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IZVORNI ZNANSTVENI RAD
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Usporedba metoda ekstrakcije RNK za molekularnu analizu u oralnoj citologiji

Comparison of RNA Extraction Methods for Molecular Analysis of Oral Cytology

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Sažetak

Svrha rada: Svrha istraživanja bila je usporediti tri metode ekstrakcije RNK iz oralnoga citološkog uzorka kako bi se pronašla najbolja tehnika izolacije s obzirom na koncentraciju i čistoću RNK koji se može koristiti u molekularnim testovima oralnih lezija, poput lančane reakcije polimeraze u stvarnom vremenu. **Materijali i metode:** Uzorci su uzeti sa sluznice oralne šupljine pacijenata bez vidljivih promjena eksfolijativnom citologijom, korištenjem četkice Orcellex Rovers Brush®. Ekstrakcija ukupnoga RNK provedena je primjenom triju metoda: 30 uzoraka ekstrahirano je primjenom sustava Trizol®, 30 sustavom Direct-zol™ RNA Miniprep i 30 sustavom RNeasy Mini Kit. Kako bi se procijenila čistoća, spektrofotometrom je izmjerena apsorpcija svjetlosti. Procijenjena koncentracija RNK dobivena je množenjem vrijednosti A260 s 40 (ng/mL). Statistička analiza obavljena je u programu GraphPad Prism 5.03. Korišteni su Studentov t-test, analiza varijance i Bonferronijev test, a razina značajnosti bila je postavljena na $p \leq 0,05$. **Rezultati:** U skupini tretiranoj Trizolom® zabilježena je najviša prosječna koncentracija, slijedi skupina tretirana Direct-zolom™ i na kraju je RNeasy. Ako se uzmu u obzir oba korištena omjera apsorpcije, u skupini tretiranoj Direct-zolom™ izmjerena je najveća čistoća, sljedeća je skupina RNeasy pa zatim Trizol®. **Zaključak:** Uzimajući u obzir sve aspekte, tj. koncentraciju, čistoću i trajanje postupka, skupina tretirana Direct-zolom™ pokazala je najbolje rezultate.

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Ključne riječi
oralna sluznica; RNK, obrada uzoraka; molekularno-dijagnostičke tehnike

Uvod

Klinički pregled i patohistološka analiza klasične su metode dijagnosticiranja potencijalno zloćudnih promjena i lezija u usnoj šupljini. Eksfolijativna citologija predložena je kao komplementarna metoda jer može dati informacije o epitelnim stanicama (1).

Introduction

Clinical examination and histopathological studies are classical methods for diagnosis of potentially malignant disorders and malignant lesions of oral cavity. Exfoliative cytology has been proposed as a complementary method, since it can provide information of epithelial cells (1).

Eksfolijativna citologija je neinvazivna, bezbolna, praktična i jeftina dijagnostička metoda (2), vrlo korisna u dobivanju RNK iz stanica oralne sluznice za ispitivanje genske ekspresije (3).

Ekstrakcija i pročišćivanje RNK do visoke kvalitete, važni su postupci za tu vrstu ispitivanja. Genska ekspresija oralnih stanica iz brisa već se istraživala (3, 4). No prema našim spoznajama još se nisu proučavale metode ekstrakcije RNK koji se može koristiti za molekularnu analizu oralnih citoloških uzoraka, iako su Pandit i suradnici 2013. ispitivali odljuštene stanica dobivene iz prikupljene sline (5). Stoga je svrha ovog istraživanja usporediti tri metode ekstrakcije RNK iz oralnoga citološkog uzorka kako bi se pronašla najbolja tehnika izolacije s obzirom na koncentraciju i čistoću RNK koji se može koristiti u molekularnim testovima oralnih lezija, poput lančane reakcije polimeraze u stvarnom vremenu (qPCR).

Materijali i metode

S bukalne sluznice 30 ispitanika u dobi između 30 i 50 godina, bez vidljivih kliničkih promjena na mjestu prikupljanja, uzeta su po tri uzorka stanica. To je obavljeno u Zavodu za oralnu medicinu, oralnu kirurgiju i implantologiju Medicinskog i stomatološkog fakulteta Sveučilišta Santiago de Compostela, u sklopu Programa za prestanak pušenja Kardiološkog instituta Sveučilišta São Paulo te u Ambulanti za oralnu medicinu Instituta za znanost i tehnologiju Sveučilišta Estadual Paulista (UNESP). Istraživanje je odobrilo galicijsko Etičko povjerenstvo za istraživanja i Etičko povjerenstvo Instituta za znanost i tehnologiju UNESP-a. Svakom ispitaniku potanko je objašnjen postupak, nakon čega je zatražen pristanak za sudjelovanje u istraživanju.

