



Mirror-polished surface of ceria-stabilized zirconia/alumina nanocomposite enhanced the adhesion strength of human gingival fibroblasts

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INTRODUCTION

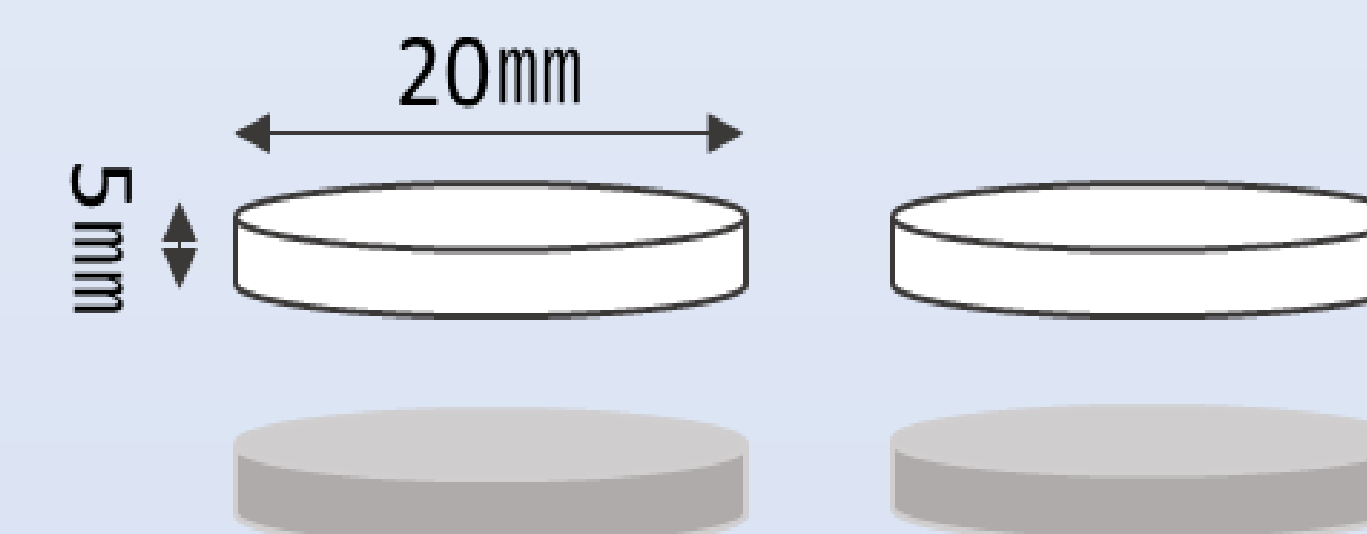
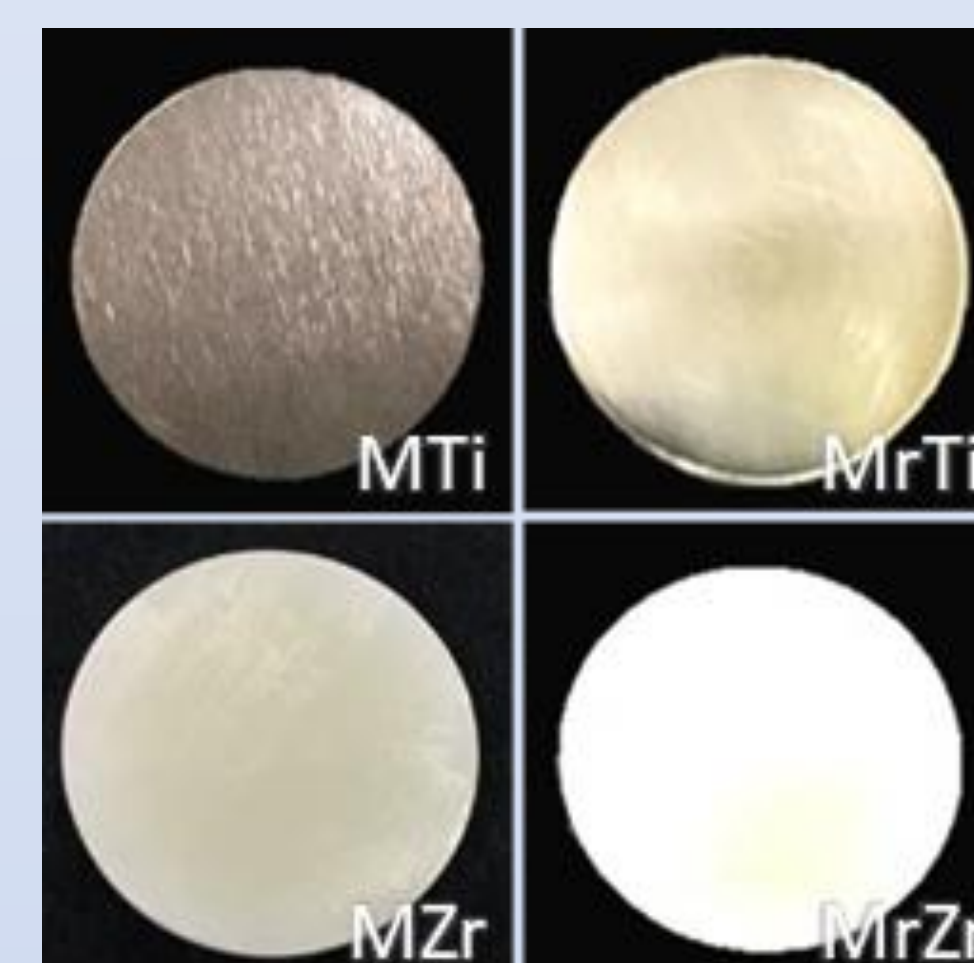
In implants, zirconium (Zr) abutments are potential substitutes to titanium (Ti) and offer better aesthetic result [1]. Furthermore, Zr can replace metals in both clinical medicine and dentistry owing to its high level of mechanical strength and excellent bioactivity [2]. In 1998, improvements in nanotechnology developed a strong ceria-stabilized tetragonal zirconia polycrystal/alumina (Ce-TZP/Al₂O₃) nanocomposite, which has an interpenetrated intragranular nanostructure. Compared with conventional 3Y-TZP, Ce-TZP/Al₂O₃ exhibits greater flexural strength and fracture hardness, making it appropriate for dental implants use [3]. Our previous studies on animals confirmed the overcome of the osseointegration strength of Ce-TZP/Al₂O₃ treated with hydrofluoric acid on acid-etched Ti implants [4]. Furthermore, we proved the improvement of gingival fibroblast biological affinity on a totally smooth surface without roughness or topographic features, such as mirror-polished Ce-TZP/Al₂O₃ surfaces (MrZr), as supported by markedly expedited cell spread, dense actin fiber formation, and upregulated expression of collagen and fibronectin, which functions as a key scaffold protein in focal adhesion plaques and is involved in linkage of integrin adhesion molecules to the actin cytoskeleton [5]. Connective tissue sealing between the transmucosal area of the dental implant surface and the soft tissue is important for the long-term stability of dental implants. This study aimed to investigate the mechanical adhesion strength of the human gingival fibroblast (HGF-1) to the ceria-stabilized tetragonal zirconia polycrystal/alumina (Ce-TZP/Al₂O₃) surface.

