bone[2–4]. The surface structure can influence the speed and effect of bone healing, and its morphology can regulate the growth orientation of cells[5]. Surface modification of magnesium alloys is to change the surface properties of metals through a series of measures, which not only improves the corrosion resistance and mechanical properties, but also enhances the biocompatibility of magnesium alloys. The biocompatibility of magnesium alloy mainly indicates that magnesium alloy, as the bone implant, will not bring adverse reactions to the body on the basis of connection, fixation and bone healing of the body. During the whole healing process, it should be firstly confirmed that magnesium alloy can support the fracture site and ensure the normal healing of the bone. Secondly, the process of corrosion degradation of magnesium alloy in the human body should be moderate, and the substances produced by corrosion degradation should not be harmful to the human body. Finally, with the ensured basic function, some surface treatments are conducted on the surface of magnesium alloy to make it conducive to cell attachment, thus further improving the biocompatibility.

Laser processing is one of the most effective methods to change metal surface properties and improve biocompatibility. Surmeneva et al[6] fabricated periodic microstructure on the surface of titanium substrates by direct laser interference patterning. And ultrathin nano-hydroxyapatite (HA) thin films were deposited using radio-frequency magnetron sputtering to form additional nanoscale grain morphology on the microstructured titanium surface, thereby generating multiscale surface structures. The results show that the HA-coated periodic microstructured titanium substrates exhibit significantly lower water contact angles and larger surface free energies. In recent years, the study of surface

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modification of magnesium alloy surface is very extensive. Shi et al[7] used femtosecond laser to process different surface microstructures on the surface of AZ31B magnesium alloy, and conducted cell culture experiments. The results show that after multiple femtosecond laser treatments, the surface coating micro-nano structure undergoes major changes in its light capture and wettability, thereby improving cell adhesion and spreading, which is beneficial to improve the biocompatibility of AZ31B magnesium alloy. Song et al[8] conducted the surface modification of magnesium alloy with eutectic composition by laser surface amorphization treatment, and investigated the effect of laser scanning speed on the microstructure and phase composition of amorphous composite coating on the surface of magnesium alloy. The results show that the biocorrosion resistance of magnesium alloy can be effectively improved by laser surface modification treatment. Sheng et al[9] used femtosecond laser to perform micro-nano processing on the surface of magnesium alloy. The treated magnesium alloy was repeatedly immersed in zinc nitrate solution and sodium hydroxide solution, and sintered in the vacuum environment. The nanostructured zinc oxide film was successfully prepared on the surface of AZ31B magnesium alloy, and its cytocompatibility was studied through in vitro cell culture experiments. The results show that the cytotoxicity of magnesium alloy with nanostructured zinc oxide film is weaker than that without film. And the biological activity and cell proliferation ability of magnesium alloy with film are stronger than those without film. Ref. [10–12] show that the surface of the magnesium alloy is quickly melted and solidified through laser processing, thereby reducing the grain size of the magnesium alloy and increasing the volume fraction of the β phase at the grain boundary. This series of operations can stimulate the formation of hydroxyapatite, increase the surface energy and improve the surface corrosion resistance of the magnesium alloy, thereby improving its biomineralization and slowing down its degradation in physiological environments. Indira et al[13] found that through laser irradiation of different media, the WE54 magnesium alloy containing rare earths was firstly irradiated by air and then by simulated body fluid (SBF) layer, releasing more calcium and phosphorus ions than that irradiated by the air alone. Surface deposition is beneficial to improve cell viability, which inspires the potential application of SBF-based films in biomedicine.

However, the effect of laser treatment on the biocompatibility of Mg-Zn-Ca alloy is rarely studied. In this study, Mg-2.0Zn-1.6Ca alloy was selected as the research object. The laser-etched morphology of Mg-2.0Zn-1.6Ca alloy was determined by observing the surface morphology of sheep ribs. The biocompatibility of magnesium alloy was explored by biological cell adhesion growth test, wettability test and cell culture experiment to determine the feasibility of magnesium alloy as the biodegradable biomedical metal material.

1 Experiment

The material used in this experiment was Mg-2.0Zn-1.6Ca alloy, which was made of 99.99wt% pure magnesium block, 99.99wt% pure zinc block and intermediate alloy Mg-25wt% Ca. This alloy was produced through alloy ratio design, melting casting, solid solution treatment and extrusion strengthening. The 350#, 600#, 1000#, 1500# and 2000# SiC paper was used step by step to remove the surface oxide layer. Then, the sample was polished on the grinding and polishing machine, cleaned with ethanol, and dried naturally at room temperature. The surface texture of the sample was processed by pulsed laser equipment (YLP-V2-1-100-30-30-30). The power of the laser was 30 W, the scanning speed was 3 mm/s, and the lasing beam diameter was 100 μm.

