Morphological changes of cornea in children with hyperopia in the immediate and remote postoperative periods after laser in situ keratomileusis according to confocal microscopy data

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Studying morphological changes of cornea in the remote postoperative period is becoming increasingly relevant with refractive surgeries being adapted for children. Purpose — comparative analysis of keratocyte density and histomorphologic changes of cornea after laser in situ keratomileusis (LASIK) and femtosecond laser-assisted LASIK (FS-LASIK) in children with hyperopia.

Materials and methods. 109 patients aged 6–17 years in 2 groups were examined with “Confoscan-4” confocal microscope. Results. In comparison with initial data, keratocyte density decreased in the immediate postoperative period after FS-LASIK and LASIK in average by 17.09%, 64.31% and 12.2% in the corneal valve, directly in the laser influence zone (interface) and in the retroablation zone respectively. After 5 years, keratocyte density decreased in the corneal valve, interface zone and retroablation zone by 12.01%, 48.71% and 8.06% respectively in comparison with initial data. A circular scar along the edge of the corneal valve was left after FS-LASIK; keratocyte density in the corneal valve and in the interface zone was in average 8.9% and 15.28% higher respectively, and twice more subepithelial nerves were noted compared to LASIK. Conclusions. In the remote postoperative period keratocyte density in the corneal decreased in average by 43% after FS-LASIK and by 46% after LASIK in comparison with initial data. No changes were seen in the morphologic state of the cornea outside the influence zone. Both the technologies are safe, however keratocyte density and the number of subepithelial nerves are higher in the corneal valve area and in the interface zone after FS-LASIK; corneal stability is assured by a circular scar formed along the valve edge.

Keywords: keratocyte density, confocal microscopy, refractive surgery in children.

Material and methods

The study included 109 patients aged 6 to 17 years (mean age 8.94±2.6 years) with hypermetropia and anisometropia of more than 4.0 Diopters who were operated in 2008–2014. All patients were examined with confocal microscopy 3 days after the surgery, and 38 of them were examined 5 years later. Manifest spherical equivalent in the study groups was in average (+) 5.21±2.13 ((+) 3.75 to (+) 6.5) Diopters before surgery. Depending on the type of surgery performed, the patients were divided into two groups. The first group included 36 patients who underwent LASIK, and the second group consisted of 73 patients who underwent FS-LASIK. Surgery goal was to reduce anisometropia, to achieve refraction balance with the fellow eye, and to establish the conditions for the amblyopia treatment. Study inclusion criteria was for children to have no severe general conditions or degenerative diseases of the cornea and other eye structures. All examinations and treatment were performed after the parents have signed the written consent in accordance to ethics principles of the Declaration of Helsinki.

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Hypermetropic LASIK was performed using MicroScan excimer laser system of 300 or 500 Hz (Troitsk, Russia) and Moria M2 mechanical microkeratome (“Moria”, France) with standard head of 90–110 μm. During the FemtoLASIK procedure, the corneal valve was formed at the depth of 100–110 μm using 60 kHz femtosecond laser system IntraLase FS (“IntraLase Corp.”, U.S.A.). Thicknesses of the cornea and the corneal valve were measured with optical coherence tomograph Visante OCT (“Carl Zeiss Meditec Inc.”, Germany). The total impact depth in the area 6.5–7 mm away from the cornea’s center was 115.34±17.57 μm on average (value range 83.43–175.6 μm).

Conorneal histomorphology was evaluated using confocal microscope ConfoScan-4 (“Nidek”, Japan). Corneal area of 460’345 μm was analyzed with the device, which provided an image sized 768’576 pixel, the scanning speed was 25 images per second with 5 μm steps. The examination was performed using Zeiss 40x NA 0.75 WD 1.9 mm lens through Carbomer immersion gel. The full corneal scan was performed in manual mode; it involved automatic calculation of endothelial cells density, and evaluation of pleomorphism and polymegathism. Keratocytes were counted manually: before the surgery in the optic zone (in anterior, middle and posterior stromal layers), after the surgery — in 3 areas of the paracentral region (in the corneal valve, in the interface and retro ablation zones). “Keratocyte density” was defined as the amount of cells in 1 mm² area. Keratocyte density was evaluated by the same specialist manually counting the keratocytes nuclei in the area under examination in endothelial cells density measurement mode. The examinations were performed before the surgery, during the early post-op period, and 5 years after the surgery.

Statistical analysis was performed using StatSoft 6.1 software. It considered conventional descriptive statistics parameters — the number of observations (n), arithmetical average (M), standard deviation (SD), and categorical data (in percentages). Due to irregular distribution of the parameters, non-parametric Mann-Whitney U-test (p< m–u) was used for independent clustering, and Wilcoxon criterion — for related groups (p< w). Differences between the sampling parameters were considered significant when the confidence level was less than 0.05.

