CHAPTER 7

Influence of USSP and Subsequent Stress Relieving on Biocompatibility and Cell Response

7.1 INTRODUCTION

This chapter presents the effect of ultrasonic shot peening (USSP) on biocompatibility of the near-β Ti–13Nb-13Zr alloy. USSP of this alloy resulted in generation of a nanostructured surface layer. Increase in wettability was observed after USSP and subsequent stress-relieving. Cell culture tests showed that MG63 cells adhered and spread more readily on the USSP treated specimen than on the Un-USSP specimen. MTT assays after 2 days of culture also indicated enhancement of cell proliferation on the USSP treated specimen than that of the Un-USSP one. MTT assay revealed no significant cytotoxicity in either substrate. The significant improvement of cell adhesion, spreading, and proliferation on the USSP treated Ti-13Nb-13Zr alloy was attributed to both grain refinement and micro-nano surface effects. These results demonstrate that USSP treatment not only improves the corrosion and fatigue resistance of the alloy but also enhances its biocompatibility, which makes it a strong candidate for applications as medical implants.

NOTE: In study related to biocompatibility and cell culture we have used three set of samples Un-USSP, USSP and USSP-SR. The duration of USSP was selected from 15 to 120 seconds.

7.2 SAMPLE PREPARATION

Disc shaped samples of 3 mm diameter and 2 mm thickness were used for the cell culture study. The samples were polished as stated above and rinsed with acetone for 15 minutes to remove contamination, if any. USSP was performed on flat surface of the specimens for the duration of 15 to 120 seconds using 3 mm steel shots and subsequently the shotpeened samples were cleaned in acetone.

7.3 OSSEOINTEGRATION

Only a few researchers have used mechanical surface treatments for Ti and its alloys and have demonstrated that the resulting nanocrystalline surfaces can improve cell adhesion, differentiation, and osseointegration. Zhao et al. used SMAT to create a nanocrystalline surface with an average grain size of around 25 nm on pure Ti, which improved cell adherence, morphology, viability, and proliferation of human osteosarcoma (Saos-2) cells [306]. Nanocrystalline surface with 20–40 nm grain size on Ti-6Al-4V alloy generated by SMAT, improved osteoblast attachment, spreading, viability, alkaline phosphatase (ALP) activity, mineralization, and osseointegration on the rabbit bone-implant interface [169,307]. Lai et al. used SMAT on pure Ti, that resulted in development of nanocrystalline surface with grain sizes of 60–80 nm, and observed increased cell adherence, spreading, proliferation, and differentiation over the treated surface [308]. Furthermore, as compared to the untreated counterpart, MG63 cell response was substantially superior on the SMAT-derived nanostructured pure Ti with surface grain size of roughly 10 nm [309].

However, it is worth mentioning that the forementioned studies mostly focused on commercially pure Ti and Ti-6Al-4V alloy, and there was limited literature on the effect of surface modification on β-Ti and related alloys using USSP technique. β-Ti

based implants have much lower elastic modulus than α+β type-Ti implants [310]. Low modulus could be expected to overcome the "stress shielding" effect caused by the mismatch of the elastic moduli between the implants and the surrounding hard tissue [120]. It is more important to improve biological effects of β-Ti alloys by USSP treatment. Based on the above fact, we investigated the possibility of creating a nanocrystalline surface layer on the Ti-13Nb-13Zr alloy using the USSP process in this study and evaluated its effects. The effect of USSP-induced grain size and associated surface property was studied on osteoblastic adhesion, proliferation and differentiation behaviour in vitro. The USSP and USSP-SR treated specimens in our study were found to be more effective for cell interactions than the Un-USSP samples of the Ti-13Nb-13Zr alloy.

As determined by XRD and microstructural characterization, following USSP, it was observed that this treatment caused surface grain refinement down to an average grain size of ~21 nm (Table 4.2). Morphological observations by SEM (Figure 4.7) and roughness measurements (Table 4.3) revealed irregular surface topography with compressive residual stress and higher roughness parameters at microscale, caused by multiple overlapping indentations due to USSP treatment. Micro-topographies have been identified as possible stimuli for osteogenic differentiation of stem cells and the production of new bone [311–313]. Several methods have been proposed for producing micro- or sub-microscale surface topography on titanium implants including sandblasting [313], acid etching [314,315], anodization [311], and sputter-deposition [316]. In this regard, the USSP treatment was thought to be a suitable option for implant surface modification due to its capability of creating micro-nano-scale surface topography that would be useful for improving bone anchoring by reinforcing the bio-mechanical interlocking phenomena.

