

Structure and microanalysis of tear film ferning of camel tears, human tears, and Refresh Plus

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Purpose: This study aimed to investigate the tear ferning pattern and chemical elements of the tear film of camel tears compared with human tears and Refresh Plus eye lubricant. Refresh Plus was used as a control because it provides a healthy ferning pattern, due to the presence of an optimum ratio of carboxymethylcellulose (CMC) sodium and electrolytes. The main research focus is elucidating the viability of camel tear film in the dry, harsh environment of the desert.

Methods: The tears were collected from five camels, five male desert workers (20–25 years old) at a small village located 100 km from Riyadh, Saudi Arabia, and five male subjects (20–25 years old) from Riyadh. A small drop (1 μ l) of tears was dried on a glass slide and observed under a light (Olympus BX1) and scanning electron microscope (Inspect S50, Field Electron and Ion Company [FEI]). Energy-dispersive X-ray spectroscopy (EDS) of the tear film and Refresh Plus were investigated with a JEOL 1400 scanning transmission electron microscope.

Results: The camel tear film pattern was surrounded by thick, peripheral, homogenous layers containing small oily droplets, particles, and tiny branches in the tear ferning. The tear ferning of the camel was grade 0–1, whereas the tear ferning of human tears and Refresh Plus was grade 1–2. The mass percentage of chloride was highest in the camel tears. The mass percentage of potassium in the camel tears was greater than that in the human tears, but it was less than that in the Refresh Plus lubricant.

Conclusions: Camel tears exhibit a better quality than human tears and Refresh Plus lubricant do. The presence of oily droplet-like structures at the periphery of tear ferning suggests that camel tear film may have a higher quality and quantity of minerals and lubricants, which may help the animal to avoid eye dryness. Future work is required to investigate the identification of the elements present in the peripheral and central part of the tear ferning.

The dromedary camel (*Camelus dromedarius*) is a native of the dry, sandy climate of Saudi Arabia. In these harsh climatic conditions, camel eyes are wet and full of tears. The tear film is a thin layer that covers the anterior part of the eye. Its presence is important for maintaining the ocular surface and providing a defense against pathogens and foreign bodies [1]. The ocular tear film can be classified into three layers, as follows: the outer lipid layer, intermediate aqueous layer, and underlying mucus layer [2,3]. The loss of the quality or quantity of tear film can result in dry eye disease [3].

The ferning phenomenon is a dendritic growth pattern of dried tear fluid. It is influenced by the secretions' electrolyte, protein, and mucus contents. When mucus is permitted to air dry on a microscope slide, a specific type of crystallization called a fern occurs, and this phenomenon has been termed “ferning.” The tear ferning test has been described as a simple test that can be used to evaluate tear film [4] and has the potential to be applied in clinical practice [3].

Rolando suggested a grading scale for tear ferning. Types I and II indicate normal tear film, while types III and IV indicate dry eye [5]. Recently, Masmali et al. [6] developed a new tear ferning grading scale, where grade ≥ 2 indicates dry eye. The application of the Masmali tear ferning grading scale has shown that the tear ferning test has good validity and reliability, and there is no change in ferning patterns during the day [6]. The tear ferning test has the right features for use in the eye clinic for dry eye diagnosis [7-9]. Pearce and Tomlinson [10] studied tear film ferning with light and electron microscopy, and they discussed the role of chemical elements, such as sodium (Na), potassium (K), chlorine (Cl), and sulfur (S), in the formation of ferning.

Few studies have performed tear ferning tests on the tear film of animals and compared their patterns with that of humans. Silav et al. [7] investigated the tear ferning of healthy horses and found Types I, II, and III ferning in these animals. The authors counted the points on the crystallized ferning pattern and correlated the pattern with other parameters to assess the ocular surface. According to these authors, the tear ferning test is easy to perform and a good tool for assessing tear film quality.

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In the present study, we investigated the quality of camels' tear film using tear ferning techniques and conducted a comparison with Refresh Plus and the tear films of humans living in desert and urban areas. "Refresh Plus" was used as a control because it contains an optimum ratio of carboxymethylcellulose (CMC) sodium and electrolytes. The ratio between electrolytes and large molecules (CMC sodium) could be the main reason for its production of healthy ferning patterns [11]. The present study also aims to elucidate the viability of the tear film in the dry, harsh environment of the desert. The chemical element analysis of the tear film ferning was performed using scanning transmission electron microscopy (STEM).

