Collagen synthesis after laser skin resurfacing of the periocular skin†

Sinteza kolagena v periokularnem področju po laserski prenovi kože†

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Abstract

Background: Laser skin resurfacing (LSR) is a procedure in oculoplastic surgery, used for the reduction of periocular rhytides. Clinical effect of the reduction of rhytides is achieved through remodeling of skin collagen. Collagen synthesis after LSR has been evaluated both by histological examination and analysis of collagen gene expression in up-to-date studies. However, these studies are comparable only to a lesser degree due to differences between examinators and histologic criteria.

The goal of this study was to assess the usefulness of *in situ* hybridization (ISH) analysis of type I collagen gene expression after Er:YAG LSR.

Patients and methods: Eyelid skin of 9 volunteer patients has been treated by Er:YAG laser with LSR parameters. Twenty-one days after the treatment the skin was excised as part of the blepharoplasty procedure. For ISH, a pro alpha (I) collagen antisense copy-DNA (cDNA) probe was designed. We prepared tissue sections and hybridized them with the radiolabelled cDNA probe. We used untreated skin areas from the same skin samples as a control group.

Results: We determined and statistically analyzed the levels of pro alpha (I) collagen gene expression in the samples collected, which varied significantly between the treated and untreated skin.

Conclusions: *In situ* hybridization analysis is a useful method in determining the site and rate of collagen gene expression after Er:YAG LSR. We suggest that this method is useful in determining the amount of collagen synthesis after laser skin resurfacing and is less observer-dependent than histological evaluation alone.

Izvleček

Izhodišča: Lasersko prenovo kože (LPK) uporabljamo v okuloplastični kirurgiji za zmanjšanje gub kože v periokularnem področju. Kožne gube lahko razdelimo na (1) fine gube, (2) mimične gube in (3) globoke gube. Za zdravljenje prvih je primerna metoda LPK, za druge injiciranje kožnih polnil ali botulinusnega toksina, za tretje je potreben kirurški poseg. Kožne gube so posledica več dejavnikov, glavna sta zmanjšanje elastičnosti kože zaradi solarno-aktinične okvare ter ponavljajoče se krčenje obraznih mišic. Ob tem se pojavljajo še individualne variacije zaradi genetskih osnov, kajenja in stresa. Kolagen v dermisu se s staranjem denaturira, stanjša, izgubi urejenost in se naguba ter s tem prispeva k nagubanosti same površine kože. Klinično opazni učinek zmanjšanja globine gub po LPK temelji na preoblikovanju kolagena v dermalni plasti kože. Učinkovitost LPK je tema številnih raziskav, kjer so avtorji ugotavljali spremembe v kožnem kolagenu s histološkimi metodami. V nekaterih raziskavah so analizirali ekspresijo genov za kolagen z metodo hibridizacije DNK, kot sta metodi Southern-blot in northern-blot, ki omogočata ugotovitev izražanja določenega gena v vzorcu tkiva in kvantifikacijo le-tega. Medsebojna primerjava objavljenih raziskav o učinkovitosti LPK je težavna zaradi razlik med ocenjevalci in histološkimi kriteriji, ki so jih avtorji uporabili.

Drugi načini ugotavljanja učinkovitosti LPK temeljijo na primerjavi neobsevane in obsevane kože oziroma analizi fotografij pred in po LPK, pri kateri se ocenjuje stopnjo nagubanosti in izgled kože klinično. Mnogo boljšo objektivnost in ponovljivost lahko dosežemo z metodo tridimenzionalne mikroskopske topografske analize kožne površine. S to metodo lahko določimo stopnjo anizometrije kožne površine s statistično

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analizo vrste, dolžine, globine in usmeritve kožnih gub.

Z metodo in situ hibridizacije (ISH) lahko natančno določimo mesto izražanja določenega gena v tkivu, hkrati lahko semi-kvantitativno določimo tudi stopnjo izražanja preiskovanega gena. Iz teh dveh razlogov smo želeli preizkusiti uporabnost metode ISH za oceno izražanja genov za kolagen tipa 1 po LPK z laserjem Er:YAG. S tem smo želeli prispevati k boljši ponovljivosti in večji objektivnosti pri ocenjevanju različnih postopkov LPK.

Bolniki in metode: V študijo smo vključili osem prostovoljk in enega prostovoljca, pri katerih je bil zaradi dermatohalaze zgornjih vek predviden kirurški poseg – blefaroplastika. Študijo je potrdila Komisija za medicinsko etiko RS v okviru raziskovalnega projekta »Delovanje laserskega sevanja na tkiva očesa in periokularnega področja«.