The surface structure of natural bone is multi-scale and complex structure. In order to clarify the surface structure of natural bone, it is necessary to conduct in-depth observation and research on natural bone. The research object used in this experiment was sheep ribs. The surface of the bone was observed and characterized by scanning electron microscope (SEM) and energy dispersive spectroscope (EDS). Before observing, surface treatment was required. The method was to randomly buy a sheep rib in the market, then take a place with the length of about 5 cm, remove a large piece of meat and periosteum on the surface of the bone, and soak it in water for 3 d. During this period, water should be changed in the morning, afternoon and evening to prevent deterioration. After 3 d, the residual periosteum, meat and tendon were softened and cleaned relatively well but still needed to be further cleaned. The treated sheep ribs were again immersed in anhydrous ethanol for 12 h. Then, the structure and tissue affecting the observation were removed, and the final sheep ribs were air-dried for 1 d. The dried sheep ribs were sawed into easy-to-observe blocks. In the sawing process, attention should be paid to the saw blade from destroying the surface structure. Finally, after disinfection, cleaning, air drying and gold spraying, they were observed by SEM.

In order to study the effect of surface texture on the wettability of magnesium alloy, the surface wettability of textured and untextured samples was evaluated by fixed droplet method using modified SBF (m-SBF) at room temperature as the test medium. The designed ion concentration of m-SBF is close to that of human plasma. Before determination, the samples were ultrasonically washed with ethanol for 5 min and then dried at room temperature. In the fixed droplet measurement, the microsyringe was used to continuously deposit the test medium droplets with the volume of 2 μL on the sample surface for 60 s. The axisymmetric droplet shape analysis profile (ADSA-P) method[14] was used to analyse the droplet profile, and the static contact angle was estimated by fitting the Laplace equation to the experimental profile. Three repeated measurements were performed at the same position to ensure the accuracy of data. The instrument and equipment model is DSA-X ROLL.

Bone tissue contains many types of bone cells. Osteoprogenitor cells are stem cells that differentiate into osteoblasts. Osteoblasts are adhesion-dependent cells with the diameter of 15–30 μm. Osteoblast proliferation is the premise of new bone formation, and the combination of implant and bone is adhesion, proliferation and differentiation of osteoblasts. Osteoblasts are very sensitive to the surface structure of micro-nano scale, which can significantly induce the biological reaction of osteoblasts[15].

In order to study the effect of surface structure on the biocompatibility of magnesium alloys, MC3T3-E1 on the sample surface was cultured in vitro to evaluate the biocompatibility of textured and non-textured samples. All samples were sterilized by autoclave under high temperature and high pressure. After thawing, MC3T3-E1 was cultured at 37 °C in the humid atmosphere of 5% CO_2 and 95% O_2 on a cell culture medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Biotech, Israel) and antibiotics (Bajotim, China) for 4 generations. The cells were seeded on the surface of magnesium alloy at the concentration of 5×10^4 cells/mL for 48 h, and the number of cells was observed. After the magnesium alloy was sterilized by alcohol, it was soaked with double antibody for 5 min and irradiated by ultraviolet for 12 h. 1×10^7 cells were spread on the surface of magnesium alloy, and the cell proportion was about 70% of the surface of the culture dish. Cell adhesion molecule (CAM, concentration of 2 μmol) was added and stained at 37 °C for 30 min. Unattached cells were washed with phosphate buffered saline (PBS) solution and observed by laser confocal microscope (ZEISS LSM 800).

2 Results and Discussion

2.1 Morphology of magnesium alloy surface

2.1.1 Observation and characterization of surface structure of sheep rib

Fig. 1 shows SEM images and groove size of sheep rib surface. It can be seen that the surface of the sheep ribs has the rough surface structure, which has grooves with different widths and spacings.

The size of the grooves on sheep rib surface was measured. It can be seen that the width of the grooves on the surface of the sheep rib is 120–500 μm. Many studies have shown that the morphology of the surface structure at the micron level (1–150 μm) will affect the growth, attachment and migration of cells. In terms of promoting osseointegration, different scales have different osteogenic effects, as shown in Table 1[16–17].

