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Ultrastructural Characterization of Hepatocellular Carcinoma Cells using Atomic Force Microscopy in Liquid and Air Conditions

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Abstract: Atomic force microscope is a powerful imaging instrument for the microscopic study of biological samples from an individual molecule to a living cell. Visualization of topology and morphology of live cells under physiological conditions with nanometer resolution is highly desirable but challenging due to the collision between the cantilever and samples. Investigations of the optimized conditions for ultrastructural characterization and surface changes of WCH-17 cells prepared with different fixation procedures were aimed at using atomic force microscopy. The experiments were set and designed based on cell culture experiments' standard protocols and procedures and atomic force microscopy analyses. Here the topological and morphological changing of WCH-17 cells under physiological conditions to fixed (air and liquid nitrogen fixation) cells were investigated using AFM imaging of live and fixed cells. Both fixation methods significantly changed the topology and morphology of the cells. Cells lose their surface smoothness and are fixed with vacuole-shaped bodies on the surfaces. The ability of AFM to use in liquid conditions makes it the most powerful device and technique for surface studies of live cells. Investigating cell surfaces can be used for many goals, from determining the molecular mechanism of cells to cell interactions or changes when treated with drugs.

Keywords: atomic force microscopy; WCH-17; ultrastructure.

1. Introduction

Atomic force microscopy (AFM) is a suitable technology for the characterization of topographical properties and mechanics of materials at the nanoscale length. AFM can image the surface topography of objects with the resolution of single atoms on a solid surface [1]. Furthermore, since AFM imaging can be performed in an aqueous condition, it can be used for biological samples such as proteins, nucleic acids, membrane lipids, and even live cells under

physiological conditions [2-7]. AFM is also applied for force measurements to estimate the strength of intra- and intermolecular bonds at the single molecule level [8-10]. In addition, AFM use for the mechanobiology measuring purpose aims to appoint the correlation of mechanical characteristics and physiological states of a living cell. [11-15].

High-resolution imaging of the cell surface is essential for gaining an understanding of the interactions that occur in communications between cells and within tissues. The usual and routine methods of AFM imaging of mammalian cells fixation or employing small concentrations of fixatives were necessary because higher structures were frequently too pliable for reproducible AFM scanning [16]. To overcome imaging problems in the dynamic system, the speed of AFM scanning has been extensively optimized by modifying each component of AFM [17,18].