MATERIALS AND METHODS

1. Sample preparation

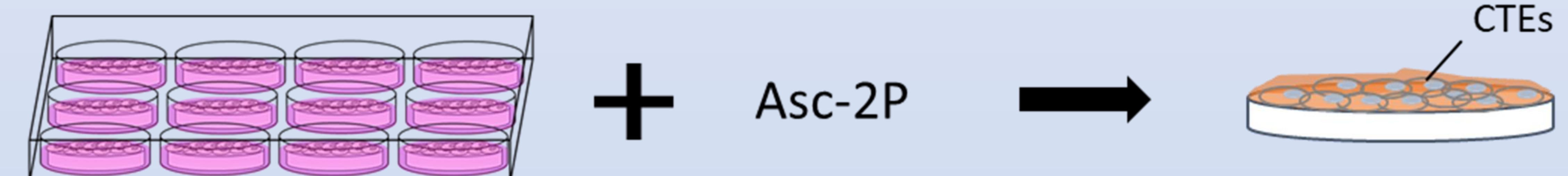
- ① Ce-TZP/Al₂O₃, Ti to produce the disks.
- ② Machined and mirror polishing of the manufactured disks.
Mirror-polished surfaces : MrZr, MrTi
Machined surfaces : MZr, MTi
- ③ Analysis of the surface properties of disks.

Surface roughness (Ra), hydrophilicity (CA)
Scanning electron microscopy (SEM),
Atomic force microscopy (AFM), Elemental analysis



2. Cell culture

- ① Cultivation of HGF-1 on the disks for 7 days.
- ② Adding Asc-2P allows fibroblasts to organize the CTEs (connective tissue equivalents : CTEs) by accumulating collagen and ECMs.



3. Assessment of the adhesion of CTEs to the substrate.

① Morphology and Talin1 expression observed by immunostaining of cultured HGF-1.

Talin1 (CellLight™ talin-green fluorescent protein (GFP) BacMam 2.0)
Actin (CellLight™ actin-red fluorescent protein (RFP), BacMam 2.0)

② RT-qPCR analysis of adhesion protein expression levels.

Talin1, Paxillin, Vinculin

③ Nano scratch test

We performed the whole experiment using a Triboindenter system (Hysitron Inc. TI950, Minneapolis, MN, USA) under constant environmental conditions (24° C, 35% H) . To detect the force of adhesion of the connective tissue by CTE on disks, we used the nano-scratch test.

① **To determine the scratch sites, we identified CTE on the disks via optical microscopy.**

② **Then, to confirm that scratching had been performed, we checked the *in situ* scanning probe microscopy (SPM) imaging before (1) and after (2) scratching.**

③ **From the first contact of the cell probe (D–x), the coefficient of friction increases with a rise in lateral force (D–y), and the maximum coefficient of friction (point y) leads to the initiation of the first cell detachment . In this study, we defined point y as the critical load value by virtue of recording a steady data compared to those after the first detachment (D–z).**