METHODS

The study was approved by The College of Applied Medical Sciences (CAMS) Ethical Committees of King Saud University, Riyadh, Saudi Arabia (ethics number: CAMS 53–35/36). The tear film pattern was investigated using a light and scanning electron microscope. Tears were collected from five camels (males) and five desert workers (males, 20–25 years old) from a small village located 100 km from Riyadh, Saudi Arabia. Tears were also collected from five male subjects (20–25 years old) living in Riyadh. The human subjects underwent ophthalmic evaluation to confirm that the ocular surfaces of their eyes were healthy. The human and camel tears were collected in the same way using a microcapillary tube (Drummond Scientific Co., Broomall, PA). Refresh Plus (Allergan) was bought from a local pharmacy shop. The main constituents of Refresh Plus are CMC sodium (0.5%), calcium chloride, magnesium chloride, potassium chloride, water, sodium chloride, sodium lactate, and hydrochloric acid.

A small drop (1 μ l) of each of the different types of tears and the artificial tears (Refresh Plus, Allergan) was placed on a glass slide at 23 °C temperature and relative humidity of less than 45%. When the tears were dried (3 h after collection), they were observed under a light microscope (Olympus BX1) and classified according to the Masmali (2015) scale [10]. The Masmali grading scale for tear ferning was used to grade the ferning patterns.

The tear film and Refresh Plus ferning patterns were observed using an Inspect S50 environmental scanning electron microscope. For scanning microscopy, 1- μ l drops of each tear type and Refresh Plus were placed on a small piece of glass. This glass piece containing the tear film droplet was stuck on the scanning electron microscope stub. The tear film was gold coated and observed under an Inspect S50, FEI Quanta 200 environmental scanning electron microscope. Digital images were captured using an xT microscope

Server software and processed with Scandium (Olympus Soft Imaging Solutions GmbH).

The energy-dispersive X-ray spectroscopy (EDS) of the tear film and Refresh Plus were performed using the JEOL 1400 STEM. The microscope was equipped with STEM Viewer software and a Valita camera. Small droplets were placed on formvar-coated, 200-mesh nickel grids and dried for 30 min. Formvar is a thin, electron-transparent layer coated on the grids. It is used to hold the sample in place when conducting electron microscopy. It is routinely used for liquid samples. The ferning pattern that formed on the formvar-coated grids was observed using STEM and then EDS element analysis. This was conducted at different regions of ferning. The microanalysis of the elements was done using the STEM Viewer software, and digital images were taken with the Valita camera.

RESULTS

Light microscopy of ferning of camel, human, and Refresh Plus tear film: Our light microscopy observations showed that the ferning patterns at the periphery and center of the tear film were extremely similar. The observation and imaging of the ferning for the light microscopy was carried out from the periphery to the center of the tear film. The peripheral part of the camel tear film pattern was surrounded by five layers (labeled 1, 2, 3, 4, and 5; Figure 1A,B). The first layer (1) was pale in color and consisted of a homogenous material, whereas the second layer (2) was dark brown and contained small oily droplets (Figure 1A,B). The third layer (3) was blueish, while the fourth layer (4) was grayish and contained tiny droplets (Figure 1A,B). Finally, the fifth layer (5) was light brown and contained droplet-like structures. The ferning pattern was present in the fifth layer, with tree-like branches with leaves present just inside this layer, (Figure 1C,D). Inside these tree-like branches, fern plant-like branching was present. Numerous secondary and tertiary branches emerged from the primary branches (Figure 1E,F). The peripheral and central ferning patterns of the camel tear film were extremely similar, containing dense, thin branches (Figure 1C-F). According to the Masmali grading scale, the tear ferning at the periphery and center could be categorized as grades 0–1 (Figure 1C-F).

The ferning patterns of the humans residing in the urban environment and the desert were similar. In both cases, the peripheral part of the tear film pattern was surrounded by three layers (1, 2, and 3; Figure 2A-C). The first layer consisted of a whitish homogenous material, while the second layer was made up of dense granular material (Figure 2C). Finally, the third layer contained fine, small ferning (Figure