**Results**

The surgery and the post-operative period had no abnormalities. Confocal microscopy performed before the surgery showed no morphological changes in the cornea. Keratocyte density in the anterior part of the corneal stroma was in average 802.94±64.7 cells/mm², in its middle part — 648.69±45.24 cells/mm², and in its posterior part — 684.89±40.5 cells/mm². The differences in this parameter between the stromal segments were statistically significant (Table 1). Endothelial cell density was 3043.15±419.6 cells/mm² in average.

According to OCT data, thickness of the corneal valve in the early post-op was 100±11.7 μm (87 to 105 μm) in the LASIK group (n=30), and 110±3.4 μm (97 to 113 μm) in the FemtoLASIK group (n=48). Biomicroscopy showed the cornea to be transparent. On the confocal microscopy image, the superficial epithelium in the LASIK group appeared with insignificant desquamation and disruption of the nuclear-cytoplasmic ratio in 33.3% and 27% of cases, respectively, and in the FemtoLASIK group — in 50% and 30% of observations, respectively. The superficial epithelium could not be seen in 66.7% of cases in the LASIK group, and in 50% of FemtoLASIK patients. All patients had edematous wing-shaped and basal epitheliocytes, unequal intercellular spaces, and the edges of their corneal valves appeared to have deep epithelialized areas.

Bowman’s membrane could be determined as an amorphous structureless formation in 26.6% of cases in the LASIK group, and in 71% — in the FemtoLASIK group, which appeared to have “fragments” of subepithelial nerves in up to 63% of cases in both groups. The corneal valve had folds variously manifested in 83% of cases in the LASIK group, and in 98% — in the FemtoLASIK group (Fig. 1, a). The number of “active” keratocytes has increased on both sides of the interface.

**Table 1. Comparative data on keratocyte density in the corneal stroma after LASIK and FemtoLASIK, cells/mm² (M±SD)**

<table>
<thead>
<tr>
<th>Time period</th>
<th>Posterior stroma (1)</th>
<th>M±SD (1:2)</th>
<th>Middle stroma (2)</th>
<th>M±SD (1:2)</th>
<th>Anterior stroma (3)</th>
<th>M±SD (1:3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before surgery</td>
<td>684.89±40.5</td>
<td>0.077</td>
<td>648.69±45.24</td>
<td>0.005</td>
<td>802.94±64.7</td>
<td>0.002</td>
</tr>
<tr>
<td>After surgery</td>
<td>628.83±19.13</td>
<td>0.5</td>
<td>309.04±84.21</td>
<td>0.29</td>
<td>637.51±94.44</td>
<td>0.43</td>
</tr>
<tr>
<td>3 days after surgery</td>
<td>Moria LASIK 591.53±70.92 (p&lt;0.22)</td>
<td>(p&lt;0.47)</td>
<td>284.96±83.13</td>
<td>0.44</td>
<td>671.8±99.27</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>FemtoLASIK 628.83±19.13 (p&lt;0.047)</td>
<td>(p&lt;0.22)</td>
<td>FemtoLASIK 284.96±83.13 (p&lt;0.07)</td>
<td>(p&lt;0.07)</td>
<td>FemtoLASIK 637.51±94.44 (p&lt;0.47)</td>
<td>(p&lt;0.37)</td>
</tr>
<tr>
<td>5 years after surgery</td>
<td>Moria LASIK 641.75±72.59 (p&lt;0.013)</td>
<td>(p&lt;0.22)</td>
<td>FemtoLASIK 366.2±82.5 (p&lt;0.007)</td>
<td>(p&lt;0.073)</td>
<td>FemtoLASIK 726.74±90.6 (p&lt;0.041)</td>
<td>(p&lt;0.37)</td>
</tr>
<tr>
<td></td>
<td>FemtoLASIK 652.01±61.62 (p&lt;0.023)</td>
<td>(p&lt;0.13)</td>
<td>FemtoLASIK 366.2±82.5 (p&lt;0.007)</td>
<td>(p&lt;0.073)</td>
<td>FemtoLASIK 726.74±90.6 (p&lt;0.041)</td>
<td>(p&lt;0.37)</td>
</tr>
</tbody>
</table>

NB. Here and in Table 2: p< m–u — a nonparametric criterion for independent Mann-Whitney groups; p< w — a nonparametric criterion for related Wilcoxon groups. Differences between the groups were considered reliable with p< m–u, p< w <0.05.
after the surgery in both groups, while in the retroablation zone they were more common in the FemtoLASIK group (Fig. 4, a, b).

