



BRIEF REPORT

REVISED Characterization of buccal cell DNA after exposure to azo compounds: a cross-sectional study [version 2; peer review: 2 approved]

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Abstract

Background: Azo compounds, containing naphthol and diazonium salts, are synthetic dyes widely used in the batik industry. Azo compounds are considered toxic when they are exposed to human tissue. The purpose of this study was to analyze buccal cell DNA exposed to azo compounds in batik workers.

Methods: A cross-sectional study involving 20 male subjects divided into two groups (n=10 group), namely azo-exposed and non-exposed (control group). Inclusion criteria were batik workers of the colouring division who have been exposed to azo for at least 5 years. Buccal cells were taken using cytobrush then DNA were isolated from buccal cell. DNA isolation was done by buccal DNA kit, while the purity and concentration of the DNA was determined using spectrophotometer and electrophoresis.

Results: The azo-exposed group revealed higher purity DNA than those in the control group. The purity of the DNA in the azo-exposed group and control group was 0.61 ± 0.93 and 0.21 ± 0.09 , respectively, while the concentration of DNA was of 59.02 and 19.35 ng/UL, respectively. The ratio at 260/280 nm was 1.84-1.94 (azo-exposed) and 1.85-1.92 (control). Principal component analysis using the first principle component (PC1) and second principle component (PC2) could successfully classify subjects in the control and azo-exposed groups.

Conclusion: Characteristics of DNA could be used as an indication of exposure to azo compounds in workers of batik industries.

Keywords

DNA, buccal cell, azo-exposed

Open Peer Review

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Author roles: **Handajani J:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation; **Tabtila U:** Methodology, Project Administration; **Rully Auliawati N:** Investigation, Methodology; **Rohman A:** Conceptualization, Resources, Software, Writing – Review & Editing

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REVISED Amendments from Version 1

We added sentences in the introduction and discussion, also explanation in the method.

Any further responses from the reviewers can be found at the end of the article

Introduction

The oral mucosa is the first defence against particles entering the body. The oral epithelial mucosa functions to protect the body from chemical, microbial, and physical challenges^{1,2}. The buccal epithelium is the thickest region in the squamous stratification epithelium. Keratinization is influenced by endogenous or exogenous factors. Exogenous factors include the use of drugs, nutritional factors, and irritant factors, such as plaque and calculus, artificial teeth, and smoking or exposure to other substances^{3,4}.

The use of azo synthetic dyes and their derivatives, especially those with benzene groups, are increasing in the batik industry^{5,6}. Azo dyes are compounds characterized with one or more azo functional groups (-N=N-), linked to benzene. They are readily reduced to hydrazines and primary amines. The benzene group in azo compounds is difficult to degrade because it takes a long time^{7,8}. Chemicals in the batik industry are known to cause irritation to the skin and eyes, and cause interference with the respiratory system⁸. Azo compounds are also known to be carcinogenic and mutagenic if they are in the environment for a long time, and they are suspected to be a source of disease^{9,10}.

Exposure to synthetic azo dyes, which are continuously inhaled by batik workers, may cause changes in the oral mucosa. Daily exposure to azo dyes needs to be analysed to assess the possibility of the risk of oral cavity abnormalities, although there have been no reports of batik workers that mention oral cavity abnormalities due to azo exposure. Exposure to azo dyes for more than 5 years in batik artisans has been known to significantly increase the frequency of micronuclei, karyolysis, and pyknosis in buccal mucosal epithelial cells¹¹⁻¹³. In addition, exposure to azo dyes significantly increases the expression of cytokeratin 5 and 19 in the buccal mucosa^{14,15}. Although previous studies stated that exposure to azo dyes significantly increased the expression of cytokeratin 5 and 19, but clinically it has not shown changes in the buccal mucosa. Until now, there is limited study on the effects of azo exposure on the buccal mucosa. The results of these studies have not yet explained the changes in buccal cell DNA exposed to azo compounds; therefore, the objective of this study was to evaluate the profile of buccal cell DNA exposed to synthetic azo dyes to determine the possibility of cellular damage.

