ACTA MEDICA (HRADEC KRÁLOVÉ)

2016, Vol. 59, No. 3

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ORIGINAL ARTICLE

Droplet Digital PCR Analysis of *GSTM1* Deletion Polymorphism in Psoriatic Subjects Treated with Goeckerman Therapy

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Summary: Goeckerman therapy (GT) represents an effective treatment of psoriasis including a combination of pharmaceutical grade crude coal tar (CCT) and ultraviolet irradiation (UV-R). Coal tar contains a mixture of polycyclic aromatic hydrocarbons. The best known carcinogenic polyaromate – benzo[a]pyrene is metabolized into a highly reactive benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE). Glutathione S-transferase M1 (GSTM1) catalyses the conjugation of drugs, toxins and products of oxidative stress with glutathione. The aim of the study is to found possible associations between *GSTM1* genotypes and the level of BPDE-DNA adducts in 46 psoriatic patients treated with GT. For genotyping, droplet digital PCR was applied. The *GSTM1* copy number was normalized to β-globin reference gene. In five *GSTM1*1/*1* subjects, the *GSTM1* to β-globin ratio moved from 0.99 to 1.03 with a median of 1.01. *GSTM1*0/*1* heterozygotes (n = 20) contained only one *GSTM1* function allele which conditioned the ratio 0.47–0.53 (median 0.50). *GSTM1*0/*0* individuals (n = 21) showed no amplification of the null variants because of the large deletion in *GSTM1*. BPDE-DNA concentrations ranged from 1.8 to 66.3 ng/µg with a median of 12.3 ng/µg. *GSTM1*0/*0* and *GSTM1*0/*1* genotypes showed non-significantly higher concentrations of BPDE-DNA adducts than the *GSTM1*1/*1* one (12.3 and 12.4 vs 7.8 ng/µg). The non-significant relationship between BPDE-DNA adducts and *GSTM1* genotypes in psoriatic patients could be associated with relatively low doses of CCT and short-term UV-R exposures used in GT.

Keywords: *GSTM1; Psoriasis; Goeckerman therapy; Genotyping; BPDE-DNA adducts*

Introduction

Goeckerman therapy (GT) represents an effective treatment of psoriasis including a combination of pharmaceutical grade crude coal tar (CCT) ointment and ultraviolet irradiation (UV-R). This therapeutic approach is applied in cases of light to moderately severe forms of psoriasis (1). CCT contains a mixture of polycyclic aromatic hydrocarbons (PAHs). The best known carcinogenic polyaromate – benzo[a]pyrene (BaP) is metabolized into a highly reactive benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) and other reactive species.

The conjugation of BaP derivatives is catalyzed by glutathione S-transferases GSTM1 or GSTP1, and UDP glucuronosyltransferases 1A10, 1A6, 1A7C or 1A9. However, BPDE also intercalates in DNA by forming covalent bond with the nucleophilic guanine nucleotide bases at the N2 position and creates the BPDE-DNA adduct (2). GSTM1 (EC 2.5.1.18) is a cytosolic enzyme which catalyses the conjugation of drugs, toxins and products of oxidative stress

http://dx.doi.org/10.14712/18059694.2016.94

with glutathione to form less reactive and more easily excreted water-soluble metabolites. To ensure high effectiveness of the conjugations, the *GSTM1* gene (chromosome location 1p13.3) is expressed in a lot of human tissues including liver and skin (3).

Previously published papers showed that genetic polymorphisms in *GSTM1* conditioned the individual response to electrophilic xenobiotic substances including PAHs. A large deletion (*GSTM1*0* null variant) occurs hereditarily in about 50% of Caucasian population. The absence of active enzyme in subjects with the *GSTM1*0/*0* genotype declines the efficiency of detoxification processes and leads to genotoxicity, toxic encephalopathy, higher cutaneous ultraviolet radiation erythemal sensitivity and risk of asbestosis or cancer (4–10). It is apparent that the activity of GSTM1 influences the level of BPDE and hence also the level of BPDE-DNA adducts. However, the results of studies focused on relationships between the GSTM1 activity and the level of BPDE-DNA adducts, are still inconsistent (11–13). Moreover, only a little is known about genotoxic and mutagenic risks associated to *GSTM1*0/*1* heterozygosity (10).

Genetic analysis of the *GSTM1* deletion polymorphism is usually performed *via* Southern blotting, long range PCR, and real-time PCR with either SYBR Green or specific hydrolytic probes (14–17). All these methods distinguish the *GSTM1*0/*0* genotype from *GSTM1*1/*1* and *GSTM1*0/*1* ones. Quantitative real-time PCR enables separation of *GSTM1*1/*1* and *GSTM1*0/*1*; both these genotypes featured by specific copy number reveal different values of cycle threshold (*Ct*). To normalize the input amount of genomic DNA, the *Ct* values are related to a reference gene with the constant copy number in the genome: *albumin*, β-globin or *RNAse P* (10, 18, 19). Despite the use of sophisticated expectation-maximisation algorithms and precisely defined cut-off *Ct* intervals for *GSTM1*1/*1*, **0/*1* and **0/*0*, the results of trimodal genotyping are not clear and strictly depend on DNA quality and PCR amplification efficiency.

Droplet digital PCR (ddPCR) is a modern technology amplifying DNA separately in thousands of nanoliter-sized oil microdroplets. After PCR, the fluorescence of each droplet is recorded and the total number of events (droplets) above the threshold is counted. Here we describe a novel approach to *GSTM1* genotyping based on droplet digital PCR. Using this technique we investigated a cohort of psoriatic patients treated with GT. In them, elevated level of BPDE-DNA adducts were previously found (20).

Material and Methods

The cohort created a group of 46 patients with chronic stable plaque psoriasis treated with GT. The group consisted of 22 males and 24 females (average age of 48 years, age span 20–82 years; 23 smokers and 23 non-smokers). Within GT therapy, dermatological ointment containing 3% of CCT was administered daily overnight on psoriatic lesions. According to the extent of lesions, 18–62% of the total body surface was covered by CCT ointment. Each morning the residues of CCT were removed from the body (using oil bath) and the patients were whole-body irradiated by UV-R. The density of used radiation was 249.75 μW/cm^2 of UV-B and 131.8 μW/cm^2 of UV-A. The effectiveness of the therapy was calculated from basic characteristics of actual disease status (erythema, desquamation, and skin infiltration) and expressed as the PASI score (Psoriasis Area and Severity Index). The study was approved by the Ethics Committee of the Charles University Hospital in Hradec Králové, Czech Republic. Written informed consent was obtained from each patient.

EDTA-treated peripheral blood specimens were collected immediately after GT. Genomic DNA was extracted from 200 μL of blood with a QIAamp DNA Blood Mini Kit (Qiagen, Germany). The level of BPDE-DNA adducts was determined by using the standard method OxiSelect BPDE-DNA Adduct ELISA Kit (Cell Biolabs, USA). The results were expressed in nanograms of BPDE-DNA adducts per microgram of DNA.

For genotyping, droplet digital PCR (QX100 Droplet Digital PCR System, Bio-Rad, USA) was applied. The *GSTM1* copy number was normalized to β-globin reference gene. The amplification mix (25 μL) contained 12.5 μL 2× concentrated ddPCR Supermix (Bio-Rad, USA), 900 nM of each primer, 250 nM of hydrolysis fluorescent probes, and 100 ng of DNA. The sequences of primers and probes were as follows: *GSTM1* forward primer 5′-CAC CTG CAT TCG TTC ATG TGA C-3′, *GSTM1* reverse primer 5′-AAG CAA GAG CAG AGA GGA GAC-3′, *GSTM1* hydrolytic probe 5′-FAM-TTC AGT CCT GCC ATG AGC AGG CAC A-BHQ1-3′, β-globin forward primer 5′-GAG GGT TTG AAG TCC AAC TCC TAA-3′, β-globin reverse primer 5′- CAG GGT GAG GTC TAA GTG ATG ACA-3′, and β-globin hydrolytic probe 5′-HEX-CAG TGC CAG AAG AGC CAA GGA CAG GT-BHQ1-3′.

The data were statistically processed by the R software version 3.22 using the "*nortest*" and "*psych*" packages. Because the Anderson-Darling test for the normality had rejected the hypothesis of a normal distribution of the BPDE-DNA adducts, nonparametric one-side Wilcoxon tests was used. Differences were considered to be statistically significant when $P < 0.05$.

Results and Discussion

Droplet digital PCR enabled identification of all three genotypes. As illustrated in Fig. 1, there were no problems to evaluate the proper *GSTM1* genotype if normalization to β-globin gene was performed. In five *GSTM1*1/*1* subjects, the *GSTM1* to β-globin ratio moved from 0.99 to 1.03 with a median of 1.01 proving the presence of two function alleles of *GSTM1* in diploid cells. *GSTM1*0/*1* heterozygotes (n = 20) contained only one *GSTM1* function allele which conditioned the ratio 0.47–0.53 (median 0.50). *GSTM1*0/*0*

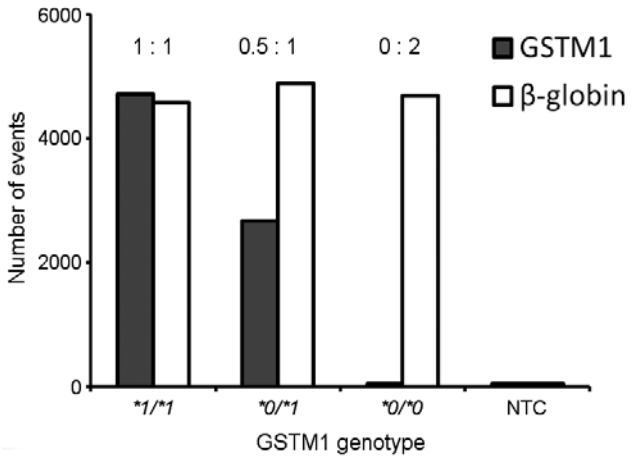

Fig. 1: The number of events recorded by ddPCR in different *GSTM1* genotypes (black) normalized to β-globin gene (white). The proper *GSTM1* to *β-globin* copy number ratio is indicated above the columns; NTC means no template control.

individuals (n = 21) showed no amplification of the null variants because of the large deletion in *GSTM1*.

The prevalence of *GSTM1*0* was 0.67, and the genotype frequencies were in agreement with the Hardy-Weinberg equilibrium. The frequency of *GSTM1*0* determined in the patients agreed with the results of other studies (10, 18).

BPDE-DNA adducts were detected in all investigated blood specimens. Their concentrations ranged from 1.8 to 66.3 ng/µg with a median of 12.3 ng/µg. No significant associations between the levels of adducts and sex or smoking were observed. *GSTM1*0/*0* and *GSTM1*0/*1* genotypes showed non-significantly higher concentrations of BPDE-DNA adducts in blood cells than the *GSTM1*1/*1* one (median values 12.3 and 12.4 vs 7.8 ng/µg, Table 1). Combinating *GSTM1*1/*1* and *GSTM1*0/*1* genotypes into one group, the difference in DNA adducts completely disappeared (median 12.2 ng/µg; $P = 0.261$). This fact could clearly justify the importance of trimodal genotyping *GSTM1*.

Our data show that the concentrations of BPDE-DNA adducts in blood cells of psoriatic subjects in GT do not significantly associate with the *GSTM1* genotype, though elevated levels of BPDE-DNA adducts in *GSTM1*0/*0* and *GSTM1*0/*1* carriers in comparison with the *GSTM1*1/*1* were apparent.

We assume that this non-significant relationship could be associated with relatively low doses of CCT and short-term UV-R exposures used in GT. In subjects exposed in industry to higher doses of PAHs, the relationship between BPDE-DNA adducts and *GSTM1* was more evident (11, 12, 21, 22). Further, in parallel with GSTM1, the BaP derivates may also be conjugated by UDP glucuronosyltransferases to corresponding glucuronides. Therefore, if we assess the association between the GSTM1 activity and the level of BPDE-DNA adducts, we have to take into account the fact that we evaluate only a part of genetically conditioned processes of activation and deactivation of BaP (23, 24). Finally, the skin of psoriatic patients was described to have lower GSTM1 catalytic activity than the normal skin (25). The loss of GSTM1 activity could lead to decreased expression of other GSTM isozyme (26) and to compensatory induction of other PAHs metabolizing enzymes, e.g., GSTP1 (27) or CYP1A1, especially in *GSTM1*0/*0* homozygotes (11, 28).

Tab. 1: DNA adducts in *GSTM1* genotypes.

***GSTM1* genotypes**	**n**	**BPDE-DNA (ng/µg)**		***P***
		Median	**Range**	
*GSTM1*0/*0*	21	12.3	1.9–66.3	*ref.*
*GSTM1*0/*1*	20	12.4	1.8–50.1	0.329
*GSTM1*1/*1*	5	7.8	7.5–49.7	0.227
*GSTM1*1/*1* or *GSTM1*0/*1*	25	12.2	1.8–50.1	0.261

ref. reference group

Conclusions

Droplet digital PCR has proved to be suitable for analysis of *GSTM1* deletion polymorphism. At the group of psoriatic patients treated with GT, we found non-significant differences in the levels of BPDE-DNA adducts, roughly corresponding to genetic polymorphisms in *GSTM1*. Taking into account all the factors mentioned above, a larger population study evaluating the associations between BPDE-DNA adducts and other xenobiotic metabolizing enzyme polymorphisms in psoriatic patients should be performed.

Acknowledgements

This work is supported by the projects PRVOUK P37/09 and PRVOUK P37/11 of Charles University, Faculty of Medicine in Hradec Králové, Czech Republic.