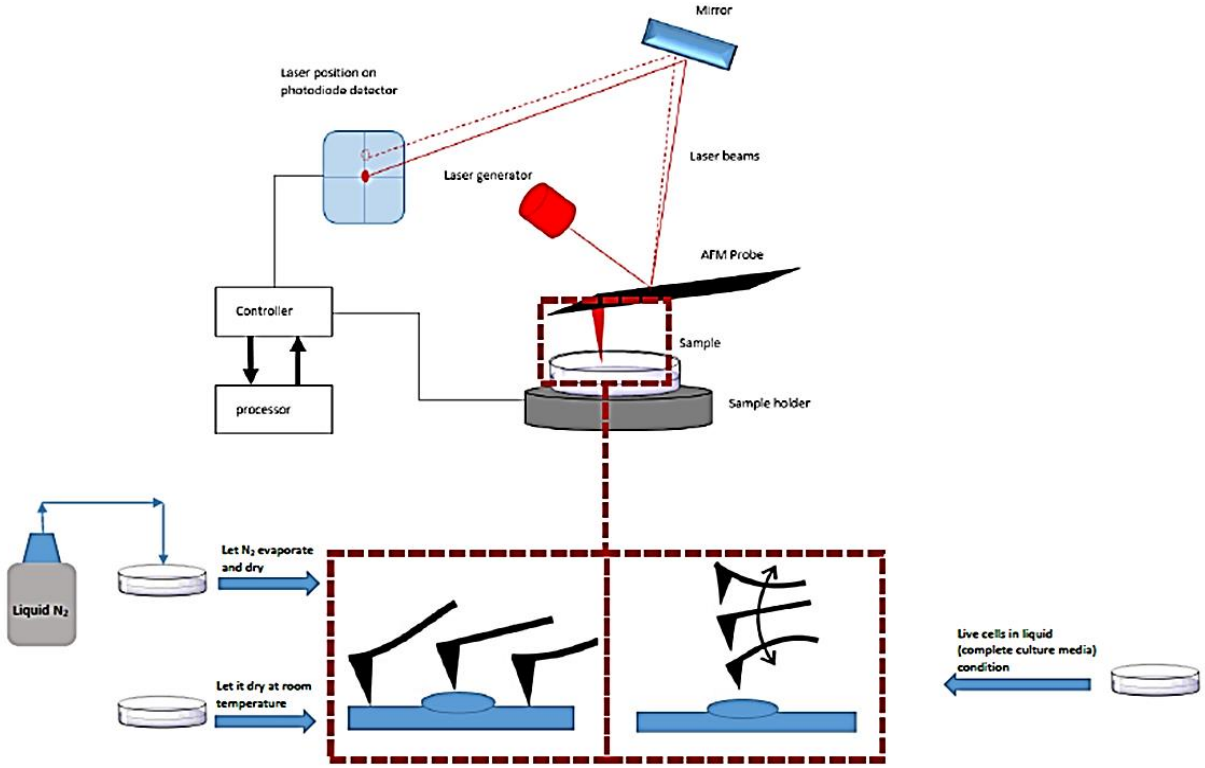

Figure 1. Schematic diagram of AFM principles, sample preparation, and modes used in this study **(a, b)**. The AFM instrument consists of a sample holder, a laser generator, a photodiode detector, a controller, a data processor (computer), and an AFM Probe. The AFM probe is composed of a cantilever with a tip attached at the end of the cantilever. The tip is the key for nanoscale imaging (whose type must change in different conditions), and the cantilever is a sensitive force measurement component. (**a**) Once fixed cells were prepared, add some liquid N2to petri dish and let all N_2 evaporate and dry, and once fixed, the cells were at room temperature. AFM imaging of fixed cells carries out in contact mode. The horizontal movement of the probe during the scanning in contact mode of AFM imaging schematically showed. (**b**) AFM imaging of live cells performed in complete culture media on intermittent contact (fluid) mode. Vertical movement of the probe during the scanning in tapping mode of AFM imaging schematically showed. The tip moves up and down, so there is no shear on the sample.

However, using conventional AFM for imaging dynamics of live biological samples has been difficult since recording an image and tip movement mostly destruct the sample takes many minutes. Developed AFM has been greatly used for material sciences (i.e., physical chemistry, chemical physics, surface science) and the characteristics of all molecular sciences fields [19-21]. Over the past 20 years, AFM has also become a standard tool in the life sciences for the study of biological phenomena such as surface topography [24–22], structural studies [25], and quantifying

biomolecule interactions [26– 30]. Hence, the microscope is a valuable complement to other characterization techniques that could be applied to the cell scaffold section.

In this study, we investigated the surface of WCH-17 cells in two conditions: Live cells without any fixation in Physiological conditions and fixed cells, to show topological changes of the cell membrane in two modes and recorded their AFM images. Figure 1 shows the schematics diagrams of AFM principles in two imaging modes.

2. Materials and Methods

2.1. Cell line culture.

WCH-17 cells (ATCC #: CRL 2082) were purchased from the Pasteur Institute of Iran. The cells were cultured in DMEM medium enriched with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO_2 based on the manufacturer's protocol.

2.2. Cell line characterization using invert microscopy.

The morphological studies of WCH-17 cells were done by inverted microscope at actual magnification 100× in two concentrations of cells 24 h after plating.

2.3. Cell line preparation for AFM microscopy.

WCH-17 cells were cultured on 40 × 11 mm cell culture Petri dishes in DMEM medium enriched with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO_2. AFM imaging was performed ≈ 24 hours after plating.

2.3.1. AFM set-up for microscopy of the cells in liquid condition.

The AFM system is housed on a vibration isolation platform. Our AFM set-up consists of the Nano Wizard 2 AFM (JPK Germany) mounted on an inverted Olympus microscope. The samples were imaged using intermittent contact (fluid) mode. The microscope was set at 150 Hz IGain, with 0.0048 PGain, and 1.0 V set point through the NanoWizard control. The cantilever was an HYDRA6V-100NG-10 probe with low-stress SiN and a V-shaped silicon tip. The rough data were converted to graphical information using a Nanoanalyzer software of the instrument. AFM imaging of live cells was performed in complete cell culture media (DMEM + 10% FBS). Fresh media reinject every 20 minutes. The temperature of the imaging solution and plate was kept at 37 °C by heating the plate holder.