Brisovi su prikupljeni posebnim četkicama Orcellex Rovers Brush® (Rovers Medical Devices, Nizozemska), bez upotrebe sredstva za ispiranje usta (6). Četkica je postavljena okomito i čvrsto pritisnuta na sluznicu. Zatim je rotirana za dvadeset potpunih okreta u skladu s preporukama proizvođača. Kod svakog ispitanika postupak je obavljen na trima različitim mjestima na desnoj strani bukalne sluznice i svaki je bris pohranjen u zasebnu epruvetu za tri različite analize. Ukupna količina stanica dobivena uzorkovanjem izmjerena je u Neubauerovoj komori i iznosila je 10^6. Kod te vrste uzoraka najčešće se pronalaze stanice intermedijarnog i površinskog sloja s malom ili nikakvom zastupljenošću bazalnih i parabazalnih stanica. Anuklearne stanice uobičajena su pojava. Kako bi se osiguralo da se u što većim količinama prikupe stanice s jezgrom iz intermedijarnih slojeva, četkica za uzorkovanje zakrenuta je dvadeset puta pri uzimanju brisa s bukalne sluznice. Uzorci su transportirani zaštićeni od svjetlosti u epruvetama bez DNaza, RNaza i pirogena, u 2 ml medija Roswell Park Memorial Instituta (RPMI) (3, 4) (Microvet, Madrid, Španjolska) i pohranjeni na -80 °C do ekstrakcije RNK. Uzorci su zamrznuti odmah nakon uzimanja kako bi se smanjila degradacija RNK. Provedeni su preliminarni testovi kako bi se usporedio medij RPMI-ja sa specifičnim otopinama za stabilizaciju RNK i rezultati su pokazali jednaku učinkovitost kao i kod otopine RNAlater®. Uzorci su zamr-

The exfoliative cytology is a non-invasive, painless, convenient and low cost diagnostic tool (2), very useful in obtaining RNA from oral mucosa cells for gene expression studies (3).

The extraction and purification of high quality RNA is an important step in such studies. Gene expression of oral cells from smears has already been studied (3, 4). However, to our knowledge, RNA extraction methods to obtain samples for molecular analysis of oral cytology samples have not yet been used, although Pandit *et al.* studied exfoliated cells via saliva collection in 2013 (5). Therefore, the aim of this study was to compare three methods of oral cytology samples RNA extraction, considering concentration and purity to establish the best technique for molecular tests of oral lesions such as real-time reverse transcriptase reaction (qPCR).

Material and methods

Three samples of cells were taken from buccal mucosa of 30 subjects aged 30-50 years, with no visible clinical changes at the site of collection, from the Oral Medicine, Oral Surgery and Implantology Unit of Faculty of Medicine and Dentistry, University of Santiago de Compostela; from the Smoking Cessation Program of Heart Institute, University of São Paulo School and from the outpatient clinic of Oral Medicine of Institute of Science and Technology, UNESP - Univ Estadual Paulista. This study was approved by the Ethics Committee in Research of Galicia and Research Ethics Committee of Institute of Science and Technology, UNESP - Univ Estadual Paulista. Informed written consent was obtained from the subjects after they had been fully explained the nature of procedures.

Smears were collected using Orcellex Rovers Brush® (Rovers Medical Devices, NL, Holanda), without use of mouthwashes (6). The Rovers® Orcellex® Brush was placed perpendicular to the area and pressed with a firm contact into the lining mucosa. It was rotated through twenty complete turns for each site to be sampled, following the manufacturer's recommendations. In each patient, this procedure was carried out in three different places of the right buccal mucosa, and placed in a different bottle for three different analyses. A total amount of cells was collected by sampling, measured in Neubauer Chamber and it amounted to 10^6. In this type of sample, the intermediate and superficial cells with rare or null appearance of basal and parabasal cells are most frequently observed. Generally, anucleate cells appear. In order to ensure that nucleated cells of the intermediate layers were collected in larger quantities, twenty rotations of the cytobrush were performed at buccal mucosa collecting sites. The samples were transported in DNase, RNase and pyrogenic free tubes, protected from light, containing 2ml of Roswell Park Memorial Institute medium (RPMI) (3,4) (Microvet, Madrid, Spain) and stored at -80°C, until RNA were obtained. The samples were frozen immediately after collection, which minimizes the RNA degradation. Furthermore, some tests were previously made to compare the RPMI with specific

znuti jer prikupljanje nije obavljeno u istom danu, pa se na taj način ograničila degradacija RNK u onima uzetima prije.