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Received: 31/05/2016
Accepted: 13/06/2016

ORIGINAL ARTICLE

Wireless Capsule Enteroscopy in Healthy Volunteers

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Summary: Introduction: The aim of our prospective study was to define endoscopy appearance of the small bowel in healthy volunteers. Method: Forty-two healthy volunteers underwent wireless capsule endoscopy, clinical investigation, laboratory tests, and completed a health-status questionnaire. All subjects were available for a 36-month clinical follow-up. Results: Eleven subjects (26%) had fully normal endoscopy findings. Remaining 31 persons (74%), being asymptomatic, with normal laboratory results, had some minor findings at wireless capsule endoscopy. Most of those heterogeneous findings were detected in the small intestine (27/31; 87%), like erosions and/or multiple red spots, diminutive polyps and tiny vascular lesions. During a 36-month clinical follow-up, all these 42 healthy volunteers remained asymptomatic, with fully normal laboratory control. Conclusions: Significant part of healthy subjects had abnormal findings at wireless capsule endoscopy. These findings had no clinical relevance, as all these persons remained fully asymptomatic during a 36-month follow-up. Such an endoscopic appearance would be previously evaluated as "pathological". This is a principal report alerting that all findings of any control group of wireless capsule endoscopic studies must be evaluated with caution.

Keywords: *Small bowel; Wireless capsule enteroscopy; Healthy volunteers; 36-month follow-up*

Introduction

Wireless capsule endoscopy represents an important progress in small intestinal imaging. During past 16 years (since 2000 (1)) it has become the leading enteroscopy method. It is now commonly used in the evaluation of obscure gastrointestinal bleeding (including iron deficiency anaemia), suspected and known Crohn's disease, small bowel tumours, complicated coeliac disease and non-steroidal anti-inflammatory drug-induced enteropathy.

The main advantage of the method is its minimal invasiveness, safety and reliability. That is why capsule endoscopy is often used both in daily clinical practice and in clinical studies on small bowel disease. Thanks to its high sensitivity capsule endoscopy is able to identify small lesions with little or no clinical significance, too. Usually, there is no problem in identifying small bowel tumours, sources of bleeding and advanced inflammatory changes (ulcers, cobble stones, inflammatory polyps, multiple erosions and aphthae). However, interpreting of tiny non-specific findings suggestive of inflammation (swelling, redness, isolated erosions) is often questionable. Mild non-specific inflammatory-like lesions were frequently described in the small bowel of healthy volunteers in many capsule endoscopy studies as pathologic ones. We found this type of lesions in 12% of a control group in our study with non-steroidal anti-inflammatory drug-induced enteropathy (2), too.

From this perspective, it is necessary to identify and define the spectrum of normal or insignificant enteroscopy findings, and consider these findings in evaluating wireless capsule endoscopy studies. The aim of this study was to assess capsule endoscopy in healthy volunteers.

Methods

Participants and design

We organized our study as prospective one and it included adult (>18 years) healthy volunteers. Exclusion criteria were any acute or chronic disease and history of use of any drugs (including non-steroidal anti-inflammatory drugs) in a month prior the study and pregnancy. Written informed consent was obtained from all participants.

A total of 42 healthy participants (29 women, mean age 42 years, median 42 years; 13 men, mean age 43 years, median 42 years) were enrolled in the study. All underwent

http://dx.doi.org/10.14712/18059694.2016.93

wireless capsule endoscopy, basic clinical investigation, laboratory tests (comprising anaemia, nutrition and inflammatory markers: blood count, reticulocytes, Coombs test, serum iron, ferritin, albumin and prealbumin, C-reactive protein, erythrocyte sedimentation rate, Helicobacter pylori stool antigen and faecal occult blood testing). All subjects completed a health-status questionnaire.

Wireless capsule endoscopy was performed in compliance with the published guidelines of the Czech Society of Gastroenterology and European Society of Gastrointestinal Endoscopy (3, 4). The EndoCapsule™ (Olympus) was used. The participants were prepared by the 12-hour fasting only, without the need of any purgative solutions. 2 and 4 hours after the capsule ingestion the subjects were allowed fluids and light meal. The position of capsule endoscope was controlled 2 hours after its ingestion using a real time viewer. Two experienced endoscopists evaluated capsule recording. Any particular findings (regardless of clinical significance or insignificance) were identified as abnormal and were described according to the CEST (Capsule Endoscopy Standard Terminology) (5, 6). Red spot was defined as a demarcated, circular, reddish, small, pinpoint mucosal mark, erosion as a superficial destruction of mucosa, denuded areas as mucosal surface without villi, aphthous lesion as a mucosal break with a pale centre and reddish halo and ulcer as an excavated lesion with its base covered with fibrin. Vascular abnormalities were lymphangiectasias (whitish dots involving individual oedematous villi), angiectasias (flat, reddish or fernlike ectatic vessels) and flebectasias (large, ectatic, bluish veins). Basic time characteristics of the investigation (gastric transit time, small bowel transit time, total time of batteries function, time of findings) were evaluated, too. Small bowel visibility, based on the degree of purification of the intestine, was scored.

Ethical approval

All procedures were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its latter amendments.

Results

Endoscopy findings

There was no clinical or technical complication observed during the study. Capsule endoscopes reached the small bowel within the first 2 hours in all healthy volunteers and completed panenteroscopy before the batteries discharge. The quality of the small intestinal mucosa visibility was good in majority of investigations; in one participant only (2.4%) the visibility was decreased due to the rich bowel content in some parts of distal ileum.

The average gastric transit time was 37 ± 33 minutes (median 27 minutes) and small bowel transit time was 262 ± 90 minutes (median 246 minutes). The total time of investigation (the battery function time) was 557 ± 93 minutes (median 571 minutes) on average. The mean evaluation time (time spent by an endoscopist to evaluate the completerecord) was 58 ± 6 minutes (median 55 minutes).

Eleven subjects (26%) had fully normal endoscopy findings. Abnormal findings were identified in 31/42 subjects (74%). The majority of these findings was described in the small bowel (27/42 persons; 64%): isolated red spots (14/42; 33%), multiple red spots (3/42; 7%), erosions (2/42; 5%), mucosal erythema in the duodenal bulb classified as bulbitis (4/42; 10%), suspected diminutive polyps (3/42; 7%), small submucosal tumour, probably a tiny lipoma (1/42) and vascular findings like angiectasias (1/42), lymphangiectasias (1/42) and small bowel flebectasia (1/42), small intestinal xanthomas (2/42; 5%). Extraintestinal findings comprised reflux oesophagitis (1/42), the endoscopic picture of erythematous gastritis (8/42; 19%), diminutive gastric polyps (2/42; 5%), and erosions (3/42; 7%). Small intestinal lesions were localised more frequently in the jejunum (16), the duodenal and ileal involvement was presented less commonly. Caecal polyp was identified in one subject. This person underwent subsequent colonoscopy with polypectomy of 4 adenomas with low-grade dysplasia. All other findings at wireless capsule endoscopy were considered as fully benign, requiring no further action.

Clinical data

All healthy volunteers were without any clinical symptoms and health problems before the inclusion and during the study period. Four people mentioned dypepsia in the past history (without need for treatment and without any other clinical consequences). All study participants were available for a 36-month clinical follow-up.

Laboratory tests

Laboratory tests were fully normal in the majority of subjects before wireless capsule endoscopy. The exception was significant increase in CRP (>10 mg/l), we found in three persons (11 mg/l, 12 mg/l, and 22 mg/l) during the initial examination. There was no significant capsule endoscopy finding in all these subjects (normal finding or isolated red spot in duodenum and ileum). There was no infectious complication subsequently diagnosed in them and control CRP levels normalized. Laboratory tests were normal after the 36-month follow-up.

Discussion

Our capsule endoscopy study revealed abnormal small intestinal findings in healthy volunteers that would be previously considered as minor but pathological ones and thus could be misinterpreted. All subjects were health professionals from our University Hospital and they were available for subsequent 36-month follow-up. All remained symp-

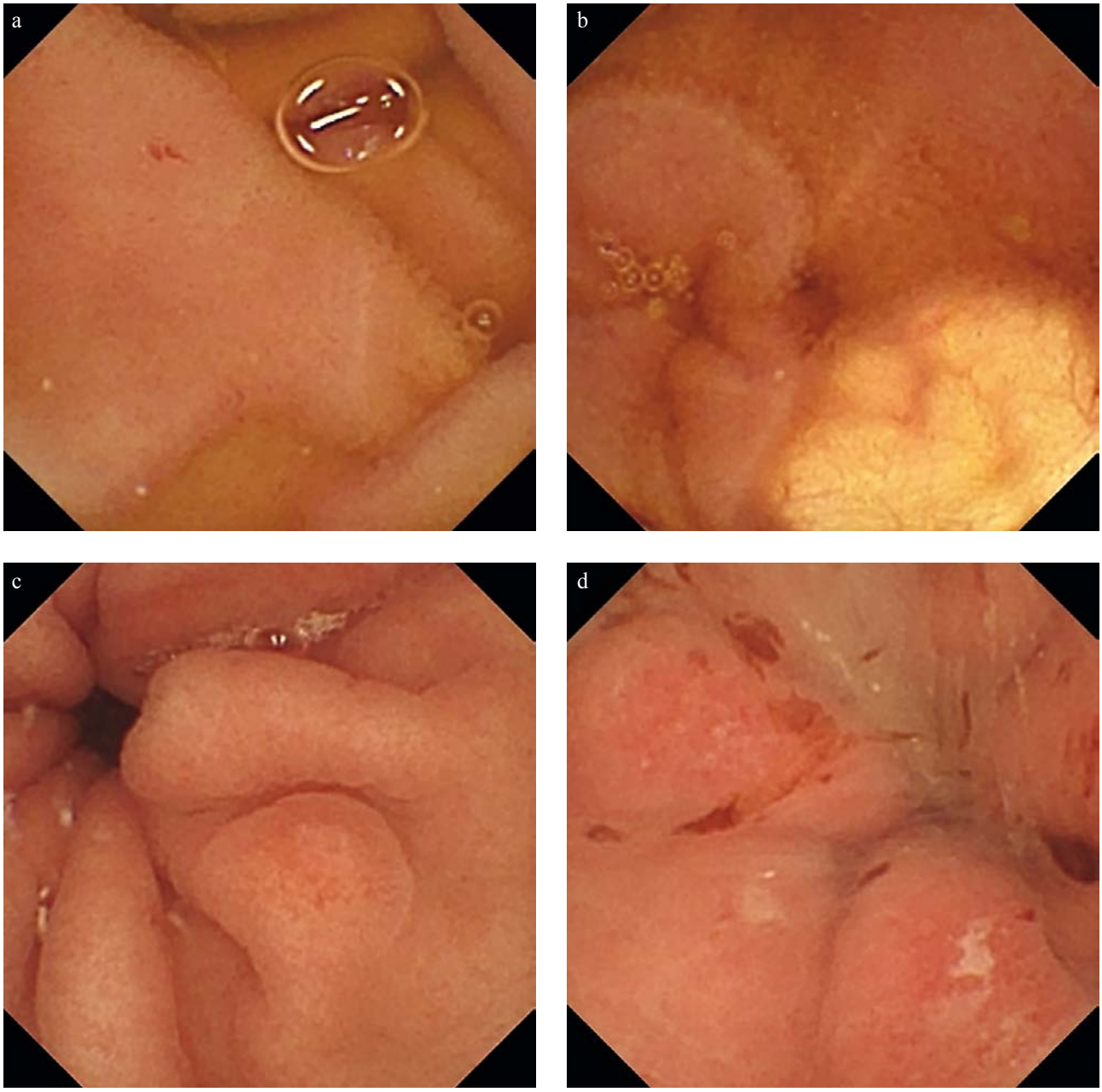

Fig. 1: CE findings: a) isolated red spot: jejunum; b) lipoma: jejunum; c) erosions: stomach; d) haemorrhagic erosions: stomach.

tom-free and had fully normal laboratory control by the end of the 36-month follow-up. Only 11 persons (26%) had fully normal finding at capsule endoscopy. The key and most important finding of our study is the high prevalence (43%) of so-called red spots in the small intestinal mucosa of healthy volunteers recognized by means of wireless capsule endoscopy.

Red spots of the small bowel were defined as demarcated, usually circular, 1–3 mm area of crimson mucosa with preservation of overlying villi and were described during the first enteroscopy procedures using sonde enteroscope (7). They were considered to be initial mucosal lesions that might progress into erosions or ulcers. This point of view has been repeatedly published without clear evidence of its basis. This terminology has been subsequently adopted by wireless capsule enteroscopy, but the definition of these lesions has been extended to any flat redness mucosa of a millimeter size, without any damage of the surface structure. Already early publications of capsule endoscopy warned against exaggerated interpretations of red spots as obvious

pathology (8). Nevertheless, red spots were believed to be a potential source of bleeding (as resembling endoscopic image of angiectasias) in some papers and therefore these studies were evaluated as positive for significant findings (9). Some authors also considered red spots as the "pre-aphthoid phase" of typical mucosal lesions in Crohn's disease (10). Graham et al. included red spots into the scoring system of non-steroidal anti-inflammatory drug-induced lesions (11), followed by Maiden et al. (12) and Caunedo-Álvarez et al. (13). These findings were repeatedly described in different control groups and/or placebo users of these studies in different rates (7–41%) (14, 11, 15). The explanation could be in overestimation of tiny mucosal lesions and terminological confusion that currently persists in the descriptions of capsule endoscopy. Wireless capsule endoscopy belongs to the most sensitive endoscopy methods with high diagnostic yield and the ability to identify diminutive, small bowel findings (optical system of currently used capsule endoscopes are theoretically able to identify objects less than 0.1 mm). Our results indicate, that true red spots (pinpoint red mucosal marks) are non-specific, clinically non-significant lesions, which can be (if isolated or infrequent) regarded as normal and should not be confused with diagnostic findings. It should be clearly distinguished of typical angiectasias, which are small (2 to 10 mm), flat, red lesions with a fern-like pattern of arborizing, ectatic vessels radiating from a central vessel (5).