Ultrasonic shot peening of the Ti-13Nb-13Zr alloy produced a nanostructured surface layer. XRD analysis and TEM micrographs revealed ultrafine grains of nano-size at the top surface. The fatigue resistance of the USSP240-treated samples was found to be improved by three times compared to the Un-USSP. Significant improvement in surface microhardness, nano-level grain formation, and related compressive residual stresses led to a considerable improvement in corrosion resistance and low cycle fatigue life of the USSP-treated samples as described in Chapters 5 and 6.

7.4 WETTABILITY TEST

The surface wettability of samples was measured using a contact angle measuring equipment. A 10 μl droplet of distilled water was suspended on the surface of samples. The observed images were captured and the contact angle of the drop on the coating was measured as shown in Figure 7.1. At least three individual measurements were performed on each specimen surface. Surface wettability studies revealed that USSP-SR samples had greater surface hydrophilicity than the Un-USSP and USSP treated samples. Wettability parameters of the different samples are shown in Table 7.1 and Figure 7.1. The Un-USSP sample showed highest contact angle, 64°, while the USSP showed decrease in contact angle. In the USSP-SR condition, there was further decrease in the contact angle.

Table 7.1 Wettability of the Un-USSP, USSP and USSP-SR samples.

Sr. No.	Sample Condition	Contact Angle (degrees)
(a)	Un-USSP	64±2.2
(b)	Un-USSP-SR	62±1.5
(c)	USSP15	49±1.8
(d)	USSP15-SR	48±1.6
(e)	USSP30	43±2.1
(f)	USSP30-SR	37±1.4