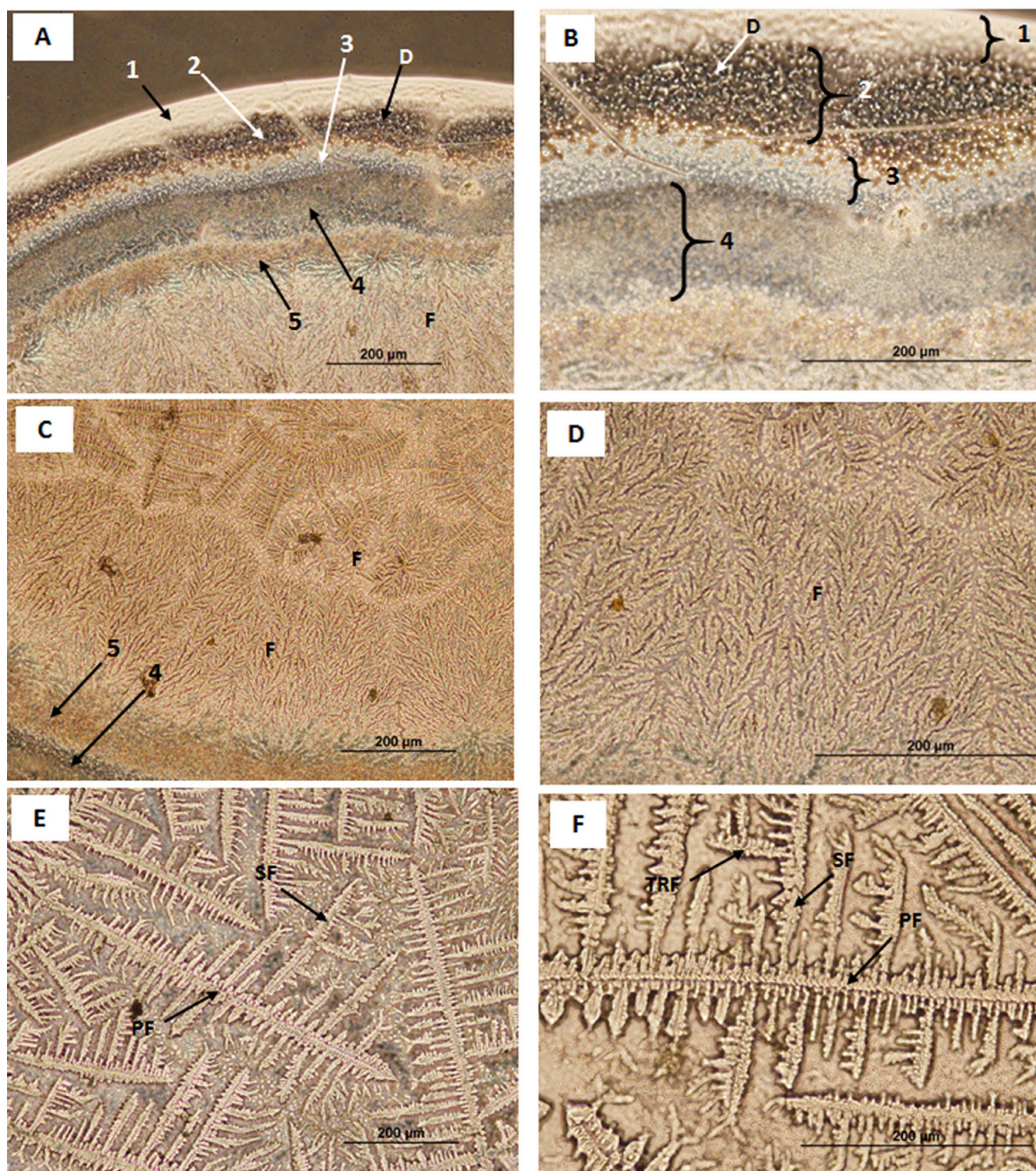


Figure 1. Light micrographs of the peripheral and central ferning pattern of camel tear film. **A** and **B**: Part of the periphery of the tear film showing the five outermost layers: 1) cream-colored homogenous layer, 2) dark brown oily droplet layer, 3 and 4) small droplet layers, 5) thick ferning pattern layer. **C**: Part of the periphery of the tear film showing a dense ferning pattern below the peripheral layers. **D**, **E**, **F**: Part of the center of the tear film showing prominent primary, secondary and tertiary branching. 1 = First layer, 2 = Second layer, 3 = Third layer, 4 = Fourth layer, 5 = Fifth layer, D = Oily droplets, F = Ferning, PF = Primary ferning, SF = Secondary ferning, TRF = Tertiary ferning.