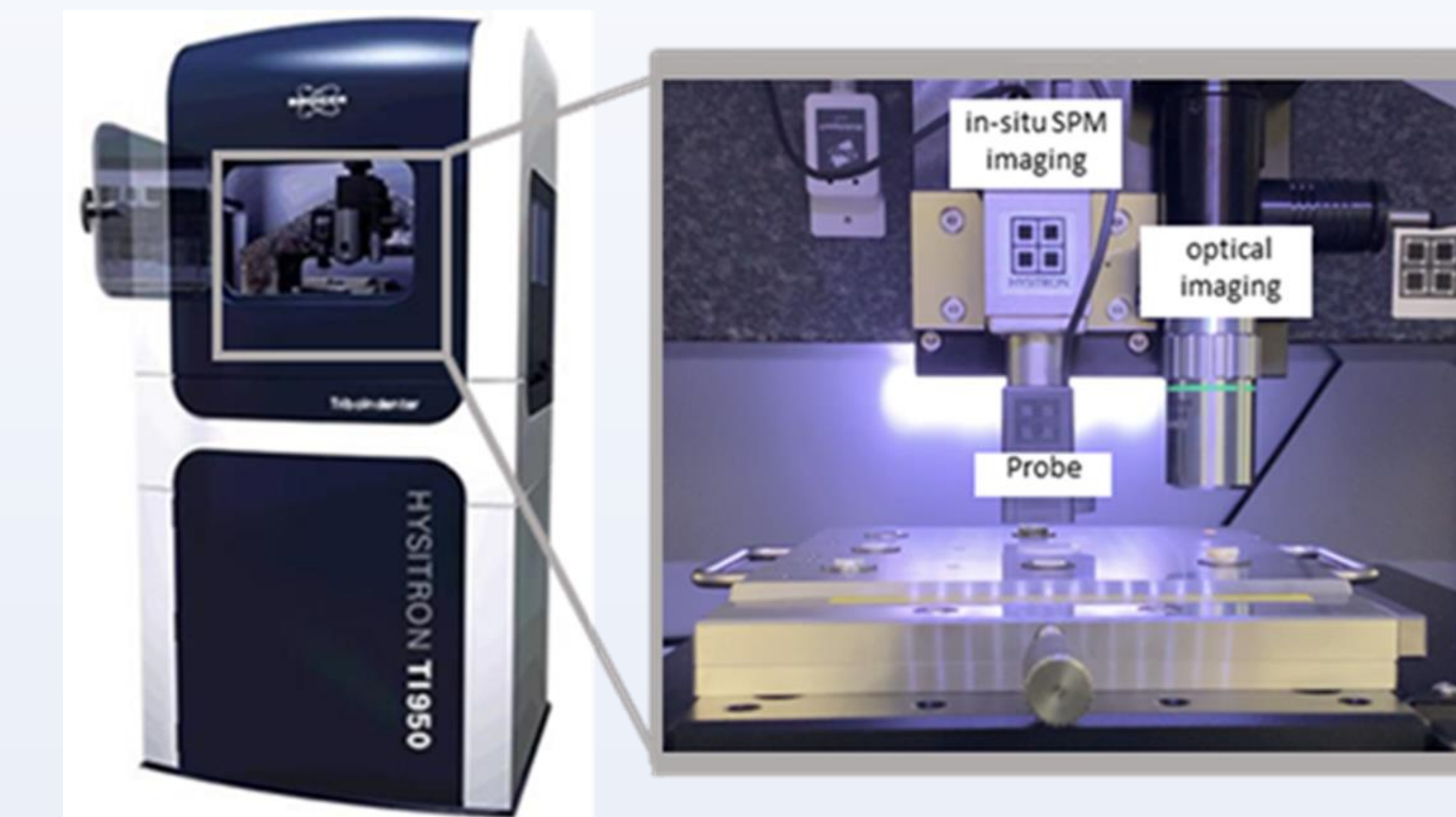
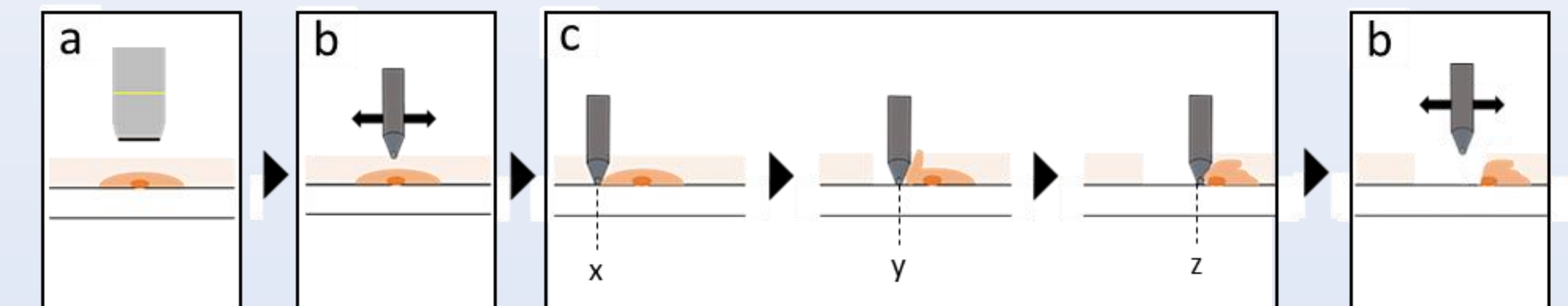
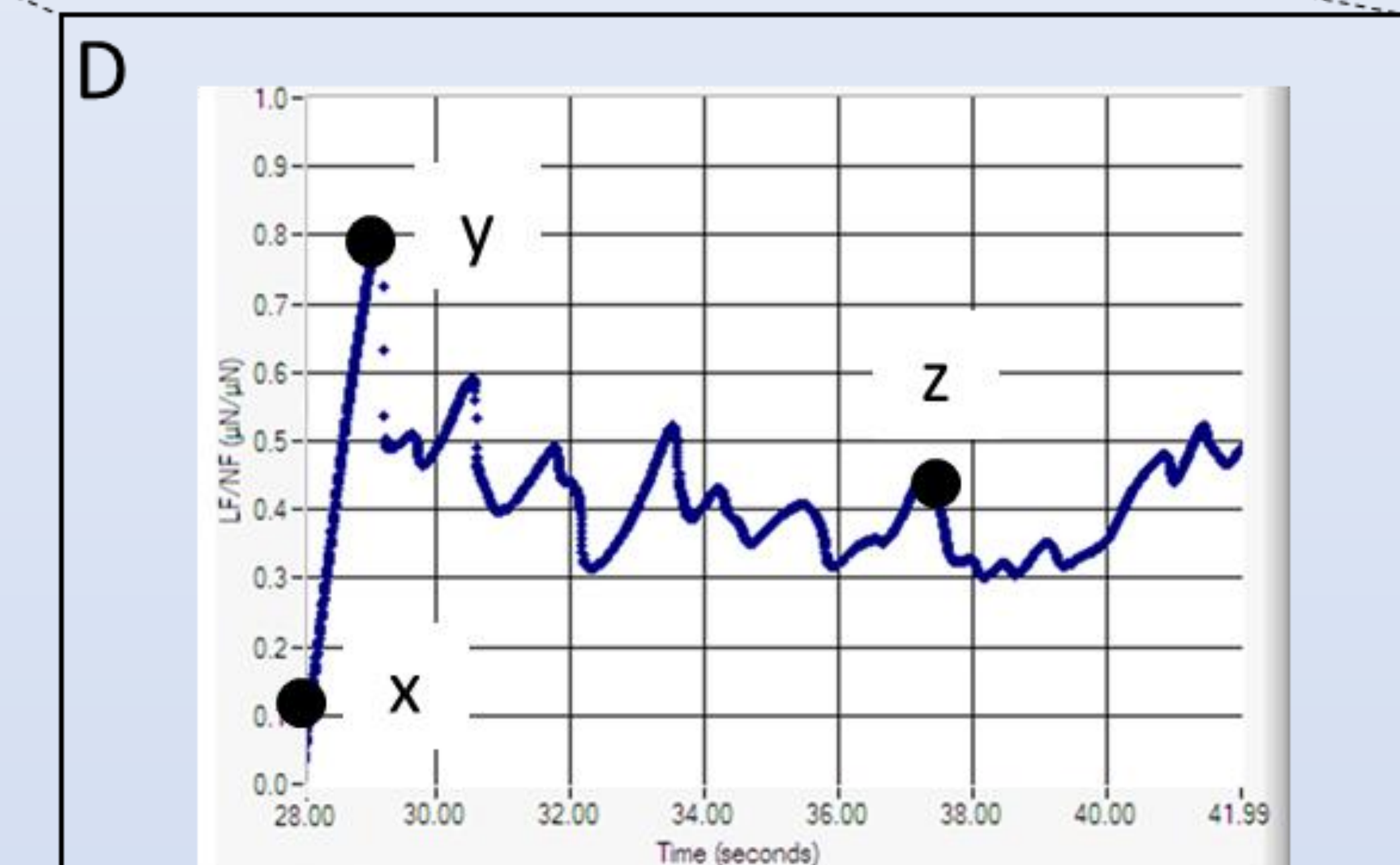
For each sample, we repeated the test four times and computed the average value as the measured value of the disk. (n=5)

