Enhanced Chondrogenic Responses of Human Articular Chondrocytes Onto Silk Fibroin/Wool Keratose Scaffolds Treated With Microwave-Induced Argon Plasma

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Abstract: Silk fibroin (SF) is a natural, degradable, fibrous protein that is biocompatible, is easily processed, and possesses unique mechanical properties. Another natural material, wool keratose (WK), is a soluble derivative of wool keratin, containing amino acid sequences that induce cell adhesion. Here, we blended SF and WK to improve the poor electrospinability of WK and increase the adhesiveness of SF. We hypothesized that microwave-induced argon plasma treatment would improve chondrogenic cell growth and cartilage-specific extracellular matrix formation on a three-dimensional SF/WK scaffold. After argon plasma treatment, static water contact angle measurement revealed increased hydrophilicity of the SF/WK scaffold, and scanning electron microscopy showed that treated SF/WK scaffolds had deeper and more cylindrical pores than nontreated scaffolds. Attachment and proliferation of neonatal human knee articular chondrocytes on treated SF/WK scaffolds increased significantly, followed by increased glycosaminoglycan synthesis. Our results suggest that microwave-induced, plasma-treated SF/WK scaffolds have potential in cartilage tissue engineering. Key Words: Cartilage tissue engineering—Chondrocytes—Microwave-induced argon plasma—Nanofibrous silk fibroin—Scaffold.

Silks are unique biomaterials for tissue engineering due largely in part to their stability, biocompatibility, slow biodegradation, and mechanical properties (1). Silk-based biomaterials have previously been demonstrated to offer exceptional benefits over conventional synthetic and natural biomaterials in generating functional tissue replacements for various mesenchymal tissues, such as bone (2), cartilage (3), and ligaments (4,5). In addition, they offer significant advantages for potential adipose tissue engineering applications as they have low immunogenicity, an absence of bioburden (6), slow degradation rates (7), and exhibit plasticity during processing (8).

An ideal tissue-engineered, porous three-dimensional scaffold that provides a framework for cells to attach, proliferate, and form an extracellular matrix (ECM) should have spatial, compositional, biocompatible, and mechanical stability properties. Furthermore, the scaffold should possess a high degree of porosity and interconnected pores giving way to cell migration, communication, proliferation, and signaling (9). To improve the mechanical stability of silk scaffolds, gelatin or collagen is often an added material (10,11).

Recently, an electrospinning technique has received much attention for fabricating ultrafine, polymeric nanofibers. The electrospinning is a facile,
efficient, and inexpensive polymer processing method for the formation of nonwoven fabrics, in which a polymer solution dissolved in a solvent is ejected through a nozzle by an electrostatic force (12). This technology offers the potential for controlling the composition, structure, and mechanical properties of biomaterials. In addition, the opportunity to generate nano- or microscale scaffolds in tissue engineering is significant, as it is believed that the nanoscale dimension provides a well-defined architecture with a high surface area-to-volume ratio (13).

Electrospun nanofibrous silk scaffolds have garnered much attention recently because the structure is very similar to that of native collagen fibers found in the ECM. The cells that are seeded on a nanofibrous scaffold adhere and grow well due to the large specific area (14,15). However, two-dimensional nanofibrous scaffolds limit the degree of proliferation and migration because seeded cells often remain in their monolayer formation after initial seeding. Therefore, we sought to improve these qualities and attempt to make three-dimensional electrospun nanofiber scaffolds with a high surface area-to-volume ratio.

Commonly, wool keratose (WK), a soluble derivative of wool keratin, can be obtained by oxidizing keratin with per-acids cleaving its cysteine linkages. WK is composed of more polar amino acids than silk fibroin (SF). Of particular interest, a larger degree of amino acid sequences naturally enhances cellular hydrophilicity than found with SF (16,17). Therefore, the hydrophilicity of WK, as well as cell adhesion specific amino acid sequences naturally enhances cellular attachment on the surface (18–20). In this study, SF and WK were blended together in a solution and electrospun, to create a substrate maximizing the beneficial qualities of both materials to achieve increased cell adhesion and chondrogenic behavior.

