THE EFFECT OF HYPOCHLORITE ON SKIN'S MRNA MARKERS IN CONTACT SAMPLES

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ABSTRACT

Background: the identification of human skin through mRNA-based profiling is useful in forensic investigations. A deliberate attempt to remove biological material, through using a variety of cleaning agents, is a problem faced by forensic scientists. Limited information exists on the effect of Hypochlorite (HOCl); cleaning agent; on human skin mRNA markers. **Aim**: This study evaluated the sensitivity of specific human genetic skin markers; LCE2D and CCL27 to different HOCl concentrations by using Real-time qPCR. **Method**: sixty four middle aged healthy volunteers (32 males and 32 females) were included in the study.**Results**: HOCl had a pronounced negative effect. The expression of the studied markers was decreased to the quarter of its normal value when the touch samples were submerged in concentrated HOCl solution.
Conclusion: sample with reduced RNA expressions might be soaked or adulterated before testing and other tests should be used to help in cellular origin determination
Keywords: forensic investigation, touch samples, mRNA skin markers, LCE2D, CCL27, hypochlorite, Real-time qPCR.

INTRODUCTION

Hypochlorite (HOCl) bleach is used world wide in homes and health care facilities. It has anti-inflammatory and antimicrobial effects. It is considered as an oxidant in addition, it is interfering with cellular signaling process**.** Diluted HOCl solutions are used in cleaning skin surface **(Rutalaet al., 1998; Huang et al., 2009).**

The outer epithelial layer of human skin is continuously developed and shed to leave epithelial cells as biological evidence. Many methods are used to identify the cellular origin of biological stains (**Goray et al., 2010).** In forensic casework, epithelial identification methodologies are not well established (**Hanson et al., 2012)**. Forensic laboratories use histological examination, UV-light, Polilight1, messenger RNA (mRNA), microRNAs (miRNAs) in identifying and differentiating epithelial stains from other biological stains (**Haas et al., 2015).**

Late cornified envelope (LCE) gene cluster is one of putative skin-specific markers. (LCE) is part of the epidermal differentiation complex and has three groups (1, 2 and 3). The first two groups are dominant in skin (**Ballantyne, 2013; Lindenbergh et al., 2013).** Chemokine (C-C motif) ligand 27 (CCL27 gene) is found in keratinocytes and has a role in skin inflammation (**Jackson et al., 2005)**. LCE2D and CCL27 can positively identify the epithelial origin of biological stains.

Cleaning agents could be used to destruct the biological stains and degrade its DNA and RNA (**Schwark et al., 2012; Visser et al., 2011).**

AIM OF THE STUDY

This study aimed to demonstrate the effect of different HOCl concentrations on mRNA expression; LCE2D and CCL27; in touch DNA samples. This effect can determine the value of using mRNA markers in identifying epithelial origin of biological stains.

METHODOLOGY

After signing an informed consent, sixty four middle aged healthy volunteers (32 males and 32 females) at the biochemistry department, faculty of medicine, Cairo University, were included in the study for one month duration. Every participant touched a slide with right index and middle fingers with 1 minute contact time.

I- Washing solutions, collection and preservation of samples:

To evaluate the effect of HOCl on the expression of skin RNA markers, the touch samples were merely submerged for 30 seconds with different concentrations of HOCl solution. These solutions were:

- Full concentrated HOCl solution: (5.25% Sodium hypochlorite, EPA Reg. No. 5813-1, Oakland, California) was used as to prepare full concentrated solution according to manufacturer's instructions.
- Half concentrated HOCl solution was prepared from the previous one.
- Distilled water at room temperature

Slides were divided into 4 groups; group (1) (control) was kept without washing, group (2) was submerged in distilled water, group (3) was submerged in half concentrated HOCl solution and group (4) was submerged in full concentrated HOCl solution. Slides were kept in a dry dark place (cartoon box) at room temperature 24°C for 2 hours. Therefore, the effect of light and humidity were minimized. Sterile dry cotton swabs were used to collect shed cells.

II. RNA extraction:

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen Inc); with DNase step to remove DNA, following the manufacturer's protocol extracted RNA was quantified by spectrophotometry. The RNA integrity was assessed using agarose gel electrophoresis and ethidium bromide staining.

III. Conversion of RNA into cDNA:

The total RNA (0.5–2 µg) was used for cDNA conversion using high capacity cDNA reverse transcription kit (K1621, Fermentas, USA) according to manufacture instruction.

IV. Quantitative real time PCR (qRT-PCR):

Real-time qPCR amplification and analysis were performed using an Applied Bio system with software version 3.1 (StepOne™, USA) using intron spanning primers with 0 duration controls.