2.4. Preparation of the cells for AFM microscopy in air condition.

2.4.1. Fixation of the cells in the air.

WCH-17 cells were cultured on 65 × 15 mm cell culture Petri dishes in DMEM enriched with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO_2. After 24 h cell, the culture medium was removed, and the Petri dish was washed with Phosphate-buffered saline (PBS) 3 times. Then let it dry and fixed overnight in the air at room temperature.

2.4.2. Fixation of the cells with liquid nitrogen.

WCH-17 cells were cultured on 65 × 15 mm cell culture Petri dishes in DMEM enriched with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO_2. After 24 h cell, the culture medium was removed, and the petri dish was washed with Phosphate-buffered saline (PBS) 3 times. Then add some liquid nitrogen to the Petri dish, giving it time to evaporate completely. Further, cells were fixed.

2.4.3. AFM set-up for microscopy of the cells in air condition.

Atomic force microscopy of the fixed cells was operated via contact mode using a JPK-AFM instrument. The microscope was set at 150 Hz IGain, with 0.0048 PGain, and 1.0 V set point through the NanoWizard control. An N-type ACTA-10 probe from silicon with 0.01-0. 025 Ω/cm was used for the experiment in air condition. Also, the rough data were converted to graphical information using the Nanoanalyzer software of the instrument.

3. Results and Discussion

3.1. Morphological confirmation of cell line.

The morphology of cells was investigated and confirmed with an inverted microscope of AFM. The cell's shape was the same at two concentrations and was similar to the previous report (Figure 2) [31].

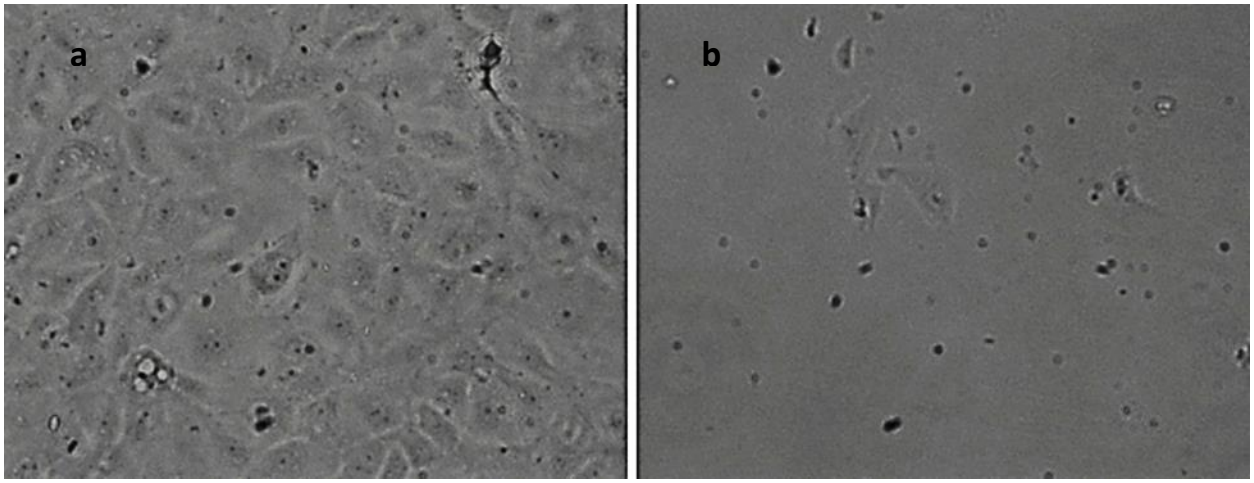

Figure 2. Morphological studies of WCH-17 cells in two congestions by inverted microscope at actual magnification 100×. (**a**) Plating 500 µL of cell suspension vs. (**b**) plating 50 µL of cell suspension in a cell culture plate. Images were recorded after 24 h.