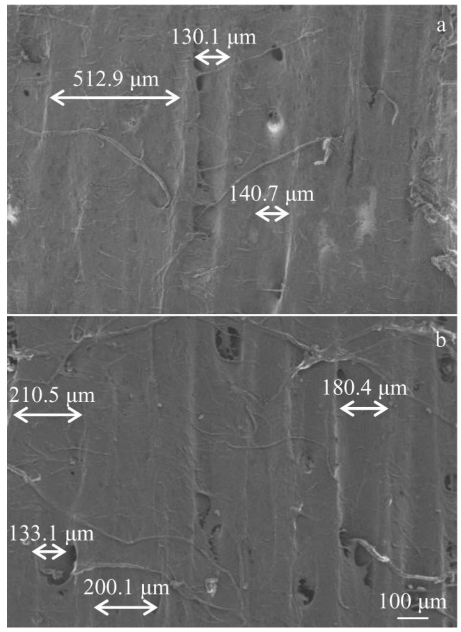

Fig.1 SEM images of sheep rib surfaces

Table 1 Relationship between size of porous implants and growth of bone tissue

Size/μm	Relationship to bone tissue growth
5–40	Allow fiber tissue to grow
40–100	Allow non-mineralized bone-like tissue to grow
>100	Bone tissue ingrowth and bone conduction

2.1.2 Laser-etched bionic microstructure

Based on the exploration of the surface of the sheep rib, the method of laser processing is used to process the pattern on the surface of the magnesium alloy. The patterns include groove and block shapes, as shown in Fig.2.

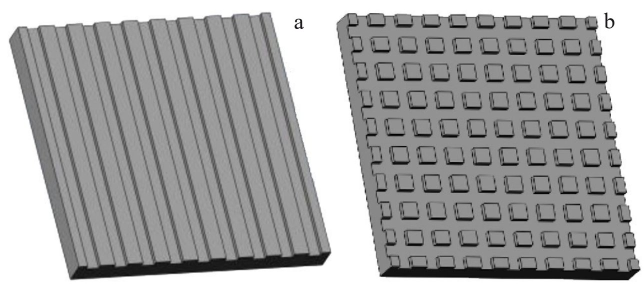

Fig. 2 Schematic diagrams of surface microstructure: (a) groove shape and (b) block shape

The frequency of the laser was set as 50 kHz, the speed was 3 mm/s, and the power was 30 W. The spacing of grooves was set as 200, 350 and 500 μm according to the width of the grooves observed on the surface of the sheep ribs. Fig.3 shows the SEM images of different laser-etched surfaces. Due to the difference in the spacing and the pattern, different morphologies appear in the area directly ablated by the laser beam. When the high-energy laser ablates on the surface of magnesium alloy, the surface rapidly vaporizes and melts, and a large amount of magnesium alloy vapor is suspended above the molten liquid magnesium alloy. When the magnesium alloy vapor accumulates to a certain extent, the violent explosion occurs, forming the downward impact on the molten liquid magnesium alloy. When the impact force is much larger than the surface tension and gravity of the liquid metal, the liquid magnesium alloy is squeezed to the sides, forming a gully. At the same time, the melted magnesium alloy has no time to volatilize, thus forming ridges on both sides of the gully due to cold deposition. Due to the difference in the spacing and the scanning path, different morphologies are formed on the surface, as shown in the Fig.3.

2.2 Surface contact angle measurement and wettability transformation

2.2.1 Basic theory of surface wettability

The most important theoretical models related to wettability are the Wenzel model and the Cassie model. Wenzel model combines the thermodynamic equation with the Young equation and proposes the "roughness factor" to modify the wettability principle of rough surfaces[3]. This model believes that liquid can fill the grooves on the solid surface, as shown in Fig. 4a. Wenzel model is only suitable for solid surfaces with uniform chemical composition and structure. In fact, the material surface is not all homogeneous in composition and structure and therefore no longer satisfies Wenzel model. Based on a large number of experiments, Cunha et al[18] proposed that the rough surface cannot be completely wetted by liquid, and some air fills the gaps on the rough surface (cassie model), as shown in Fig.4b.

2.2.2 Contact angle measurement and wettability analysis

Since the wetting angle will change with time, the shooting time intervals are 0, 30 and 60 s. Fig. 5 shows the contact angle evolution with time for the groove-shaped and block-shaped surfaces prepared by microsecond laser. The magnitude of the contact angle of groove-shaped and block-shaped surfaces prepared by microsecond laser is shown in Fig.6.