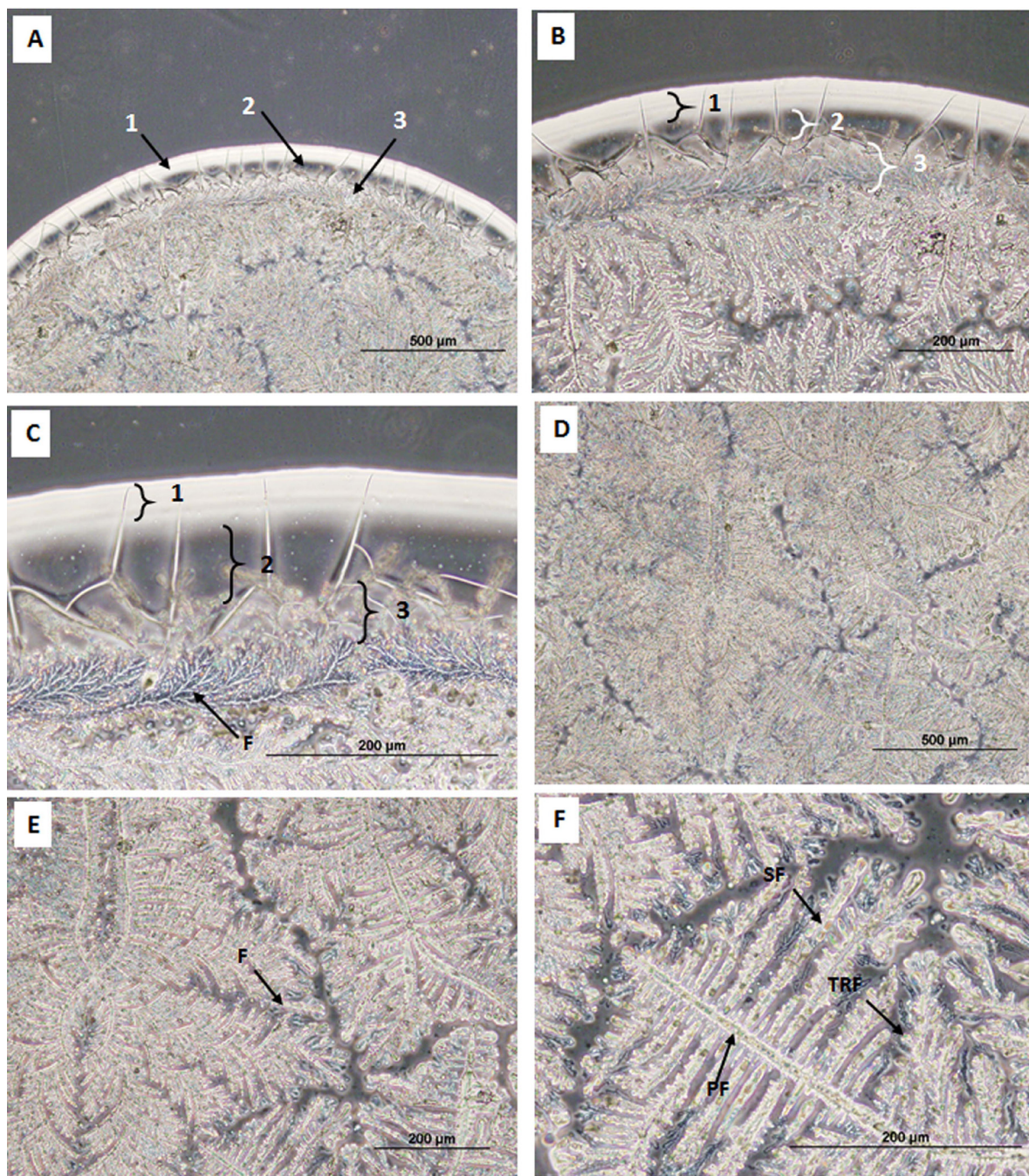


Figure 2. Light micrographs of the peripheral and central ferning patterns of desert human tear film. **A, B:** Peripheral part of human tear film showing peripheral ferning surrounded by three layers: 1) white homogenous layer, 2) dark brown homogenous layer, 3) granular layer with network like structure. **C:** "Tree branch" ferning pattern at the periphery of tear film below the peripheral layers. **D, E, F:** Ferning pattern of the center of the human tear film showing prominent primary, secondary, and tertiary branching. 1 = First layer, 2 = Second layer, 3 = Third layer, F = Ferning, PF = Primary ferning, SF = Secondary ferning, TRF = Tertiary ferning.