3.2. AFM imaging analysis of live cells in physiological conditions.

The AFM analysis of a single WCH-17 live cell in liquid condition showed a smooth and uniform surface, and the overall shape of the cell was similar to that inverted microscope one. Cells are approximately about 25 µm. The micrographs are shown in Figure 3.

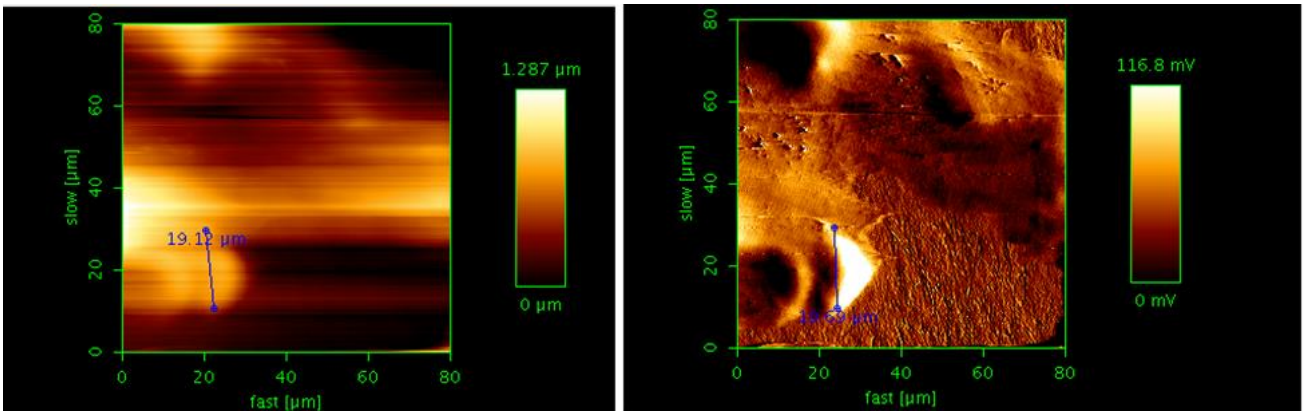

Figure 3. AFM image of live WCH-17 cells in physiological condition on intermittent contact (fluid) mode. Left: Representative AFM height measured-trace image. Right: Representative AFM error-trace image.

3.3. AFM imaging analysis of air-fixed cells.

AFM micrographs of air-fixed WCH-17 cells showed that the smooth texture of the cells' surface got changed. They had protrusions and depressions that are recognizable in the images shown in Figure 4. The main shape of the cell also changed. Other parameters, including surface roughness, were recorded (Table 1). Micrographs represent that surface roughness was increased compared with live cells. Additionally, cell size decreased when it was fixed.