Methods

Participants

The method was *cross-sectional* to compare subjects exposed and not exposed at the same time. We conducted the study

in batik industries (for exposed group) and non-batik industries (for control group) in Yogyakarta-Indonesia from May to August 2019. The procedure of this study was approved by Research Ethics Committee of the Faculty Dentistry, Universitas Gadjah Mada (Ethical Clearance No.00107/KKEP/FGK-UGM/EC/2019).

Participants of exposed group were from batik industries in Yogyakarta Indonesia whereas participants of the control group were students and staff at the Faculty of Dentistry, Universitas Gadjah Mada, Indonesia. For the exposed group, batik factories were identified from a list online and information about the study was sent to the manager of the factories (letter No. 5189/UN1/FGK/Set.KG1/PT/2019 from Universitas Gadjah Mada to the factories), who allowed the researchers to interview their workers. For the control group, information about this study was sent to students at our university that asked them to participate in the study. All participants agreed to participate by providing written informed consent.

Information collected from the participants were age, past medical and dental history, occupational history, lifestyle (smoking and alcohol consumption) and if they wore a dental apparatus. Oral Hygiene Index-Simplified (OHI-S) were calculated from calculus index (CI(S)) and debris index (DI(S)): OHI-S = CI(S) + DI(S). Interpretation: 0 - 1.2 is good; 1.3 - 3.0 is fair; and 3.0 - 6.0 is poor¹⁶.

Inclusion criteria were aged between 18 and 45 years old (age group most likely to be working), male (to provide continuity among participants), OHI-S status of 'good', worked in colouring batik for a minimum of 5 years (for exposed group), and did not work in coloring batik (for control group).

Study size was calculated according to Notoatmodjo¹⁷

$$n = \frac{Z1 - \alpha/2P(1 - P)}{d}$$

n = number of samples

Z1- α /2 = the Z value at 95% degree of significance is 1.96

P = proportion of subject azo-exposed around 50% (0.5)

d = degree of deviation to population, by 5% (0.05)

Based on the formula, $n = 9.8 \approx 10$. In this study, the number of subjects for each group is 10 participants.

Data collection

Participants were asked to rinse out their mouths first to remove debris in the oral cavity. Buccal epithelial cell harvesting was carried out using the smear method using sterile foam Tipped Swab (Product Code: PW1174, Himedia, India). Swab was done by turning in the direction of at least 360° in the buccal mucosa then put in a microtube. Samples were transported in the microtube with 1x PBS to the lab. Sample collection was carried out at the batik factories for the exposed participants and at the university for the non-exposed participants.

DNA isolation

DNA isolation was done following the protocol from HiPurA™ Buccal DNA Purification Kit (Product Code: MB531; Himedia, India). Briefly, the buccal swab sample was placed into a 2.0 ml microcentrifuge tube, 400 µl of resuspension solution was added, and the tube was centrifuged at 14,000 rpm for 5 minutes. The pellet was discarded and the supernatant was transferred to a new collection tube. 20 µl of Proteinase K solution (20 mg/ml) was added to the tube containing the supernatant, and this was vortexed for 10–15 seconds. 20 µl of RNase A solution (20 mg/ml) was added, and the tube was again vortexed for 10–15 seconds. The sample tubes were incubated for 2 minutes at room temperature (15–25°C).

The lysis reaction was done by added 400 µl of lysis solution to the tube, which was vortexed thoroughly for a few seconds to obtain a homogenous mixture. Samples were incubated at 55°C for 10 minutes. For the binding step, 400 µl of ethanol (96–100%) was added to the lysate, which was then mixed thoroughly by vortexing for 5–10 seconds. The lysate was added to the HiElute Miniprep Spin Column (Capped) and samples were centrifuged at 6,500 x g (10,000 rpm) for 1 minute. The flow-through liquid was discarded, and the procedure was repeated with any remaining lysate. A prewash was performed by adding 500 µl of diluted prewash solution to the column and centrifuged at 6,500 x g (10,000 rpm) for 1 minute. The flow-through liquid was discarded and the same collection tube was re-used with the column.

Subsequently, samples were washed by adding 500 µl of diluted Wash Solution to the column and centrifuged at 12,000–16,000 x g (13,000–16,000 rpm) for 3 minutes to dry the column then the flow-through was discarded and a new uncapped 2.0 ml collection tube was placed in the column. DNA Elution was done by pipetting 150 µl of the Elution Buffer directly onto the column without spilling on the sides. The samples were incubated for 1 minute at room temperature and centrifuged at >6500 x g (10,000rpm) for 1 minute to elute the DNA. Storage of the eluted purified DNA was done at 2–8°C for short-term (24–48 hours) or -20°C for long-term storage.