Za ekstrakciju RNK iz uzoraka eksfolijativne citologije primijenjene su tri tehnike. Trideset uzoraka ekstrahirano je tehnikom Trizol® (skupina Trizol®) (Life Technologies, Madrid, Španjolska), 30 tehnikom Direct-zol™ RNK sustavom Miniprep (Zymo Research, SAD) (skupina Direct-zol™) i 30 sustavom RNeasy Mini Kit (Qiagen, SAD) (skupina RNeasy). U svakoj skupini brisovi su prikupljeni od 10 istih pušača, 10 bivših pušača i 10 nepušača. Budući da pušenje uzrokuje keratinizaciju i promjene u sazrijevanju stanica (6), autori smatraju da je ta činjenica mogla utjecati na količinu RNK.

Svi postupci provedeni su u skladu s preporukama proizvođača. Za ekstrakciju u skupini Trizol®, uzorci su nakon homogenizacije centrifugirani i inkubirani Trizolom®. Dodano je 200 µl kloroforma, a zatim su obavljeni homogenizacija i centrifugiranje. Vodena faza prenesena je u novu epruvetu; dodano je 500 µl 100-postotnog izopropilnog alkohola, homogenizirano je inverzijom i centrifugirano. Supernatant je odbačen, a talogu je dodan 1 ml 75-postotnog etanola, a zatim je slijedila homogenizacija i centrifugiranje. Supernatant je uklonjen, talog je isušen i dodano je 20 µL vode bez nukleaza tretirane dietilpirokarbonatom (DEPC) (Life Technologies, Madrid, Španjolska). Ukupna količina ekstrahiranog RNK (1 µg) tretirana je DNazom I (Ambion Inc., Carlsbad, SAD).

Za ekstrakciju u skupini Direct-zol™, talogu je dodan 1 ml reagensa TRI-Reagent® uz potpunu homogenizaciju, nakon čega je u otopinu izravno dodan 1 ml 100-postotnog etanola i sve je homogenizirano. Uzorak je zatim stavljen u kolonu Zymo-Spin IIC™ i centrifugiran. Kolona je premještena u novu epruvetu, a ona s filtratom je bačena. Uzorci RNK tretirani su DNazom. Nakon toga dodano je 400 µl Direct-zola™, provedeno je centrifugiranje, odbačen je filtrat i ovaj postupak je ponovljen. Dodano je 700 µl pufera RNK za ispiranje i sve je centrifugirano. Kolona je pažljivo prenesena iz epruvete za skupljanje u epruvetu bez RNaza, dodano je 20 µl DEPC-vode (Life Technologies) nakon čega je slijedilo centrifugiranje.

Za ekstrakciju u skupini RNeasy uzorci su centrifugirani te je nakon uklanjanja supernatanta staničnom talogu dodano 350 µl pufera RTL koji je homogeniziran 1 minutu miješalicom Vortex; dodano je 350 µl 70-postotnog etanola i homogenat je stavljen u kolonu te je centrifugiran, nakon čega je odbačen filtrat. Dodano je 700 µl pufera RW1 nakon čega je slijedilo centrifugiranje te je filtrat odbačen. Zatim je dodano 500 µl pufera RPE, provedeno je centrifugiranje te je filtrat odbačen. Nakon toga postupak je ponovljen. Kolona je stavljena u novu epruvetu za skupljanje bez poklopca i 1 minutu centrifugirana maksimalnom brzinom. Kolona je prenesena u epruvetu s poklopcem i dodano joj je 20 µl DEPC-vode (Life Technologies) te je slijedilo centrifugiranje. Ukupna količina ekstrahiranoga RNK (1 µg) tretirana je DNazom I (Ambion Inc., Carlsbad, SAD).

Jedna mikrolitra RNK svakog uzorka korištena je za mjerenje apsorpcije svjetlosti pri valnim duljinama 260 (A260) i 280 (A280) nanometara (µm) u spektrofotometru Na-

solutions to RNA stabilization and these results showed the same performance of RNAlater®. Also, the samples were frozen since collecting was not all made on the same day; in order to limit a degradation of RNA, the samples were collected before.

Three techniques were used for RNA total extraction from exfoliative cytology. 30 samples were extracted by Trizol® technique (Trizol® group) (Life Technologies, Madrid, Spain), 30 samples were collected using the Direct-zol™ RNA Miniprep system (Zymo Research, CA, USA) (Direct-zol® ™ group) and 30 samples were extracted using RNeasy mini kit (Qiagen, CA, USA) (RNeasy group). In each group, the smears were collected from the same 10 smokers, 10 ex-smokers and 10 nonsmokers. Since tobacco use is associated with an increase in keratinization and cell maturation changes (6), the authors believed that it could alter the RNA amount.

All procedures were performed according to manufacturer´s recommendation. For Trizol® group extraction, the samples were centrifuged and incubated with Trizol® after homogenization. 200µL of chloroform was added, homogenized and centrifuged. The aqueous phase was transferred to a new tube, avoiding contact with the interface; 500µL of 100% isopropyl alcohol was added to it, homogenized by inversion and centrifuged. The supernatant was discarded without disturbing the pellet. 1mL of 75% ethanol was added to it, homogenized and centrifuged. The supernatant was removed and the pellet dried and 20µL of diethylpyrocarbonate (DEPC)-treated nuclease-free water (Life Technologies, Madrid, Spain) was added to it. The total extracted amount of RNA (1 µg) was subsequently treated with DNase I (Ambion, Inc., Carlsbad, CA, USA).