Scratch protocol

Probe : 0.5μm spherical
Scratch range : 10μm
Scratch speed : 0.5μm/s
Vertical Force : 1000μN

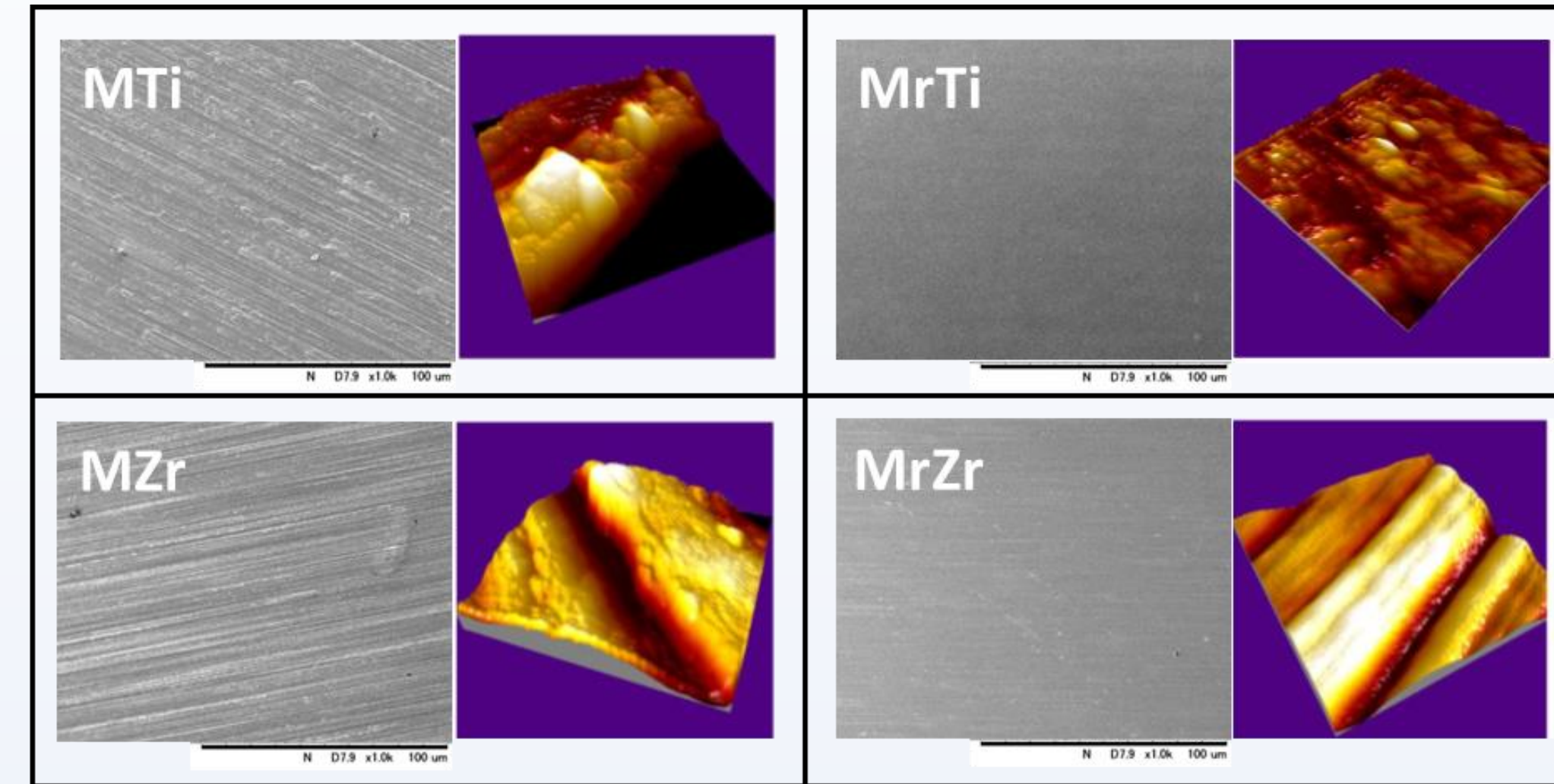
4. Statistical analysis

To evaluate the effect of the substrate on cell spread, protein synthesis, and critical load values, we applied two-way analysis of variance. For multiple comparisons, we performed the Tukey test. Significance was considered for $p < 0.05$.