Evaluation of DNA characteristics

Purity and concentration of DNA buccal cells were characterized using electrophoresis and spectrophotometer. Agarose gel was prepared in concentration of 2%. Agarose (Biotechnology Grade, 1st Base, Singapore; 1 gr) was added to 50 ml

Tris/Borate/EDTA (TBE) buffer, then put in microwave for around 2 minutes until completely dissolved. After agarose solution was cooled to about 50°C (around 5 minutes), this was poured into a gel tray with the well comb in place and then let sit at room temperature for around 20 minutes until agarose gel had completely solidified. Loading dye 1µl was prepared on parafilm then added 5 µl DNA, aspirated in the micropipette, put into the agarose well. Agarose gel was placed into the gel box (electrophoresis unit), then filled gel box with 1x TBE until the gel was covered. Electrophoresis was run at 100 mA and then visualized with Florosafe DNA Stain (Genetika Science, PT. Genetika Science Indonesia) using a UV transilluminator. Concentration of DNA buccal cell were measured using a spectrophotometer. Purity of DNA was analysed using spectrophotometer at 280 and 260/280 nm.

Data analysis

Data analysis was performed using stat Shapiro-Wilk and Levene's test, to see if the data were normal and homogenous. Mann-Whitney U test was used to describe the comparison between azo-exposed group and the control group. Statistical measurement was performed using IBM SPSS Statistics v22. The classification of the azo-exposed group and control group was performed using chemometrics of principal component analysis (PCA) using Minitab version 17. P<0.05 was taken as significant.

Results

Characteristics of study participants are described in [Table 1](#).

The presence of high-molecular weight DNA was evaluated by gel electrophoresis and visualized using a UV illuminator. [Figure 1](#) shows that bands for high-molecular weight DNA only appeared for the azo-exposed group, but it did not appear in control group. There was no significant difference between groups for the purity of the DNA at $A_{280\text{ nm}}$ ($p=0.076$), ratio $A_{260/280}$ ($p=0.718$), or the concentration of the DNA ($p=0.076$) ([Table 2](#)).

PCA could successfully classify participants in azo-exposed and control groups. The score plot for the first principal components (PC1) and second principle component (PC2) is shown in [Figure 2](#). In addition, the loading plot for the evaluation of variables contributing to the separation is shown in [Figure 3](#). The concentration of DNA contributed the most in PCA, as it was the farthest variable from the initial points (0.0).

Table 1. Demographics of azo-exposed batik workers and control group (non-exposed).

Group	Total, n	Male gender, n	Mean age, years	Oral Hygiene Index-Simplified rating of 'good', n	Smoking, n	Alcohol consumption, n	Wearing dental apparatus
Azo-exposed	10	10	27.6	10	3	0	0
Control	10	10	18.1	10	2	0	0

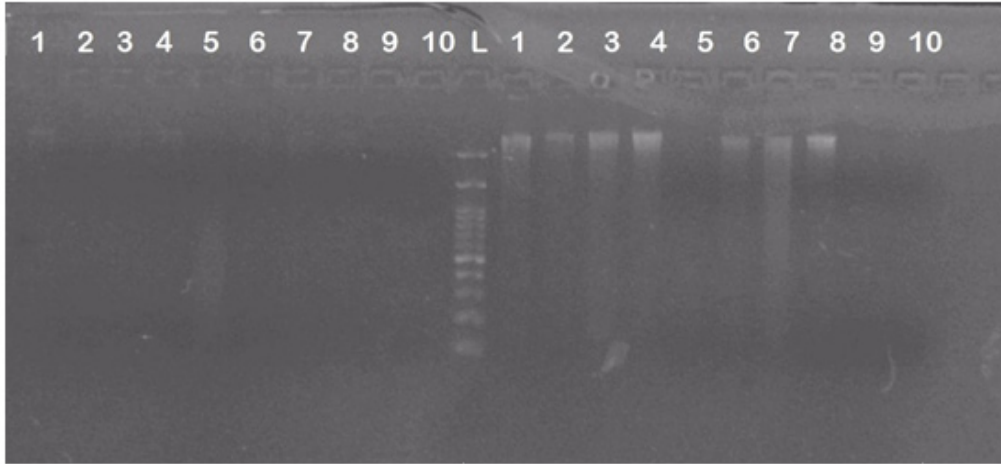


Figure 1. DNA electrophoresis obtained from buccal epithelial cell control (left 1–10) and azo-exposed (right 1–10) from ladder (L).