The real time- PCR reaction mixture was 50µl and consisted of 25 µl SYBR Green Mix (2x), 0.5 µl kidney cDNA, 2 µl primer pair mix (5 pmol/ µl each primer), 22.5 µl H_2O. The used primers' sequences were as follows

	Primer sequence
LCE2D	**Forward: 5′ - TCTGTGCTTTTGCATGTGAC -3′** **Reverse: 5′- GGACCACAGCAGGAAGAGAC -3′**
CCL27	**Forward: 5′-AGCACTGCCTGCTGTACTCA -3′** **Reverse: 5′-TTCAGCCCATTTTCCTTAGC -3′**
Glyceraldehyde 3-phosphate dehydrogenase GAPDH	**Forward: 5′- CTCCCATTCTTCCACCTTTG-3′** **Reverse: 5′- CTTGCTCTCAGTATCCTTGC-3′**

PCR program's set up was; 2 min. at 50°C (1cycle), 10 min. at 95°C (1cycle), 15 sec at 95°C → 30 sec at 60°C → 30 sec at 72°C (40 cycles) and 10 min. at 72°C (1cycle). The level of expression of each target mRNA was normalized relative to the expression of GAPDH (internal housekeeping gene) in that sample using the ΔCt method. Relative differences in mRNA expression among groups were determined using the comparative Ct (ΔΔCt) method.

Statistical methods:

Data were coded and analyzed using the Statistical Package for Social Sciences (SPSS) version 21. Data was described in terms of mean ± standard deviation. Comparison of quantitative variables was done using t-test and repeated Measures ANOVA P value less than 0.05 was considered statistically significant.

RESULTS

Sixty four participants (32males and 32 females) participated in this study. Their mean age was 31.6±4.6 years. LCC27and LCE2D mRNA expression levels were 1.026±0.05 and 1.127±0.4 respectively. Regarding mRNA expression levels, there was no significant difference between the male and female studied groups. Table (1).

Table (1): Gender, age, LCC27 and LCE2D expression among the studied groups

Mean ±SD	**Male**	**Female**	**P value**
Age (31.6±4.6)	**32.80±4.89**	**31.00±4.85**	**0.420**
LCE2D (1.026±0.05)	**1.02±0.7**	**1±0.5**	**0.748**
LCC27 (1.127±0.4)	**1.2±0.4**	**1±0.4**	**0.306**

Submerging the samples in water or different HOCl concentrations decreased LCE2D and LCC27 expressions. Table (2) fig.1. The highest LCE2D and LCC27 expressions (0.52±0.86 and 0.6±0.16) were found in samples submerged in water.

Table (2): The effects of different concentrations of HOCl bleach on the expression of LCC27 and LCE2D in the studied samples.

Mean ±SD	**Air**	**H2O**	**Half diluted**	**Full concentrated**
LCE2D	**31.6±4.6**	**0.52±0.86**	**0.32±0.11**	**0.22±0.12**
LCC27	**1.026±0.05**	**0.6±0.16**	**0.43±0.58**	**0.26±0.63**

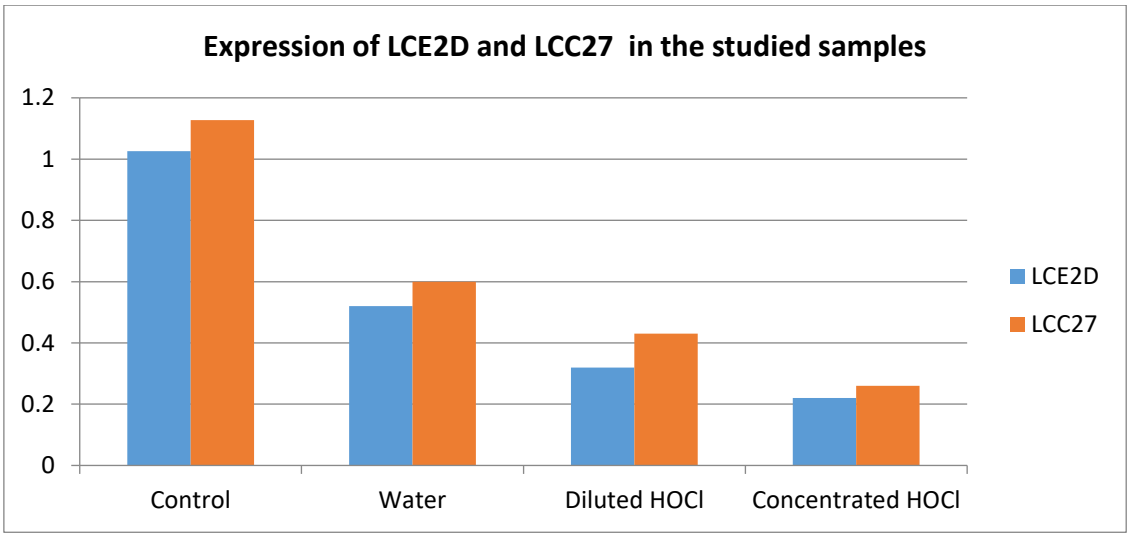

Figure (1): The expression of skin LCE2D and LCC27 marker after using different concentrations of chlorinated bleach.

There was significant difference in LCE2D expression and LCC27 expression in different submerged media (P value<0.05).