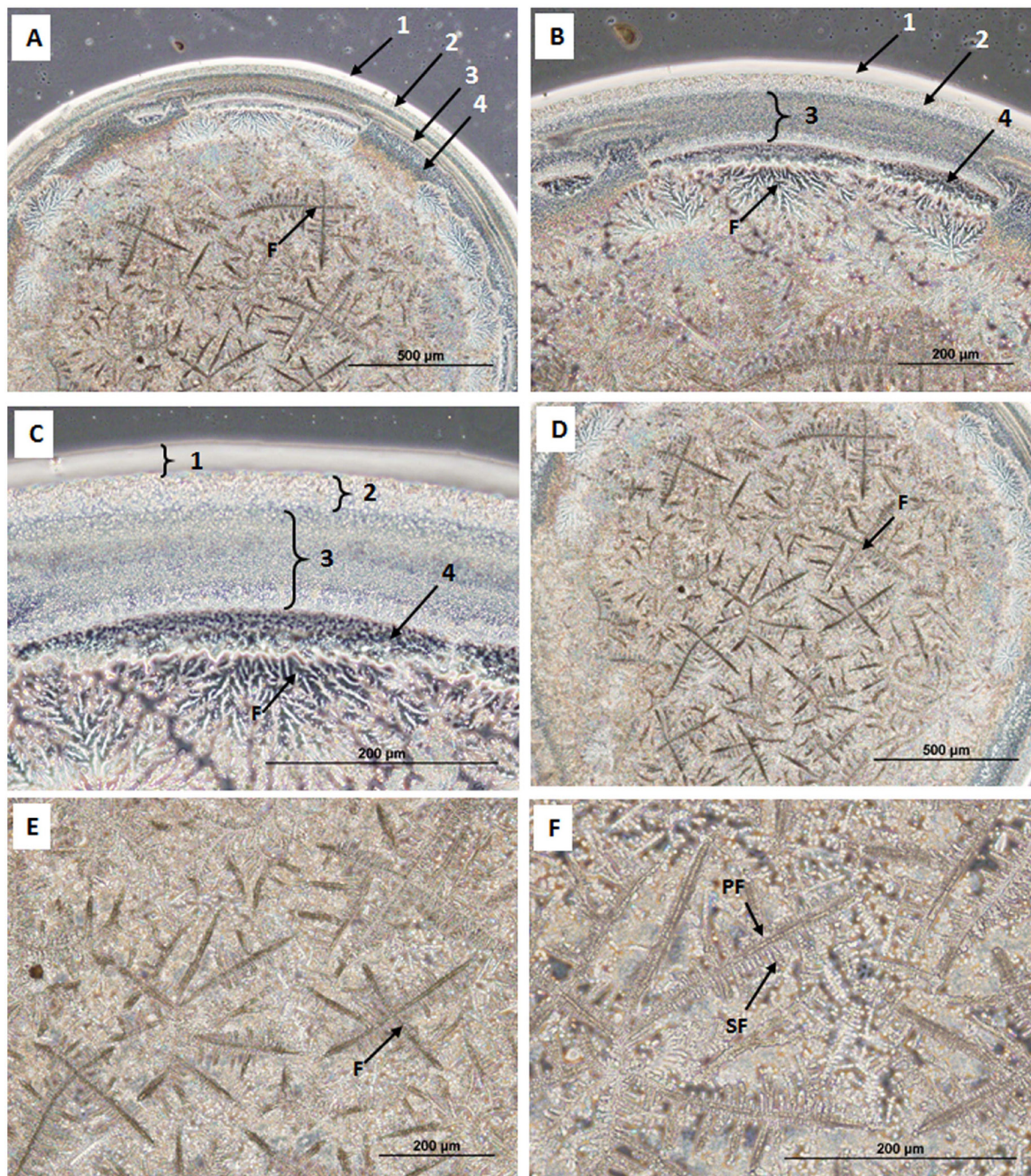


Figure 3. Light micrographs of the peripheral and central ferning patterns of Refresh Plus. **A, B, C:** Peripheral part of Refresh Plus surrounded by four layers: 1) outermost layer, white in color and homogenous; 2) granular white layer; 3) thick granular layer; 4) granular dark brown layer. Just below the fourth peripheral layer, the ferning contains only primary branches. Away from the periphery, the ferning pattern has secondary and tertiary branching. **D, E, F:** Ferning pattern in the central part of the Refresh Plus lubricant containing secondary and tertiary branching. 1 = First layer, 2 = Second layer, 3 = Third layer, 4 = Fourth layer, 5 = Fifth layer, F = Ferning, PF = Primary ferning, SF = Secondary ferning.