The surface modifying effect of plasma treatment is confined within the outermost surface layer, hence preserving the subsurface composition and bulk properties. A typical method of plasma treatment is to introduce hydrogen peroxides to the surface either by inert gas followed by an exposure to oxygen, or by a corona discharge treatment (21). A major drawback with the oxygen and air plasma treatment is material degradation caused by chain scission (22). However, the argon plasma treatment results in topographical changes at the nanoscale level (23). Argon plasma can introduce an oxygen functionality to the surface and oxygen-modified surfaces lead to subsequent incorporation of polar groups, which improves hydrophilicity without affecting bulk characteristics (23,24). This facilitates adhesion and improves the biocompatibility of the material. Moreover, argon plasma treatment of hydrophobic surface materials (e.g., polyethylene) has been shown to produce a hydrophilic surface with a decreased contact angle (22,25).

The purpose of this study was to investigate the effects of microwave-induced argon plasma treatment on the attachment, proliferation, and cartilage-specific ECM formation of neonatal human knee articular chondrocytes (nHAC-kn) onto a three-dimensional, porous electrospun nanofibrous SF/WK scaffold.

**MATERIALS AND METHODS**

**Preparation of SF/WK**

SF was obtained by soap–soda degumming as previously described (26). *Bombyx mori* cocoons were boiled in an aqueous solution of marseillus soap 0.3 (wt %) and sodium carbonate 0.2 (wt %) for 1 h to remove sericin (degumming), and were then rinsed with distilled water. Degummed SF was dissolved in a ternary solvent system of CaCl2/H2O/EtOH (mole ratio = 1/8/2) solution at 85°C for 3 min. The solution was then dialyzed (cellulose acetate membrane MWCO: 12 000) against distilled water at room temperature for 3 days, after which the solution was then lyophilized. WK was obtained by oxidizing wool keratin with performic acid. Scoured Merino wool hairs were immersed in performic acid (HCOOOH) at 0°C for 24 h (liquor ratio = 1:25). The soluble fraction was then separated and dried at ambient temperature. All other chemicals were used without further purification.

**Fabrication of three-dimensional SF/WK scaffold**

The three-dimensional porous nanofibrous SF/WK scaffold was fabricated via electrospinning followed by the salt-leaching method. Dope was prepared by dissolving SF and WK in 98% formic acid for 4 h. The blend ratio was 50/50 and dope concentration was 12%. Impurities and bubbles in the solution were removed by filtration and brief vacuum. For electrospinning, the dope was put into a 10 mL syringe with a 22-gauge stainless steel syringe needle connected to a high voltage power supply (Chungpa EMT Co., Ltd., Seoul, Korea). A voltage of 10 kV was applied across a distance of approximately 20 cm with a constant flow rate of 0.2 mL/h as controlled by a metering pump (KD Scientific, Inc., Holliston, MA, USA). Electrospinning was carried out at room temperature with 60% humidity. Porous scaffolds were fabricated by collecting electrospun SF/WK nanofiber dispersions in a methanol (99.5%) bath. After electrospinning, the methanol, which was used as a dispersing
medium and coagulant simultaneously, was replaced by 1,4-dioxane. NaCl particles (diameter 300–500 μm) were then added to the dispersion as porogens. The mixture was mildly stirred to ensure a homogenous dispersion of the NaCl particles, poured into a glass vessel (cylindrical shape) and lyophilized. It was then cross-linked with glutaraldehyde vapor in a sealed chamber for a day and subsequently immersed in 0.1 M glycine in a 0.2 M sodium carbonate buffer (pH 9.2) for 24 h to neutralize the toxicity of glutaraldehyde. The mixture was then washed with phosphate-buffered saline (PBS) several times. In this process, the NaCl particles were completely dissolved out and the porous structure formed. Finally, the washed SF/WK scaffold was lyophilized again and cut into disks (9 mm in diameter and 1.5 mm in thickness). Structural characteristics of the electrospun SF/WK scaffolds are shown in Table 1.

### Surface modification of SF/WK scaffolds by microwave-induced argon plasma

SF/WK scaffolds were treated with a 2.45 GHz, waveguide-based, microwave-induced argon plasma system at atmospheric pressure for 12.4 seconds, as described previously (27). This system consisted of a 1 kW magnetron power supply, a WR-284 copper waveguide, and an applicator including both tuning and nozzle sections. Argon was used as the working gas in the plasma system at a gas flow rate of approximately 100 L/min at 8 kgf/cm².