Magnesium alloy is a hydrophilic material. Due to the influence of surface roughness, the apparent contact angle which is greater than 90° shows hydrophobicity. After laser treatment, the apparent contact angles are all less than 90°, indicating hydrophilicity. By dropping 2 μL SBF on six

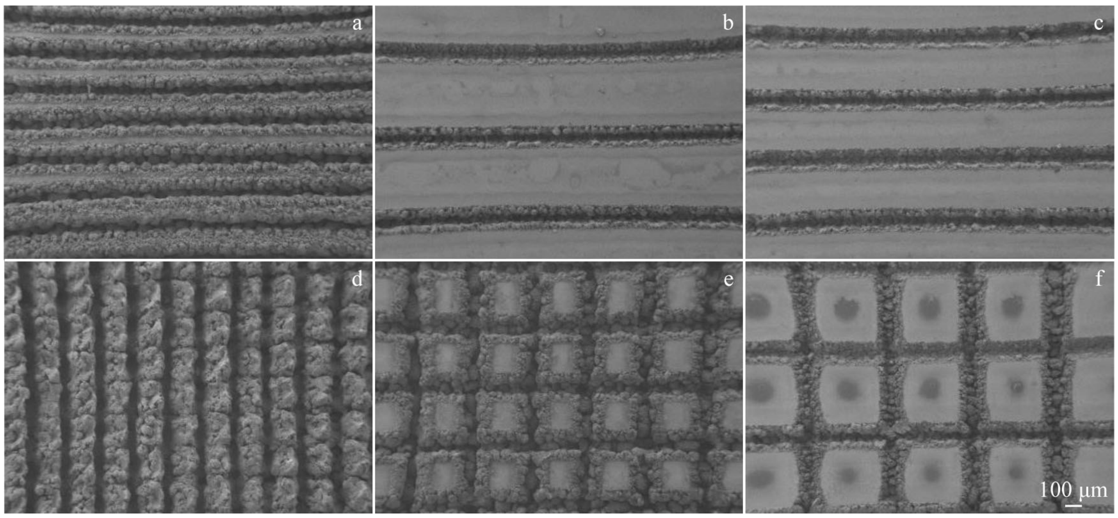

Fig.3 SEM images of groove-shaped (a–c) and block-shaped (d–f) laser-etched surfaces with spacing of 200 μm (a, d), 350 μm (b, e), and 500 μm (c, f)

surfaces, the surface contact angles were tested. Results show that as the surface spacing of the groove structure increases, the surface contact angle gradually increases and the surface hydrophilicity gradually weakens. When the surface spacing of the groove structure is 200 μm, the surface contact angle reaches 27°, showing superhydrophilicity. This is because there is the large capillary effect in the direction of the parallel groove structure, which enables the droplets to overcome the

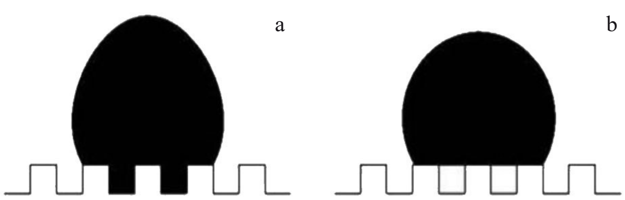

Fig.4 Schematic diagrams of wettability model: (a) Wenzel model; (b) Cassie model

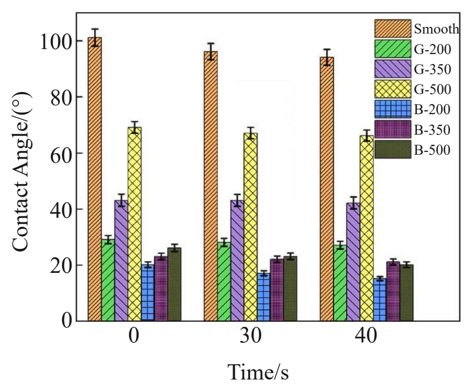

Fig.5 Contact angle of different surfaces (smooth surface is indicated by Smooth; groove-shaped surfaces with spacing of 200, 350 and 500 μm are indicated by G-200, G-350 and G-500, respectively; block-shaped surfaces with spacing of 200, 350 and 500 μm are indicated by B-200, B-350 and B-500, respectively)