For the Direct-zolTM group extraction, 1ml of TRI-Reagent® was added to pellet and homogenized completely. 1ml of ethanol 100% was directly added to the solution and homogenized. The sample was added to Zymo-Spin IIC™ column with the collection tube and centrifuged. The column was moved to a new collection tube and the collection tube containing the filtrate was discarded. The RNA samples were treated by DNase. After, 400µl Direct-zol™ RNA, a prewash was added to the column, and centrifuged. Subsequently, the filtrate was discarded and this step was repeated. 700µl RNA wash buffer was added to the column and it was centrifuged. The column was carefully transferred from the collection tube into the RNase-free tube, 20µl DEPC-treated nuclease-free water was added to it (Life Technologies, Madrid, Spain) and it was centrifuged.

For RNeasy group extraction, the samples were centrifuged after removing the supernatant. 350µL of RTL buffer was added to the cell pellet and homogenized with vortex for 1 minute; 350µL of 70% ethanol was added, and this homogenate was transferred to the column with the collection tube and centrifuged, discarding the filtrate. 700µL of buffer RW1 was added to the column, centrifuged and the filtrate was discarded. 500µL of RPE buffer was added to it, centrifuged and the filtrate was discarded. Then, this step was repeated. The column was placed in a new collection tube without lid and centrifuged at maximum speed for 1 minute.

noDrop 1000 (Thermo Scientific, Wilmington, Delaware). Procijenjena koncentracija RNK dobivena je množenjem vrijednosti A260 (ng/ml) s 40. Čistoća koja upućuje na kvalitetu RNK procijenjena je omjerima A260/A280 i A260/A230, pri čemu vrijednosti omjera A260/A280 između 1,8 i 2,0 i vrijednosti omjera A260/A230 oko 1,7 upućuju na nekontaminirani RNK. Odgovarajuća prazna otopina korištena je za baždarenje spektrofotometra. Integritet je provjeren elektroforezom na 1-postotnom gelu agaroze.

Statistička analiza podataka obavljena je u sklopu programa GraphPad Prism 5.03 te su rezultati prikazani kao medijan, prosječna vrijednost i standardna devijacija. Statistička značajnost između dviju skupina određena je u programu SPSS (v.20.0) Studentovim t-testom i analizom varijance (ANOVA); statistički značajnom razlikom smatrala se p vrijednost od < 0,05. Za optimalno usklađivanje varijabli korišten je Bonferonijev test.

Rezultati

Prosječne koncentracije, standardne devijacije i rankovi dobiveni svakom tehnikom nalaze se u tablici 1.; dodatno su prikazane prosječne vrijednosti, standardne devijacije i maksimalne varijacije omjera A260/A280 i A260/A230.

Uzorci su dobiveni od 24 muškarca i 6 žena. Čistoća (omjer A260/A280) dobivena u skupini Direct-zol™ bila je veća kod muškaraca (prosječno = 1,90, SD = 0,064) nego-

The column was inserted into a tube with lid and 20µL of DEPC-treated nuclease-free water was added (Life Technologies, Madrid, Spain) and centrifuged. Total RNA extracted (1 µg) was treated with DNase I (Ambion, Inc., Carlsbad, CA, USA)

One microliter of RNA was used to measure the absorbance at 260 (A260) and 280 (A280) in nanometer scale (nm) in NanoDrop 1000 Spectrophotometer for each sample (Thermo Scientific, Wilmington, Delaware). The estimated RNA concentration was obtained by multiplying by 40 the value of A260 (ng/mL). The purity, which indicates the quality of RNA, was assessed by the A260/A280 and A260/A230 ratios, where A260/A280 ratio values between 1.8 and 2.0 and A260 / A230 ratio values close to 1.7 suggest RNA free of contamination. An appropriate blank solution was used to zero the spectrophotometer. Integrity was checked by electrophoresis, performed using 1% agarose gel.

Statistical analyzes of the data were performed using GraphPad Prism 5.03 software and the data were presented by median, mean and standard deviation. The significance between two groups was determined, using SPSS (v.20.0), with the Student's t and ANOVA test; p value, $p<0.05$ was considered to indicate a statistically significant difference. The Bonferroni test was used to obtain the optimal fit between the variables

Results

Concentration average, standard deviation and range obtained in each technique used may be observed in Table 1 along with average, standard deviation and maximum variation of A260/A280 and A260/A230 ratios.