(g)	USSP60	44±1.6
(h)	USSP60-SR	42±1.3
(i)	USSP120	46±2.2
(j)	USSP120-SR	37±1.2

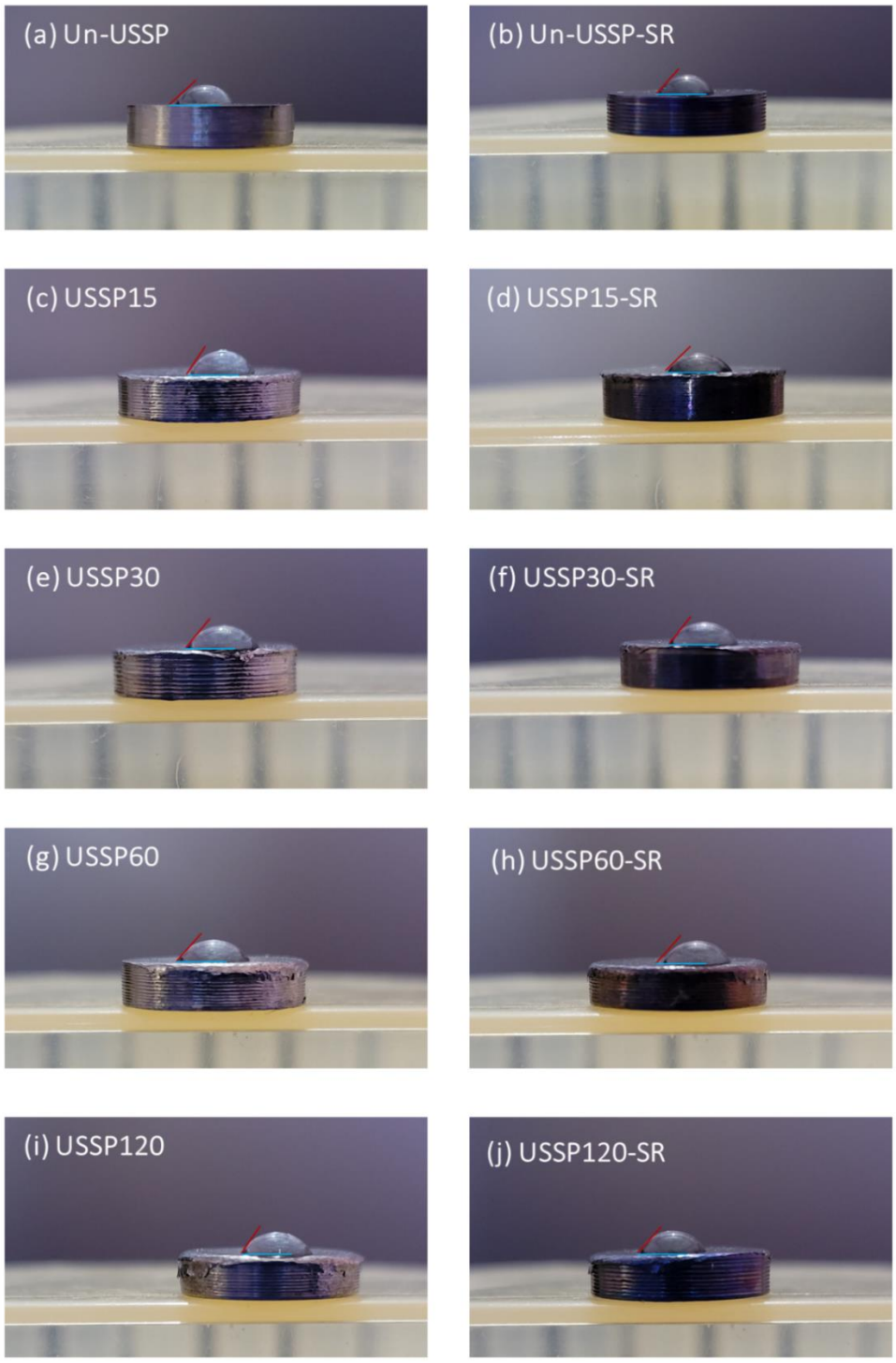

Figure 7.1 Contact angles formed by distilled water droplets, applied on the surface of the Un-USSP, USSP and USSP-SR samples.

7.5 CELL CULTURE

Fluorescent microscopy images of MG-63 human bone osteosarcoma cells cultured on various specimens to examine the effect of surface topography on cell adherence are shown in Figures 7.2 to 7.5. On all of the samples, there is a noticeable increase in cell coverage as the culture duration progresses, indicating a steady cellular proliferation with time. The proportion of cell attachment and spreading in the USSP15 and USSP30 samples is similar. In the USSP treated samples cells are found to proliferate with incubation time compared to Un-USSP. Figures 7.2 and 7.3 demonstrate cell culture images of various Un-USSP and USSP samples after 2 and 4 days of incubation, where blue color indicates nuclei staining; red color shows actin cytoskeleton filaments staining**.** Extensive growth of cells can be observed on the surfaces of the USSP treated samples, following 2 days of incubation or culture.

Even after 4 days of incubation cells are found to be fully scattered in all the USSP treated conditions while in the Un-USSP condition cells are not proliferating. All the available surface area had good coverage of cells for the both USSP and USSP-SR samples. However, after 4 days of cell culture, a distinct difference can be observed in the cell coverage. The cell culture images in Figure 7.3 of the USSP15, USSP30 and USSP60 samples, show significantly higher cell coverage compared with that of the Un-USSP and USSP120 samples. After 4 days of exposure, it is very difficult to differentiate the cell coverage, as in both the cases, there was interconnection of cytoskeleton filaments. Numerous studies have revealed higher osteoblast adhesion and proliferation on nano-rough surfaces compared to materials with no surface modification [102,317], while many others have shown no significant link [318,319].

Fluorescent microscopic images of cells cultured on stress relieved condition of the Un-USSP and USSP samples are shown in Figures 7.4 and 7.5 after 2 and 4 days of incubation. A significant increase in cell proliferation can be observed with stress relieving conditions of samples. After stress relieving more cells are found proliferating in the Un-USSP and USSP treated samples. All the USSP treated samples had good coverage of cells. After 4 days of culture a distinct difference can be seen in the cell growth of all the USSP treated samples. Cell coverage was found to increase with the USSP duration. When compared to the Un-USSP sample, the USSP treated samples have much greater cell coverage on the surface. Cell coverage on the USSP-treated surface increased gradually as the USSP duration increased from 15 to 120 seconds. The USSP-SR samples had a very high cell coverage of MG-63 cells, indicating that the stress-reduced USSP samples have a greater cell proliferation rate than the Un-USSP samples.