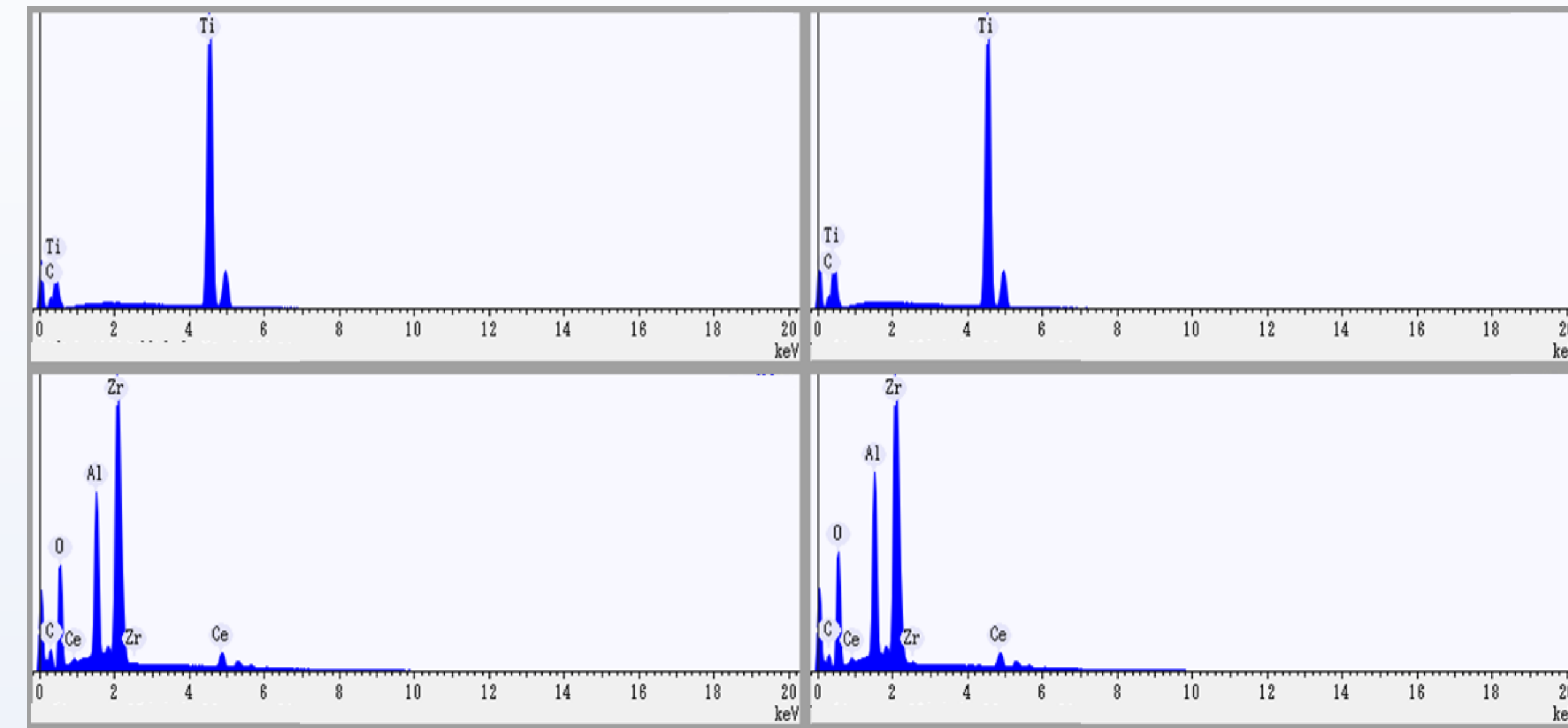
RESULT

Surface morphology



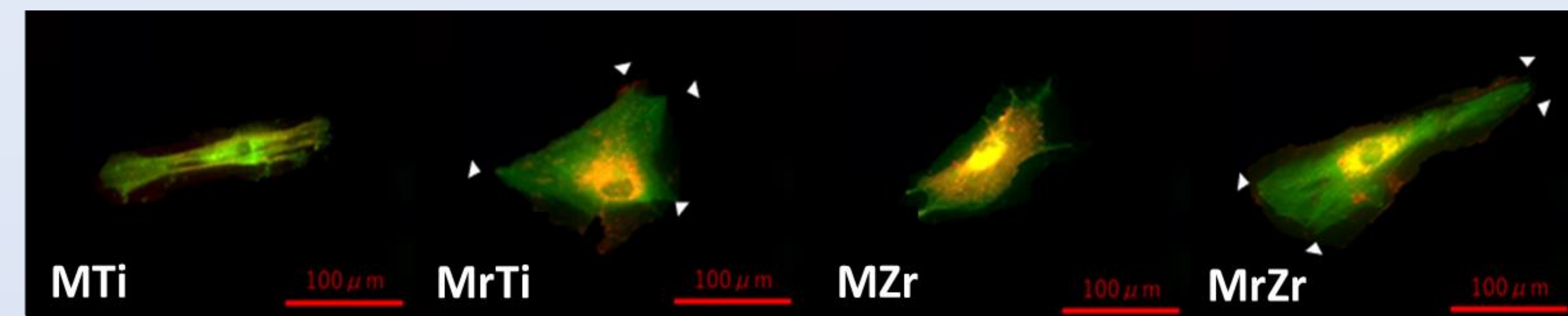
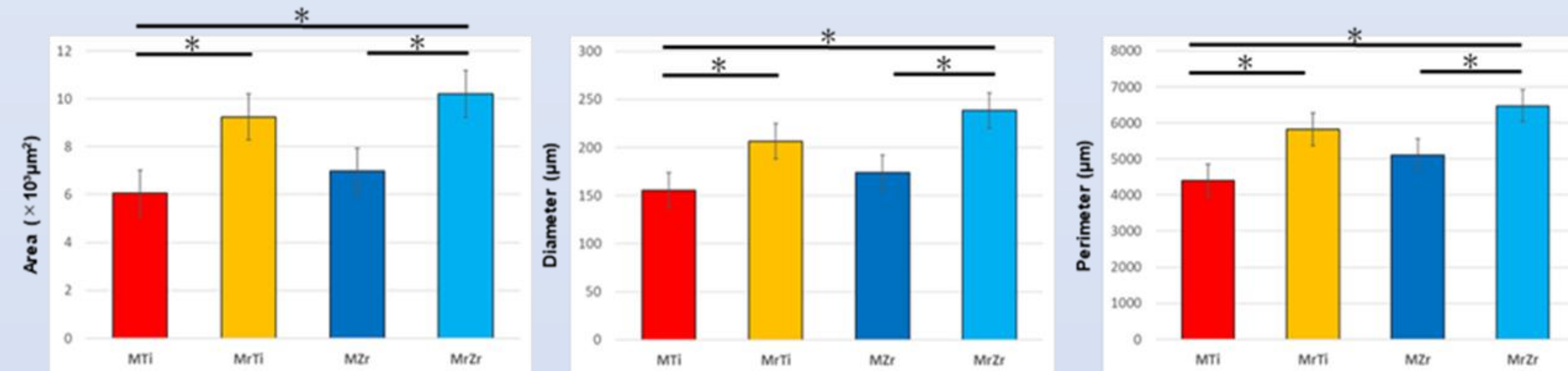
Different surface roughness profiles of Ti and Ce-TZP/Al₂O₃ disks characterized by SEM and AFM. Left : SEM Right : AFM

- The surface morphologies of both MTi and MZr disks were relatively smooth and flat, with some typical residual traces left from the grinding process .
- In contrast, the SEM images of MrTi and MrZr disks showed very smooth and flat morphologies, without topographical or rough features.
- The Ra values of MTi and MZr were 0.98 and 0.93 μm , respectively. Also, the Ra values of MrTi and MrZr were 0.02 μm .
- All sample surfaces of Ti and Zr disks were hydrophilic .