Samples were collected from 24 men and 6 women. Purity (A260/A280 ratio) obtained in Direct-zol™ group was higher in male subjects (average = 1.90, SD= 0.064) than in

Tablica 1. Informacije o koncentraciji i omjerima A260/A280 i A260/A230 za svaku tehniku
Table 1 Information about concentration: A260/A280 and A260/A230 ratios obtained by each technique.

	Concentration average (ng/µL)	Concentration range	A260/A280 average	A260/A280 range	A260/A230 average	A260/A230 range
Trizol	421.54 ± 424.35	50.53 - 1788.25	1.77 ± 0.11	1.61 - 1.99	0.75 ± 0.41	0.23 - 1.87
Direct-zol RNA MiniPrep	143.15 ± 114.36	38.66 - 440.31	1.8693 ± 0.19	0.88 - 1.99	1.69 ± 0.32	0.9 - 2.15
RNeasy mini kit	42.303 ± 57.13	15.41 - 337.36	1.939 ± 0.07	1.77 - 2.09	0.166 ± 0.16	0.05 - 0.9

Tablica 2. Koncentracija prema navici pušenja u skupinama Trizol®, Direct-zol™ i RNeasy MINIKIT
Table 2 Concentration in each tobacco subgroup: Trizol® group, Direct-zol™group and RNeasy mini kit group.

	Konzumiranje duhana • Tobacco	Prosječna koncentracija (ng/µL) • Average Concentration (ng/µL)
Trizol	Nepušač • No smoker	201.99 ± 116.26
	Bivši pušač • Ex smoker	792.41 ± 545.59
	Pušač • Smoker	270.21 ± 192.95
Direct-zol RNA MiniPrep	Nepušač • No smoker	66.84±48.26
	Bivši pušač • Ex smoker	176.70±139.14
	Pušač • Smoker	185.93±103.43
RNeasy MINIKIT	Nepušač • No smoker	73.10±93.37
	Bivši pušač • Ex smoker	29.66±13.51
	Pušač • Smoker	24.13±4.44

li kod žena (prosječno = 1,73; SD = 0,42) (t = 2,065, p = 0,048). Za ostale varijable nisu zabilježene razlike među skupinama.

S obzirom na varijablu pušenja, ANOVA je pokazala statistički značajne razlike za kvantitativnu varijablu koncentracije RNK u skupinama Trizol® (F = 8,983, p = 0,001) i Direct-zol™ (F = 4,06, p = 0,029). Koncentracije u podskupinama nalaze se u tablici 2.

Da bi se postigla optimalna prilagodba varijabli, ispitanici su podijeljeni u skupine pušača (trenutačni i bivši pušači) i nepušača (osobe koje nikada nisu pušile i one koje su pušile u jednom trenutku i nakon toga prestale). U tom slučaju, omjer A 260/A280 u skupini RNeasy bio je veći kod nepušača (prosječno = 1,96; SD = 0,06), negoli kod pušača (1,90 ± 0,09; F = 4,507, p = 0,043).

Rasprava

Oralna eksfolijativna citologija jest proučavanje i tumačenje obilježja odljuštenih epitelnih stanica oralne sluznice. Metodu su razvili Papanicolau i Traut (1941.) (7) za dijagnostiku cervikalnih neoplazija, a poslije je korištena u dijagnostici bolesti usne šupljine (8). Citološki uzorci mogu se promatrati u nekoliko pristupa primjenom qPCR-a, uvijek radi razumijevanja promjena tkiva (3, 9,10),

Citološki brisovi jednostavno se prikupljaju, praktični su i jeftini (2), a mogu dati korisne informacije o epitelnim stanicama (1) jer one koje se nalaze u površinskim slojevima epitela sadržavaju podatke o promjenama koje su se dogodile tijekom staničnog sazrijevanja (6). Zato citologija može biti korisna u otkrivanju ranih promjena kod pušača i u praćenju promjena (6), a može biti i neinvazivna tehnika prikupljanja uzoraka za proučavanje genske ekspresije u toj skupini pacijenata i drugim rizičnim skupinama.

Svrha ovog istraživanja bila je pronaći najbolju tehniku ekstrakcije RNK, uključujući i čimbenik smanjenja vremena i troškova, kako bi se dobili uzorci oralnih lezija potrebnih za qPCR.

Metoda u stvarnom vremenu (qPCR) značajno je proširila primjenu i opseg testova lančane reakcije polimeraze (11) koji se smatra *zlatnim standardom* za proučavanje prijepisa određenog skupa gena. Stoga metoda qPCR mora ispuniti visoke zahtjeve pouzdanosti, osjetljivosti i ponovljivosti (12). Kvantifikacija ekstrahiranoga RNK smatra se važnim korakom (13) jer su potrebne jednake količine RNK u analizi kada se uspoređuju različiti uzorci.