Table 2. Mean and standard deviation of the purity and concentration of buccal cell DNA from azo-exposed and control group.

Group	n	Purity of DNA (optical density)		Concentration of DNA (ng/uL)
		A ₂₈₀	A _{260/280} (range min-max)	
Azo-exposed	10	0.61 ± 0.93	1.89 ± 0.07 (1.84–2.07)	59.02 ± 87.08
Control	10	0.21 ± 0.09	1.89 ± 0.05 (1.85–1.92)	19.35 ± 4.41

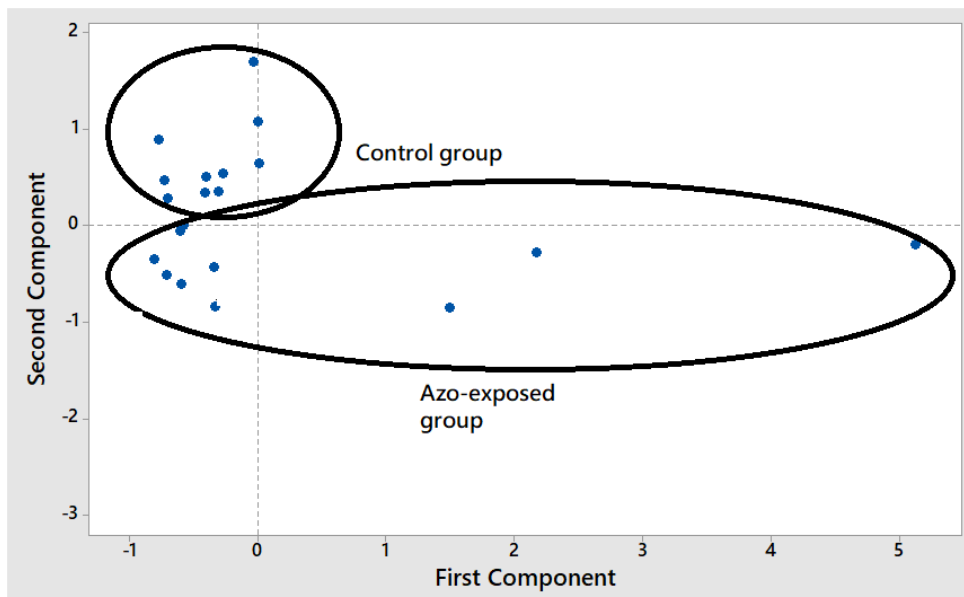


Figure 2. Score plot for the first principal components (PC1) and second principle component (PC2) for participants in the azo-exposed group and control group.