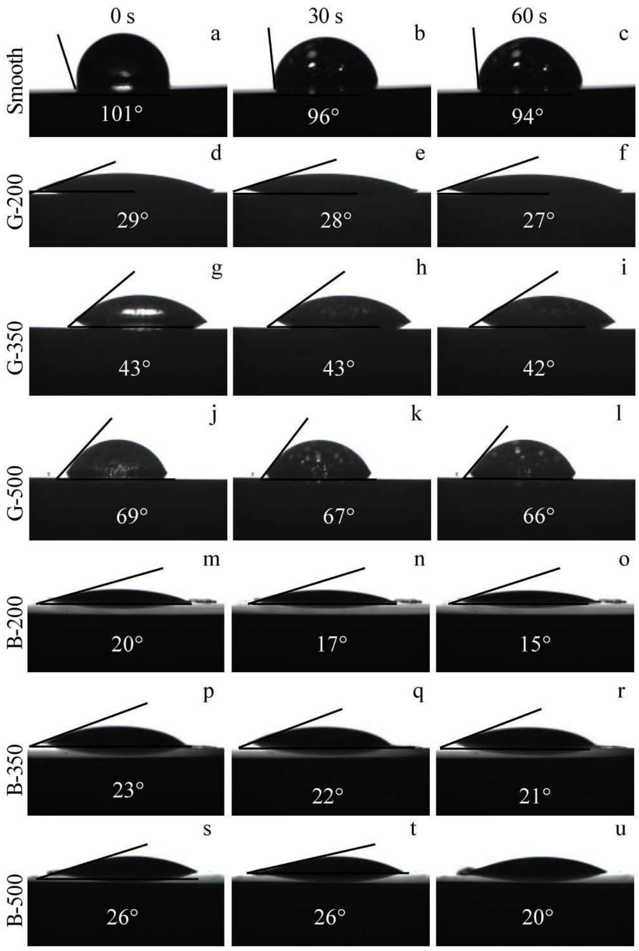

Fig.6 Contact angle observation diagrams with different time intervals of different surfaces: (a−c) smooth surface; (d−l) groove-shaped surface; (m–u) block-shaped surface

resistance caused by the uneven inner wall and the interfacial tension between the droplets and the inner wall of the groove, showing superhydrophilic properties. When the spacing of the groove structure decreases, its capillary effect increases, which ultimately affects the surface contact angle[7].Generally, the wetting angle of the block morphology is smaller than that of the groove morphology, indicating that the block morphology has better hydrophilicity.

The morphology of droplets on the studied surface is not only affected by the physical (roughness) and chemical (composition) characteristics of the material surface, but also by the combined action of surface force (surface tension), volume force (mainly gravity) and external excitation (atmospheric pressure, vibration, etc), so the wetting state of the material surface will change. The hydrophobic state of the magnesium alloys surface prepared by laser is unstable, and the surface hydrophilicity gradually increases over time. This transformation is mainly attributed to the rough micro-nano structure surface accelerating the chemical reaction between the magnesium alloy and the liquid droplets. The upward movement of the generated gas generates the reaction force that promotes the downward movement of the liquid-gas interface, and the released heat causes the increase in system temperature and the decrease in interfacial tension. At the same time, the solid matter formed by the chemical reaction also affects the interfacial tension.

2.3 Cell adhesion with different morphologies

Since the groove-shaped and block-shaped surfaces with the spacing of 200 μm are corroded too fast during cell culture, chemical reactions occur rapidly after contact with the cell culture medium. So the pH value of surface changes rapidly and toxicity increases. The surface corrosion is serious, as shown in the marked areas in Fig. 7, and it is not conducive to cell growth. Therefore, in cell adhesion research, only the groove-shaped and block-shaped surfaces with the spacing of 350 and 500 μm were studied.

The results of laser confocal microscopy observation show that compared with the untreated smooth surface, the block-shaped surface with the spacing of 350 μm has the optimal cell attachment, and the depressions and ridges are covered with cells. Fig. 8 shows the cell staining images of groove-shaped and block-shaped surfaces at different positions. According to the groove-shaped morphology with the spacing of 350 μm (Fig 8a−8b), it can be clearly seen that the cells grow along the groove, and the cell density is larger than that of the surrounding smooth surface. As shown in Fig.8c−8d, it can be clearly seen that the cells grow along the block-shaped morphology, and the cell density is relatively large. The cell density on the surface with the spacing of 500 μm is significantly lower than that with the spacing of 350 μm. Based on the experiment test results in Fig.6, the final contact angles of the G-350, G-500, B-350 and B-500 samples are 42° , 66° , 21° and 20° , respectively, all of which show hydrophilic surfaces. Moreover, the block-shaped surface has better hydrophilicity than the grooved surface. Generally, hydrophilic surfaces have better cell adhesion than hydrophobic surfaces[19]. To sum up, the block-shape surface with the spacing of 350 μm is most suitable for cell growth.