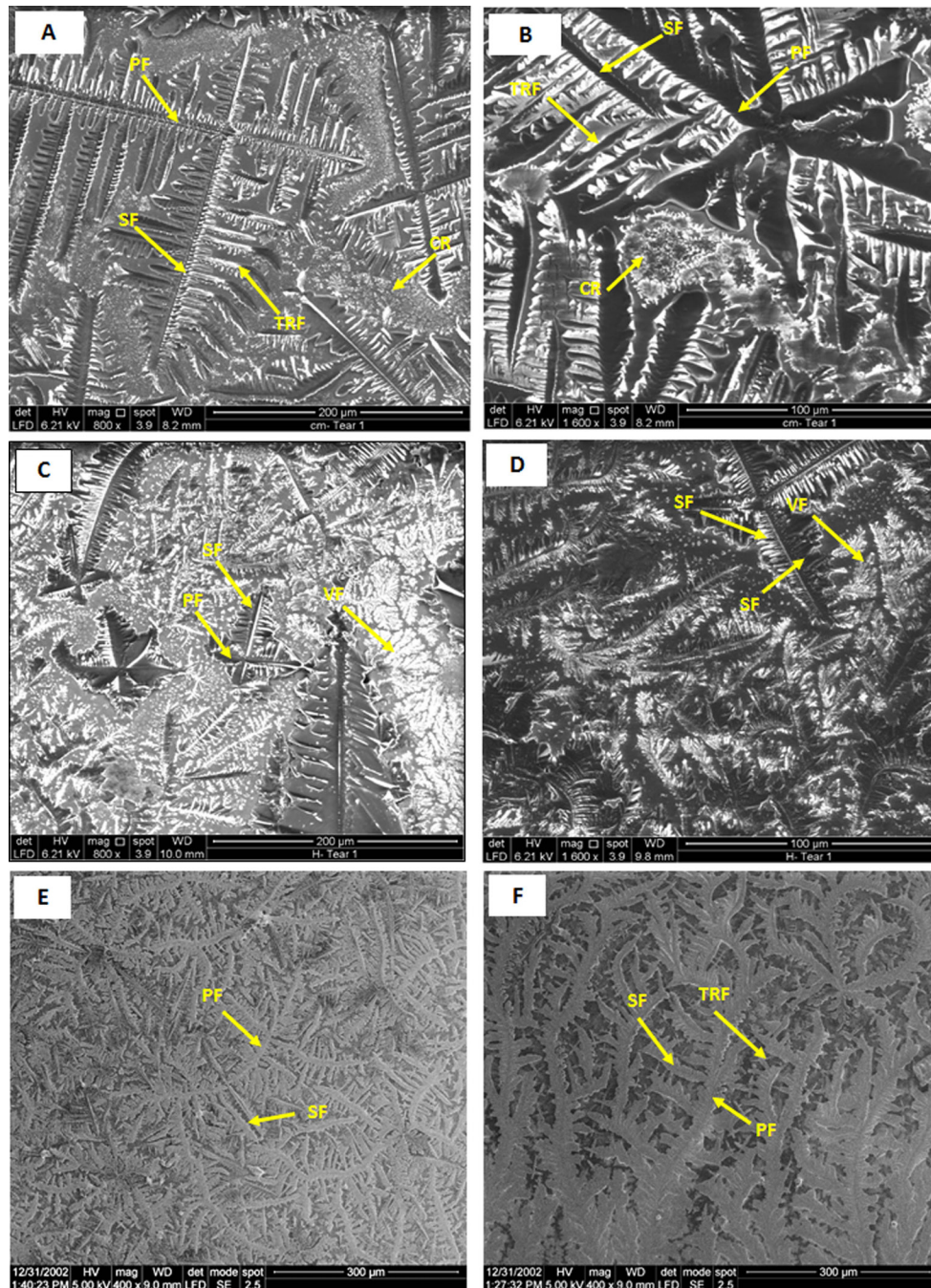


Figure 4. Scanning electron micrographs of the ferning pattern of camel tear film, human tear film, and Refresh Plus lubricant. **A, B:** Part of the camel tear film showing primary (pf), secondary (sf), tertiary (trf), and fine ferning; **C, D:** Human tear film containing primary (pf) secondary ferning (sf) and fine ferning. **E, F:** Refresh Plus ferning showing primary and secondary branching. 1 = First layer, 2 = Second layer, 3 = Third layer, 4 = Fourth layer, 5 = Fifth layer, CR = Crystals, PF = Primary ferning, SF = Secondary ferning, TRF = Tertiary ferning.

2C). Just inside the third layer, the ferning was like the branching of a tree, and this was different from the central ferning (Figure 2C). The secondary and tertiary branches of ferning shoots from the primary branches were dense and thin (Figure 2C-F). According to the Masmali grading scale,

the ferning pattern in the center and periphery could be categorized as grades 1–2 (Figure 2A-D).

In Refresh Plus, the ferning pattern was surrounded by four layers (1, 2, 3, and 4; Figure 3A-C). The first, outermost layer (1) was a whitish homogenous layer, while the

second (2) and third (3) layers were granular (Figure 3A-C). The fourth layer (4) was dark brown and formed a network structure (Figure 3C). The ferning patterns in the periphery and center were similar. The branching of the secondary and tertiary branches was not clear and defined. The peripheral

and central ferning patterns were thick and belonged to grade 2 (Figure 3E,F).

Scanning microscopy of tear ferning of camel and human tears and Refresh Plus: Scanning microscopy showed further fine secondary and tertiary (offspring) branches in the tear