Lesions identified by means of capsule endoscopy in our study can be divided from a clinical point of view into the two main groups: findings with a possible clinical potential and clinically non-significant objects. Angiectasias, bulbitis and erosions can be included into the first one. Small bowel angiectasias are the most common vascular anomalies found within the gastrointestinal tract especially in the older patients. Although it can cause obscure gastrointestinal bleeding, angiectasias are often diagnosed incidentally and remains clinically silent for many years; the indication for treatment is bleeding or iron deficiency anaemia. We identified small typical angiectasias in the jejunum and ileum of a 51-year-old man without any symptoms or laboratory signs of anaemia. Sporadic erosions were found in another 2 healthy volunteers. Erosions are non-specific mucosal reaction on different etiopathogenetic factors (Crohn's disease, infectious enteritis, drug-induced damage etc.). The aetiology of these isolated erosions in our study was probably non-inflammatory as the surrounding mucosa was normal and they were no laboratory markers of systemic inflammatory reaction. The first erosion was observed in a 50-year-old woman in the distal ileum. This area may be prone to mechanical and ischaemic injury due to motility and repetitive constriction of ileo-caecal region. In the second case haemorhagic erosions in the proximal duodenum (postbulbar region) were found in a 34-year-old woman. The possible aetiology is questionable; it could be inbalace of aggressive and protective mucosal mechanisms. Another clinically non-significant findings observed in our study were suspected, tiny (<3 mm) intestinal polyps (in distal ileum explained as focal nodular lymphoid hyperplasia), with no malignant potential and no risk of complications and therefore with no need for further investigation or treatment. The only one identified submucosal tumour has the appearance of lipoma (yellowish translucent, smooth, circumscribed). Flebectasias, lymphangiectasias and xanthomas are frequently diagnosed during capsule enteroscopy and they need no specific treatment. Extra-intestinal findings can be an important part of capsule endoscopy in asymptomatic persons, potentially significant with possible further complications. Although capsule endoscopy is dedicated for the small bowel investigation, visualized parts of the oesophagus, stomach and caecum should be evaluated and all extra-intestinal findings should be described, too (16). The most frequent findings were erythematous and erosive gastropathy in our study. Caecal polyp diagnosed by means of capsule endoscopy was removed during subsequent colonoscopy. Small gastric polyps were identified as suspected fundic gland polyps with no need of treatment.

Conclusions

Significant part of healthy subjects had abnormal findings at wireless capsule endoscopy. These findings had no clinical relevance, as all these persons remained fully asymptomatic during a 36-month follow-up. Such an endoscopic appearance would be previously evaluated as "pathological". This is a principal report alerting that all findings of any control group of wireless capsule endoscopic studies must be evaluated with caution.

Acknowledgements

This study was supported by research project IGA NT 13532-4/2012 from the Ministry of Health, Czech Republic.

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Received: 17/05/2016
Accepted: 09/06/2016

ORIGINAL ARTICLE

Risk Factors of Acute Pancreatitis in Oral Double Balloon Enteroscopy

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Summary: Double balloon enteroscopy (DBE) was introduced 15 years ago. The complications of diagnostic DBE are rare, acute pancreatitis is most redoubtable one (incidence about 0.3%). Hyperamylasemia after DBE seems to be a rather common condition respectively. The most probable cause seems to be a mechanical straining of the pancreas. We tried to identify patients in a higher risk of acute pancreatitis after DBE. We investigated several laboratory markers before and after DBE (serum cathepsin B, lactoferrin, E-selectin, SPINK 1, procalcitonin, S100 proteins, alfa-1-antitrypsin, hs-CRP, malondialdehyde, serum and urine amylase and serum lipase). Serum amylase and lipase rose significantly with the maximum 4 hours after DBE. Serum cathepsin and procalcitonin decreased significantly 4 hours after DBE compared to healthy controls and patients values before DBE. Either serum amylase or lipase 4 hours after DBE did not correlate with any markers before DBE. There was a trend for an association between the number of push-and-pull cycles and procalcitonin and urine amylase 4 hours after DBE; between procalcitonin and alfa-1-antitrypsin, cathepsin and hs-CRP; and between E-selectin and malondialdehyde 4 hours after DBE. We found no laboratory markers determinative in advance those patients in a higher risk of acute pancreatitis after DBE.

Keywords: *Acute pancreatitis; Deep enteroscopy; Device assisted endoscopy; Double balloon enteroscopy; Hyperamylasemia; Small intestinal disorders*

Introduction

Double balloon endoscopy (DBE) is a method of enteroscopy that was introduced in 2001 (1–3).

The system consists of enteroscope and over-tube; both have a soft balloon at their tips. Both balloons can be alternately inflated and deflated by an air balloon-pump controller. DBE is based on a new insertion technique in which these two balloons are operated in combination, and the endoscope is inserted while simultaneously shortening the intestine.

Acute pancreatitis is the most feared complication in oral DBE. Despite a 15-year experience, the causal reason of acute pancreatitis remains uncertain. There are many hypotheses explaining this fact: direct trauma of the pancreas caused by the pressure of an endoscope against the vertebral column, the disorders in microcirculation during the procedure, increase in intraluminal duodenal pressure during enteroscopy caused by inflation of the two balloons, reflux of duodenal fluids into the pancreatic duct, timing of the procedure and others. We published a prospective study concerning this risk (Kopáčová et al. Gastrointest Endosc 2007; 66(6): 1133–1138) (4). In our contemporary project we continue in priority investigation of known or supposed protective and risk factors (rehydration, oral DBE, time of procedure, number of cycles, the depth of intubation, CO_2 insufflation) in correlation with serum and urine amylase, lipase and hs-CRP and some possible plasmatic markers of a higher risk of acute pancreatitis (malondialdehyde, procalcitonin, S 100 proteins, cathepsin B, pancreatic secretory trypsin inhibitor (PSTI; also known as serine protease inhibitor Kazal-type 1 (SPINK 1) or tumour-associated trypsin inhibitor (TATI), lactoferrin, E-selectin and alfa-1 antitrypsin (A1AT). Our project assumes the outcome of possibility to identify high risk patients for DBE-associated acute pancreatitis.

As DBE is a lengthy procedure, a large volume of air is usually insufflated leading to significant distension of the small bowel. Indeed, one of the main technical challenges of DBE is the formation of distended bowel loops and acute angulations with increasing amounts of gas intra-luminally. Carbon dioxide (CO_2), unlike air, is rapidly absorbed from the bowel. Bowel insufflation with CO_2, instead of air, enhances patients comfort and decreases the need for sedation

ACTA MEDICA (Hradec Králové) 2016; 59(3):84–90
http://dx.doi.org/10.14712/18059694.2016.95

(5–7). We have used CO_2 insufflation in DBE procedures regularly since 2007; since we had no complications with hyperinflation, the comfort of the patient rapidly increased and this type of insufflation is helpful for easier and deeper insertion of the scope, because the absorption of CO_2 is 150-times faster than absorption of air in the bowel. A randomised, double-blind trial showed that insufflation with CO_2 is safe, reduces patient discomfort, and significantly improves intubation depth (8).

A combination of water with simethicone is used routinely to do away with bubbles in the intestine. During withdrawal of the endoscope and during therapeutic interventions, spasmolytics might improve visualisation of the small-bowel mucosa by reducing motility of the small bowel (5, 6).

A venous access is obtained before the procedure. All patients are monitored during the procedure; oxygen saturation, heart rate, and blood pressure are monitored. Intravenous crystaloids are administered during DBE. Conscious sedation is thought to be sufficient for DBE (5, 6). It seems to be much better in DBE in comparison with general anaesthesia according to our experience. Abdominal pain of the patient is a very important warning signal, and it is necessary to terminate the procedure immediately in that case. Intense pain may be a sign of inadequate pressure on the pancreas and poses a high risk of post-DBE pancreatitis (4, 7, 9, 10). We use small intravenous repetitive doses of midazolam and pentazocine for conscious sedation (batch-wise).

The duration of the procedure and the discomfort for the patient caused by oral passage of the over-tube require deep analgo-sedation. The cost of the procedure is high (the over-tube and balloons are designed for single use). The procedure requires an experienced endoscopist and fluoroscopy, especially at the beginning, during a learning period (11).

Material and Methods

A total of 117 DBEs in 94 patients were scheduled for our recent study yet (50 men and 44 women, mean age 52 years) under deep conscious sedation (midazolam and pentazocine). The mean time of DBE was 80 min. (range 30–180 min.), the mean number of push-and-pull cycles was 13 (range 1–45). Thirty healthy volunteers without DBE (9 men and 21 women, mean age 41 years) created a control group.

The DBE investigations were performed standardly by an oral approach in our in-patients. The choice of the endoscope (therapeutic or diagnostic) was based on the indication. Over-tube was used in all cases. The conscious sedation was used (midazolam, pentazocine). Crystaloids were given intravenously during the DBE (depending on the time of the procedure 500–1,000 mL F1/1; 500 mL per hour).

Blood and urine samples were collected before the DBE procedure and 4 and 24 hours after the DBE. Besides standard routine investigations (amylases, lipase, hs-CRP), several laboratory markers were also investigated, their list is provided bellow. Abdominal pain was evaluated using a three-step scale (no pain, moderate and significant pain).

Hs-CRP was quantified using imunoturbidimetry on Roche/Hitachi MODULAR P (Roche, Germany), detection range 0–5 mg/L.

Malondialdehyde was measured using photometry – spectrophotometer Secomam S.500P (TrigonPlus, Czech Republic), detection range 0.26–1.07 μmol/L.

Procalcitonin was assessed by means of enzyme-linked immunosorbent assay kit (ELISA, USCN Life Science Inc., USA), detection range 31.2–2,000 pg/mL.

S 100 proteins were quantified using electrochemiluminiscence (ECLIA) – sandwich reaction on automatic immunoanalyzer Elecsys 2010 (Roche, Germany), detection range < 0.105 μg/L.

Cathepsin B was measured using enzyme-linked immunosorbent assay kit (ELISA, USCN Life Science Inc., USA), detection range 0.312–20 ng/mL.

Serine peptidase inhibitor Kazal type 1 (SPINK 1) was quantified using enzyme-linked immunosorbent assay kit (ELISA, USCN Life Science Inc., USA)**,** detection range 1.56 – 100 ng/mL.

Lactoferrin was analyzed by means of enzyme-linked immunosorbent assay kit (ELISA, USCN Life Science Inc., USA), detection range 0.312–20 ng/mL.

E-selectin was measured using enzyme-linked immunosorbent assay kit (ELISA, USCN Life Science Inc., USA), detection range 39–2,500 pg/mL.

Alfa-1-antitrypsine (A1AT) using immunoturbidimetry on Roche/Hitachi MODULAR P (Roche, Germany), detection range 0.9–2.0 g/L.

Laboratory markers were also investigated in a control group of clinically healthy volunteers without DBE.

Study approval and confidentiality of data obtained

The project received a full approval from the local Ethics Committee (joint committee of the University Teaching Hospital and Faculty of Medicine at Hradec Králové). For all data obtained, all personal identification information was deleted in compliance with the laws for the protection of confidentiality of the Czech Republic.

Results

Data was processed by means of statistical software for these analyses (SigmaStat; Jandel Corp., Erkrath, Germany), using descriptive statistics, paired t-test, Mann-Whitney rank sum test and Pearson product moment correlation.

We investigated supposed protective and risk factors (rehydration, oral DBE, time of procedure, number of cycles, the depth of intubation, CO_2 insufflation) in correlation with serum and urine amylase, lipase and hs-CRP and some possible plasmatic markers of a higher risk of acute pancreatitis

(malondialdehyde, procalcitonin, S 100 proteins, cathepsin B, pancreatic secretory trypsin inhibitor (PSTI; also known as serine protease inhibitor Kazal-type 1 (SPINK 1) or tumour-associated trypsin inhibitor (TATI), lactoferrin, E-selectin and alfa-1 antitrypsin (A1AT) in 117 DBEs (3 times – basal, after DBE in 4 and 24 hours) and 30 volunteers (only basal).

The age range of volunteers and patients were slightly different; mean 44 years (range 28–64 years) in healthy volunteers and 52 years (18–84) in patients. We did not find any correlations of followed parameters with age, so we find this difference insignificant.

We find only correlation of age and pain after DBE. The patients without pain (n = 89) were significantly older than patients with moderate (n = 22; p = 0.038) or significant (n = 6; p = 0.020) pain. Duration of procedure was significantly shorter in patients without pain (n = 89) than in patients with moderate (n = 22; p = 0.041) or significant pain (n = 6; p = 0.007).

We have not recorded any DBE-associated acute pancreatitis in this series of 117 DBEs.

In healthy controls, serum amylase correlated with serum lipase (r = 0.514; p = 0.004), alfa-1-antitrypsin correlated with hs-CRP (r = 0.503; p = 0.005), serum cathepsin correlated significantly with E-selectin (r = 0.467; p = 0.009) and with serum lipase (r = 0.495; p = 0.005).

In our 117 patients serum amylase and lipase rose significantly with the maximum 4 hours after DBE ($p < 0.001$ and $p < 0.001$) while in hs-CRP we found maximum in 24 hours after DBE ($p < 0.001$) – see Figures 1–4. There was a significant correlation between significant pain and serum amylase ($p < 0.039$) and lipase ($p < 0.005$).