Važno je napomenuti da se uzorak RNK dobiven citologijom djelomično razgrađuje i može se spustiti ispod razine detekcije transkriptaze (13). Nadalje, znatne količine RNaza nalaze se u slini (14), pa se RNK degradira u uvjetima *in vivo* zbog prirodne regulacije kao odgovora na vanjske podražaje (13). Zato postupak treba provesti pažljivo kako bi se smanjila razgradnja uzorka prije ekstrakcije i analize (5).

Vrijednost pH i ionske jakosti otopina kojima se koristimo za spektrofotometrijsku analizu mogu znatno utjecati na kvalitativne i kvantitativne odrednice nukleinske kiseline. Na primjer, voda koja sadržava RNaze može promijeniti omjer

female subjects (average = 1.73; SD= 0.42) (t = 2.065, p = 0.048). There were no differences between other variables in each group.

The tobacco variable was measured using ANOVA and the distribution is statistically significant for quantitative variable RNA concentration in Trizol® group (F = 8.983, p = 0.001) and in Direct-zol™ group (F = 4.06, p = 0.029). Concentration in each subgroup can be observed in Table 2.

To achieve the optimal adjustment of variables, the tobacco smokers variables were considered as tobacco smokers (smokers now and ex-smokers) and nonsmokers (persons who have never smoked and those who smoked at one time and subsequently quit). In this case, A260/A280 ratio in RNeasy group was higher in non-smokers (average= 1.96; SD=0.06), than in smokers (1.90± 0.09; F = 4.507, p = 0.043).

Discussion

Oral exfoliative cytology includes the study and interpretation of the features cells exfoliated from the oral mucosa. It was first developed by Papanicolaou and Traut (1941) (7) who studied the cells from precancerous and cancerous lesions of the cervical mucosa in an attempt to diagnose cervical neoplasia. After that, oral cytology was used for the diagnosis of oral cavity diseases (8). The cytological samples can be studied by taking different approaches but the researchers should always make efforts to understand the tissue changes (3, 9, 10), by qPCR.

The cytological smears are easily collected. They are practical and low cost (2) and may provide information about epithelial cells (1) since the cells of more superficial layers of the epithelium store information about the changes occurred during the cellular maturation process (6). Therefore, cytology may be helpful in detecting early changes in smokers and in biomonitoring (6). Thus, it can be used as a non-invasive collection technique to study genes expressions in this type of patients and other risk groups.

The aim of this study was to find the best RNA extraction technique, including minimization of time and cost to obtain samples of oral lesions utilized in qPCR.

Real-time technology (qPCR) has significantly extended the use and scope of PCR assays (11) and it is considered the gold standard technique to study transcript levels of a specific set of genes. Therefore, qPCR is a technique with high demand that has to assure high reliability, sensitivity and reproducibility [12]. RNA quantification of the extracted samples is considered an important step (13), because it is necessary to use the same RNA amounts in an analysis when comparing different samples. Naturally, it should be accurately quantified.

It is important to note that the RNA sample obtained by cytology is partially degraded and it creates low-levels of transcripts detection (13). Furthermore, significant amounts of RNases are present in the saliva (14) and RNA degrades markedly *in vivo*, owing to the natural regulation of mRNAs in response to environmental stimuli (13). Therefore, the process should be executed carefully to minimize sample degradation, before extraction and analysis (5).

A280/A260 istog pripravka RNK (15). Zato je u ovom istraživanju korištena voda bez nukleaza tretirana DEPC-om koja se dodavala otopljenom RNK svim tehnikama.

Najveća prosječna koncentracija zabilježena je u skupini Trizol® nakon čega je slijedila skupina Direct-zol™. Najniža prosječna koncentracija izmjerena je u skupini RNeasy. Otapanje i ekstrakcija Trizolom® razvijeni su kao opći postupak za deproteinizaciju RNK. Taj je postupak posebno korištan kada stanice ili tkiva imaju znatnu količinu endogenih RNaza ili ako nije moguće odvajanje citoplazmatskoga RNK iz nuklearnog RNK (16).

Trizol® je monofazna otopina fenola i gvanidin-izotiocijanata koja istodobno otapa biološki materijal i denaturira proteine. Nakon otapanja, dodatak kloroforma uzrokuje odvajanje faza – protein ekstrahira u organsku fazu, DNK se otapa u međufazi, a RNK ostaje u vodenoj fazi (16).

Proizvođač navodi da je Direct-zol™ RNA Miniprep sustav za izolaciju kvalitetnoga RNK s pomoću reagensa Trizola brzom rotacijom kolona, i može iz ljudskih epitelnih stanica izvući čisti RNK. U ovom istraživanju prosječno dobivena koncentracija RNK bila je dovoljna za provedbu PCR-a, a uzorci RNK imali su visoku kvalitetu.