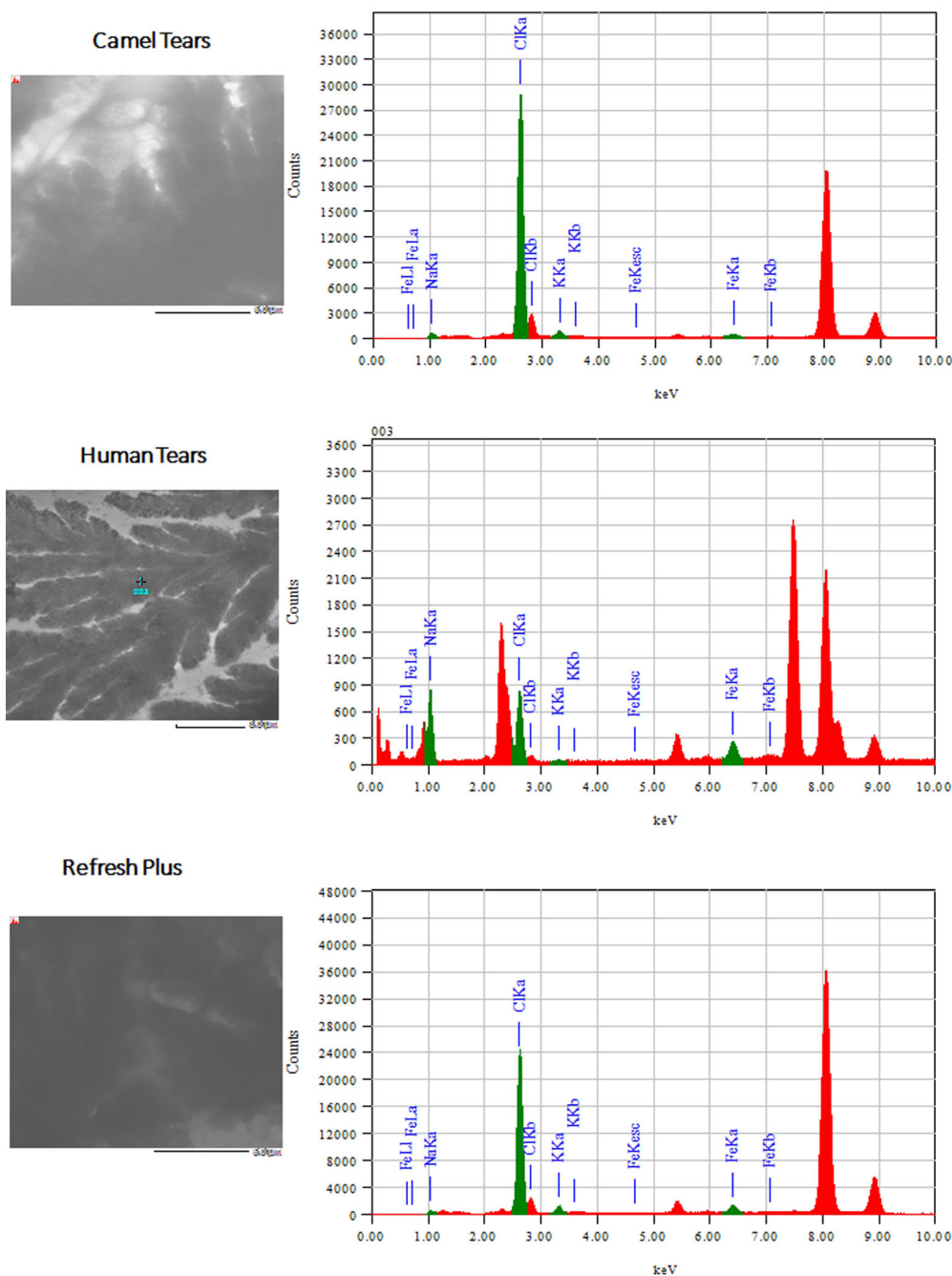


Figure 5. Energy-dispersive X-ray spectroscopy (EDS) analysis of dendrites in tear film showing peaks of the mass percentage of chloride and potassium in camels, humans, and Refresh Plus lubricant. The tear samples were placed on nickel grids. The unlabeled peaks are nickel (Ni) from the nickel grids. The labeling of the peaks from backgrounds, such as nickel grids, were excluded. ClKa = Chlorine at K α , FeKa = Iron at K α , FeLa = Iron at L α , K = Potassium, KKa = Potassium at K α , K Kb = Potassium at K β .

ferning of camel tears, human tears, and Refresh Plus (Figure 4A-F). The offshoots of branches of the ferning pattern in the camel tears (Figure 4A,B) were thinner compared with those in human tears (Figure 4C,D) and Refresh Plus (Figure 4E,F). There were tiny crystals between the branches of the tear ferning (Figure 4A,B).

The microanalysis of the elements took place from the periphery to the center of the tear film. The analysis of the elements was performed at a different part of the ferning. The EDS analysis showed that the mass percentage of chloride was highest in the camel tear ferning compared with the human tears and Refresh Plus (Figure 5). The mass percentage of potassium in the camel tear ferning was greater than that in the human tears, but it was less than that in the Refresh Plus lubricant (Figure 5).

DISCUSSION

Our observations showed that the branching pattern in camel tears was more prominent and dense than that in human ferning, especially in the tertiary branches. The branching in the outermost layer of the tear film was denser in camel than human tears. Furthermore, there was a difference between the central and peripheral ferning of the camel tear film. The tear film ferning of humans living in the desert and city were similar to each other.

In the camel, the ferning pattern of the tear film was surrounded by five layers (1, 2, 3, 4, 5; Figure 1A), whereas in human and Refresh Plus, it was protected by three and four layers, respectively. It is documented that sulfur-containing amino acid residues, namely cysteine and methionine, are present in the proteins and protein backbone of mucin [10]. This leads us to think that macromolecules like mucin and proteins are present at the periphery of tear ferning [10]. We consider that the peripheral layers of the tear film ferning were formed from the sulfur-containing proteins and mucin.