MTi and MrTi were composited with Ti 87.673 and 88.923 wt%. A small amount of carbon and oxygen was detected on the surface of MTi and MrTi. As described previously, Ce-TZP/Al₂O₃ consists of three elements, namely, ZrO₂, Al, and Ce. Both MZr and MrZr showed the highest contents of Zr (40.1 and 40.6 wt%, respectively). The contents of Al increased from 8.26 to 9.4 wt% after the grinding process. No significant differences were observed in Ce between MZr and MrZr (6.6 wt%). Additionally, the contents of C decreased from 13.62 to 9.35 wt%. Finally, the contents of O increased from 31.99 to 34.1 wt% between MZr and MrZr

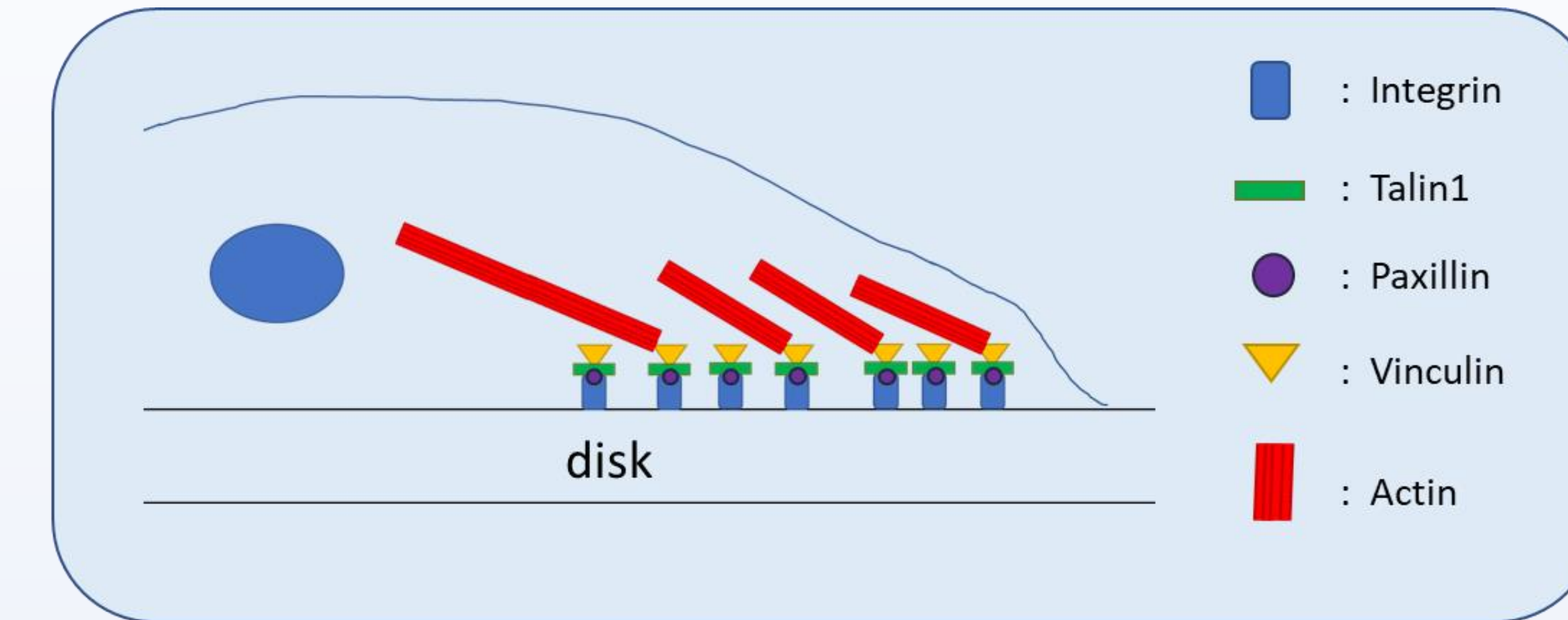
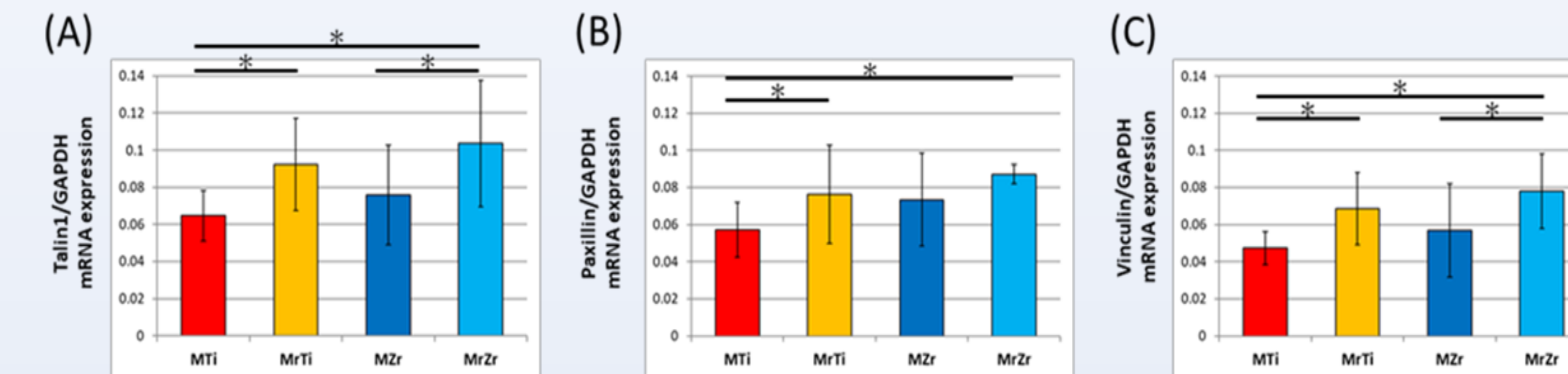
Cell morphology and spreading on Ce-TZP/Al₂O₃



Fluorescence phase contrast microscopy of the spreading, cytoskeletal composition, and talin1 distribution of HGF-1 cultured on Ti and Ce-TZP/Al₂O₃ for 7 days.