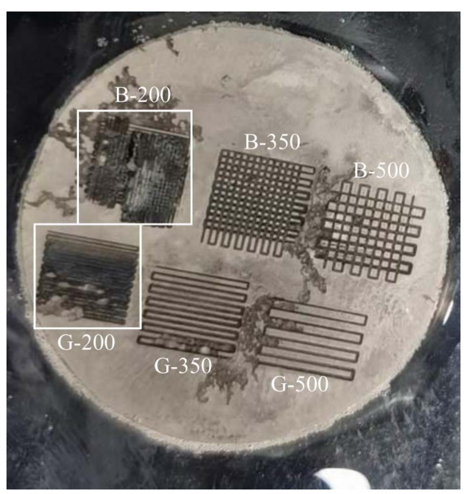

Fig.7 Corrosion maps of groove-shaped and block-shaped surfaces with different spacing

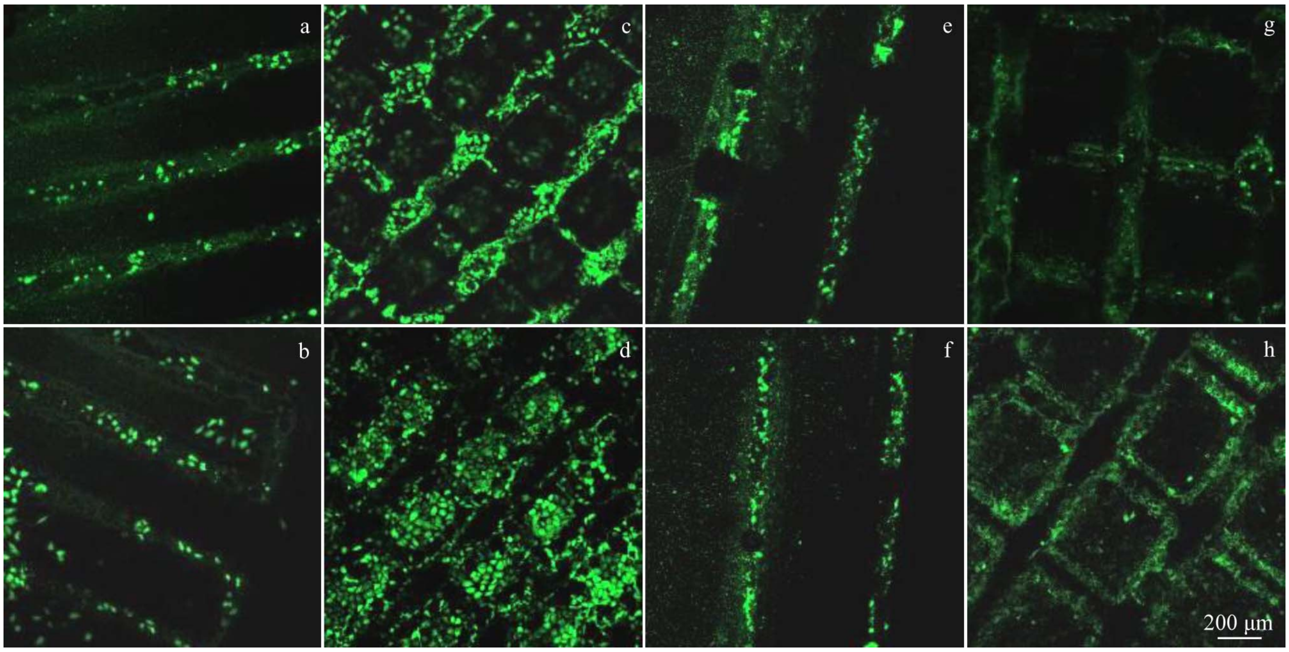

Fig.8 Cell staining images of groove-shaped (a–b, e–f) and block-shaped (c–d, g–h) surfaces at different positions with spacing of 350 μm (a–d) and 500 μm (e–h)