Serum cathepsin and procalcitonin decreased significantly 4 hours after DBE compared to healthy controls and patients' values before DBE (p = 0.018 and p = 0.031); see Table 1 for details. There was a trend for an association between number of push-and-pull cycles and procalcitonin (r = −0.384; p = 0.011) and urine amylase (r = 0.313; p = 0.043) 4 hours after DBE; between procalcitonin and alfa-1-antitrypsin (r = 0.358; p = 0.021), cathepsin (r = 0.362; p = 0.020) and hs-CRP (r = 0.358; p = 0.021); and between E-selectin and malondialdehyde (r = 0.364; p = 0.019) 4 hours after DBE. Either serum amylase or lipase 4 hours after DBE did not correlate with any markers before DBE.

We did not identify any marker to recognise high risk patients for DBE-associated acute pancreatitis. As the main risk factor was identified time of procedure (number of push and pull cycles) and the pain during procedure.

Discussion

In general, acute pancreatitis is a very heterogenous group of different aetiology and pathogenesis. Several predisposing factors, including genetic ones, were identified. The aim of our current project was to investigate several laboratory markers to identify patients in a higher risk of DBE-associated acute pancreatitis. Some interesting findings were revealed but no clear "high-risk" factor was identified. Procalcitonin surprisingly decreased after DBE. This decline was consistent and statistically significant. Explanation for this phenomenon is difficult. Procalcitonin, a propeptide of calcitonin, is an acute phase reactant that has been investigated extensively as an early marker of severe acute pancreatitis and/or its infective complications (12). This significant fall of procalcitonin in our study might be at least partly explained by distribution changes due to "preventive" saline infusion during DBE to secure proper

Tab. 1: Laboratory investigations in healthy control volunteers and in patients before and after double balloon enteroscopy.

Parameter (Mean ± StdDev)	**Controls**	**DBE-0h**	**DBE-4h**	**DBE-24h**
Cathepsin B (pg/L)	14.9 ± 20.0	10.1 ± 14.9	7.2 ± 12.9	9.1 ± 14.8
Lactoferrin (pg/L)	495 ± 413	725 ± 553	708 ± 711	840 ± 824
E-selectin (μg /L)	27433 ± 15861	26865 ± 18721	23274 ± 16465	25973 ± 17074
SPINK 1 (pg/L)	46.7 ± 21.6	43.6 ± 43.7	43.6 ± 52.6	37.7 ± 34.9
Procalcitonin (μg /L)	71.7 ± 19.4	121.6 ± 118.6	67.6 ± 54.9	92.3 ± 81.9
S100 (μg/L)	0.05 ± 0.02	0.05 ± 0.04	0.07 ± 0.08	0.05 ± 0.03
Alfa-1-antitrypsin (g/L)	1.37 ± 0.28	1.63 ± 0.43	1.63 ± 0.43	1.52 ± 0.42
hs-CRP (mg/L)	1.16 ± 1.69	3.02 ± 8.90	8.13 ± 14.97	6.15 ± 11.50
Malondialdehyde (μmol/L)	0.27 ± 0.19	0.34 ± 0.28	0.26 ± 0.17	0.28 ± 0.21
Serum amylase (μkat/L)	1.02 ± 0.33	0.99 ± 0.37	3.65 ± 2.96	1.80 ± 1.29
Urine amylase (μkat/L)	0.83 ± 0.87	1.77 ± 1.55	7.41 ± 10.64	4.03 ± 3.75
Serum lipase (μkat/L)	0.67 ± 0.23	0.72 ± 0.55	4.40 ± 5.86	1.01 ± 1.13

Notes: DBE-0h: investigation before double balloon enteroscopy. DBE-4h: investigation 4 hours after double balloon enteroscopy completed. DBE-24h: investigation 24 hours after double balloon enteroscopy completed.

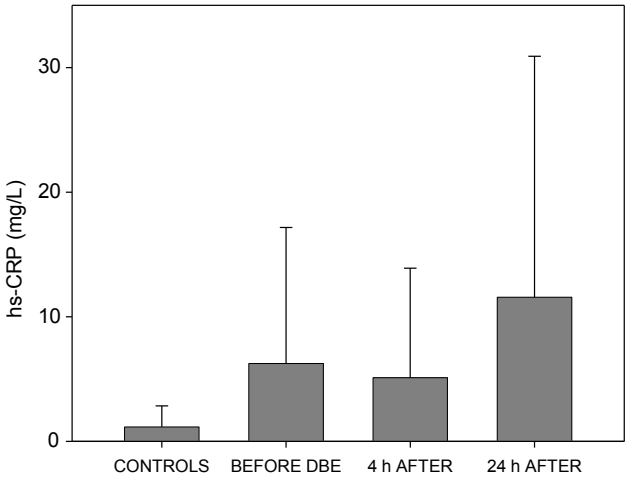

Fig. 1: Serum hs-CRP (mean + standard deviation) in healthy control subjects and in patients before double balloon enteroscopy (DBE-0h) and 4 hours and 24 hours after double balloon enteroscopy was completed (DBE-4h; DBE-24h).

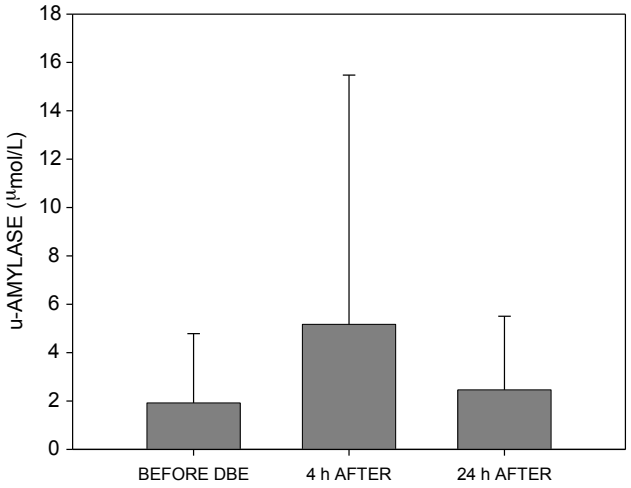

Fig. 2: Serum amylase (mean + standard deviation) in patients before double balloon enteroscopy (DBE-0h) and 4 hours and 24 hours after double balloon enteroscopy was completed (DBE-4h; DBE-24h).

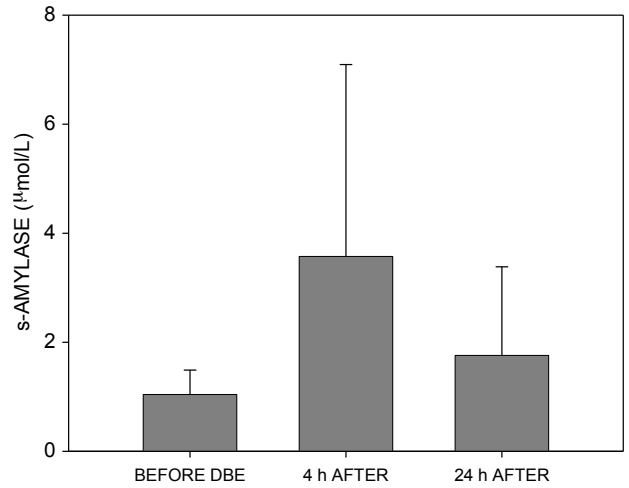

Fig. 3: Urine amylase (mean + standard deviation) in patients before double balloon enteroscopy (DBE-0h) and 4 hours and 24 hours after double balloon enteroscopy was completed (DBE-4h; DBE-24h).

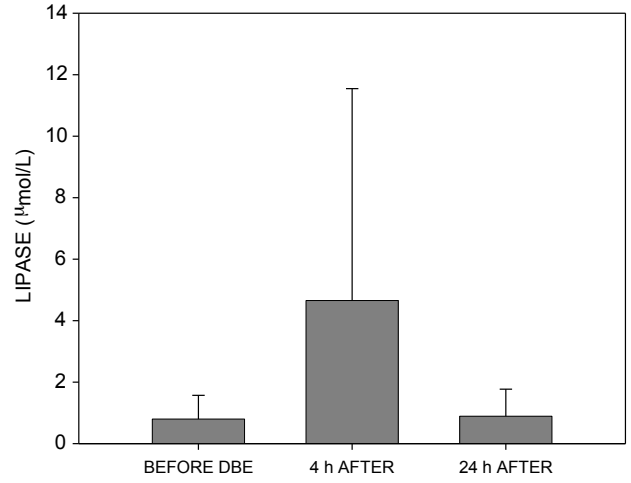

Fig. 4: Serum lipase (mean + standard deviation) in patients before double balloon enteroscopy (DBE-0h) and 4 hours and 24 hours after double balloon enteroscopy was completed (DBE-4h; DBE-24h).

microcirculation in the pancreas. Our results suggest that DBE does not stimulate proinflammatory cascade.

DBE has been reported as a safe endoscopic technique (13), the number of severe complications being mentioned ranging from 0 to 1.4% (14–16). However, abdominal pain lasting 1–2 days occurred in 9% of patients in one study (17) or even in 20% according to the another one (14). Abdominal discomfort slaking within 72 hours was reported in 50% of patients after a DBE procedure (18).

In some series on DBE, no complications during or after DBE were reported (19–27). But reading these articles carefully, one will find in Pata et al. (25) 13% oral bleeding, 2% broken tooth and 2% respiratory depression due to aspiration. Moreover, some patients in the initial setting of 36 patients developed pancreatic-type abdominal pain and under prospective following 6 of next 48 patients (12.5%) developed acute pancreatitis and another 6 hyperamylasemia (25). Similarly in a large multicentre study of Domagk et al. (26) no adverse events are declared in the abstract, but mean pain one hour after examination on the 100 mm visual scale was 12.2 and mean pain after 24 hours was 2.4 (26). Are there really difference in complication rate be-

tween particular endoscopy units or the difference is only in the investigation of the patient after endoscopy and his/her follow up?

Major (severe) complications such as pancreatitis, bleeding and perforation have been reported in approximately 1% of all diagnostic DBE whereas the complication rate for therapeutic procedures is about 5% (28).

Minor complications are sore throat, oedema of the uvula, and abdominal discomfort. Some casuistic complications were described in the literature: balloon dislocation, segmental enteritis after argon plasma coagulation (29), intestinal necrosis after epinephrine injection (30), paralytic ileus (31) etc. The complication rate of diagnostic procedures is low (0.4–0.8%) according to the literature (32–35). The overall complication rate of therapeutic DBE is about 3–4%. However, difficult therapeutic endoscopic procedures (e.g. resection of large polyps) may increase the risk up to 10% (5, 6, 32–34). The perforation rate is significantly elevated in patients with postsurgical anatomy undergoing diagnostic retrograde DBE examinations (36).

The overall complication rate is reported in about 1.7% of patients in an international multicentre survey in 2,362 DBE procedures. The complications were rated minor in 0.9%, moderate in 0.3% and severe in 0.6% of procedures. The complication rate is significantly higher in therapeutic procedures in comparison with diagnostic ones (4.3% versus 0.8%). An exception to this rule is acute pancreatitis, the most common complication in diagnostic DBE procedures. Acute pancreatitis was reported in 0.3% of DBEs (32, 37).

A report from the National German DBE Register showed an overall complication rate of 1.2% in a large series of 3,894 DBE procedures. The incidence of acute pancreatitis was also 0.3% in this report (33, 34).

A publication by May et al. (29) evaluated acute complication rate of DBE in 353 patients. Only therapeutic procedures are evaluated with a complication rate of 3.4%. No acute pancreatitis was reported (29).

On the other hand, a recent prospective study was published by Zepeda-Gómez et al. with incidence of acute pancreatitis of 3% (38). According to Pata et al. pancreatitis after DBE was observed in 12.5% in their series (39).

In general, DBE is associated with a higher complication rate compared with standard endoscopic procedures (36).

A complication of endoscopy is defined as any event that negatively changes the health status of the patient, and that occurs during the 30-day period after the investigation. Complications are usually categorised as minor when requiring up to 3 days of hospitalisation, moderate when requiring 3–10 days and major or severe when requiring more than 10 days of hospitalisation, and/or an endoscopic, radiological or surgical intervention, and/or contribute to the death of the patient (32, 40). Procedure-related mortality is defined as mortality within 30 days of DBE (32).

It is possible categorized into three groups: 1) those common to other endoscopic techniques (perforation, bleeding), 2) related to sedation administered during the procedure (respiratory depression, aspiration, pneumonia) and 3) complications specifically associated with DBE (acute pancreatitis) (5, 6, 28). The complication rate is about 9% for minor complications and less than 1% for major ones (28). The most discussed complication is acute pancreatitis after DBE (35, 37). In diagnostic procedures via the anterograde approach, acute pancreatitis is the most common and most severe complication (5, 6). The very first post-DBE acute pancreatitis was reported by Honda et al. in 2006 (41).

The causal mechanism of post-DBE acute pancreatitis is uncertain; there are several theories in the literature: direct trauma of the pancreas caused by pressure of the endoscope against the vertebral column in the oral procedure, the disorders in microcirculation during the procedure, increase in intraluminal duodenal pressure during the endoscopic procedure caused by inflation of the two balloons, reflux of duodenal fluids into the pancreatic duct leading to acute pancreatitis. No one of these hypotheses brought the total explanation of the pathogenesis (9, 10, 28). Whereas the increase of amylase and lipase levels after DBE occurs in the significant percentage of patients (30 – 50%) when systematically measured (25, 38, 42).), the rate of post-DBE acute pancreatitis is much lower, about 0.2–0.5% (25, 42). Hyperamylasemia and hyperlipasemia are common conditions after DBE. Asymptomatic hyperamylasemia may occur in nearly half of DBE procedures (4, 9, 10, 41, 43–45). The incidence of hyperamylasemia and pancreatitis after single-balloon enteroscopy or spiral enteroscopy seems to be comparable to that after DBE (45–47).