- We observed the morphology and cytoskeletal organization of HGF-1 on various Ce-TZP/Al₂O₃ and Ti surfaces under a fluorescence phase contrast microscope (BZ-X710; Keyence, Osaka, Japan).
- After 7 days of culture, the cells on mirrored surfaces consistently showed significantly higher values than those on machined surfaces for the following cytomorphometric parameters: cell area, perimeter, and diameter. The shapes of HGF-1 on both machined surfaces have several common characteristics including spindle-shaped and small protrusions . In contrast, HGF-1 deposits on both mirrored surfaces were widely spread with large protrusions (white arrows).
- Fluorescence phase contrast microscopy images, after anti-talin1 staining , labeled with GFP, and overlaid with images of actin expression, showed that fibroblasts on both surfaces expressed talin1 in their cytoplasm and at the tip of their extended cellular projections at 1-week incubation.

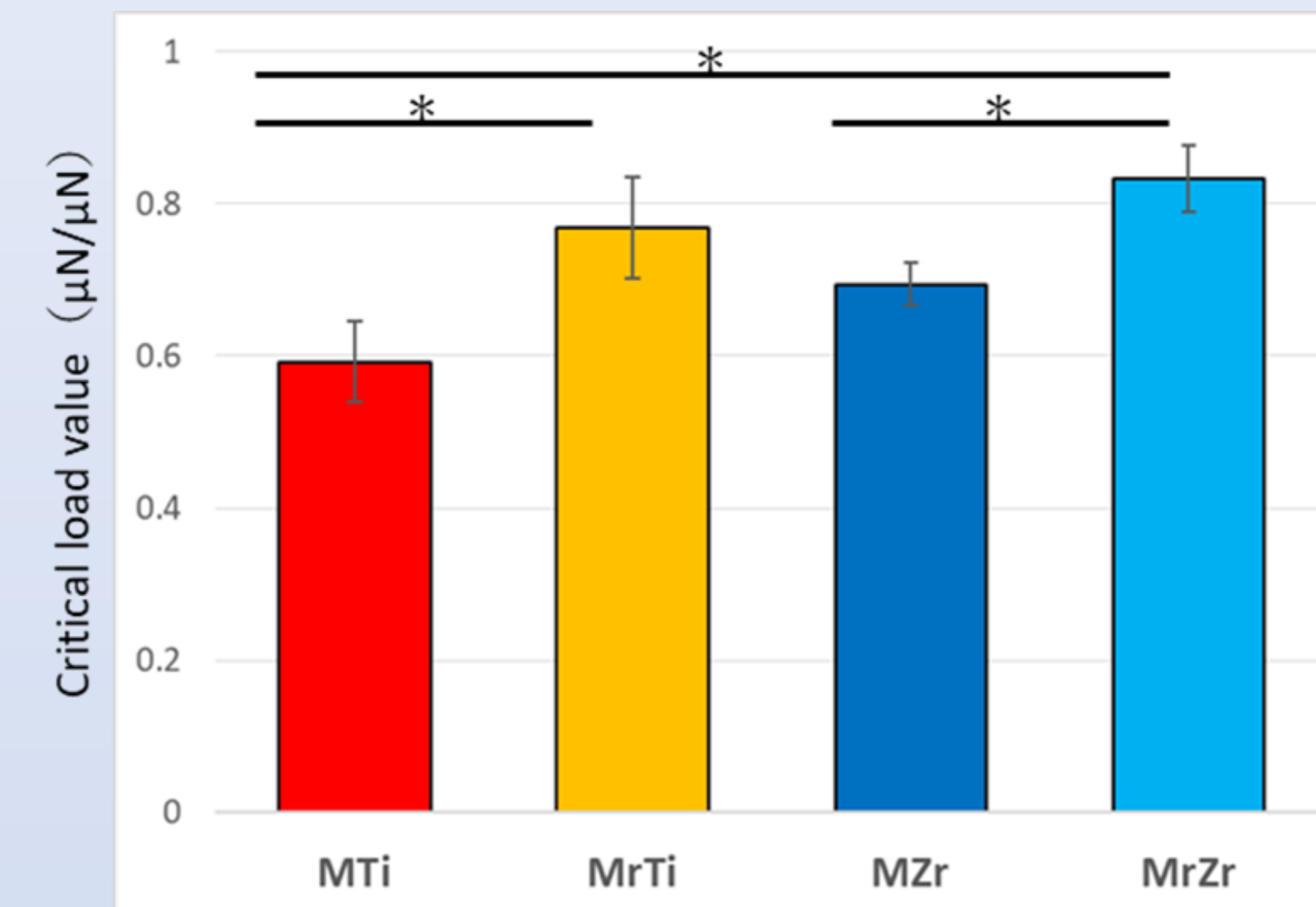
mRNA expression of adhesive protein



Expression levels of mRNA for talin1 (A), vinculin (B), and paxillin (C) in HGF-1 cultured on each disk for 7 days.

- The gene expression of focal adhesion proteins represents both the development of focal adhesion and the activation of subsequent signal transduction. First, the gene expression of talin1 upregulated equally the result of confocal image, so it reflects the widespread of cell.
- Expression of talin1 and vinculin in an individual fibroblast was substantially about 1.5 times significantly higher on MrZr than on MZr at 7-day incubation. Similarly, the identified expression on MrTi was around 1.4 times significantly higher than that on MTi .
- In contrast, paxillin expression levels were not significantly different between MZr and MrZr.

Nano-scratch test



The average critical load values of the four test substrates with standard deviations (n=5).

- At 7-day incubation, the mean value required to initiate detachment of CTE on MrZr was 1.3 times significantly greater than that on MZr ($0.83 \pm 3 \mu\text{N}$ to $0.64 \pm 4 \mu\text{N}$, respectively).
- Similarly, the force level on MrTi remained almost the same as Ce-TZP/Al₂O₃ even at 7-day incubation ($0.78 \pm 4 \mu\text{N}$), yet it was significantly greater than that on MTi ($0.61 \pm 1 \mu\text{N}$). The force on MrZr tended to be insignificantly higher than that on MrTi. ($p < 0.05$)

These results suggest that the CTEs on MrZr show higher critical load values than those on MZr, and are therefore more strongly attached to the substrate. CTEs on MrZr showed a higher critical load value compared to MZr, suggesting stronger adhesion to the substrate.