It is found that the chemical composition, wettability, morphology and roughness of the material surface will affect cell compatibility[20]. Therefore, the reasons for cell differences will be explained through these aspects below. Severe oxidation reaction occurs on the surface after laser treatment, resulting in the formation of loose magnesium oxide on the surface. Based on the strong activity of magnesium alloy, the same oxidation occurs on the surface of untreated magnesium alloy. Even if there is a trace amount of calcium and zinc burning loss in the process of laser ablation, it can be ignored because of its very low content on the surface. Therefore, the effect of chemical composition on the cytotoxicity of different microstructure surfaces can be excluded firstly[7]. The most critical factor is the difference of surface structures which makes the difference in surface roughness. First of all, after laser treatment, the surface area increases, which increases the contact area with nutrients and it is conducive to cell adhesion and growth. Secondly, based on the adherent growth characteristics of cells, the geometric microstructure constructs the environment conducive to cell growth. Finally, the groove-shaped surfaces are conducive to the deposition of cell secretions[20]. The above three points are the main factors leading to the difference in cytotoxicity between the geometric surface and the base metal surface in this experiment. The surface structure also affects cell behavior and the interaction between cells. At present, there is no unified conclusion on the mechanism of action between microstructure and cells. It is worth mentioning that the corrosion rate of the magnesium alloy surface in the medium is very fast, and the increase in the microstructure surface area will accelerate the reaction rate, but it is not infinite increase. After reaching a certain number, it will also inhibit corrosion. In other words, the size of the structure also has a certain limit. The interaction between surface microstructure and cells is carried out in the complex system, which still needs to be explored. As the implantable biological material, magnesium alloy still needs to be studied, mainly including cell metabolites, cell differentiation and in vivo cell compatibility. In summary, the laser preparation of magnesium alloy surface microstructure is a good method to improve cell adhesion, but the mechanism of different micro/nano structures still needs further study.

2.4 Corrosion resistance of magnesium alloys in different environments

With the background of magnesium alloy as the bone grafting plate after implantation into the human body, the laser-processed magnesium alloy was tested through the surface morphology observation, elemental detection, comparison with NaCl solution and comparison with that processed by PBS solution to investigate the corrosion resistance under different solutions. Fig.9a shows SEM images of the laser-processed magnesium alloy whose surfaces were corroded under cell culture medium. It can be seen that the surface grooves are covered by a large number of precipitated crystals. This crystal covering phenomenon can alleviate the corrosion of magnesium alloy to a certain extent. Elemental testing of the magnesium alloys surface after immersion in cell culture medium reveals that the relative content of elemental oxygen is 63.38wt%.

In order to study the corrosive effect of cell culture medium on the surface of magnesium alloy, the NaCl solution as control group was added. The surface is observed, as shown in Fig.9c. It can be seen that the corrosion is severe, but there is no precipitation of crystals because only sodium and