Hyperamylasemia and hyperlipasemia after DBE are usually asymptomatic and do not present the immediate risk factor of acute pancreatitis (9, 10). The only identified factors increasing risk of post-procedure acute pancreatitis are duration of the procedure (i.e. number of push-pull cycles) and interval time between the first and the second inflation of balloons according to some authors (9, 10, 39). Learning curve seems to be another risk factor; about 50 procedures are needed to acquire enough experience (28). We had four cases of acute pancreatitis among our first 267 oral DBE procedures; it was number 24, 50, 57 and 256 in chronological order. Classification system for acute pancreatitis was discussed in our previous paper (10).

Our centre has long-term experience (since 1994) with both push-enteroscopy (48) and intra-operative enteroscopy (10, 49–51). We have never registered acute pancreatitis as a complication of either push-enteroscopy or intra-operative enteroscopy in our setting. However, acute pancreatitis as such a complication of push-enteroscopy, caused by an overtube, was described previously by other authors (52). Acute pancreatitis was even described after uneventful upper and lower gastrointestinal endoscopy (53–55). Blackwood et al detected asymptomatic hyperamylasuria in 6.6% of patients undergoing gastrointestinal endoscopy (56).

Pelletier et al. (57) studied the prevalence of hyperamylasemia 2 hours and 24 hours after upper gastrointestinal endoscopy in 50 consecutive patients. In the 2-hour sample,

hyperamylasemia was observed in nine patients (18%), in the 24-hour sample in five patients. Pelletier et al. conclude that the cause of hyperamylasemia may be due to hypersalivation during the procedure (57). In our opinion, hypersalivation cannot affect the serum amylase level in such a way (most of the saliva runs out of the mouth during endoscopy and is not swallowed). Furthermore, it cannot affect abdominal pain or pancreatic lipase elevation (9).

There have been about 100 published cases of post-DBE acute pancreatitis on PubMed so far (10, 14, 32–37, 39, 41, 44, 46, 58–62, 64).

In agreement with the Dutch study, we believe that post-DBE pancreatitis is underestimated in retrospective studies on an outpatient basis. It is hard to say how many patients with self-limited abdominal post-DBE pain had mild acute pancreatitis because of inadequate follow-up. Especially retrospective questionnaire-based surveys might be at risk from an inaccurate report or inclusion bias (59). As the distinction between clinically mild pancreatitis and hyperamylasemia with transient abdominal discomfort is somehow arbitrary, it seems likely that the underdiagnosis of post-DBE pancreatitis might have occurred, especially in out-patients. In our opinion, mechanical stress on the pancreas seems to be presumable. Traumatic injury of the pancreas seems to be the main cause of acute pancreatitis (14, 41, 44, 59, 64).

Another important point is prevention of post-DBE pancreatitis. We use parenteral hydration during the oral procedure and after it. The usual dose is 1 litre of saline solution during a 2-hour procedure. We presume that hydration could improve blood supply to the splanchnic region, and secure pancreatic microcirculation and post-procedure recovery. The use of proteinase inhibitors such as gabexate mesylate in the prevention of post-endoscopic pancreatitis has been disappointing (65, 66). There are some studies of intravenous nitroglycerine (67), ulinastatin (68, 69), somatostatin (65, 66, 70, 71), rectal diclofenac (68), and other drugs in prevention of post-procedure pancreatitis, but the results are not significant.

Conclusions

Our current results support our previous hypothesis that endoscope-induced mechanical straining during DBE is the most important factor responsible for the increase of amylase and lipase or even for progression to acute pancreatitis. We found no laboratory markers that would identify in advance those patients in a higher risk.

Acute pancreatitis is a feared complication of oral DBE (100 cases of acute pancreatitis have been described in the literature so far).

Acute pancreatitis is the most common severe complication seen after diagnostic oral DBE (complications of therapy itself prevail in therapeutic procedures).

Hyperamylasemia and elevation of pancreatic lipase after DBE seems to be a common condition. Association with acute pancreatitis is supposed to be possible, but not obligatory.

The complication rate of acute pancreatitis is reported at about 0.3% of DBEs according to large studies, almost solely after the oral DBE. Drawbacks and possible bias of those studies are that they are mostly retrospective, a substantial part of DBEs were performed on an outpatient basis and the follow-up of these patients was inadequate.

In all patients with abdominal pain during the procedure and/or after the oral DBE, diagnosis of acute pancreatitis should be considered and treatment should be provided in good time, identically as in post-ERCP pancreatitis.

Conscious sedation seems to be more favourable in comparison with general anaesthesia due to monitoring of the patient's pain during the procedure.

Intense pain during the procedure may be a sign of inadequate pressure on the pancreas and pose a high risk of post-DBE pancreatitis.

CO_2 insufflation during DBE is highly recommended as it prevents over-inflation of the small bowel, however, possible preventive relationship to post-DBE pancreatitis has not been determined yet.

Acknowledgements

This study was supported by research project IGA NT 13414-4/2012 from Ministry of Health, Czech Republic.

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Received: 11/05/2016
Accepted: 15/06/2016

ORIGINAL ARTICLE

Effect of Riboflavin/UVA Collagen Cross-linking on Central Cornea, Limbus and Intraocular Pressure. Experimental Study in Rabbit Eyes

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Summary: The Purpose of present study was to investigate the effect of riboflavin/ultraviolet-A-induced collagen cross-linking (CXL) on central cornea, limbus and intraocular pressure (IOP). This was an animal experimental study. The right corneas of 10 rabbits were ultraviolet-A irradiated (3 mW/cm^2 for 30 minutes) after de-epithelialization and instillation of 0.1% riboflavin / 20% Dextran drops. Left corneas served as controls. Samples were examined histologically one month postoperatively. Before and after treatment, IOP measurements were recorded bilaterally. At central cornea of eyes underwent CXL keratocyte repopulation, normal arrangement of collagen fibres and a statistically significant change in fibres diameter were detected, compared to controls. At limbus area, there were not any significant histological differences after CXL. There was no statistically significant difference between pre- and postoperative IOP in all eyes.
Results indicate no pathological effects on central cornea and no significant changes in limbus and IOP after CXL in rabbit eyes, so this method might be a safe therapeutical option. Further clinical investigations may be needed.

Keywords: *Corneal Crosslinking; Intraocular pressure; Limbus; Cornea*

Introduction

It is known that the biomechanical properties of the cornea depend on the characteristics of the collagen fibers (1–3). The surgical technique of corneal collagen cross-linking (CXL), using ultraviolet-A (UVA) irradiation and riboflavin, affects the hydration of the cornea, the apoptosis of the keratocytes and the diameter of the collagen fibers. The CXL-associated biomechanical and enzymatic changes make the cornea stiffer and more compact after treatment (4–8). It should, also, be noted that both the instillation of riboflavin and the irradiation are applied up to the area of limbus. Among the possible additional local impact of CXL treatment are the changes in the structure of the trabecular meshwork and/or of the Schlemm's canal. The latter could be associated with a reduction of the aqueous humor outflow facility by acting in a way similar to Argon LASER Trabeculoplasty (ALT).

Based on the aforementioned hypothesis and evidence, we report our experience with experiments in rabbit eyes. Primary objective of the study was to assess potential morphological changes in the limbal area induced by CXL, which could affect the aqueous humor outflow and the IOP values. To our knowledge, no published data are available, regarding the impact of CXL on the limbus area and the trabeculum.

Materials and Methods

This experimental study was conducted at the Laboratory of Experimental Surgery and Surgical Research of Democritus University of Thrace, Greece, following the institutional and national guide for the care and use of laboratory animals (Trial number: EL71 BIO1), as well as principles of animal maintenance described by the Association for Research in Vision and Ophthalmology.

Ten healthy male New Zealand white albino rabbits [mean age of 5 months and mean weight of 3.6 ± 0.3 (Mean ± SD) Kg] were used for this single-center and single-surgeon experimental study. The corneal epithelium of the right eyes was mechanically removed and a 0.1% riboflavin in 20% Dextran 500 solution was instilled to the cornea for

http://dx.doi.org/10.14712/18059694.2016.96

30 minutes, until the stroma was completely penetrated and aqueous was stained yellow. Then, the corneas were UVA-irradiated for 30 minutes (wavelength of 365 nm, irradiance of 3 mW/cm^2) (UV-X^{TM} Illumination System Version 1000, IROC AG, Zurich, Switzerland). Instillation of riboflavin drops was continued during irradiation, in order to sustain the necessary concentration of the riboflavin. Moreover, balanced salt solution (BSS) was applied every 6 minutes to moisten the cornea. The left fellow eyes served as intra-individual controls, remaining completely untreated (4 eyes), with epithelial debridement alone (3 eyes) or with epithelial debridement plus the application of the 0.1% riboflavin / 20% Dextran solution (3 eyes). This was performed to exclude an influence of the epithelial abrasion or associated hydration changes in the examined tissue and especially in the trabeculum. Before all of the procedures, the rabbits were anaesthetized with subcutaneous injection of a mixture of ketamine hydrochloride (50 mg/Kg) and xylazine hydrochloride (5 mg/Kg). After CXL treatment, drops of ofloxacin, fluorometholone and diclofenac nitrate were applied on both eyes of the rabbits, until complete re-epithelization of the cornea was detected by slit-lamp biomicroscopy. Before and one month after treatment, in all animals, IOP measurements were recorded bilaterally, using the Tonopen-XL (Reichert Inc., NY, USA), after topical anaesthesia (tetracaine drops), since pharmaceutical substances that are commonly used in general anaesthesia cause immediate and significant reduction of IOP (9).

The animals were sacrificed one month after completion of the experiments and both eyes were enucleated. After fixation, the corneas were excised along with a 2 mm scleral rim, in order to maintain normal hydration conditions and to minimise oedema and striae, and bisected. The first section of every bisected cornea was prepared for scanning electron microscopy (SEM) and the second for histologic examination (light microscopy) by trichrom staining (CytoLogix Masson Trichrome Stain Kit, Cambridge, MA). The study was observer masked.

Results

Light microscopy (Figures 1–3) showed that there was complete re-epithelialization of all corneas that underwent epithelial debridement. The CXL effect on treated corneas was established by the detection of the changes in both number and size of gaps between collagen bundles, particularly in their central area, where the treatment was focused. Limbus of the CXL treated corneas seemed to have nor-

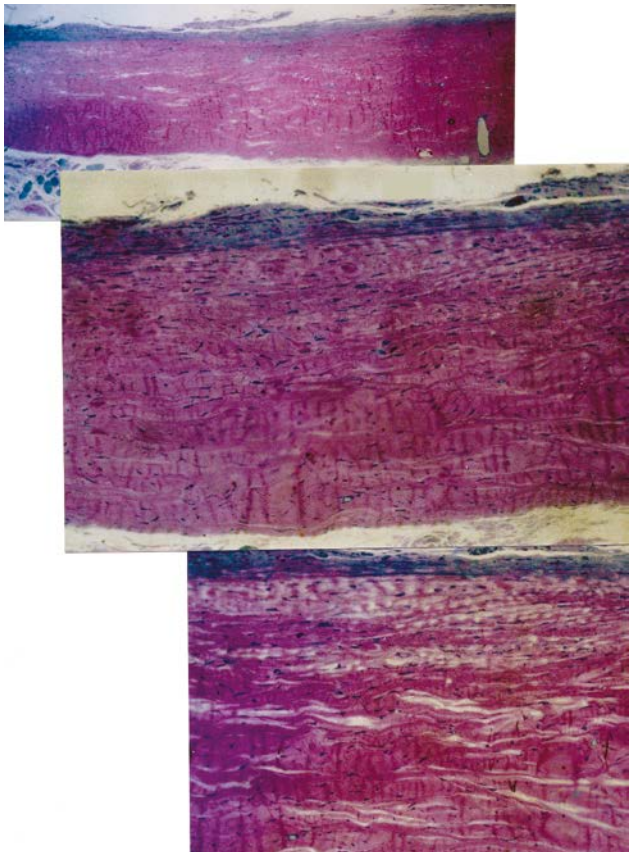

Fig. 1: Light microscope – Untreated cornea (control).

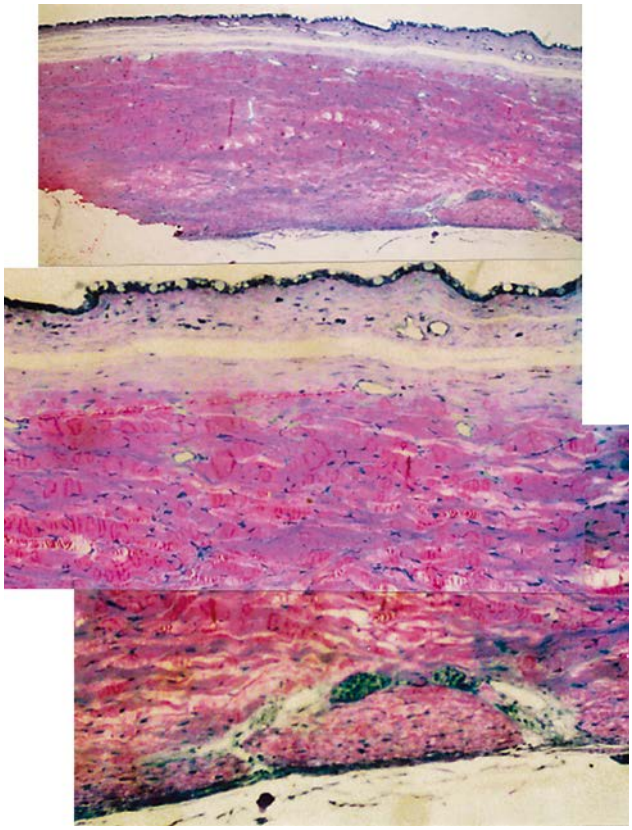

Fig. 2: Light microscope – Cornea underwent de-epithelialization and riboflavin solution instillation (control).