In the interface zone after the surgery, both groups appeared to have a variety of particles of different sizes and reflective power (Fig. 3, a, b). A large amount of chaotically distributed hyperreflective particles was observed in the LASIK group. The interface zone had transparency loss: in the LASIK group, prominent loss of transparency was observed in 19% of cases, moderate — in 20.5%, insignificant — in 60.5%; in the FemtoLASIK group, the phenomenon was prominent in 45.3% of cases, moderate — in 36.7%, and insignificant in 18% of cases. In patients of both group, the retroablation zone stroma appeared to have thin and dichotomously divided thickened stromal nerves.

In the interface zone, keratocyte density changed in the LASIK group to an average of 309.04±84.21 cells/mm², which was 52.36% lower than baseline; in the FemtoLASIK group, it decreased to an average of 284.96±83.13 cells/mm², 56.07% lower than baseline. Keratocyte density in the corneal valve decreased by 20.6% compared with baseline (to average 637.51±94.44 cells/mm²) in the LASIK group, and by 20.8% in the FemtoLASIK group (to average 636±86.4 cells/mm²). In the retroablation zone, keratocyte density decreased to 628.83±19.13 cells/mm² in the LASIK group — 8.18% lower than baseline, and to an average of 591.53±70.92 cells/mm² in the FemtoLASIK group, which was 13.63% lower than baseline. The differences in keratocyte density in the corneal valve, as well as in the interface and retroablation zones between the groups were statistically unreliable (see Table 1).

Five years after the surgery, biomicroscopy showed transparent corneas in all patients who had undergone LASIK (n=16) and FemtoLASIK (n=22). Applying lateral illumination, however, it revealed a circular scar along the edge of the corneal valve after FemtoLASIK.
Fig. 2. Confocal microscopy picture. Fibers of the subepithelial nerve plexus.
a — five years after the surgery, FemtoLASIK group; b — five years after the surgery, LASIK group (marked with arrows are nerve fibers).

Fig. 3. Confocal microscopy picture. Corneal stroma in the ablation zone.
a — third day after the surgery, FemtoLASIK group; b — third day after the surgery, LASIK group; c — five years after the surgery, FemtoLASIK group; d — five years after the surgery, LASIK group (inside the circles are hyperreflective inclusions).
Fig. 4. Confocal microscopy picture. Corneal stroma in the retroablation zone.

a — third day after the surgery, FemtoLASIK group; b — third day after the surgery, LASIK group; c — five years after the surgery, FemtoLASIK group; d — five years after the surgery, LASIK group (inside the circles are “active” keratocytes).

Fig. 5. Confocal microscopy picture. The edge of the corneal valve.

a — five years after the surgery, FemtoLASIK group; b — five years after the surgery, LASIK group (marked with arrows is the edge of the corneal valve).
According to confocal microscopy, subepithelial nerves were present in the visual field — in average 2–3 fibers after LASIK (Fig. 2, b) and 5–6 fibers after FemtoLASIK (Fig. 2, a). In the LASIK group, only singular hyperreflective inclusions were present (see Fig. 3, d), while in the FemtoLASIK group they were absent (see Fig. 3, c).

In the interface zone, keratocyte density remained decreased in both groups: in the LASIK group, it was 46.05% lower compared with baseline (average 349.91±88.78 cells/mm² in average), in the FemtoLASIK group — 43.54% lower (366.2±82.5 cells/mm² in average). Keratocyte density in the corneal valve increased by 8.9% in the FemtoLASIK group compared with the LASIK group — 43.54% lower (366.2±82.5 cells/mm² in average). In the LASIK group, only singular hyperreflective inclusions were present (see Fig. 3, d). The differences in keratocyte densities in the interface zone and in the corneal valve between the groups were statistically unreliable (see Table 1).

In contrast to the LASIK group, single “active” keratocytes were observed in the retroablation zone after FemtoLASIK (Fig. 4, c, d). The edge of the corneal valve in the LASIK group had uneven diastasis across its width (Fig. 5, b), while in the FemtoLASIK group it was even across the width, its valve edge was hyperreflective, and it was filled with epithelial plug (see Fig. 5, a). Keratocyte density in the retroablation zone in the FemtoLASIK group increased by 4.8% compared with the LASIK group, where it amounted to mean 652.01±61.62 cells/mm² — 4.8% less than baseline, while in the FemtoLASIK group the density was 641.75±72.59 cells/mm² being 6.3% less than baseline. However, the differences in mean keratocyte densities between the groups were statistically insignificant (see Table 1).

According to confocal microscopy, the density of endothelial cells after the surgery remained within age norms in both groups for all follow-up periods. Evaluation of pleomorphism and polymegathism of corneal endothelium in children revealed absence of any reliable changes in both the early and the late post-op follow-ups.