21 dni pred posegom smo odvečno kožo vek obsevali z laserjem Er:YAG z enakimi nastavitvami kot pri LPK. Odstranjeno kožo smo zamrznili v tekočem dušiku pri -90°C. Za postopek ISH gena za kolagen tipa 1 smo pripravili specifično protismiselno DNK (cDNA) in jo hibridizirali z vzorci tkiva. Označene tkivne rezine smo inkubirali v svetlobno neprodušno kaseto s fotografskim filmom in ga nato razvili, da smo iz njega lahko odčitali rezultate poskusa. Prav tako smo na objektna stekelca s tkivnimi rezinami nanesli fotografsko emulzijo za neposredno primerjavo rezultatov ISH in histološke ocene tkivnih rezin. Kot kontrolna skupina za primerjavo so nam služili neobsevani predeli kože iz istih vzorcev kože vek.

Rezultati: Z metodo ISH smo dokazali tvorbo novega kolagena tipa 1 v vzorcih kože po LPK z laserjem Er:YAG. Določili smo mesto izražanja gena za kolagen tipa 1 v tkivnih rezinah vzorcev obsevane kože v povrhnjem dermisu do globine 200 µm. Semikvantitativno smo določili stopnjo izražanja gena za kolagen tipa 1, ki je bila značilno višja (do 20x) v vzorcih obsevane kože v primerjavi z vzorci neobsevane kože.

Zaključki: Dokazali smo uporabnost metode ISH za dokazovanje tvorbe kolagena v koži po obsevanju z laserjem Er:YAG z nastavitvami za LPK. Povečanje izražanja gena za kolagen tipa 1 je tolikšne stopnje in sega do take globine, da lahko povzroči izboljšanje videza kože z zmanjšanjem globine kožnih gub. Menimo, da je metoda ISH uporabna za primerjavo učinkovitosti različnih metod LPK. Poglavitna prednost te metode je, da je manj odvisna od kriterijev opazovalca kot sama histološka analiza in omogoča semikvantitativno oceno stopnje izražanja gena za kolagen tipa 1.

Background and objective

Laser skin resurfacing (LSR) is a dermatosurgical laser procedure, used for the reduction of fine periocular and facial rhytides (wrinkles). Facial wrinkles can be divided in three main groups: fine wrinkles, expression wrinkles and grooves, and creases. LSR procedures are indicated for fine wrinkles; botulinum toxin and filler product injections are used for mimetic wrinkles and grooves; but creases can only be treated with removal or retension/lifting surgery.[1] Two main mechanism of ageing cause wrinkles: the reduction of skin elasticity due to solar elastosis and actinic damage; and repeated contractions of fine facial muscles. Additionally, individual variability is caused by genetic predispositions, smoking and stress. Changes in dermal collagen take form of denaturation and loss of orientation of the fibres, thinning and folding of the collagen dermal layer.[2] The mode of action of LSR is on this dermal collagen layer. Partial skin collagen denaturation is achieved by delivery of laser energy, which causes an inflammatory response in the skin, which in turn leads to collagen remodeling and new collagen synthesis. The ultimate effect of this procedure is smoothening of the skin surface and the reduction of wrinkles. Various laser types are used for LSR, most notably the CO_2 and Er:YAG laser. Alternatively, other energy sources have been introduced in the last few years, e.g. radiofrequency waves (RF) and intense pulsed light (IPL) sources.

Since the introduction of LSR, many studies investigated the effectiveness of various LSR procedures. The amount of collagen and the arrangement of its fibrils were histologically evaluated in most of these studies.[3-11] Other studies rely on the evaluation of skin texture by either half-face comparison or

photographic analysis, and arbitrary score systems are used for different degrees of skin wrinkles. When comparing these studies, the problem of differences between histological criteria, score systems and examinators arises, leading to significant interobserver differences. It is therefore difficult to objectively assess the effectiveness of different laser resurfacing techniques used today, and to make an objective comparison.

Three-dimensional microscopic topographic analysis of the skin surface is a quantitative method, where degree of anisometropy, type, length, depth and orientation of wrinkles can be evaluated.[12,13]

More recent studies use the methods of imunuhistofluorescence for the analysis of specific proteins (e.g. collagen) and gene--expression analysis, e.g. Southern-blot and Northern-blot techniques. These techniques enable us to measure the rate of activation of a specific gene in a tissue sample.

In situ hybridization is a method, which enables us to detect the amount of synthesis of a specific protein at a pretranslational level as well as the site of synthesis in a tissue section. This method has been used in dermatopathology since 1988, and the rate of collagen synthesis in a variety of pathological processes has been studied.[14-16] Examples include keloids, surgical scars, psoriasis, scleroderma, photoaged skin, and connective tissue diseases, which were studied *in vitro* and *in vivo*.[17-28] A few studies have been made where gene expression after irradiation of the skin with laser was analyzed with Southern-, Western- or Northern-blot DNA expression techniques. However, to our best knowledge, none of these studies used the method of *in situ* hybridization in combination with Er:YAG LSR. *In situ* hybridization offers one major advantage over other gene expression techniques: the exact site of expression of the challenged gene in the tissue can be determined. Additionally, the amount of gene expression can be assessed semi-quantitatively.