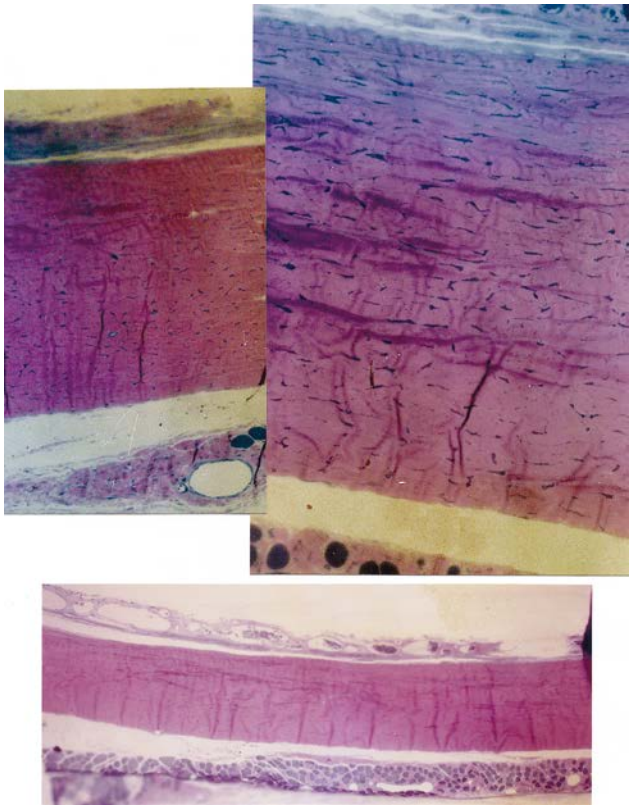

Fig. 3: Light microscope – Cornea underwent CXL.

mal appearance. Furthermore, no observable pathological changes, presence of inflammation or angiogenesis could be detected on the periphery of the corneas, including controls.

Scanning electron microscopy (Figures 4–9) showed that in all specimens that underwent debridement, a complete recovery of the corneal epithelium with a relative degree of diversity was observed. Also, in places, there was a rupture of intercellular bridges to some extent. All corneas that served as controls demonstrated a normal collagen fiber pattern with an unremarkable keratocyte appearance. On the other hand, the corneas that were treated with the CXL technique, demonstrated keratocyte repopulation of the anterior stroma, but with some acellular areas in between, as well as keratocyte apoptotic changes, such as the formation of apoptotic bodies, chromatin condensation and cell nuclei shrinkage, particularly in the periphery of the irradiated area. The structure and the arrangement of the collagen fibers seemed to be normal, with an exception of some areas of the anterior stroma, where the collagen fibers were not very well-organized in bundles. On the contrary, deeper in the stroma of the CXL treated corneas, the collagen fiber pattern was similar to that observed in controls. The distances between the collagen fibers could not be precisely evaluated due to the dehydration of the tissue before observation. However, even after dehydration, in the anterior area of central corneas, where the application of CXL treatment was focused, the changes in spaces between fibers and bundles due to therapy were evident to some extent, with these distances to seem relatively shorter compared to the rest of the cornea. In addition, there were not any significant differences at the limbus area between CXL treated eyes and controls, regarding both collagen fibers morphology and distances between collagen bundles. Finally, endothelial cells and their intracellular connections had a normal appearance in all tissue samples.

The electron microscope digital images from central cornea and limbus area of all samples were transferred to a computer screen. The collagen fiber diameters were marked manually and calculated with the help of a special semiautomatic software program (Image tool). In each image, the

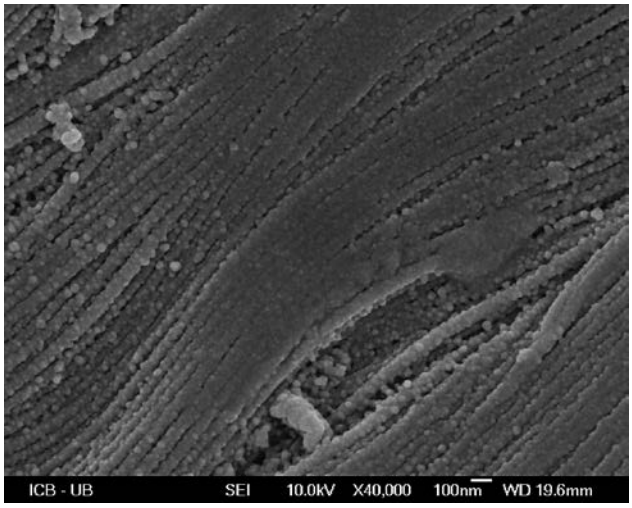

Fig. 4: SEM – Untreated cornea (control): Collagen fibers are organized in bundles. Some collagen fibers are collapsed due to fixation (arrows).

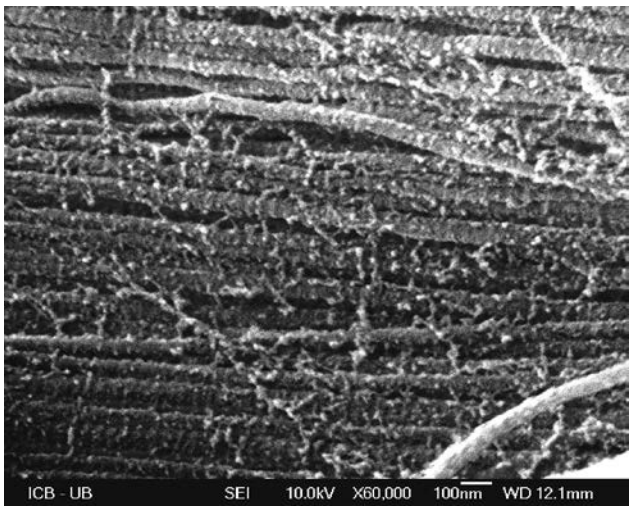

Fig. 5: SEM – Cornea underwent de-epithelialization (control): Collagen fibers with small fibrils of proteoglycans.

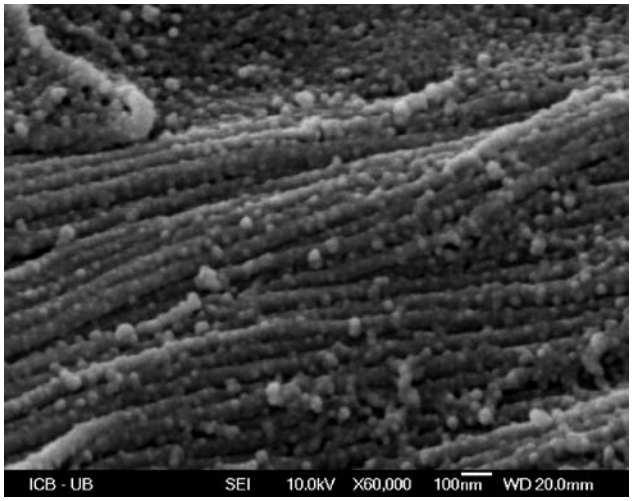

Fig. 6: SEM – Cornea underwent de-epithelialization and riboflavin solution instillation (control): Collagen fibers and granular substance (arrow).

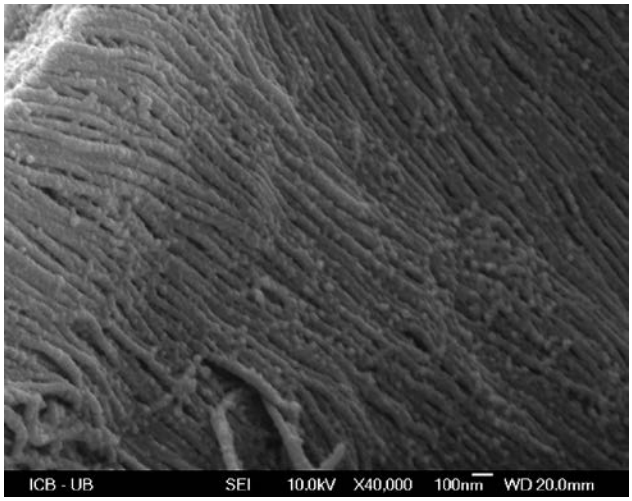

Fig. 8: SEM – Cornea underwent CXL: Sample from anterior stroma close to limbus. At some points collagen fibers are not yet well-organized in bundles and there are rests of granular extracellular substance (arrow).

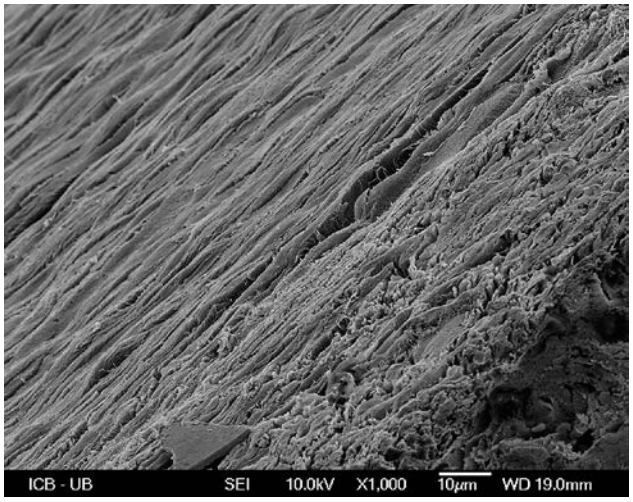

Fig. 7: SEM – Cornea underwent CXL: Sample from anterior stroma. Right arrow shows the epithelium and left arrow shows the stroma.

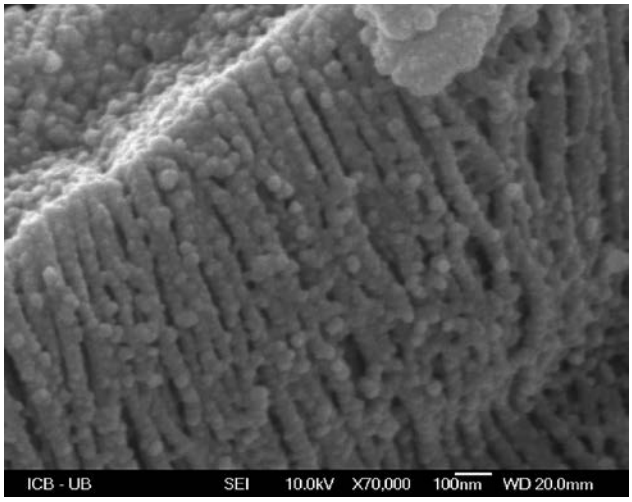

Fig. 9: SEM – Cornea underwent CXL: Sample from limbus, horizontal and perpendicular section over collagen fibers (arrows).

diameters of 20 contiguous fiber section profiles were measured. The change in thickness and morphology of collagen fibers was estimated, comparing the corneas underwent CXL treatment with controls (Table 1). Mean (± SD) preoperative and postoperative IOP measurements of all eyes included in this study are shown in Table 2. Statistically significant difference at 0.05 level was detected using the independent samples t-test and the Wilcoxon Signed Rank Test.

Discussion

It is known that the structure of the rabbit corneal stroma is similar to human one, therefore, it was considered as the ideal substitute tissue for this experimental study. However, there are several differences between human and rabbit eye that have to be mentioned. The collagen fibers, which are observed in the rabbit cornea, are thinner than those in the human one. Also, other types of collagen fibres, than Type I, are found in greater frequency than in the human cornea, such as the Type VI, which usually occurs in scarring (10).

The results of this study confirmed a smooth reconstructive process following CXL treatment. Active inflammation was not observed. The epithelium regenerated rapidly after treatment and its mild non-specific differentiations seem to have no clinical significance. Regarding keratocytes, their number was normal, although some of them, especially

Tab. 1: Preoperative and postoperative collagen fiber diameter measurements (nm).

	Without any treatment	De-epithelialization		De-epithelialization + Riboflavin		CXL	
	(Mean ± SD)	(Mean ± SD)	p	(Mean ± SD)	p	(Mean ± SD)	p
Central Cornea	24.7 ± 2.5	26.0 ± 2.4	0.110	25.5 ± 3.1	0.421	31.4 ± 3.5	<0.001*
Limbus	53.5 ± 2.9	53.2 ± 2.7	0.715	53.3 ± 2.9	0.717	52.3 ± 3.9	0.268

*Significant at 0.05 level

Tab. 2: Preoperative and postoperative IOP measurements (mmHg).