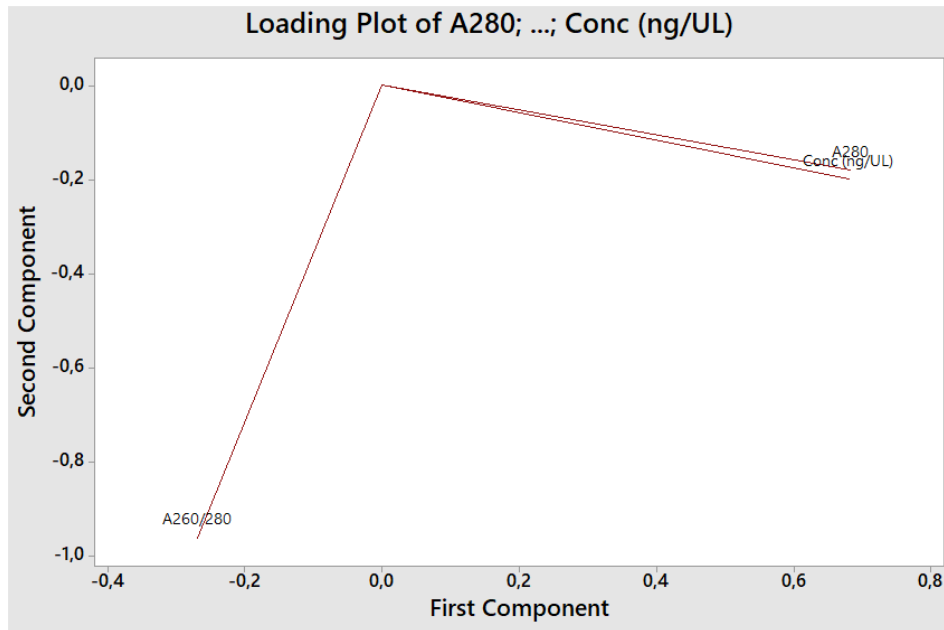


Figure 3. Loading plot of principal component analysis using the purity of DNA at 280 nm, the concentration of DNA, and the ratio of absorbance values at 280 and 260 nm ($A_{280/260 \text{ nm}}$).

Discussion

The method of this study was done using exfoliative buccal epithelial cells by swab tip or cytobrush then purity and concentration of the DNA was analysed. The exfoliative method was non-invasive. One of the important procedures in the study for DNA extraction was the collection method of the sample. According to Mulot *et al.*¹⁸, cytology brushes (cytobrush) are the most appropriate method and provide good quality cell collection compared to mouthwashes, swabs, or collected from saliva.

In the present study, DNA electrophoresis revealed a band for high-molecular weight DNA in azo-exposed group only (Figure 1). This result indicated that the concentration of the DNA from buccal epithelial cells in azo-exposed group was higher than controls. However, we noticed that not all samples in the azo-exposed group revealed a band. This may be because of the low concentration of DNA in the collected buccal epithelial cells. Another possibility, azo may cause DNA breakage in the human cell. In accordance with previous study, the component azo dye Disperse Orange 1 may cause apoptosis and DNA breakage in HepG2 cell derived from human hepatoma¹⁹.

This result was supported by our spectrophotometer measurements (Table 2), showing that DNA concentration in the control group was lower than in the azo-exposed group. DNA quality may have been affected by collection and isolation methods. This result showed the mean OD 260/280 ratio was 1.89 both in the azo-exposed and control groups, which indicates that the bulk of the proteins were removed successfully.

According Desjardins and Conklin that pure nucleic acids typically was in yield a 260/280 ratio of ~1.8 for DNA²⁰.

The standard deviation for the purity of the DNA at A280 nm and the concentration of the DNA (Table 2) in azo-exposed groups was higher than the mean. This indicates that the purity of the DNA from azo-exposed participants varied, which may be due to exposure of azo that has induced DNA damage. According to Ferraz *et al.*¹⁹, the azo dye, Disperse Orange 1, which is used in textiles, induces a frameshift mutation and cytotoxic effect in the human hepatoma cell line HepG2. Mutagenicity was shown by enhanced nitroreductase and o-acetyltransferase, which are important enzymes in mutagenicity. This result was also supported by a previous study that showed that azo dye exposure increases the number of micronuclei, karyolysis, pyknosis, and expression of cytokeratin 5 and 19 in oral epithelial cells¹¹⁻¹⁵. However, these results have not yet revealed how the mechanism of DNA damage occurs in oral epithelial cells due to azo exposure.

In order to classify participants into azo-exposed and control groups, principal component analysis (PCA) was used. PCA is capable of projecting the initial variable data in reduced dimensions defined by principal components (PCs). The value corresponding to the PC is known as score plot^{21,22}. PCA was done in this study using three variables, namely the purity of DNA at 280 nm, the concentration of DNA, and the ratio of absorbance values at 280 and 260 nm ($A_{280/260 \text{ nm}}$). Our results showed that the azo-exposed group could be separated successfully and easily differentiated from control group using PC1 and PC2 score plots (Figure 2). The loading plot of PCA was

performed to evaluate the variables having the most significant contribution to the separation and classification of participants as azo-exposed and controls. The loading plot can explain the projection of variables used during PCA in the same plane as the score plot²³. The absolute value of loading in the variables explains the importance of the contribution of each region. Therefore, the further the variables are from the origin of the variable point, the larger the contribution of that variable to the PCA model^{24,25}. The results of the loading plot indicated that all three variables made a significant contribution to the PCA model.