Our goal was to explore the usefulness of ISH in determining collagen synthesis after laser skin resurfacing with the Er:YAG laser.

Patients and methods

Eight female and one male volunteer, aged between 54 and 62 years, participated in this study. Their informed consent was obtained, and the study received clearance by the National Medical Ethics Committee. All of the patients had Fitzpatrick skin type 2 and blepharochalasis, for which blepharoplasty was indicated. None of the patients had a history of previous skin disease or laser/surgical treatment of the involved area. The eyelid skin was treated with laser under local anesthesia with 2 % Xylocaine. On day 21 after laser treatment elective surgery – blepharoplasty was performed by standard surgical protocol. At the time of surgery the treated skin was excised as part of the procedure and immediately frozen in dry ice (frozen CO_2, -90°C) and stored for further analysis.

We used a 2940 nm Er:YAG laser (Fidelis M320A, Fotona d.d., Slovenia) with 'smooth' mode parameters: energy fluence 1.0, 1.25, 1.50 and 1.75 J/cm2; six pulses per packet; 550 µs/pulse, 250 ms/packet; single pass, no overlapping; spot size 5 mm; repetition rate 20 Hz. There were 2 areas of irradiated skin per eyelid, giving together four areas, one for each selected laser energy fluence.

The tissue sections were cut with a microtome in sections of 2 micrometer thickness and placed on a microscopic glass slide. We used an oligonucleotide (45 base-pair) DNA probe for in situ hybridization, labelled with radioactive sulphur 35. We designed a specific probe for alpha 1 procollagen chain (COLA1), which together with procollagen alpha 2, form collagen type I. The nucleotide sequence for alpha 1 chain antisense probe is: gttcttggtctcgtcacagatcacgtcatcgcacaacacct tgc, base pairs 360 to 316, GC/AT ratio: 23/22. The probe was tested for specificity and sensitivity for the target gene; a comparison to other genes was performed using the MeSH browser. The labeled probe on microscopic glass, tissue sections and radiosensitive film were incubated for 10 days and a second set of tissue sections on microscopic glass and labeled probe was covered with film emulsion directly.

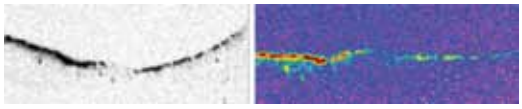


Figure 1 (left): Untreated vs. treated skin. ISH with probes COLA1 and COLA2. Note the gap – untreated zone.
Figure 2 (right): Relative signal strength image of ISH with COLA1 probe. Gap – untreateated tissue, right – effect of single laser spots.

After this period, a microscopic analysis of the tissue sections covered with film emulsion was performed. Additionaly, a photographic scanner was used to measure the density of silver grain in the developed film.

Results

The expression of pro alpha 1 collagen type I mRNA was significantly higher in the treated tissues compared to untreated tissue ($p < 0.01$). The expression of this mRNA was limited to the subepidermal layer, which correlates well with the histological findings of extracellular matrix thickening. The zone of enhanced collagen expression extended up to 200 µm deep into the dermis. We also noted proliferation of fibroblasts in the same layer. The amount of mRNA expression was also correlated with the energy fluencies used in laser skin resurfacing. At fluencies of 1.00 and 1.25 J/cm^2 scarce mRNA expression in a limited number of fibroblasts was noted, whereas at fluencies of 1.50 and 1.75 J/cm^2 a strong signal was measured, which was correspondingly stronger at the latter energy fluency (Figures 1 and 2).

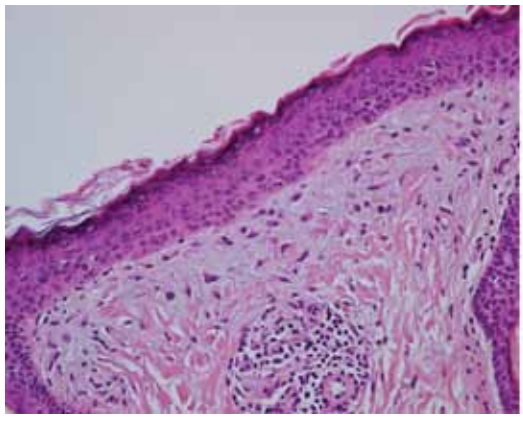
Discussion

We successfully proved new collagen synthesis after Er:YAG LSR in eyelid skin by ISH method. To our knowledge, this is the first study to unequivocally show new collagen synthesis after Er:YAG LSR. New collagen synthesis has been speculated on, and deducted from analysis of the thickness of collagen fibrils, but no direct proof existed. Opponents to the idea of new collagen synthesis after LSR argued that there was only re-arrangement of collagen fibrils. Other authors believe the skin tightening effect is due to shrinkage of the epidermal layer or scarring in the subepidermal layer.[6,7,9,10] Concerning scar formation, collagen type V is typical for scars, whereas collagen type I and III are less present. The cDNA probe that we used to label the mRNA for procollagen alpha 1 chain is specific for collagens type I and III.