Group	IOP pre-operatively	IOP post-operatively	p
	(Mean ± SD)	(Mean ± SD)	
CXL	18.5 ± 3.3	20.3 ± 3.8	0.416
De-epithelialization + Riboflavin	22.5 ± 0.7	19.6 ± 0.6	0.180
De-epithelialization	18.6 ± 0.3	22.8 ± 0.3	0.737
Without any treatment	17.4 ± 5.1	20.2 ± 1.4	0.655

those located in the anterior stroma, demonstrated apoptotic features. In previous experimental studies, keratocyte apoptosis was found due to the de-epithelialization or the UVA (11–13). The clinical significance of keratocytes apoptosis is unclear. Maybe, it is associated with scarring, clouding or thinning of the cornea (14–16). This loss can be restored by repopulation due keratocyte migration from the adjacent tissue (11, 17). However, in our study, we observed no change in the transparency or thickness of the cornea after CXL. Regarding collagen fibers, there were no significant changes of collagen pattern and collagen fibers morphology, with an exception of some areas of the anterior stroma, in which the collagen fibers were not very well organized. This may be due to the fact that the sacrifice of the animals took place in a relatively short time after treatment. Our results showed that there is a statistically significant increase in the diameter of collagen fibers of the central cornea, indicating the effect of the CXL treatment on this area, but there was not any similar observation at the limbus area. Also, a previous study by Wollensak et al has shown a statistically significant increase in corneal collagen fiber diameter, as a result of riboflavin/UVA-induced collagen cross-linking (6). The reason for this is that the induced cross-links push the collagen molecules apart, resulting in an increased intermolecular spacing and diameter of the collagen fibers (18). However, over the years, the intrastromal processes caused a regression of the fiber thickness to baseline and a normalization of the cornea. In addition, the influence of fixation on corneal collagen has been examined systematically by others. They found an increase of the collagen fiber diameter through cross-linking induced by the fixative glutaraldehyde and a reduction in fiber diameter by the embedding resin. The two opposite effects cancel each other out, so collagen pattern is not affected by corneal fixation (19). In our series, all of the specimens underwent the same fixation, so that the relative differences between the specimens cannot have been influenced by tissue processing anyway. Also, in previous studies, there was a cytotoxic effect of CXL treatment on the endothelium, when the radiation that reached there was greater than 0.36 mW/cm^2. Using intensity of UVA on the surface of the cornea of 3 mW/cm^2, this happens, only when the thickness of the cornea is less than 400 μm (1, 20). In this study the endothelium remained intact, since the corneas remained transparent throughout the whole postoperative period.

Regarding limbal area our results suggested that there are not any significant differences in the distribution and morphology of collagen fibers in this area between treated and untreated corneas. There is, certainly, the case that with this cutting method and observation using SEM, we have more reliable images from the limbus rather than from the trabecular meshwork. However, we can make the assumption that, if there are no changes at the limbus area, the changes at the trabeculum are rather improbable, since the trabeculum is deeper. The rabbit cornea thickness averages 0.37 mm at the center and 0.45 mm near the limbus. Also, the cross-linking effect is not distributed homogenously over the corneal depth. The maximum effect is detected in the anterior 200–300 mm of the cornea, because of the UVA-absorption by the photosensitizer riboflavin (7, 21). This depth-dependent strengthening effect correlates with riboflavin corneal diffusion.

IOP values were measured using Tonopen-XL, since it is the tonometer of choice for measuring IOP in rabbits within the range of IOP 3 to 30 mmHg (22). There was not any statistically significant difference between preoperative and postoperative IOP measurements. The IOP results after CXL plead for the assumption that there are no significant changes of the trabecular meshwork. This result, also, leads to the conclusion that CXL treatment might be a safe therapeutical option for patients, without influencing IOP and nerve condition.

Conclusion

In conclusion, we can say that the cornea responds well to the application of the CXL and no damage of its structure and functionality is observed. However, further experimen-

tal and clinical studies with longer follow-up are necessary to indicate, if there are or not any significant changes at limbus area and IOP measurements after CXL, by changing some of the parameters of treatment. This information will likely be a useful tool in the design and duration of treatment and is one of our future targets.

Acknowledgements/Disclosure

No financial support was received for this study.

The authors have no actual or potential conflict of interest, including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, this work.

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Received: 08/06/2016
Accepted: 23/08/2016

CASE REPORT

Thyroid Gland Metastasis from Cancer of the Uterine Cervix: An Extremely Rare Case Report

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Summary: The thyroid gland is a relatively uncommon site for a metastatic disease, although it is richly supplied with blood. The metastases may originate from various primary sites, mainly kidney, lung, head and neck, and breast. Thyroid metastasis from cervical carcinomas is extremely rare; and only a few cases have been previously reported in the literature. In patient with thyroid nodules and an oncological history, the possibility of thyroid metastasis should be seriously considered. Despite the rarity of the metastasis of cervical carcinoma to the thyroid, it is difficult to say appropriate treatment approach for these lesions. When managing such patients, decision-making should balance the possibility of gaining long-term survival against estimation of the aggressiveness of the disease and its possible complications. Here, a case of thyroid metastasis from a squamous cell carcinoma of the uterine cervix presenting with cervical mass and difficulty in swallowing and its treatment is reported.

Keywords: *Metastasis; Thyroid gland; Cancer of the uterine cervix*

Introduction

Worldwide, uterine cervix cancer is the most common gynecologic malignancy among women with an estimated 527,600 new cases in 2012 and tends to have a favorable prognosis (1, 2). It spreads predominantly by direct local extension and lymphatic metastasis. The most common metastatic sites are the vagina, parametrium, and pelvic lymph nodes (3). Metastasis of cervical carcinoma to the thyroid is extremely rare and only a few cases have been previously reported (3–5).

Although the thyroid gland has a rich blood supply, clinically detectable tumor metastases to the thyroid are uncommon, accounting for approximately 1.4% to 3% of all thyroid malignancies (4, 6). Moreover, numerous large autopsy series have reported an incidence ranging from 1.9% to 24% in patients who died due to malignancies at other primary sites (7). Herein, we report a case of thyroid metastasis from a squamous cell carcinoma of the cervix presenting with painless cervical mass and difficulty in swallowing and its treatment in a 56-year-old woman.

Case report

A 56-year-old woman with a six-month history of uterine cervical squamous cell carcinoma treated with total abdominal hysterectomy and bilateral salpingo-oophorectomy plus combining adjuvant radiotherapy and chemotherapy was admitted to our clinic with bilateral palpable masses on the thyroid. The complaints of the patient were swelling on the neck and severe difficulty in swallowing. On physical examination, a diffusely enlarged thyroid and a 3-cm moderately hard nodule were detected in the right lobe without palpable cervical lymphadenopathy. The other systemic evaluation was unremarkable except for mild hypertension. The patient had no other significant past medical history and no family history.

Preoperative thyroid function tests showed the following results: free triiodothyronine (fT_3): 5.6 pmol/L (3.8–6), free thyroxine (fT_4): 13.9 pmol/L (7–16), and thyroid-stimulating hormone (TSH): 0.02 μIU/mL (0.34–5.6). Thyroglobulin: 128 ng/mL (1.4–78), anti-Tg: 1.6 IU/mL (0–4), and anti-TPO: 0.25 IU/mL (0–9). Ultrasonographic thyroid examination revealed bilateral and multiple nodules with micro- and macrocalcifications that the largest was heterogeneous, hypoechoic, and approximately with a diameter of 34 mm in right lobe. An ^{18}F-fluorodeoxyglucose (FDG)-positron emission tomography (PET/CT) scan was performed to further evaluate any possible sites of metastatic disease. The whole body ^{18}F- FDG PET/CT scan demonstrated significant uptake in the right upper lobe of the lung, lumbar spine, paraaortic and paratracheal lymph nodes. Multiple bilater-

http://dx.doi.org/10.14712/18059694.2016.97

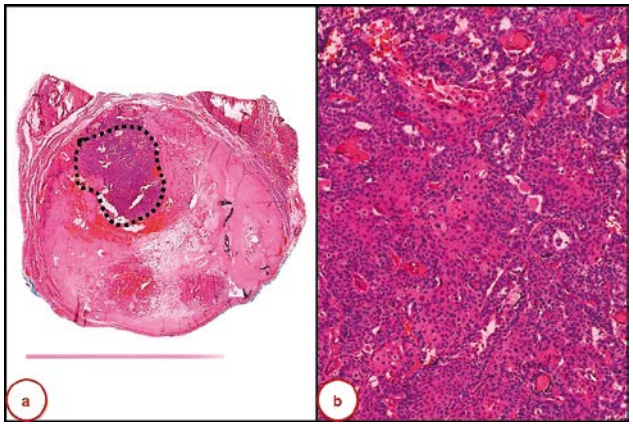

Fig. 1: (a) A well demarcated area (dotted) of metastatic tumor was observed within a benign nodule (0.6×, HE); (b) Tumor islands were composed of atypical squamous cells (15.2×, HE).

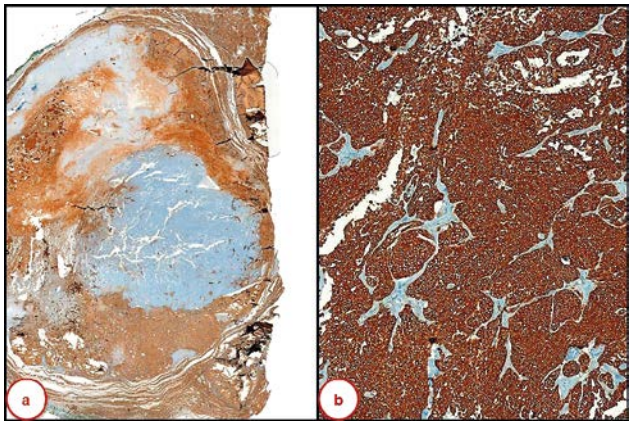

Fig. 2: (a) Tumor cells were negative by thyroglobulin immunostaining (0.8×, thyroglobulin); (b) Tumor cells were strongly CK5/6 positive (8.6×, CK5/6).

al thyroid nodules were also seen with no hypermetabolic activity and the biggest one, which was in the right lower pole, was calcified and about 3 centimeters in diameter as well.

In view of the rapid growth and progressing symptoms of dysphagia and difficulty in swallowing, palliative surgery was planned and the patient subsequently underwent total thyroidectomy and central lymph node dissection. Macroscopically, multiple nodules were observed in both lobes and isthmus, the largest located in the right lobe measuring 32 mm in diameter and showing hemorrhagic areas. On histopathological examination, multiple benign nodules were observed. On the right lobe, two areas measuring 3 mm and 10 mm were noted, within two different thyroid nodules (Figure 1). These areas were observed as sharply demarcated foci, within benign thyroid nodules, showing islands of atypical mitotically active squamous cells. Immunohistochemical staining revealed positive status for cytokeratin 5/6, cytokeratin 19, CEA, HBME-1, galectin-3, p63, and estrogen receptor whereas negative for thyroglobulin, thyroid transcription factor-1 (TTF-1), progesterone receptor, calcitonin, synaptophysin, CD56, and chromogranin A in these foci (Figure 2). p53 and Ki-67 were expressed in 5% and 30% of tumor cell nuclei, respectively. In situ hybridization for human papilloma virus was also negative. These foci were considered as metastasis from cervical squamous cell carcinoma. The patient was discharged in good general condition on postoperative day 2. The clinical status of the patient rapidly deteriorated and she died five months after the diagnosis of thyroid metastasis.

Discussion

Clinically evident metastases to the thyroid gland are generally considered rare, commonly associated with concurrent metastatic lesions to other organs and usually indicate a poor prognosis. Despite the high prevalence in autopsy series, metastases represent approximately 1.4% to 3% of all malignant tumors of the thyroid (6, 7); possibly due to a high concentration of oxygen and iodine in the thyroid tissue which may impair the ability of metastatic cells to settle and develop (8). The high intrathyroidal vascular flow also probably plays a role in impeding the adhesion and implantation of metastatic tumor emboli (9).

Recently, Hegerova et al. identified 97 patients with a metastatic solid neoplasm of the thyroid gland. In this analysis, 22% of cases were metastases from renal cancer, 22% from lung, 12% from head and neck, 11% were from breast cancer, and 9% from esophagus (6). Less commonly, metastases originated from skin, neuroendocrine, and ovary/uterus. Thyroid metastasis from uterine cervical squamous cell carcinomas is extremely rare; and to the best of our knowledge, only a few cases have been previously reported in English-language literature (3–5). The median and mean age at discovery of clinically significant metastases to the thyroid are 63 and 59 years, with a female predominance (6, 10). The mean latency between diagnosing primary malignancy and its metastases to the thyroid gland is variable 53 to 70 months (6, 10). The mean interval between diagnosing primary tumor and discovering its metastases to thyroid is longest in patients with sarcomas (75 months) and shortest in lung cancer (4.5 months) (10). In the present case, the latency was about six months.

The majority of patients with a thyroid metastasis present with an asymptomatic thyroid nodule and it is difficult to determine whether the tumor is primary or secondary. Neither ultrasonography nor scintigraphy provides specific data as cold nodules on radioiodine uptake studies or as a heterogeneous and hypoechoic mass on ultrasonographic examination (9). Thyroid function tests usually show no abnormalities and most patients are euthyroid (87.6%). Occasionally, some patients may develop a transient changes in thyroid hormones, so called non-thyroidal illness or euthyroid sick syndrome (10). The laboratory parameters of

this condition characterized mainly by low serum fT_3, with normal or low fT_4 and normal or suppressed levels of TSH. With the advanced diagnostic imaging technology such as ^{18}F- FDG PET/CT, an increasing number of incidental cases of metastatic disease to the thyroid gland are likely to be detected (8). Fine-needle aspiration cytology is able to make the differentiation between benign and malignant thyroid diseases with a high negative predictive value, high specificity (100%), and sensitivity (94%) and help avoid unnecessary surgery (6, 7, 9). Immunohistochemically, negative staining with anti-thyroglobulin and anti-calcitonin antibodies would favor a metastatic tumor. Moreover, in patients with an oncological history of presenting with thyroid nodules, the possibility of thyroid metastasis should always be considered (8). Patients with previously known multinodular goiter and follicular adenoma have also been associated with an increased incidence of metastases to thyroid (8). In the present case, metastatic tumor deposits occupied only a small proportion of the benign thyroid nodules.