A one–way repeated measured analysis of variances (ANOVA) indicated a significant evidence to accept that the submerged media affected the expression levels of LCE2D (Wilks' Lambda 0.028, F=7.3, P <0.05) and expression levels of LCC27 (Wilks' Lambda 0.081, F=35.9, P <0.05). In close relation, the difference between these pairwise groups (1,2/1,3/1,4/2,3/2,4/3,4) were significant (P value <0.05).

DISCUSSION

Forensic genetics is an alternative approach for identifying the cellular origin of crime scene's biological stains. Tissue-specific messenger RNA (mRNA) markers are used to detect forensically relevant human body fluids (**Visser et al., 2011).** Skin-specific mRNA markers, late cornified envelope gene 2D (LCE2D) and chemokine (c-c motif) ligand 27 (CCL27) are considered as highly sensitive and abundant biomarkers (**Hanson et al., 2012; Ballantyne, 2013).**

Cleaning products can damage the biological samples and make their identification through the currently used forensic analysis procedures difficult (**Castelló et al., 2012).**

Multiple concentrations of HOCl solution, were used to remove biological stains like fingerprints from surfaces (**Thomasma & Foran, 2013),** were tested to detect the sensitivity of skin mRNA markers to this agent. The present results showed that submerging samples in HOCl solution, greatly affect the two studied skin mRNA markers; (LCE2D and LCC27). With reducing the concentration of HOCl, the expression of these markers increased and presented in values equal to third of their normal values. Moreover, the pure water affected the studied skin markers. These data declared that soaking the crime scene's biological stains in pure water with or without HOCl significantly decreased the expression of mRNA skin markers, (LCE2D and LCC27), and making their detection difficult.

Ruissen et al. (1998) found that the keratinocyte gene expression was induced in vitro after application of anionic detergents. These findings were

due to differentiation switching from the normal one to hyperproliferative/ inflammatory one in the living keratinocyte. They also demonstrated that isolated RNA was degraded due to the detergents' cytotoxicity and they couldn't isolate detectable amount of RNA by northern blots technique. They concluded that detergents' cytotoxicity could be graded according to the amount of isolated intact RNA. Regarding our study HOCl is considered as moderate cytotoxic detergents.

We were in the same line with a study conducted by **Harris et al. (2006),** who found that the cleaning agent insignificantly decreased the quality of DNA profiles isolated from washed blood soaked materials.

Wood et al. (1996) stated that the expression of mRNA has been enhanced in skin samples treated with irritant substance.

CONCLUSION

Expression of skin's markers was reduced after HOCl treatment. Hence, samples with reduced RNA expressions might be soaked or adulterated before tested, so other tests should be used to help in cellular origin determination before excluding skin source e.g. micro RNA, immune-based assays and DNA methylation (**Jennifer & Fei, 2013).**

In our study there was a lack of diversity in the type of substrates tested. However, glass slides were used as an example for smooth non-porous surfaces. More researches using non-porous substrates is needed to detect difference in skin cells attachment and release from them. Qiagen Mini Kit was used to recover RNA. However, this study focused mainly on identifying HOCl effect on skin-specific RNA markers.

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تأثير مادة حمض الهيبوكلوريت علي مستقبلات الحمض النووي الريبوسومي للجلد في عينات الأتصال

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الملخص العربي

معلومات اساسيه: يعتبر الأستعراف علي أنسجه جلد الأنسان من خلال الحمض النووي الريبوسومي من عمليات الفحص المفيدة في الطب الشرعي و يعتبر التعمد في إزاله المواد البيولوجيه عن طريق المنظفات من المشاكل التي تواجه علماء الطب الشرعي. كما توجد معلومات ضئيلة عن تأثير حمض الهيبوكلوريت (مادة منظفة) علي دلالات الحمض النووي الريبوسومي لجلد الأنسان .

الهدف: هذا البحث يقيم حساسيه الدلالات الجينيه المتخصصه لجلد الانسان LCE2D وCCL27 لتركيزات حمض الهيبوكلوريت المختلفه بواسطه R-T qPCR

النهج: أربعة وستون متطوع متوسطي العمر وبصحه جيدة من (الذكور ٣٢، ٣٢ الاناث) مضمن في البحث.

النتيجة: وجد أن حمض الهيبوكلوريت له تأثير سلبي ملحوظ. وان التعبيرالجيني للدلالات الجينية تحت الفحص قد قلت الي ربع مقدارها الطبيعي عند انغماس العينات ملموسة السطح في حمض الهيبوكلوريت المركز.

الخلاصة: إن العينات التي بها انخفاض في التعبير الجيني للحمض النووي الريبوسومي من الممكن ان تكون قد أنغمست أو غشت قبل عمليه الأختبار وأن ثمه أختبارات اخري يجب أن تستخدم لتحديد المصدر الخلوي.

الكلمات الدالة: الحمض النووي الريبوسومي-عمليات الفحص- حمض الهيبوكلوريت-LCE2D -CCL27 -R-T qPCR .