We have also shown the exact site of new collagen synthesis, which is the upper dermal layer and extends up to 200 µm deep, which is more than enough to produce a clinical effect. We have proved the presence of myofibroblasts in the same layer, which is a further proof of new collagen synthesis.

There are two main methods of evaluation of the effectiveness of LSR as described in the literature. The first is by examining the patient's skin texture and smoothness before and after treatment by (in-)dependent observers, either in a clinical setting or on the basis of photographs. In most of the studies, some sort of arbitrary score system or gradation of the results is used, which are usually referred to as excellent, good, and poor. Such scoring systems have several flaws: in many cases, there is no definition of the grading system, which therefore relies on the subjective judgement of the observer. A direct consequence is a very low inter-observer repeatability of the results. A new approach to the evaluation of skin texture is the method of three-dimensional topography; a 3D-relief of the skin is acquired by means of different microscopic techniques: mechanical, laser, interferometry and fringe projection microscopy. The acquired topographic data are analysed and a degree of skin fold anisometropy, orientation and volume can be determined.[12,13] This method offers the advantage of objectivity and reproducibility, but has not been used for evaluation of the periocular skin.

The second method is the analysis of histological sections obtained from skin grafts or volunteers' skin after LSR. Most observers rely on the measurement of the depth of the collagen denaturation layer and zone of collagen remodelling. In both cases, there is no sharp demarcation line between 'denaturated' and normal collagen or between the zone of collagen remodelling and unchanged subepidermal layer. From the analysis of pictures and our own experience we know that there is a large 'grey' zone, which could be part of either measured or unmeasured zone.[3-11]



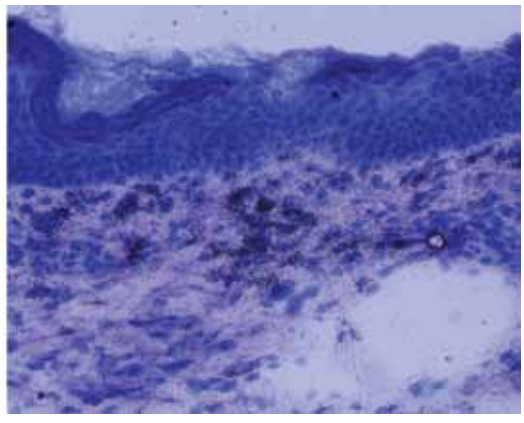


Figure 3 (left): HE stained section of eyelid skin 21 days after LSR, 100x magnification.
Figure 4 (right): Methylen-blue stained tissue section of eyelid skin 21 days after LSR, 200x magnification. Black dots represent signal from cDNA probe for collagen type 1.

This important question of repeatability of data analysis and inter-observer repeatability can be avoided with collagen analysis using the method of ISH.

We have additionally measured the rate of new collagen production by densitometric analysis of the tissue sections. There is an up to 20x increase in collagen production on day 21 compared to baseline values before laser treatment. We have chosen this three-week interval between laser treatment and tissue sampling as it corresponds to a peak in collagen synthesis according to other studies on collagen expression after laser treatment with Northern-blot analysis, as well as according to our own unpublished data. This is contradictory to the argument of scar formation, which develops at a much later stage after tissue injury – weeks to months after the stimulus. We also noted a correlation with laser energy fluence. To our best knowledge, this study shows the method of ISH as the most objective method for the evaluation of LSR effectiveness for new collagen synthesis, with a low intra- and inter-observer bias.

This method has its own deficiencies as well. First, it is a time-consuming procedure, where a specific oligonucleotide probe has to be designed and labeled with a radionuclide such assulphur 35, which was used in our case. Special laboratory equipment as well as staff training is mandatory.

Conclusions

The method of in situ hybridisation is a useful method in determining the site and rate of mRNA expression of pro alpha collagen type I in samples after laser skin resurfacing. We suggest that this method is therefore useful in determining the amount and site of collagen type I synthesis, which would make it possible to compare different lasers used for skin resurfacing, as well as the effectiveness of other, non-laser skin resurfacing methods. ISH of collagen type 1 facilitates the quantification of results with greater objectivity compared to histological and clinical evaluation. Our goal in the future is to correlate, if possible, this method with long-term clinical results.

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