There is no clear consensus about the surgical procedure that should be performed in patients with thyroid metastases (9). To date, however, no conclusive survival advantage following surgery has been proven for metastases to the thyroid gland which represent advanced systemic disease (11, 12). In most cases, it is suggested that metastases to the thyroid are associated with a poor prognosis. Although controversial, radical treatment for isolated metastases can be curative and an aggressive surgical approach has been recommended by many authors (6, 7, 9). For other patients with metastatic neoplasm of the thyroid gland which manifestation of a widely metastatic disease, the aim of the surgery must be palliative such as the prevention of asphyxia and other local complications (9). Adjuvant chemotherapy and/or radiotherapy may be considered as palliative treatment for local control of the disease. In our reported case, because of the tumors rapid growing with possible airway compression palliative surgery was planned and the patient subsequently underwent total thyroidectomy.

In conclusion, although metastatic diseases to the thyroid gland are uncommon, it is important for the pathologists, endocrine surgeons, and oncologists to be able to recognize and make the differentiation between the other more common thyroid malignancies. Due to the rarity of these lesions it is difficult to determine the survival advantage of surgical intervention for patients with metastasis to the thyroid. Because of metastatic thyroid tumor from uterine cervix carcinoma is extremely rare, the decision-making should balance the possibility of gaining long-term survival against the risk of complications, worsening the outcome and decrease in quality of life. It should be noted that most of the thyroid metastases present in the context of widespread metastatic disease and the metastatic diseases to the thyroid tend to behave more aggressively.

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Received: 04/05/2016
Accepted: 28/07/2016

CASE REPORT

Anomalous Medial Branch of Radial Artery: A Rare Variant

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Summary: Radial artery is an important consistent vessel of the upper limb. It is a useful vascular access site for coronary procedures and its reliable anatomy has resulted in an elevation of radial forearm flaps for reconstructive surgeries of head and neck. Technical failures, in both the procedures, are mainly due to anatomical variations, such as radial loops, ectopic radial arteries or tortuosity in the vessel. We present a rare and a unique anomalous medial branch of the radial artery spiraling around the flexor carpi radialis muscle in the forearm with a high rising superficial palmar branch of radial artery. Developmentally it probably is a remanent of the normal pattern of capillary vessel maintenance and regression. Such a case is of importance for reconstructive surgeons and coronary interventionists, especially in view of its unique medial and deep course.

Keywords: *Radial artery; Variation; Loop/Anastomosis; Superficial palmar branch of radial artery; Flexor carpi radialis*

Introduction

The main vessel of the upper limb, the brachial artery, divides at the level of the neck of the radius into two terminal branches – the radial and ulnar arteries. These arteries through further branching supply the forearm and hand along with the associated joints.

The widespread use of the forearm flap of the radial artery (RA) for reconstructive surgeries of head and neck as well as use of the RA as a route for transradial coronary intervention (TRI) has resulted in an interest in the variations of RAs. During reconstructive surgeries it is the aberrant anatomy which may affect the surgical outcome. Anomalies in the RA system although rare include hypoplasia, stenosis, tortuous configuration or presence of a radioulnar loop (3, 25). However the most frequently encountered anomaly is the high origin of RA or a superficial RA which accounts for 78% of all variations of RA (23).

We describe a rare anomalous medial branch of RA forming an unusual rectilinear anastomosis between the proximal RA and superficial palmar branch of RA (SPbr) coursing around the flexor carpii radialis muscle (FCR).

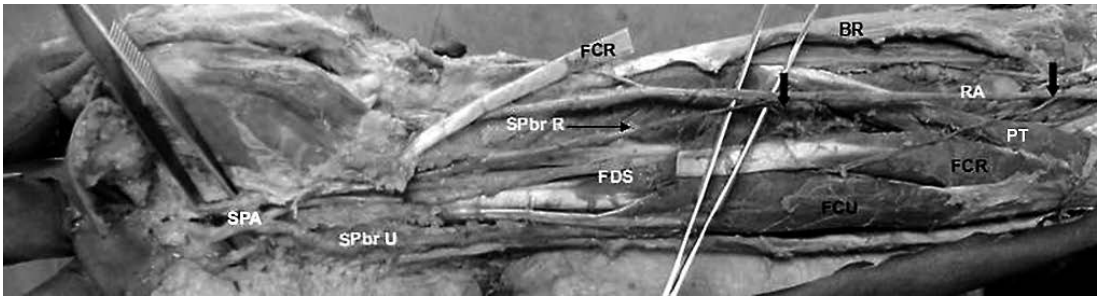

Fig. 1: Photograph of the dissection of the right forearm depicting the anomalous long slender branch (arrow), arising from the medial aspect of radialartery (RA), running anterior to the belly of flexor carpii radialis (FCR). Note the vessel courses posterior to the muscle, spirals around it and emerging at its lateral border to anastomose (arrow) to the high rising superficial palmar branch (SPbr), forming an arterial sling around the FCR. FCR – Flexor carpii radialis, BR – Brachioradialis, PT – Pronator teres, FCU – Flexor carpii ulnaris, FDS – Flexor digitorium superficialis, SPbrU – superficial palmar branch of ulnar artery, SPA – superficial palmar arch.

ACTA MEDICA (Hradec Králové) 2016; 59(3):100–103
http://dx.doi.org/10.14712/18059694.2016.98

Case Report

While dissecting the right forearm of a 72-year-old male cadaver for undergraduate teaching we encountered a unique variation of RA. The RA arose normally from the brachial artery at the level of neck of the radius. It descended into the forearm medial to the FCR and anterior to pronator teres under cover of brachioradialis. Just distal to the elbow it gave rise to a normal radial recurrent artery which ascended to anastomose with radial collateral branch of profunda brachii. Distally, while still in the cubital fossa, it gave an anomalous long slender branch, which ran medial to the RA, anterior to the belly of FCR. Near the musculotendinous junction of FCR, the vessel coursed posterior to the muscle, spiraling around it, emerging at its lateral border to anastomose to the SPbr, forming an arterial sling around the FCR. The SPbr itself, originated at a higher level from the RA in the middle third of the forearm. This artery descended in the forearm along the RA to the wrist. While the RA ran on to the dorsum of the wrist, the SPbr coursed through the thenar muscles to complete the superficial palmar arch with the ulnar artery, which was otherwise normal in location and branching (Figures 1, 2, 3). The left limb did not show any anomalies.

Discussion

The above mentioned case presented with a unique variation, wherein an anomalous medial branch arose from the RA in the proximal part of the forearm, spiraled around the FCR and formed a rectilinear anastomosis with a high rising SPbr, just distal to its origin from RA. The SPbr ran parallel to the RA in the distal third of the arm, entered the palm superficial to flexor retinaculm to form superficial palmar arch with normally arising superficial branch of an ulnar artery.

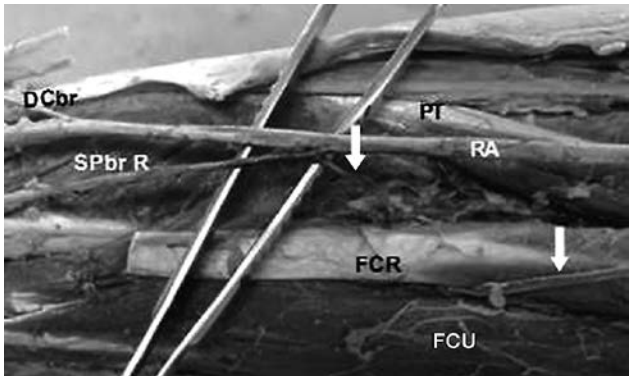

Fig. 2: Note the anastomoses of the anomalous medial branch to SPbr in the middle third of the forearm.

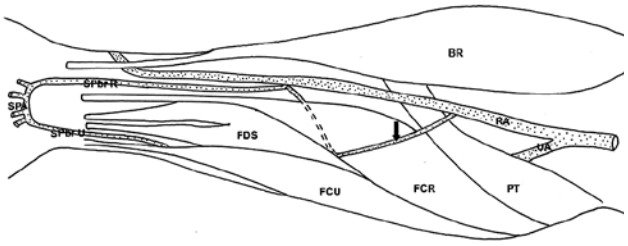

Fig. 3: Diagrammatic representation of the anomaly.

Anastomosis seen between vessels have been postulated to be atavistic remnants of embryological capillaries. They maybe between the superficial and deep set of vessels with an incidence of 1–6%, mainly present around the elbow and are usually incidental findings (14, 17, 18, 19). Blood vessels of the upper limb are now postulated to arise from a capillary plexus in the limb bud which undergoes differentiation as it moves distally. While some of these capillaries are retained and undergo differentiation and enlargement, the others regress (20). These anastomosis have been classified on basis of their length (long/short), caliber (large/slender) and form (sling like/rectilinear) (17). Since they are present in the region of cubital fossa, another classification groups them on the basis of site of origin and number of recurrent radial arteries(121) or whether the anastomosis were present anterior or posterior to the bicipital tendon (14). In our case, the anastomosis is rectilinear in form, slings around FCR and is present in the forearm instead of the cubital fossa or arm.

Normally the RA, during its course through the forearm, gives rise to a radial recurrent artery just distal to the elbow in addition to muscular and cutaneous branches. This 'normal anatomy' of RA was seen in 92 % of cases studied by Nasr (2012) in a gross cadaveric study. Commonly encountered anomalies of RA include variation in its course – superficial or a deeply placed, variable origin, duplication or complete absence of the vessel or its septocutaneous perforator branches (13, 16, 19). There are very few reports of anomalous branches arising from RA .These anomalous branches described in literature are laterally places and have a superficial course (1, 15, 22). The only described medial branch was by Bhatt et al. (2009) which arose from the RA 1.5cms distal to its origin; however this vessel remained superficial to the FCR supplying the skin and fascia of the distal third of the forearm. Further description of the course of the vessel was not mentioned by the authors due to limited dissection during surgery. The aberrant branch in our case is a rare medial branch however in contrast to previousy reported case by Bhatt et al. (2009), this branch courses around the FCR and formed an anastomosis between RA and SPbr (2).

Honma et al., 2008, also found arterial rings but around the tendon of biceps brachii in the cubital fossa connecting the brachial artery to RA near its origin (6). While evaluating the impact of RA anomalies on transradial coronary intervention, loops were also observed by Lo et al. 2011. These loops were present between the RA and brachial artery/ulnar artery and mostly involved the proximal radial artery just below the brachial bifurcation. A recurrent radial artery (occasionally two) arose from the apex of the loop in all cases.

The presence of these RA loops was associated with high procedural failure rate (37.1%, p = 0.001).

Microvascular techniques help to effectively transfer the flap from its site of origin to donor repair site. However these techniques require accurate knowledge of the normal or variant anatomy of the forearm to avoid inadvertent injury or complications during and after the procedure. To raise the radial forearm flaps the surgeon needs to dissect carefully between the brachioradialis laterally and the FCR medially. It is here that a surgeon may encounter the anomaly mentioned here. The above mentioned anomaly also needs to be kept in mind while harvesting palmaris longus. The palmaris longus which lies medial to the FCR is also harvested for lip or mid-face reconstruction (7).

Inadvertent injury to the high rising SPbr as in this case when present should be borne in mind as ligation of it would compromise the superficial palmar arch especially in a handful of individuals where there is no communication between superficial and deep arches.

Nasr (2012), studied the origin, course and branching pattern of radial artery in 100 upper limbs wherein he found the SPbr to be variant in only 6 limbs. A high rising SPbr is a rare finding, with an incidence of 5–7% (4, 16). The SPbr is a vessel which is consistently present in the forearm although they maybe variations associated with them. Flaps involving the SPbr of radial artery are used for forefinger reconstruction (8). A high arising SPbr running parallel to the RA may sometimes be mistaken for the parent vessel and cannulated during invasive blood pressure monitoring. Since radiocepahalic arteriovenous fistulas are end (of the vein) to side (of the artery), such anomalies are unlikely to impact surgical technique or efficacy.

The radial artery, as a virtue of its superficial location which is away from the major nerves of the upper limb, is regarded as a useful vascular access site for coronary procedures like transradial coronary angiography and transradial coronary intervention. It is also one of the the preferred vessel for coronary artery bypass grafting as it offers long term patency (26). During these procedures the artery is cannulated 1–2 cm proximal to the radial styloid process. Difficulties arise due to presence of bilateral brachioradial arteries anastomosing with a brachial artery through a sling like loop (5), or due to radioulnar loop (0.9%) or due to presence of tortuosities and variations (9, 10) .The reported technical failure with transradial interventions is 1–5%, attributed to variant anatomy, arterial spasms and inablity to puncture (10). These anomalies are not contraindications for interventions; however they result in difficulty in introducing guidewires especially in unidentified variant anatomy. Transradial coronary interventions when attempted on such patients result in increased risk of dissections and perforations (11, 25). It has been observed that the loop like anomalies when present in the limb can be circumvented with appropriate instrumentation and manupilation on the table but lead to increased patient discomfort which may sometimes hinder further catheter advancement and in some cases alternate routes needed to be explored (10). Lo et al., 2011 observed higher procedural failure rates in patients with radial artery anomaly (10). Keeping these risks in mind, it is advisable for the operating surgeon to routinely screen the upper limb for a detailed vessel anatomy prior to intervention.

Clinical tests for vascular patency, such as Allens Test would probably be unaffected in most instances. The close proximity of the RA and SPbr at the wrist would not allow selective compression and hence not change the result. However a Doppler study may indicate the presence of two vessels if used, as is increasingly the norm.

Conclusion

A medial anomalous branch of radial artery is a rare variation. Further its re-anastomosis to the high rising superficial palmar branch has not been reported before. Awareness of such vessel is of utility to the reconstructive and peripheral vascular surgeon during harvesting the radial artery or raising a radial flap.

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Received: 26/04/2016
Accepted: 22/08/2016