Conclusion

Buccal cell DNA of batik workers exposed to azo compounds had higher purity of DNA, concentration of DNA and absorbance ratio at 260/280 than buccal cell DNA of controls (not exposed to azo compounds). Principal component analysis, based on score plot, could successfully classify participants as controls and azo-exposed individuals. The characteristics of DNA

could be used as an indication of exposure to azo compounds in workers in batik industries.

Data availability

Underlying data

Figshare: Raw data-Dana Masyarakat 2019, <https://doi.org/10.6084/m9.figshare.12733055.v5>²⁶.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license \(CC-BY 4.0\)](https://creativecommons.org/licenses/by/4.0/).

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Open Peer Review

Current Peer Review Status:  

Version 2

Reviewer Report 22 September 2020

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Boy M. Bachtiar 

Department of Oral Biology and Oral Science Research Center, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia

In general, the author's response, in my opinion, can be accepted.

My concern is only regarding the DNA concentration that had been put into the agarose well. According to the author's response, they put the DNA in volume of 1uL into the agarose well. However, it is still not clear how much DNA concentration has been in 1uL (volume).

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 04 September 2020

<https://doi.org/10.5256/f1000research.28470.r70435>

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Boy M. Bachtiar 

Department of Oral Biology and Oral Science Research Center, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia

Title: I suggest "Risk assessment of DNA damage exposed to azo compound"

Introduction

- I suggest, in the introduction section, the author needs to write a strong background in this study. Please add the fundamental reason behind it. For example: whether the author has observed/found that the population (batik worker) were consisting of those with and without a "clinical symptom" of cellular damage.
- Therefore, the objective of this study was to compare the profile of buccal cell DNA exposed to synthetic azo dyes between the batik worker with and without clinical symptoms of cellular damage.

Methods

- I think this study did not need to use control of those who come from not exposed subjects.
- There is no explanation of the concentration of DNA that was put into the agarose well?

Results

- It would be better if the characteristics of study participants incorporated with pictures showing the buccal mucosa of participants with and without clinical symptoms.

Discussion

In general, this section needs to be revised. Please do not repeat the result but discuss it to answer the research question (this needs to clearly explain in the introduction section).

Specific questions:

"This result was supported by our spectrophotometer measurements (Table 2), showing that DNA concentration in the control group was lower than in the azo-exposed group. DNA quality may have been affected by collection and isolation methods. This result showed the mean OD 260/280 ratio was 1.89 both in the azo-exposed and control groups, which indicates that the bulk of the proteins were removed successfully."

- In the second paragraph, "DNA electrophoresis revealed a band for high-molecular weight DNA in azo-exposed group", indicating the DNA concentration was higher in BEC in azo exposed people. What does it mean? I only show the same band derived from either group. How much of the DNA concentration was added into the well of agarose?
- Similarly, "This result was also supported by a previous study that showed that azo dye exposure increases the number of micronuclei.....,However, the author needs to explain what is the obtained data they have to support this statement.
- Also, "not all samples in the azo-exposed group revealed a band. The author argues that the unrevealed band could be due to low concentration of DNA in the collected buccal epithelial cells". I suggest, that they need to explain this result with the possibility of the relationship between the unrevealed DNA and azo exposure.

Conclusion

- Please revise the conclusion. It should be related to this study's aim. Particularly, what does the author mean with the "characteristics of DNA"? It could be used as indication (indicator?) of clinical symptom of azo exposure? The meaning is not clear.

References

- Some references (no 11, 12, and 13) can be searched. Please use references that have been indexed in database.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 12 Sep 2020

Juni Handajani, Universitas Gadjah Mada, Yogyakarta, Indonesia

Title: I suggest "Risk assessment of DNA damage exposed to azo compound"

Answer:

Thank you for your suggestion.

In this study, we did not know yet the occurrence of DNA damage, but it was to examine the change of DNA characteristics. We need further study to identify possible DNA damage.

Introduction

I suggest, in the introduction section, the author needs to write a strong background in this study. Please add the fundamental reason behind it. For example: whether the author has observed/found that the population (batik worker) were consisting of those with and without a "clinical symptom" of cellular damage.

Therefore, the objective of this study was to compare the profile of buccal cell DNA exposed to synthetic azo dyes between the batik worker with and without clinical symptoms of cellular damage.