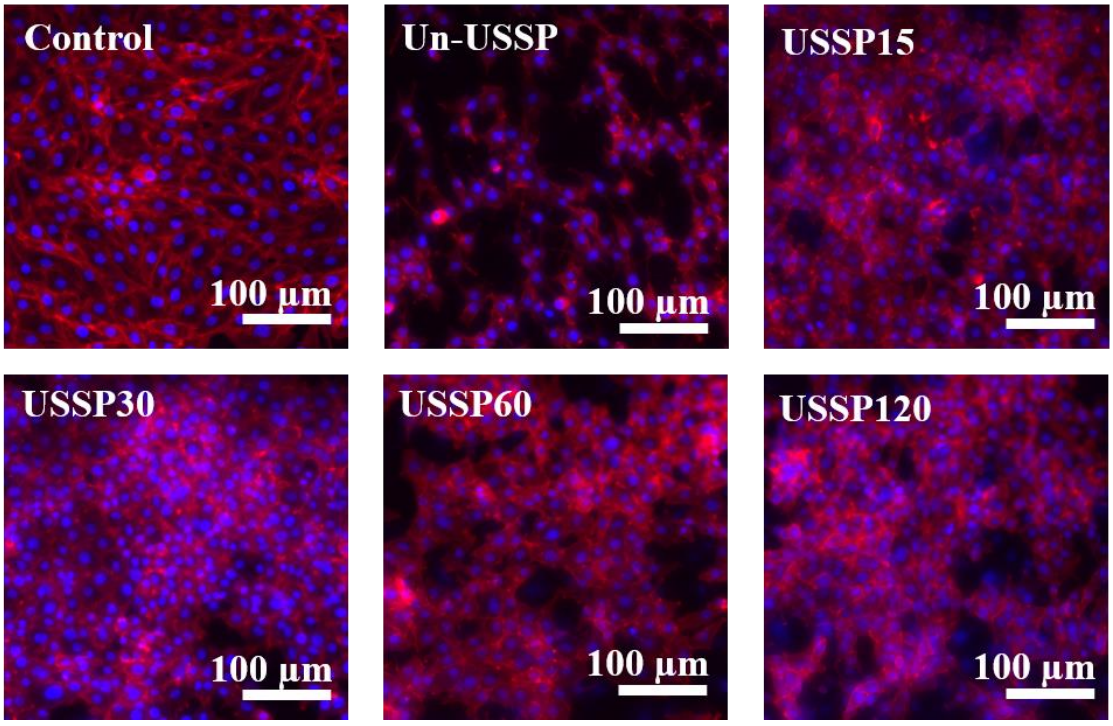

Figure 7.2 Cell culture images of on the various Un-USSP and USSP samples after 2 days of incubation. Blue color: nuclei staining; red color: actin cytoskeleton filaments staining.