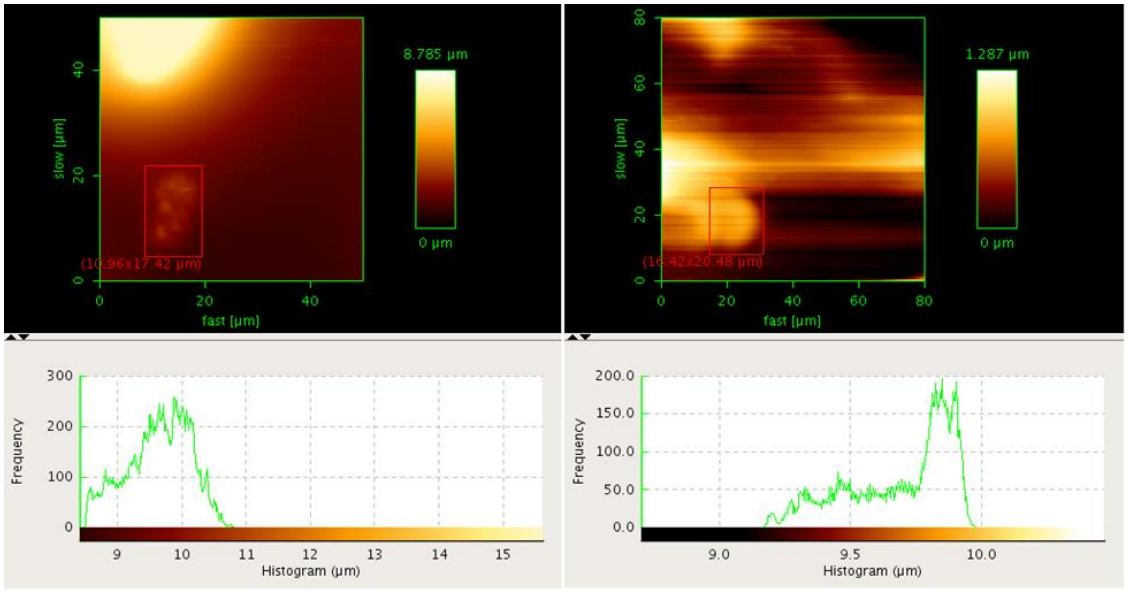

Figure 4. AFM image and micrograph of air-fixed WCH-17 cell on contact mode(left) and live WCH-17 cell (right). Note: the scan ranges are different. The AFM image scan range of fixed cell is 60 µm, and the AFM image scan range of live cell is 80 µm

3.4. AFM imaging analysis of Liquid N2 fixed cells.

The AFM analysis of Liquid N_2 fixed WCH-17 cells revealed a topographically vacuolated cell's surface again, but more obvious protrusions and depressions in comparison with the fixed cells under air conditions. N_2 Fixed cell morphology also changed from a curved triangle to a circular shape. Micrographs also showed increased surface roughness compared with live cells (Table 1) (Figure 5).

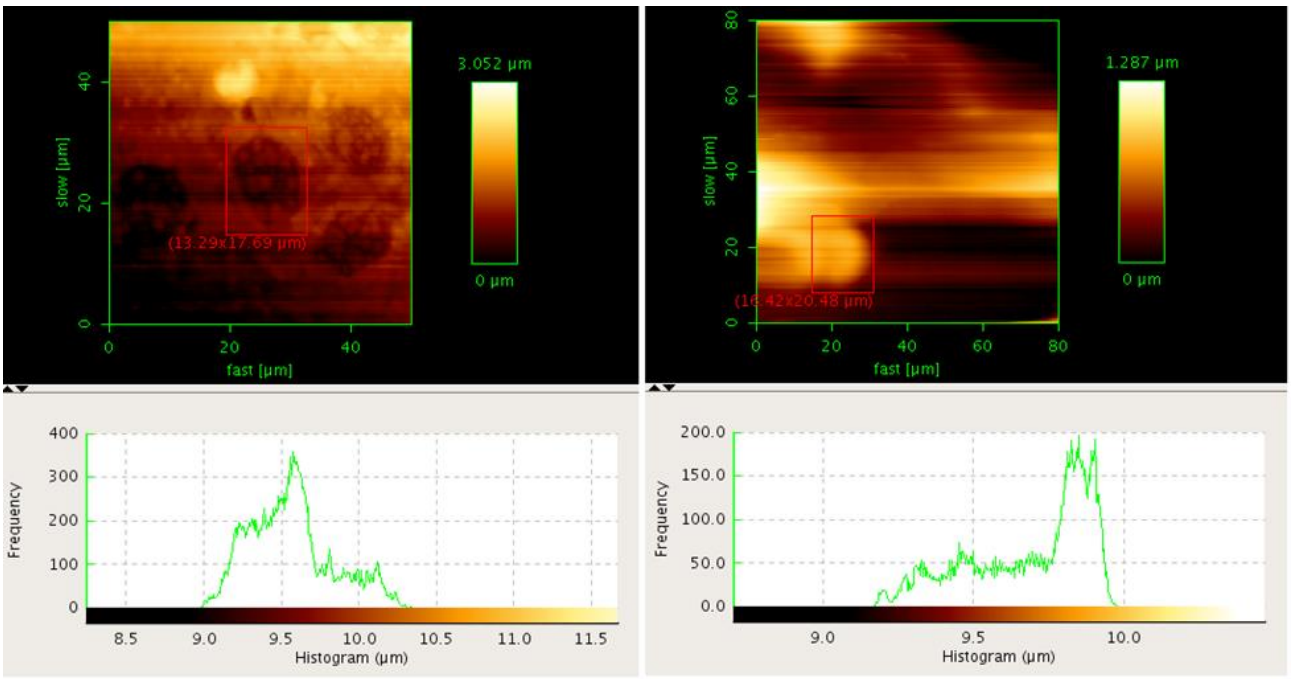

Figure 5. AFM images and micrograph of WCH-17 fixed cells with N_2 on contact mode (left) and live WCH-17 cell (right). Note: the scan ranges are different. The AFM image scan range of fixed cell is 60 µm, and the AFM image scan range of live cell is 80 µm.