The differences in parameters in various examined areas between the groups being statistically unreliable, we performed an analysis of generalized data obtained after the surgeries. Keratocyte density immediately after the surgery in the interface zone was reduced in average to 231.49±70.38 cells/mm², in the retroablation zone — to 601.18±62.74 cells/mm², and in the corneal valve — to 665.70±57.83 cells/mm², which was lower than baseline by 64.31%, 12.22% and 17.09%, respectively. In the late post-op follow-up, keratocyte density increased in all zones; in the interface zone, it amounted to mean 332.71±80.39 cells/mm², in the retroablation zone — to 629.68±65.89 cells/mm², and in the corneal valve — to 706.46±65.1 cells/mm², remaining lower than baseline by 48.71%, 8.06% and 12.01%, respectively (Table 2).

In summary, keratocyte density in the early post-op in the interface, retroablation zones and in the corneal valve decreased in average by 17.09%, 64.31% and 12.22%, respectively, while in the late follow-up it ended up lower than baseline by 12.01%, 48.71% and 8.06%, respectively. There were no statistically reliable differences in keratocyte density in the examined zones between the groups. However, according to data obtained with confocal microscopy, in the FemtoLASIK group there were in average 2 times more subepithelial nerves in one area unit within the vision field, and keratocyte density was in average 8.9% higher in the corneal valve, 15.28% higher in the interface, retroablation zones and in the corneal valve, 15.28% higher in the interface zone, compared to the LASIK group. Furthermore, there was a circular scar along the valve edge after FemtoLASIK, providing positional stability for the valve. Morphological state of the cornea outside the impact area was intact, and the density of endothelial cells remained within normal range during the whole follow-up.

**Discussion**

When estimating the amount of keratocytes, the results are known to depend on the type of confocal microscope and the method of counting. Images obtained with confocal microscope systems Confoscan-4 (‘Nidek’, Japan) and Heidelberg HRT-III RCM (‘Heidelberg Engineering GmbH’, Germany) differ from images acquired with tandem scanning confocal microscopes in higher contrast between keratocyte nuclei and the background.
which contributes to efficiency of the counting. Manual calculation of keratocyte density is subjective in nature, and its results notably vary from ones obtained using the automatic method [10, 11].

According to published data, amount of keratocytes varies in different layers of the stroma and have no clear correlation to age [12–14]. In our previous studies, we have established differences in keratocyte density and in increase rate of keratocytes numbers 3–5 years after keratorefractive surgeries in children compared to adults, which may be associated with specificity of rehabilitation in them [15, 16]. A number of authors believe that in adults, density of keratocytes after LASIK decreases the fastest during the first year after the surgery, stays reduced in the long-term post-op, and does not return to the baseline values [17]. Further research showed reduction of the keratocyte count in the corneal valve and retroablation zone after 3 years by 26% and 36%, respectively, and after 5 years — by 37% and 42%, respectively, compared to baseline [18, 19]. Our own analysis showed reduction of keratocyte density in the corneal valve and in the interface and retroablation zones 5 years after the surgery by 16.33%, 46.05%, and 4.8%, respectively, compared to baseline.

According to literature data, subepithelial innervation does not change with age [20]. Innervation recovery after LASIK was noted to undergo slower — by 34% compared to baseline after 3 years, and fully recovered after 5 years [21]. Some authors consider the method used to create the corneal flap does not affect the speed of cornea reinnervation or the recovery of its sensitivity after LASIK [22]. In our own study, confocal microscopy showed difference between the groups in keratocyte density and amount of subepithelial nerve fibers in the field of vision (albeit, statistically unreliable) 5 years after the surgery, which can be attributed to the technique used for creation of the corneal valve.

Earlier literature reported a loss of endothelial cells during the first year after laser keratomileusis [23]. We could not confirm any loss of endothelial cells even 5 years after the surgery with all parameters remaining within age norm. Another point of view suggests that yearly loss of endothelium after LASIK is the same as yearly loss of endothelial cells in healthy, unoperated corneas [24, 25].

One important long-term feature that appears after applying the femtosecond laser is a circular scar along the edge of the corneal valve, which distinguishes FemtoLASIK from LASIK and is consistent with the data from other sources [26–28]. This not only assures stability of the corneal valve, but also plays an important role in prevention of biomechanical weakening of the whole cornea.

Conclusions

Over the long-term follow-up, reduction of keratocyte density in the interface zone compared with baseline amounted to 43% after FemtoLASIK and 46% — after LASIK. Outside the laser impact area, no changes in the morphological state of the cornea were observed.

Both techniques are safe, but the amount of subepithelial nerves and the keratocyte density in the corneal valve and the laser impact areas are higher after Femto-LASIK, while the cornea’s stability is ensured by the formation of a circular scar along the valve’s edge.

Author contributions:
Study conception and design: I.K., O.Sh.
Acquisition and processing of data: O.Sh.
Statistical analysis: O.Sh.
Drafting of manuscript: O.Sh.
Critical revision: I.K., N.P.

The authors declare that there are no conflicts of interest.

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38
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