### Scanning electron microscopy (SEM) and quantitative pore structure analysis

The surface morphology of three-dimensional porous SF/WK scaffolds treated with or without microwave-induced argon plasma was observed under a scanning electron microscope (Hitachi S-4700, Hitachi, Tokyo, Japan). The scaffolds were mounted and sputter-coated with gold/platinum using an ion coater (E1010, Hitachi) and then observed at an accelerating voltage of 15 kV. The irregularity of the three-dimensional pore sizes resulted in variable sizes, which led to using an estimation technique to generate pore sizes. The pore size was estimated by measuring equivalent circle diameter (ECD) according to ASTM F2603. ECD is the diameter of the circle that has the same area as the pore cross-section. ECD can be calculated by Eq. 1:

\[
ECD = \left( \frac{4A}{\pi} \right)^{1/2}
\]  

Here, A is the cross-sectional area of the pore. The actual cross-sectional area of the pore was measured by AutoCAD 2006 (Autodesk, Inc., San Rafael, CA, USA) using SEM images of the SF/WK scaffolds. The average pore size was determined by measuring at least 20 pores.

The scaffold porosity was calculated by Eq. 2:

\[
\text{Porosity} (\%) = \frac{V_B - V_S}{V_B} = 1 - \frac{\rho_B}{\rho_S}
\]

where \(V_B\), \(V_S\), \(\rho_B\), and \(\rho_S\) are bulk volume, skeleton volume, bulk density, and skeleton density of the scaffold, respectively. \(\rho_B\) was easily obtained by measuring the scaffold volume and mass. \(\rho_S\) was determined by measuring the difference of the masses in the air and in liquid (n-hexane). The detailed method for porosity calculation was introduced in our previous report (17). Five total specimens were measured and are presented with an error means standard deviation in Table 1.

### Water contact angle measurement

To examine the effects of the argon plasma treatment on the hydrophilicity of SF/WK hybrid scaffolds, the surface of the scaffold was characterized by static water contact angle measurements using the sessile drop method. In the sessile drop measurement, approximately 10 water droplets were placed on the dry surface of each composite. The contact angles of water on both of the composites were detected at room temperature using a SEO contact angle analyzer (Phoenix 300A, Surface Electro Optics Co. Ltd., Suwon, Korea) equipped with a special optical system and a charge-coupled device camera.

### Cell culture and conditions

Neonatal human knee articular chondrocytes (nHAC-kn, Lonza, Walkersville, MD, USA) were cultured in a chondrocyte basal medium (Lonza) supplemented with 5% fetal bovine serum (Lonza), chondrocyte growth factors (1 mL R3-IGF-1, 2.5 mL bFGF, 1 mL insulin, 0.5 mL GA-1000, 0.5 mL transferrin per 500 mL; Lonza), and a 1% antibiotic antifungal solution (including 10 000 units penicillin,
10 mg streptomycin, and 25 g amphotericin B per mL, Sigma-Aldrich Co., St. Louis, MO, USA) at 37°C and 5% CO2 in a humid environment. To examine the effects of plasma treatment on the cellular behaviors of nHAC-kn, cells were seeded onto SF/WK scaffolds treated with or without plasma, incubated for 4 h with an initial cell density of 2.0 × 10^5 cells per scaffold for attachment assays, and 1, 3, or 7 days with an initial cell density of 1.0 × 10^4 cells per scaffold for proliferation assays.

Cell attachment and proliferation assays

The MTT assay (reduction of 3-[4,5-di-methylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to form a purple formazan product) was used to determine cell attachment and proliferation on the SF/WK scaffold. nHAC-kns cultured onto three-dimensional porous SF/WK scaffolds, treated with or without argon plasma, were incubated with 0.5 mg/mL MTT in the last 4 h of the culture period and tested at 37°C in the dark. The cells were washed twice with phosphate-buffered saline upon removal of the MTT media. The produced formazan salts were then dissolved with dimethylsulphoxide, and an absorbance was determined at 570 nm by an enzyme-linked immunosorbent assay reader (SpectraMax 340, Molecular Device, Sunnyvale, CA, USA).