Prema navodima proizvođača, sustav RNeasy Mini Kit omogućuje pročišćavanje RNK iz male količine početnog materijala. Taj je set korišten u istraživanjima u kojima se primjenjuje qPCR (3,4). No mala ukupna količina RNK koja se dobije onemogućuje ispitivanje nekoliko gena u istom uzorku.

Omjer apsorpcije pri valnim duljinama od 260 i 280 nm (omjer A260/A280) često se rabi za procjenu čistoće pripravaka RNK i DNK. Maksimalna apsorpcija nukleinskih kiselina i proteina upravo je pri 260 i 280 µm. Omjer A260/A280 koristi se kao mjera čistoće i općenito je prihvaćeno da *čisti* RNK ima omjer oko 2,0. Slično se, kao pokazatelj onečišćenja, koristimo apsorpcijom pri valnoj duljini od 230 nm, pa je omjer A260/A230, pri čemu se vrijednosti omjera za *čisti* RNK kreću u rasponu od 2,0 do 2,2 (15). U ovom istraživanju zabilježen je prosječni omjer A260/A280 u skupini RNeasy i bio je vrlo blizu 2 (1,939), ali prosjek u skupinama Direct-zol™ i Trizol® bio je manji – 1,8693 i 1,77. Prosječan omjer A260/A230 najbliži iznosu 2 zabilježen je u skupini Direct-zol™ (1,69), a prosjek u skupinama Trizol® i RNeasy bio je manji – 0,75 i 0,166. Dakle, najvišu čistoću RNK s obzirom na dva omjera apsorpcije, imala je skupina Direct-zol™, a slijedile su je skupine RNeasy i Trizol®.

Sustavi Direct-zol™ RNK Miniprep i RNeasy Mini Kit, metode su kojima se koriste kolone za pročišćavanje dok uklanjaju otpad i onečišćenja kroz precipitate u kojima se koriste alkohol i silicij. To može biti ograničenje jer velika količina citoloških ostataka može u cijelosti ili djelomice začepiti membranu. S druge strane, metoda Trizol® ima veliko ograničenje jer uvelike ovisi o pipetiranju zbog vodene faze koju izravno uklanja operater.

Jedna od prednosti metode Direct-zol™ jest uključivanje tretiranja DNazom. Važno je istaknuti da je uzorak RNK tretiran DNazom kako bi se spriječilo onečišćenje DNK-om (13).

Želimo istaknuti i neke rezultate – koncentracija RNK bila je veća kod nepušača i bivših pušača negoli kod pušača.

The pH and ionic strength of the solutions used for spectrophotometric analysis can influence substantially the qualitative and quantitative determinations of nucleic acids. For example, the RNA solubilized water can alter the A260/280 ratio of the same RNA preparation (15). In this study, DEPC-treated nuclease-free water to solubilized RNA was used in all techniques.

The highest average concentration was observed in the Trizol® group, followed by the Direct-zol™ group. The lowest average concentration in RNeasy group was observed. Trizol® solubilization and extraction method was developed as a general method for deproteinizing of the RNA. This method is particularly advantageous in situations where cells or tissues have a considerable amount of endogenous RNases or when the separation of cytoplasmic RNA from nuclear RNA is unpractical (16).

Trizol® is a monophasic solution of phenol and guanidinium isothiocyanate which, simultaneously, solubilizes biological material and denatures the protein. After the solubilization, the addition of chloroform causes the phase separation, where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase (16).

According to the manufacturer, Direct-zol™ RNA Miniprep system is a quick spin column purification of high-quality total RNA directly from Trizol® and it can extract RNA purified from human epithelial cells. In this study, an average amount of RNA concentration suitable for PCR studies was observed and RNA samples are of high quality.

According to the manufacturer, the RNeasy Kit is designed to purify RNA from small amounts of starting material. This kit has been used in studies of qPCR (3, 4). However, the low amount of total RNA obtained hinders the implementation of studies of several genes in the same sample.

The ratio of absorbance at 260 and 280nm (the A260/280 ratio) is frequently used to assess the purity of RNA and DNA preparations. The maximum absorbance for nucleic acids and proteins is 260 and 280 nm, respectively. The A260/A280 ratio has been used as a measure of purity. Generally, it is accepted as "pure" for RNA the ratio about 2.0. Similarly, another contamination absorbance has 230nm and the A260/A230 ratio values for "pure" are in the range of 2.0-2.2 [15]. In this study, the average A260/A280 ratio observed in the RNeasy group was very close to 2 (1.939), but the average of Direct-zoL™ and Trizol® groups was lower: 1.8693 and 1.77, respectively. The average A260/A230 ratio observed in the Direct-zol™ group was the nearest to 2 (1.69), and the average of the Trizol® and RNeasy group was lower: 0.75 and 0.166, respectively. Therefore, it was observed that the RNA Direct-zol™ group has the highest purity, followed by RNeasy and Trizol® groups, considering the two ratios.