4. Discussion

AFM was invented in 1986 [1], and it could advance the state of the sciences (such as physics, chemistry, biology, and medicine) through the history of nanotechnology. This microscope contours a surface by balancing the forces between a probe tip and the surface. This microscope began to be adapted to work over a wide range of temperatures and in any environment. The ability to probe surfaces with exceptional signal-to-noise ratio at the sub-nanometer scale has led to the development of a wide range of AFM-related measurements that use a variety of probes to sense local interactions and manipulate matter. The flexibility of AFM for imaging, probing, and manipulating materials has made it the most versatile tool in nanotechnology. The possibility of working in liquid media and at room temperature has pushed this microscope into biology, leading to the analysis of biomolecules and cells at (sub)nanometer scale [32-37]. Using conventional AFM also has limitations. Although AFM can be applied in liquid conditions, it may damage the biological samples by tip movement while scanning the specimens. Nevertheless, AFM results are strong, reliable data to identify surfaces for biological research.

Our study used AFM imaging for live and fixed WCH-17 cell characterization and cell surface changing while cells were fixed in air and liquid conditions. In physiological conditions, AFM imaging of live cells revealed that the morphology of cells is in agreement with previous reports [31]. In contrast, AFM images of fixed cells in both air and liquid N2 conditions showed total changes in shape. In chemical fixation methods, there is a possibility of interaction with the cell membrane and loss of the original morphology and topology of the cell [16]. While the methods we used minimizes these interactions.

Another characteristic that was measured was surface roughness which increased in fixed cells (Table 1). Increasing roughness in air-fixed cells was more than in the liquid N_2-fixed ones. The observed variation in roughness may be related to different fixation methods, in agreement with L.W. Francis *et al.* report [16].

Table 1. Parameters measured by AFM in live, air-fixed, and N_2 liquid-fixed cells.

Parameter	Live cell	Air fixed cell	Liquid N_2 fixed cell
Average Roughness Ra (nm)	154.8	346.6	189.4
RMS Roughness Rq (nm)	203.4	491.4	270.0
Peak-to-Valley Rt (nm)	805.3	2306	1362
Length (µm)	26.1	17.4	16.6
Width (µm)	16.4	10.9	12.2
Thickness (µm)	0.8	1.8	1.0

We used intermittent contact mode (tapping mode) in the present study for imaging live cells in physiological conditions. Contact mode can damage or deform the biological samples [23], so the original data may have been missed. There was some limitation in our AFM technique. It is very difficult to record a high-resolution image of live cells under physiological conditions, in a liquid environment, with conventional AFM. The cell membrane is dynamic in physiological conditions when cells are live and may change during the recording of one image to the next from the same area (back and forth scanning), which takes several minutes. Therefore, the second scan of an area of the plate, which reduces noise and raises resolution, was not possible. This problem may have been solved by design and using modified tips [28].

However, the AFM study of live cells in their physiological state is a very powerful and remarkable technique in determining superficial nanodegradation, including in the diagnosis of cancers, in determining the biological pathways of diseases that depend on cell surface receptors (38), and the effect of chemical drugs on microorganisms [6,39].

4. Conclusions

AFM has played an increasingly important role in cell imaging and the study of the mechanical properties of cells. Despite recent development in AFM techniques, there are still many problems to be solved before the application of AFM technology can be fully realized in action (for example, in the diagnosis of cancer). On the other hand, using AFM for biological purposes like live cell imaging has some limitations. For example, the sample may be mobilized in liquid condition or destroyed by tip movement. Despite all the limitations, AFM characterization is valuable nanoscopic data to investigate biomolecule properties. Cell surface changes are a valuable trace in detecting cellular mechanisms in the face of other substances (for example, biomolecules to synthetic nanomaterials). In this respect, AFM can be considered the most powerful method for cellular studies.

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Conflicts of Interest

The authors declare that there are no conflicts of interest in this study.

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