Answer:

We added in introduction:

Although previous studies stated that exposure to azo dyes significantly increased the expression of cytokeratin 5 and 19, but clinically it has not shown changes in the buccal mucosa. Until now, there is limited study on the effects of azo exposure on the buccal mucosa.

Methods

I think this study did not need to use control of those who come from not exposed subjects.

Answer:

In this study we need control to compare between azo-exposed group and without exposed-azo.

There is no explanation of the concentration of DNA that was put into the agarose well?

Answer:

We added explanation:

Loading dye 1µl was prepared on parafilm then added 5 µl DNA, aspirated in the micropipette, put into the agarose well

Results

It would be better if the characteristics of study participants incorporated with pictures showing the buccal mucosa of participants with and without clinical symptoms

Answer:

We apologize, we did not take pictures of the buccal mucosa each subject. All subject did not show any clinical symptoms.

Discussion

In general, this section needs to be revised. Please do not repeat the result but discuss it to answer the research question (this needs to clearly explain in the introduction section).

Specific questions:

"This result was supported by our spectrophotometer measurements (Table 2), showing that DNA concentration in the control group was lower than in the azo-exposed group. DNA quality may have been affected by collection and isolation methods. This result showed the mean OD 260/280 ratio was 1.89 both in the azo-exposed and control groups, which indicates that the bulk of the proteins were removed successfully."

Answer:

We added explanation:

According Desjardins and Conklin that pure nucleic acids typically was in yield a 260/280 ratio of ~1.8 for DNA.

In the second paragraph, "DNA electrophoresis revealed a band for high-molecular weight DNA in azo-exposed group", indicating the DNA concentration was higher in BEC in azo exposed people. What does it mean? I only show the same band derived from either group. How much of the DNA concentration was added into the well of agarose.

Answer:

The results from DNA electrophoresis showed band in azo-exposed group while band was not seen in control group. When prepared the electrophoresis, we used loading dye to visualize the DNA. We added 5 µl DNA into loading dye. If we compared to DNA ladder, a band indicated that concentration in azo-exposed group is higher than in control group.

Similarly, "This result was also supported by a previous study that showed that azo dye exposure increases the number of micronuclei.....,,....However, the author needs to explain what is the obtained data they have to support this statement.

Answer:

The results of this study have not yet revealed how the mechanism of DNA damage occurs in oral epithelial cells due to azo exposure even previous study showed there was some alteration in the buccal cell e.g. increases the number of micronuclei, karyolysis, pyknosis, and expression of cytokeratin 5 and 19 in the epithelial cell may be due to dye exposure.

Also, "not all samples in the azo-exposed group revealed a band. The author argues that the unrevealed band could be due to low concentration of DNA in the collected buccal epithelial cells". I suggest, that they need to explain this result with the possibility of the relationship between the unrevealed DNA and azo exposure.

Answer:

We added explanation:

Another possibility, azo may cause DNA breakage in the human cell. In accordance with previous study, the component azo dye Disperse Orange 1 may cause apoptosis and DNA breakage in HepG2 cell derived from human hepatoma

Conclusion

Please revise the conclusion. T should be related to this study's aim. Particularly, what does the author mean with the "characteristics of DNA"? It could be used as indication (indicator?) of clinical symptom of azo exposure? The meaning is not clear.

Answer:

The result of this study was a preliminary study, and we have successfully to classify participants as controls and azo-exposed individuals based on score plot. We also characterized DNA of buccal cell according purity and concentration DNA, and absorbance

ratio at 260/280.

References

Some references (no 11, 12, and 13) can be searched. Please use references that have been indexed in database.

Answer:

We revised some references by adding link.

Competing Interests: We have no conflict of interest

Reviewer Report 04 September 2020

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In this study, authors analysed buccal cell DNA exposed to azo compound in batik workers. And, they showed that the characteristics of DNA could be used as an indication of exposure to azo compound in workers of batik industries.

Although the number of subjects, 10 for each group, was small, the authors performed statistical comparisons. And they provided sufficient Tables and Fig.s for the conclusion. Also, descriptions of Introduction, Methods, Results and Discussion were reasonable. Therefore, I think that this article is acceptable as Brief Report.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral Microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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