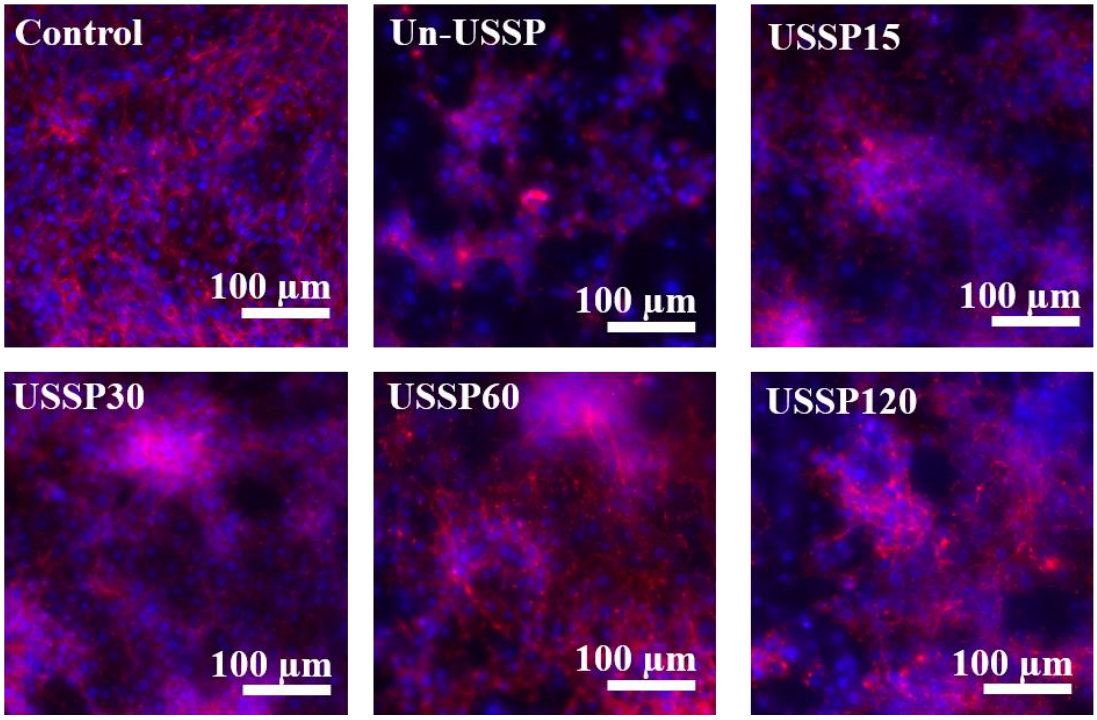

Figure 7.3 Cell culture images of on the various Un-USSP and USSP samples after 4 days of incubation. Blue color: nuclei staining; red color: actin cytoskeleton filaments staining.

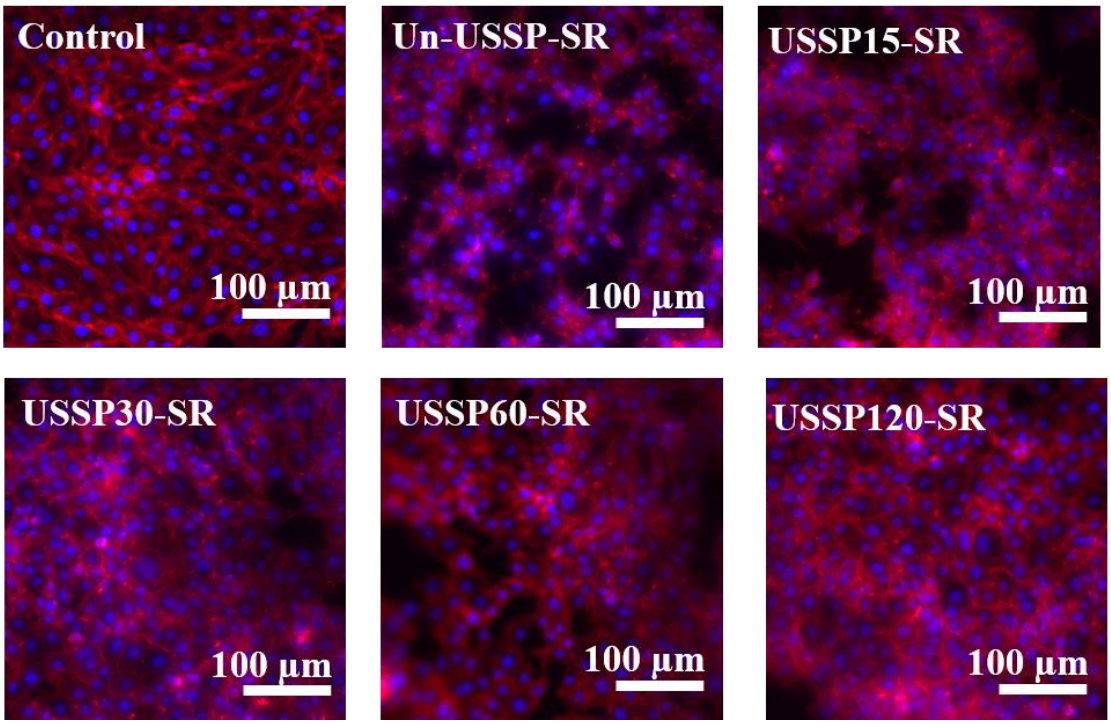

Figure 7.4 Cell culture images of on the various USSP-SR samples after 2 days of incubation. Blue color: nuclei staining; red color: actin cytoskeleton filaments staining.