Additionally, the morphologies of cells grown onto the nontreated or plasma-treated SF/WK scaffolds were observed after 7 days of incubation by SEM. In brief, the scaffolds were washed with 0.1 M cacodylate buffer (pH 7.4) to remove unattached cells. Attached cells were fixed with a 2.5% glutaraldehyde solution overnight at 4°C, dehydrated with a series of increasing concentrations of ethanol solutions and then vacuum-dried. Scaffolds were mounted and sputter-coated with gold/platinum using an ion coater, and then observed with a scanning electron microscope (Hitachi S-800) at an accelerating voltage of 20 kV.

Glycosaminoglycan (GAG) assay

GAG assay content was determined by a dimethylmethylene blue (DMMB) dye binding assay (Blyscan kit, Biocolor Ltd., Newtownabbey, Northern Ireland) according to the manufacturer’s instructions. Chondroitin sulfate, provided with the kit, was used as the GAG standard. Briefly, the SF/WK scaffolds cultured with nHAC-kn were washed with PBS and digested overnight at 37°C in papain solution (1 mg/mL, Sigma-Aldrich). The digested solution was mixed with DMBB periodically at 25°C for 30 min. Solutions were then centrifuged at 10,000 g for 10 min and the supernatant (unbound dye) was discarded. The remaining pellet (insoluble GAG-dye complex) was suspended in the provided dissociation reagent, and absorbance measurements were taken at 656 nm.

Statistical analysis

All variables were tested in triplicate for each experiment and every experiment was repeated twice (n = 6). Quantitative data were expressed as mean ± standard deviation. Statistical comparisons were carried out with a Student’s t-test. A value of P < 0.05 was considered statistically significant.

RESULTS

Surface structure and morphology of the three-dimensional porous SF/WK scaffolds

SEM images of surface morphologies of nontreated and plasma-treated SF/WK scaffolds are shown in Fig. 1. The images revealed that both scaffolds had uniformly distributed pores and a high porosity (about 91%) with relatively uniform pore sizes (390–550 μm). Studies relate that an electrospun nanofibrous scaffold has more than 90% porosity, which indicates a highly porous structure (14).

Plasma treatment increased the pore depth and the surface roughness. Also, plasma treatment resulted in differences in pore shape. Before treatment, pores were shaped like inverted cones and after plasma treatment, pores were cylindrical, as noted with SEM observation (Fig. 1a,b). The fibers of plasma-treated scaffolds were morphologically similar to nontreated scaffolds as apparent in the high magnification view meaning that plasma treatment did not adversely affect fibers of the scaffold (Fig. 1c,d).

Hydrophilicity of the SF/WK scaffold treated with plasma

To determine the effects of plasma treatment on the hydrophilicity of SF/WK scaffolds, the water contact angle was measured (Fig. 2). The water contact angle of scaffolds significantly decreased because of the plasma treatment. The measured angle was found to be six times lower after treatment than before treatment (Table 2).

Cellular behavior of nHAC-kn on SF/WK scaffolds treated with plasma

Cellular behavior of nHAC-kn seeded onto plasma treated and nontreated SF/WK scaffolds was monitored and cell attachment and proliferation were investigated. Attachment of cells onto the scaffold increased by plasma treatment, although the difference was not statistically significant. The value of
plasma treated scaffold was approximately 1.3 times higher than non-plasma-treated scaffold (Fig. 3).

The proliferation of nHAC-kn on the SF/WK scaffold was investigated at 1, 3, and 7 days after seeding. Proliferation rates were not significantly increased by plasma treatment. However, the rate of cell growth markedly increased as the incubation period increased (3 and 7 days) in the plasma-treated group. Similar increases in cell growth were not observed in the non-plasma-treated group (Fig. 4).