The camel is a native of dry, harsh weather conditions. Its ability to withstand dry and semidry weather could be due to the presence of antibody molecules that have only two heavy chains, making them smaller and more durable [8]. Camel eyes are protected by two rows of eyelashes and three eyelids, which provide protection from the sand and the harsh weather conditions [9]. The camel's cornea constitutes 35% epithelium and 60% stroma [12], and the thick epithelium protects the stroma from drying out in the arid weather [12]. To lubricate the thick epithelium of the cornea, the camel tear film presumably has a special composition of proteins, lipids, and minerals.

The abundance of proteins in the tears of humans and other animals has been analyzed previously [13,14]. Lysozymes were observed in the tears of humans and domestic animals (sheep, goats, llamas, cattle, horses, dogs, and rabbits) [13,14]. Furthermore, the lysosomes in humans are synthesized in the acinar cells of the lacrimal glands and constitute 20–40% of the total tear protein [15,16]; they exhibit antibacterial activity and hydrolyze the glycosidic bonds of certain Gram-positive bacteria [15,16].

Shamsi et al. [17] investigated tear proteins, namely lysozyme, lactoferrin, lipocalin, and vitelline membrane outer layer protein 1 (VMO1), in cow, sheep, human, and camel tears. The authors reported that the cow and sheep tears had a lower concentration of lysosomes compared with the human and camel tears. The lipocalins were identified only in the cow and sheep, whereas VMO1 was found in the sheep and camel tears but not in the human and cow tears. Furthermore, higher VOM1 levels were found in summer tears compared with winter tears [18]. It has been suggested that VOM1 expression may serve to maintain the ocular surface by keeping the tear film stable [19,20]. We think that VOM1 may lubricate the cornea in the harsh and dry climate to protect it from infection.

The camel tear film ferning was surrounded by thick layers of homogenous material containing oily, droplet-like structures. These structures were not observed in the human tears or Refresh Plus. It has been reported that tears contain phosphatidylcholines (PCs), sphingomyelins (SMs), wax esters (WEs), and free cholesterol (Chos) [21]. In addition to PCs and SMs, the presence of triacylglycerides (TAGs), ceramides (Cers), and phosphatidylethanolamines (PEs) in tears has also been observed [22]. Recently, Lam et al. [23] reported the presence of a novel lipid amphiphile, cholesteryl sulfate (CS), in human tears. We speculate that the oily droplets present in the outer peripheral layer of the tear ferning may be composed of the lipids present in the tears, inhibiting evaporation, and therefore, dryness of the eye. The presence of highly oily droplets may protect camel tears from drying out in the hot, dry climate.

The tear ferning pattern is thought to be formed due to the concentration and interaction of electrolytes, especially sodium and chloride, with macromolecules, such as tear film mucins and proteins [24]. This concentration, especially the ratio of monovalent Na and K to divalent Ca and Mn, is the key to the formation of tear ferning [25]. Pearce et al. [10] also suggested that the balance of Na, K, and Cl—rather than the level of a single element—is responsible for the ferning formation. The authors further suggested that mucin and proteins are not part of the fern structure; instead, their

prohibition from macromolecules helps the formation of ferning to progress. Golding et al. [11] supported the view that fern crystals are mainly composed of sodium chloride and potassium chloride, without any participation of protein and mucin, contradicting their [24] previous hypothesis.

The structure analysis showed that the camel tear ferning was better than that of the Refresh Plus lubricant. The scanning electron microscope showed that the tertiary and quaternary branches were fine in the camel tears compared with the human tears and Refresh Plus. These observations suggest that the elements responsible for the ferning pattern were more prevalent in the camel tears than in the human tears and Refresh Plus. Our present study showed the presence of higher concentrations of chloride and potassium in camel tear ferning. It is thought that Cl and K may facilitate the healthy formation of tear ferning in the camel. To our knowledge, EDS results on Refresh Plus have not been published. However, our elemental analysis showed the presence of elements (Cl, K) that were present in the Refresh Plus ingredients in EDS.

The tear ferning of Refresh Plus was dense and thin compared with that of the human tear film. The ingredients reported in Refresh Plus are CMC sodium (0.5%), calcium chloride, magnesium chloride, potassium chloride, purified water, sodium chloride, and sodium lactate. We think that the quantities of these components or their relationships are greater in the camel tears compared with the Refresh Plus and human tears, resulting in the higher grade of ferning in camel tears. We speculate that the formation of the outer oily droplets and homogenous layers in camel tear ferning could be formed due to the presence of the higher content of lipid, mucin, and multiple sulfur-containing proteins, such as lactoferrin, lipocalin, and VOM1. Healthy tear film helps camels to protect their eyes from dryness in the harsh, arid climatic conditions. Future work is required to investigate the identification of the droplet-like structure and chemical elements present in the peripheral layers and central part of the tear ferning.

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