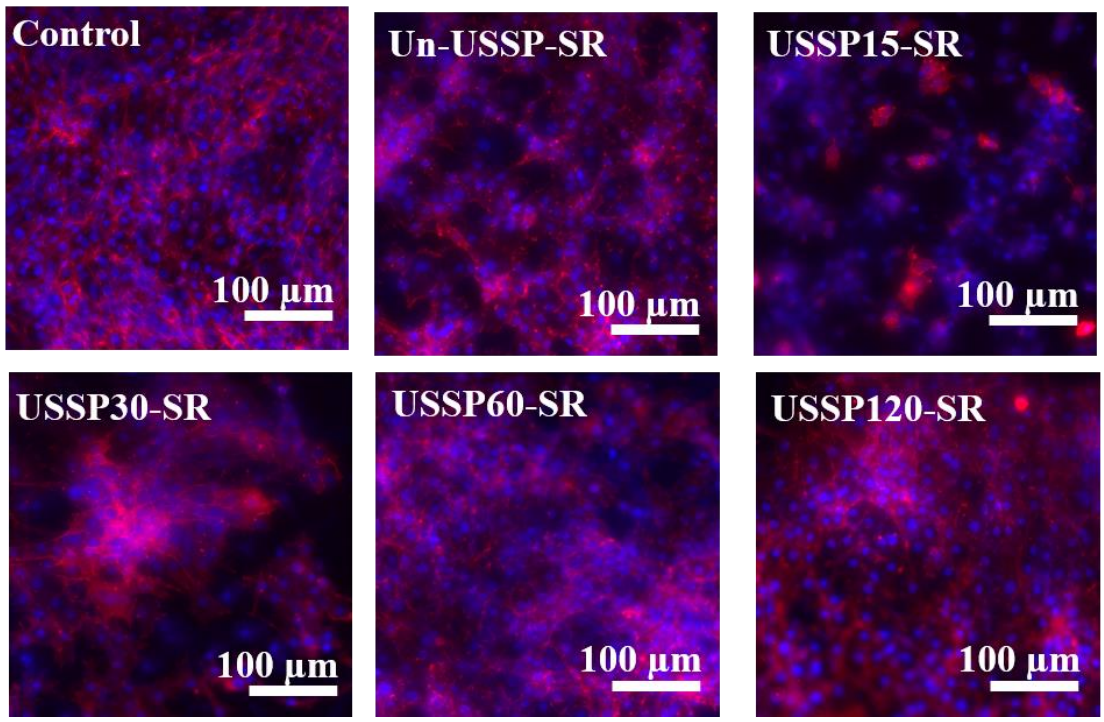

Figure 7.5 Cell culture images of on the various USSP-SR samples after 4 days of incubation. Blue color: nuclei staining; red color: actin cytoskeleton filaments staining.

7.6 MTT ASSAY

The MTT test was used to investigate the viability and proliferation of MG-63 osteoblast-like cells on the Ti-13Nb-13Zr alloy samples. After two-days incubation period, histograms of cell viability/proliferation for MG-63 cells under various conditions of the samples are shown in Figure 7.6. The histograms clearly showed progressive rise in cell proliferation with the duration of USSP treatment for all the conditions. The mean percentage of relative cell viability of the MG-63 cells on the Un-USSP sample shows that it increased from ≈ 25% to 36% after stress relieving. The cell proliferation in the USSP120 sample was highest ≈ 64%.