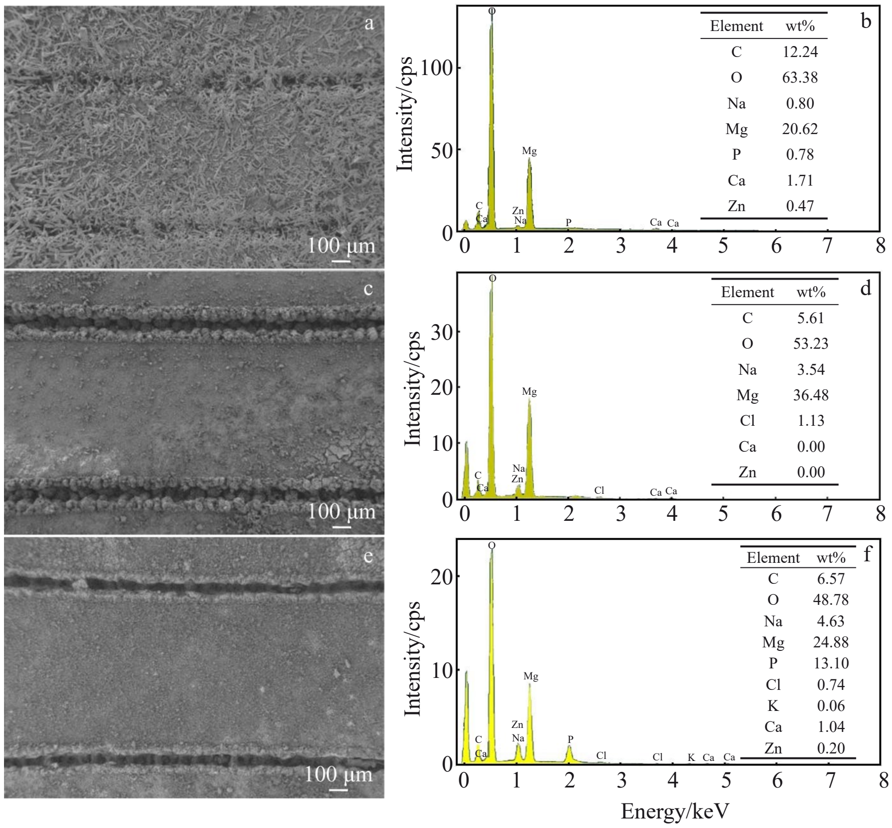

Fig.9 SEM images and corresponding EDS analysis of magnesium alloy surface in different environments: (a–b) cell culture medium, (c–d) NaCl solution, (e–f) PBS solution

chloride ions are present in the NaCl solution and no crystal-like substances are formed. At the initial stage of contact between magnesium alloy and NaCl solution, the main manifestation is the active dissolution of Mg on the surface of the alloy in water, and the specific reaction formulae are as follows:

$$Mg \rightarrow Mg^{2+} + 2e^- \quad (1)$$

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad (2)$$

$$Mg^{2+} + 2OH^- \rightarrow Mg(OH)_2 \quad (3)$$

However, pitting corrosion subsequently occurs on the surface of the magnesium alloy due to the high content of Cl^- in the NaCl solution. The reaction formula is as follows:

$$Mg(OH)_2 + 2Cl^- \rightarrow MgCl_2 + 2OH^- \quad (4)$$

The magnesium alloy after the same treatment is immersed in PBS solution and its surface is observed, as shown in Fig. 9e. It can be seen that the corrosion is lighter compared with that in NaCl solution and there is no crystal-like substance. Pitting corrosion subsequently occurs on the surface of the magnesium alloy due to the high content of Cl^- and HPO_4^{2-} in the PBS solution. The reaction formula is as follows:

$$Mg^{2+} + HPO_4^{2-} + OH^- \rightarrow Mg_3(PO_4)_2 + H_2O \quad (5)$$

As the corrosion proceeds, some corrosion products are deposited on the surface, which inhibits the further development of pitting corrosion, and the magnesium alloy after immersion in PBS solution has more corrosion products deposited on the surface than that in NaCl solution, so the corrosion degree is lighter.

Elemental testing of magnesium alloy surface immersed in NaCl solution and PBS solution found that the relative content of oxygen element is reduced, as shown in Fig.9d and 9f. The reason is the displacement reaction between magnesium alloy and hydrogen ions in solution, the formation of magnesium hydroxide, and insoluble magnesium ion salts combined with magnesium and chloride ions, such as $MgCl_2$. This process can also consume the chloride ions in the solution, thus reducing the combination of oxygen and chlorine, resulting in the decrease in the relative content of oxygen element. In contrast, PBS solution contains ions such as HPO_4^{2-}, which can combine with Mg^{2+} to form insoluble phosphate precipitates, such as $Mg_3(PO_4)_2$, resulting in relative decrease in elemental oxygen amount in solution.

3 Conclusions

1) The wetting angle increases with the increase in the spacing of the groove. In general, the wetting angle of the surface with block morphology is smaller than that with groove morphology, and the block morphology has better hydrophilicity.

2) Through the cell adhesion test, compared with the smooth surface, the B-350 surface has the optimal cell adhesion, and the depressions and bulges are covered with cells. The block morphology with the spacing of 350 μm is more suitable for cell growth.

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激光加工镁合金表面形貌的生物相容性

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摘　要：对镁合金表面进行激光加工改性，通过观察羊肋骨表面形貌，确定激光刻蚀形貌为沟槽。通过接触角测试，探究镁合金表面不同形貌的润湿性。通过细胞粘附试验，探究不同形貌的镁合金表面对细胞粘附、生长、迁移的影响。结果表明，田形形貌润湿角比沟槽形貌小，田形形貌的镁合金表面有更好的亲水性；与光滑表面相比，田形表面细胞附着性好，凹陷及凸起处均铺满细胞，这说明经过激光加工处理获得的显微图案有利于生物相容性的提高。

关键词：镁合金；激光表面改性；仿生；润湿性；细胞粘附

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