These results were confirmed by SEM micrographs, showing the cellular morphology of nHAC-kn cultured for 7 days on SF/WK scaffolds treated with or without plasma. Cells on the non-plasma-treated scaffolds were locally attached and partially covered the surface. In contrast, cells on the plasma-treated scaffolds almost formed a single layer while maintaining the natural original morphology of

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**TABLE 2. Water contact angle of SF/WK scaffolds**

<table>
<thead>
<tr>
<th>SF/WK scaffolds</th>
<th>Water contact angle, $\theta$w</th>
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<tbody>
<tr>
<td>Non plasma treated</td>
<td>$70.4 \pm 5.9$</td>
</tr>
<tr>
<td>Plasma treated</td>
<td>$12.2 \pm 1.8^*$</td>
</tr>
</tbody>
</table>

Results are reported as mean ± standard deviation ($n = 10$). The data was analyzed by Student’s $t$-test, and values marked with asterisks are significantly different from the nontreated control ($^* P < 0.05$).
articular chondrocytes. Also, cells on the plasma-treated scaffolds demonstrated highly organized patterns and shapes (Fig. 5).

**GAG synthesis of nHAC-kn onto SF/WK scaffold**

The surface of the SF/WK scaffold was modified by plasma treatment and the effect on GAG synthesis of nHAC-kn was determined by biochemical analysis. Cells on the plasma-treated SF/WK scaffold showed significantly higher GAG synthesis than those on the non-plasma-treated SF/WK scaffold at 3 and 7 days (Table 3). However, no significant differences existed between the two treatment groups at the onset of seeding (i.e., after 4 and 24 h). GAG synthesis on the scaffold also increased in a time-dependent manner, irrespective of plasma treatment.

**DISCUSSION**

Plasma treatment can improve scaffold fabrication techniques by introducing surface modifications to enhance tissue engineering material designs (28). In addition, plasma treatment is a convenient method for modifying the surface properties of materials, such as hydrophilicity, surface energy, charge, and roughness without dramatically affecting the bulk properties. As shown in Fig. 1, plasma treatment did not adversely affect the fibers of scaffolds, though the plasma-treated scaffold had significantly altered morphology compared with the non-plasma-treated surface. Scaffold roughness and pore depth increased after argon plasma treatment, as well as a distinctive alteration in the shape of the pores after treatment. Before plasma treatment, the pore shape looked similar to inverted cones, but after plasma treatment, the pore shape looked cylindrical. These properties may be more suitable for cell growth because they not only provide a larger space in which the seeded cells can live, but might also make cell migration easier. Indeed, it is well-known that pore size, as well as the degree of pore interconnectivity of polymer scaffolds are important parameters of scaffold design that determine the fluid flow through the porous scaffold (29).

The hydrophilicity of SF/WK scaffolds increased as a result of plasma treatment. Hydrophilic scaffolding provides more benefits for cell adhesion and growth than do hydrophobic scaffolds. Cell attachment and proliferation on the scaffold increased due to plasma treatment, although these results were not significant. In the plasma-treated group, the rate of cell growth increased markedly over the incubation period (7 days), whereas an increase in growth rate was not observed with the control group. This evidence suggests that the surface of the plasma-treated SF/WK scaffold was more favorable for cell spreading, growth, and proliferation than the nontreated scaffold. A recent study reported that the use of plasma treatment and acrylic acid grafting can successfully introduce hydrophilic functional groups onto the surface of electrospun nanofibrous scaffolds and these surface-modified scaffolds provide significant improvement with regards to cell attachment and proliferation in vitro (30). Moreover, it was shown that polycaprolactone and hydroxyapatite surface-modified nanofibrous scaffolds possess significant potential for the mineralization of osteoblasts in bone tissue engineering (31).

As seen by SEM images, cells on the non-plasma-treated scaffold covered the surface, but cells on the plasma-treated scaffold formed an almost complete monolayer while maintaining their natural articular chondrocyte morphology. These results suggest that surface-modified scaffolds are more effective at

**FIG. 3.** Attachment of nHAC-kn on non-plasma-treated and argon plasma-treated SF/WK scaffolds. The results are reported as a percentage of control ± SD (n = 10).

**FIG. 4.** Proliferation of nHAC-kn on non-plasma-treated and plasma-treated SF/WK scaffolds. Results are reported as a percentage of control ± SD (n = 10).
maintaining natural cellular morphology upon cell attachment, whereas the nontreated SF/WK forced the cells to alter their favored shape. SF/WK scaffolds also show more mechanical stability in wet conditions (32). Furthermore, the ability of electrospun silk matrices to support the attachment, spreading, and growth of bone marrow stromal cells in vitro, combined with the biocompatibility and biodegradable properties of the silk protein matrix, suggest that these biomaterial matrices may be useful as scaffolds for tissue engineering (33).