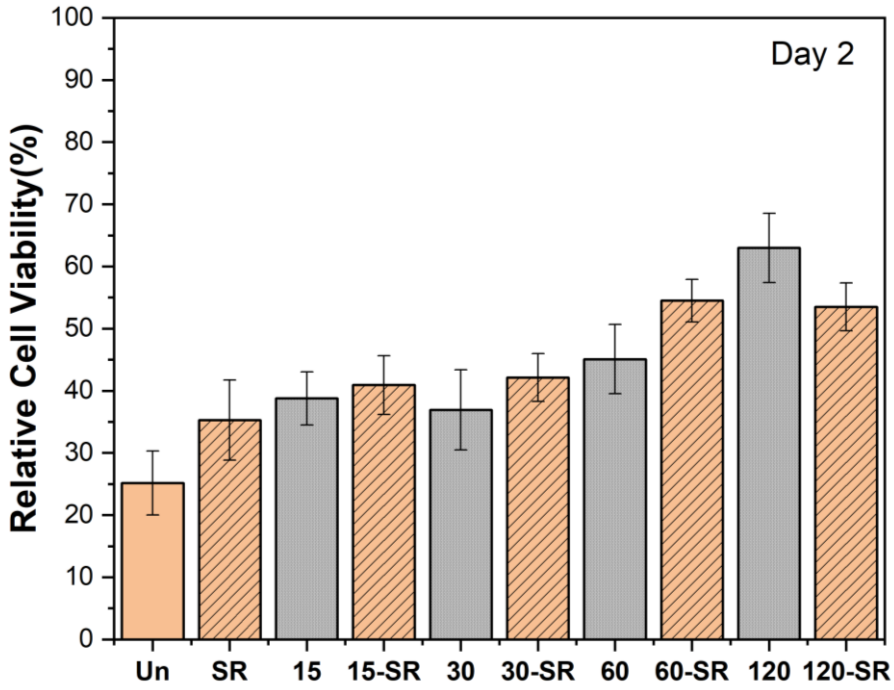

Figure 7.6 Histograms showing relative cell viability measured for the Un-USSP, USSP and USSP-SR conditions. The absorbance of the control for the second day culture was used as a reference for all the samples in this experiment.

These findings show that ultrasonic shot peening improved osseointegration. Since osteoblasts rely on anchoring, increasing surface roughness would allow them to adhere more often and tightly, with a faster rate of attachment and perhaps permanence. All the stress-relieved samples have shown improvement in the relative cell viability except the USSP120 sample.

7.7 DISCUSSION

Surface roughness and wettability are known to have major impact on cellular response. It has been suggested that, by increasing surface roughness on a sub-micron scale it might improve cell adhesion, proliferation, and differentiation [320,321]. Furthermore, it is commonly acknowledged that hydrophilic surfaces, as opposed to hydrophobic surfaces, promote early phases of cell adhesion, proliferation, differentiation, and bone mineralization [195].

Hydrophilicity is connected to surface energy of the material and has a direct impact on its interactions with proteins, cells, and micro-organisms. These interactions, in turn, affect the osseointegration process [140,322]. The greater the surface's hydrophilic character, the smaller the contact angle [323]. When the liquid is water, if the angle of contact created between the surface and the drop is less than 90°, the surface is hydrophilic; otherwise, it is hydrophobic [324,325]. Hydrophilic surfaces have higher cell adhesion, which aids in osseointegration. According to this criterion, all of the materials examined in this investigation were hydrophilic. However, hydrophilicity isn't the only factor that influences how cells interact with the surface of a biomaterial. Surface wettability is a measure of surface energy that is strongly connected to the significant increase in the grain boundaries during grain refinement process, such as USSP. Because of higher surface energy and reactivity, nano-grained surfaces have higher wettability than coarse-grained surfaces. These surfaces have the capacity to regulate protein absorption that interposes cell adhesion, as well as adjust and boost subsequent cellular activities [326].

A contact angle less than 65° is typically considered as a hydrophilic surface, whereas one more than 65 is regarded as hydrophobic [312]. As a result, the surface of the Un-USSP samples was slightly hydrophobic, whereas the USSP and USSP-SR samples were strongly hydrophilic (Table 7.1).