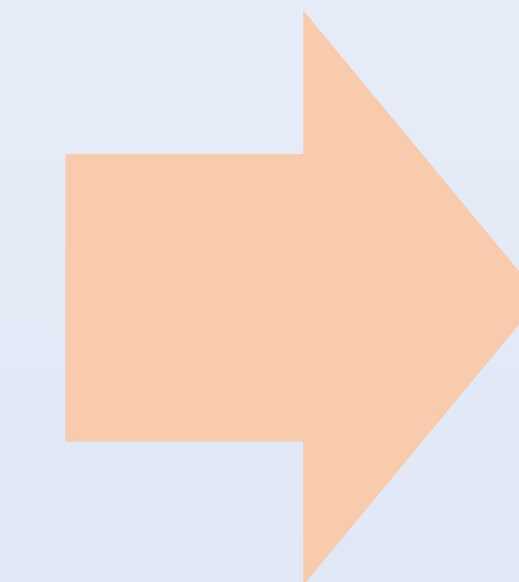
DISCUSSION

- Mirror polishing treatment modified adhesion strength and the cellular adhesive properties of individual fibroblasts. For example, the critical load values needed to detach CTE from Ti and Ce-TZP/Al₂O₃ were, respectively, 1.29 and 1.32 times higher on mirror-polish surfaces than on roughened surfaces at 7-day after culture. These results clearly indicate that cell retention is strongly increased on mirror polished surfaces, resulting in greater resistance to shear and compressive force that might cause cell detachment even in the final stage of cell adhesion.
- The expedition and advancement of cell spread accompanying focal adhesion assembly by Talin1, paxillin and Vinculin and cytoskeletal development may contribute to the enhancement of the capability of fibroblasts to adhere to mirror-polished surfaces, which have unique characteristics such as hydrophilicity, extremely smooth with micro-grooves created during the polishing process.
- The region at which fibroblasts become completely detached was unclear in molecular levels, even though traces of individual cells were detected by *in situ* SPM after delamination. The cell–material interactive mechanism underlying bio-activation on mirror-polished surfaces needs to be fully clarified. Clarification of how interfacial signal transmission from material to cell results in fibroblastic affinity and adhesion strengthening on mirror-polished Zr and Ti surfaces would be of great interest.

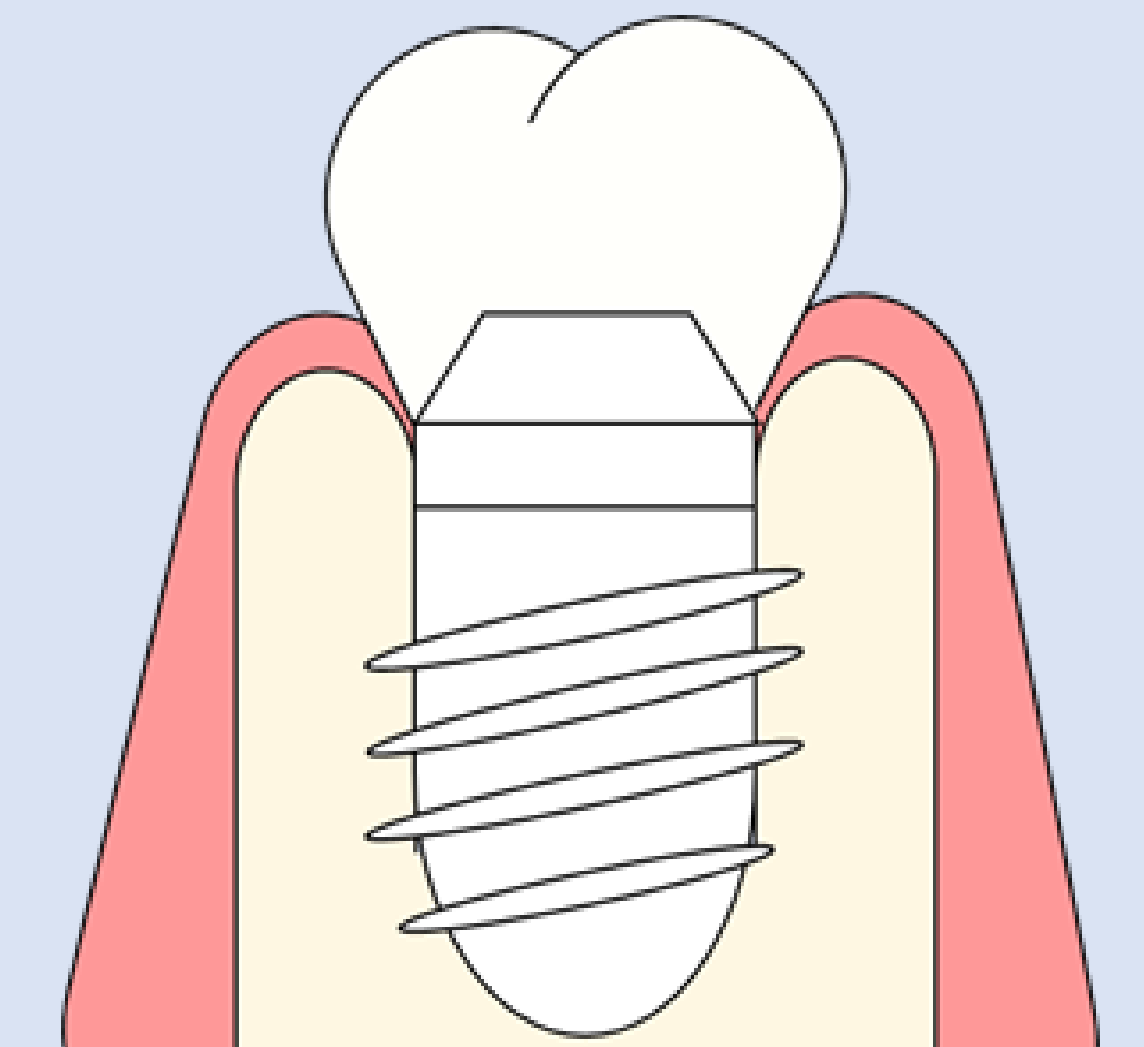
CONCLUSION

In the present study, MrZr compared to MZr ...

- ① **HGF-1 were more spread out and showed cytoskeletal development**
- ② **Increased expression of talin1, vinculin and paxillin**
- ③ **Critical load values at which CTEs start to delaminate were significantly higher**



It was suggested that Ce-TZP/Al₂O₃ with mirror-polished surface could acquire stronger soft tissue sealing properties.



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