Recently, it has been shown that the adhesion and redifferentiation of human chondrocytes can be regulated by controlled changes in substrate surface

![FIG. 5. SEM images of nHAC-kn on SF/WK scaffolds. (a) nHAC-kn cultured for 1 day on non-plasma-treated SF/WK scaffolds; (b) nHAC-kn cultured for 1 day on plasma-treated SF/WK scaffolds; (c) nHAC-kn cultured for 7 days on non-plasma-treated SF/WK scaffolds; (d) nHAC-kn cultured for 7 days on plasma-treated SF/WK scaffolds.](image)

**TABLE 3. GAG synthesis of nHAC-kn onto SF/WK scaffolds according to incubation time**

<table>
<thead>
<tr>
<th>Time</th>
<th>Non-treated</th>
<th>Plasma-treated</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAG synthesis (μg/scaffold)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>0.288 ± 0.020</td>
<td>0.360 ± 0.030</td>
<td>0.20</td>
</tr>
<tr>
<td>1 day</td>
<td>0.625 ± 0.009</td>
<td>0.567 ± 0.120</td>
<td>0.64</td>
</tr>
<tr>
<td>3 days</td>
<td>0.744 ± 0.081</td>
<td>0.985 ± 0.071</td>
<td>0.02*</td>
</tr>
<tr>
<td>7 days</td>
<td>0.964 ± 0.099</td>
<td>1.174 ± 0.076</td>
<td>0.04*</td>
</tr>
</tbody>
</table>

Results are reported as a mean ± standard deviation (*P < 0.05 vs. nontreated, Student’s t-test, n = 10). Cells on the plasma-treated SF/WK scaffold showed significantly higher GAG synthesis (P < 0.05) than those on the non-plasma-treated SF/WK scaffold at 3 and 7 days.
chemistry and composition following glow discharge gas plasma treatment (34). Successful cartilage tissue engineering requires cells capable of undergoing chondrogenic differentiation upon treatment with appropriate biochemical factors and a three-dimensional porous scaffold that provides a favorable environment for chondrogenic cell growth and new cartilage specific ECM formation. In this study, plasma-treated SF/WK scaffolds exhibited much more GAG synthesis, suggesting higher human chondrocyte adhesion and cartilage specific ECM formation than the non-plasma-treated scaffold. Moreover, a recent report showed that cartilage regeneration using a fibroin sponge and a stirring chamber improved the potential of articular cartilage tissue engineering (35).

Polymer surfaces are commonly modified by chemical means using plasma treatment, adsorption of a single ECM protein component onto the polymer surface from a solution, or by a combination of these methods. The disadvantages of chemical modifications include the use of organic solvents and/or harsh chemicals. A three-dimensional, aqueous-derived biomaterial scaffold has also been reported, but it had decreased hydrophilicity and a higher SF concentration (32). Plasma treatments not only do not use harsh chemicals, but the hydrophilicity and adhesive properties of the surface can be modified with the choice of plasma gas or vapor, and exposure time.

CONCLUSIONS

This study demonstrated that nHAC-kn cultures on SF/WK scaffold that was surface-modified by a microwave-induced argon plasma treatment had significantly increased hydrophilicity, cellular attachment and proliferation, and chondrocyte-specific ECM synthesis. These argon plasma-treated nanofiber-based scaffolds were characterized by nanoscale texturing, a favorable condition for cell attachment, growth, and proliferation. Cell density was found to be a critical step in the redifferentiation of culture-expanded nACH-kn in the three-dimensional SF scaffolds (3). The relatively low density used in mesenchymal stem cell-based constructs was not sufficient to fully induce the redifferentiation of culture-expanded nACH-kn (36). The surface modification of scaffolds by argon plasma treatment solved these issues because a more favorable condition for cell attachment and proliferation was generated. Further, in vivo animal studies and eventually clinical trials in human patients will be necessary to fully elucidate the clinical relevance of these studies.

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REFERENCES