High cell growth was found in the USSP treated samples (Figures 7.2 and 7.3) which could be associated with surface nano-structuring, optimum surface roughness and presence of high positive potential on the surface [187]. It has been reported that during the initial stages of osseointegration, the previously formed bones are resorbed and creation of lacunae (gap/pits) of 100 μm diameter occurs. These macroscale surface

modulations naturally attracts cells [312]. The nanostructures formed on the implant surface keep proteins in a configuration that is favourable to their bioactivity. These proteins are important for cell adhesion and differentiation [327]. It is well established that cell adherence and viability over the implant surface are linked to protein adsorption over the surface. Cells are regulated indirectly via surface-adsorbed proteins rather than direct signaling from the nano-surface [327]. The USSP treated samples have higher positive potential on surface. This may lead to preferential adhesion of proteins. These attached proteins can also aid in enhanced cell viability. In comparison to negative potential, the non-negative potential on the surface promotes cell spreading [328]. Cell death has been found to be caused by negative potential on the surface of titanium [329]. According to Aizawa et al. [330], a properly modelled cell potential could affect protein production as well as gene expression and hence cell proliferation. Earlier studies also confirm the beneficial effect of positive potential on implant surface [331,332].

A TiO_2 oxide layer is responsible for exceptional corrosion resistance of titanium, chemical inertness, and biocompatibility. This oxide layer forms naturally when the material is exposed to oxygen and has a thickness of 4 to 12 nm depending on the Ti alloy. The most frequent polymorph variations of titanium oxide, rutile and anatase have a tetragonal structure, almost the same band-gap energy of 3.0 and 3.2 eV, respectively, and similar biocompatibility due to the production of OH^- groups on the surface. Rutile has a more compact crystal structure and is more stable at high temperatures [333–335]. Anatase is employed for biomimetical deposits of bonelike apatite [333], although rutile is better since it is blood compatible material.

The properties of surface oxide layer can affect biocompatibility of titanium significantly. Thus, various surface oxidation methods have been used to create a

functional implant surface by altering the surface properties of the native passive layer to improve osseointegration, such as electrochemical treatment (anodic oxidation) [336], chemical (acid and alkali) treatment [315], plasma spray deposition [337], sol-gel formation [338], ion implantation [181], and thermal oxidation [339]. Thermal oxidation, during stress-relieving process based on the production of a thick and hard oxide on titanium surfaces, is thought to be a reasonably simple and cost-effective process among them. Temperature and time are the most important factors in achieving a good TiO_2 coating during this process. It has been observed that anatase film can bind calcium and phosphate ions from the physiological environment to produce a hydroxy apatite coating. The rutile coating on titanium, on the other hand, was linked to both basic and acidic hydroxyl groups on the surface, as well as surface energy. The combination of rutile and anatase may be responsible for the improved osteogenic characteristics of biomaterials. The presence of anatase and rutile in titanium may aid to boost its osteogenic activity. A decrease in contact angle was observed after stress-relieving treatment of samples. Wang et al. have also shown similar decrease in contact angle after thermal oxidation of titanium [340]. A porous structure over implant is generally preferred because such a surface has larger contact area. Also, the cells are capable to migrate inside such pores and inter-penetration helps in better osseointegration [256]. The oxidized film formed over the treated surface may provide such porous structure to help the process of osteointegration. There are numerous nucleation sites for oxide films due to defects generated during USSP. The resulting oxide film formed on such surfaces of the USSP-SR samples would be porous, giving beneficial effect for cell response [341]. The increase in cell viability following stress relieving of the Un-USSP and USSP treated samples may also be attributed to relieving of associated compressive stresses in the surface region [342].

7.8 CONCLUSIONS

The following conclusions can be drawn from this chapter:

1. A significant increase in cell proliferation is observed with increase in USSP duration.
2. After 48 hours of incubation there is about 160% increase in cell viability of the MG-63 cells from the 120 s USSP treatment.
3. The cell proliferation is found maximum in the USSP 120 condition due to increased roughness and formation of nano-structures with high positive potential.
4. Improvement in cell viability in the USSP-SR samples can be attributed to the increased osteoblast anchorage due to combined effect of roughness and formation of biocompatible oxide layer on the surface via stress-relieving treatment.
5. A good amount of cell coverage is observed in fluorescence microscopy images of the USSP and USSP-SR samples compared to Un-USSP samples.
6. The present study shows that USSP treatment on alloy surface has significantly changed the surface architecture to nano level, which led to enhanced osteoblast cell adhesion and proliferation.