The Direct-zol™ RNA Miniprep system and RNeasy minikit are methods which use column purification to eliminate waste and contaminants through precipitation using alcohol in the silica that is inside the column. This may be a limitation, since much cytology debris may obstruct the membrane either partially or completely. On the other hand, the Trizol® method has certain limitations, since it depends greatly on the pipetting due to the aqueous phase when it is directly removed by the operator.

Veći stupanj keratinizacije bukalne sluznice pušača (17) može biti povezan s nižom koncentracijom RNK pronađenom u trima korištenim metodama.

Budući da je u skupini RNeasy omjer A260/A280 bio veći kod nepušača negoli kod pušača, potrebna su istraživanja koja bi to razjasnila.

Prema navodima proizvođača, reagens Trizol koji se upotrebljava u Trizolu® i Direct-zolu™, uglavnom se sastoji od fenola. Može biti otrovan ako se proguta, u doticaju s kožom, ako se udahne izaziva teške opekline kože i ozljede oka, nadražuje dišne puteve, sumnja se da uzrokuje genetska oštećenja, oštećuje organe nakon duljeg ili ponavljanog izlaganja te dugotrajno šteti i onečišćuje vodeni okoliš. Važno je napomenuti da s njime treba oprezno raditi, te je obvezno korištenje zaštitne opreme i pravilno odlaganje. RNeasy Mini Kit ne sadržava fenol.

Zaključak

Ovo je preliminarno istraživanje i potrebna su dodatna istraživanja kako bi se dobilo više informacija o kvaliteti uzoraka. S obzirom na dva korištena omjera apsorpcije svjetlosti, u skupini Direct-zol™ zabilježena je najveća čistoća, a pratile su je skupine RNeasy i Trizol®. Uzimajući u obzir sve aspekte, tj. koncentraciju, čistoću i trajanje postupka, skupina Direct-zol® postigla je najbolje rezultate. Osim toga, taj se preparat jednostavno i brzo primjenjuje. No Trizol® je jeftiniji.

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One of the advantages of Direct-zol™ kit is the inclusion of the treatment with DNAse. It is important to note that the RNA sample has been treated with DNase to avoid genomic-DNA contamination (13).

Furthermore, we would like to highlight some findings: RNA concentration was higher in non-smoking and ex-smokers than smokers. Higher keratinization of buccal mucosa of smokers (17) may be related to RNA lower concentration found in the three methods which were evaluated.

Considering that A260/A280 ratio was higher in non-smokers than in smokers belonging to RNeasy group, further studies to clarify these findings are needed.

According to the manufacturer, the Trizol reagent used in the kits Trizol® and Direct-zolTM is mainly composed of phenol. It may be toxic if swallowed. In contact with skin or if inhaled it may cause severe skin burns and eye damage, respiratory irritation. Besides, it is suspected of causing genetic defects, may cause damage to organs through prolonged or repeated exposure and is harmful to aquatic life with long lasting effects. It is noteworthy that it should be used carefully and disposed properly. Personal protective equipment is recommended. The RNeasy minikit does not contain phenol.

Conclusion

This is a preliminary study and additional studies are necessary to give more information about the quality of the samples. Direct-zolTM group had the highest purity, followed by RNeasy and Trizol® groups, considering the two ratios used. Considering the aspects such as concentration, purity and time spent in the procedures, Direct-zol® group obtained the best results. Moreover, this kit can be easily implemented, since its use does not require any special training. Finally, Trizol® is a lower- cost option.

Conflict of interest

The authors declare no conflict of interest.

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Abstract

Objective of work: The aim of this study was to compare three methods of RNA extraction for molecular analysis of oral cytology to establish the best technique, considering its concentration and purity for molecular tests of oral lesions such as real-time reverse transcriptase reaction. **Material and methods:** The sample included exfoliative cytology from the oral cavity mucosa of patients with no visible clinical changes, using Orcellex Rovers Brush®. The extraction of total RNA was performed using the following three techniques: 30 samples were extracted by Trizol® technique, 30 by the Direct-zol™ RNA Miniprep system and 30 by the RNeasy mini Kit. The absorbance was measured by spectrophotometer to estimate the purity. The estimated RNA concentration was obtained by multiplying the value of A260 (ng/mL) by 40. Statistical analysis of the obtained data was performed using GraphPad Prism 5.03 software with Student t, analysis of variance and Bonferroni tests, considering $p \leq 0.05$. **Results:** Trizol® group revealed higher average concentration, followed by Direct-zol™ and Rneasy group. It was observed that the RNA Direct-zol™ group had the highest purity, followed by RNeasy and Trizol® groups, allowing for the two ratios. **Conclusion:** Considering all aspects, concentration, purity and time spent in the procedures, the Direct-zol™ group showed the best results.

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