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Judul Artikel : Waste anesthetic gases have a significant association with deoxyribonucleic acid (DNA) damage: A systematic review and meta-analysis of 2,732 participants

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Penulis : Mayang Indah Lestari, Krisna Murti*, Iche Andriyani Liberty, Zen Hafy, Violantina Linardi, Muhammad Khoirudin, Tungki Pratama Umar

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EXPLANANATION OF STATEMENT, COMMENT AND RESULT OF REVISED PAPER FROM

REVIEWER 2

The highest thanks to reviewer who have patiently and carefully examined and revised our article titled: **Waste Anesthetic Gases Have a Significant Association with Deoxyribonucleic Acid (DNA) Damage: A Systematic Review and Meta-Analysis of 2,732 Participants**
We are very happy to receive your comment that you have presented in our article. We realize that there is a shortcoming in our article based on your review. Finally, we could construct better article with your substantially important issue addressed to us.

Introduction

1. **Correction: of having, explain shortname, in, also no cell culture is needed, ed**
   Adjustment: Thank you for your corrections. All of them have been addressed.

Method

1. **Correction: and their DNA damage asessment, re-evaluate**
   Adjustment: Thank you for your corrections. All of them have been addressed.

2. **This should be included in themain text. the words that were the same for all the databaes should be written first and then additional words for each database, althiugh I do nit understand why all the databases werenot checked on the same way. you did not explain the time limitation (from which to which year).**
   Adjustment: Thank you for your corrections. We have moved the table to the main file as instructed. We need to do adjustment for some databases, for example, Science Direct only has the capability to include 8 parameters for the checking, and our keywords must be adjusted accordingly. Time limitation (2002 to 2022) is mentioned just before the protocol registration (we just do filter on each database, not in search terms). However, we moved it to the electronic search section.

3. **Strange**
   Adjustment: Thank you for your comment. We have changed it (also highlighted by reviewer 3 about duplicate writing of high risk of bias). We also noted a problem on citation previously. All of them have been addressed.

4. **And all those studies had control and exposed group?**
   Adjustment: Yes, we only included studies with this trait. It is also revised in the eligibility criteria.

Result

1. **Correction: M, 24-35**
   Adjustment: Thank you for your corrections. All of them have been addressed. Reference number has changed slightly.

2. **In your search you did not mention halothane, etc. (specific type of gases)**
   Adjustment: Thank you for your comment. We are sorry for not including these type of gases. However, waste anesthetic gases is a common terms employed in these particular subjects and already inclusive of these gases. This is a useful suggestion for the future systematic reviews to include it when possible.

3. **Who has made those limits?**
   Adjustment: Thank you for your comment. We have changed the regulation as also highlighted by reviewer 3 to use the standard from the National Institute for Occupational Safety and Health (NIOSH). It has been corrected and lead to different value in the revised manuscript.

Discussion

1. **estblished by whom?**
   Adjustment: Thank you for your comment. We have changed the data and mentioned it as regulated by the National Institute for Occupational Safety and Health (NIOSH).

2. **this is totally opposite from the sentence before???**
   Adjustment: Thank you for highlighting this issue. We have removed the sentence due to the confusion made by it and lack of coherence.

Conclusion

1. **you should also mention that there are many operations including ionizing radiation with these WAGs that can also make possible synergistic effects in DNA damage**
   Adjustment: Thank you for your useful suggestion. We have added this information at the end of the conclusion section.

Figure

1. **there should be explanations on x and y, with units**
   Adjustment: Thank you for your comment. We have added the explanation as instructed and edited the figure.

EXPLANANATION OF STATEMENT, COMMENT AND RESULT OF REVISED PAPER FROM

REVIEWER 3

The highest thanks to reviewer who have patiently and carefully examined and revised our article titled: **Waste Anesthetic Gases Have a Significant Association with Deoxyribonucleic Acid (DNA) Damage: A Systematic Review and Meta-Analysis of 2,732 Participants**

We are very happy to receive your comment that you have presented in our article. We realize that there is a shortcoming in our article based on your review. Finally, we could construct better article with your substantially important issue addressed to us.

1. **The International Agency for Research on Cancer (IARC) has classified the anesthetic agents as group 3 carcinogens (e.g., not classifiable as to their carcinogenicity to humans). However, In this paper, the authors explained, many studies show the genotoxicity of IAs following occupational exposures. These findings must highlighted in the different sections of the manuscript. I think the reason for writing a review articles controversial findings about carcinogenicity these agent. The authors only brought positive studies but not all studies in this field. The authors should reach a conclusion about the carcinogenicity of these agents according to different studies. Why are these substances still in the group 3 of carcinogenicity despite all these positive studies?**

   Adjustment: Thank you for your comment, and we really appreciate these specific comments from the reviewer. We have described the rationale that although WAGs have been classified as non-carcinogenic, excessive WAGs exposure is still happening worldwide due to the lack scavenging system. Meanwhile, DNA damage is not only associated with cancer (several impact of chronic exposure to WAGs have been described in the revised manuscript), which is not becoming the focus on our study and need further examination. It is also described in our study results when approximately half of included studies reported higher than recommended level of volatile anesthetics and more than half (16) of the studies did not report any information about WAGs concentration (did not presented in our manuscript), which can describe higher than recommended value further. For the concern of not including all studies, (1) we only included studies between 2002 to 2022; (2) we already used hand-searching to manually detect studies that may be unindexed in five databases used in this study to prevent the non-inclusion of the potential study. To support it, we have provided more detailed Prisma study selection flow diagram.

Abstract

1. **The first sentences must be rewritten.**

   Adjustment: Thank you. We have rewritten the first sentence.

2. **Please, use "DNA damage indicator" instead of "DNA damage parameter" in the result section.**

Adjustment: Thank you. We have rewritten the phrases in the abstract and in the main text.

3. **Conclusion must be rewritten. what means six parameters?**
   Adjustment: Thank you. We have specified the conclusion writing.

Introduction:

1. **Page 5, line 31: " It is not clear what means a sentence of "On susceptible individuals, WAGs exposure can develop into a malignancy". Please re-write it.**
   Adjustment: Thank you for highlighting this issue. We have removed the sentence and and add new sentences highlighting long term impact of WAGs.

2. **Page 5, line 42: Please, write the full name of "MNA" examination. it means micronucleus assay?**
   Adjustment: Thank you for highlighting this issue. We have edited it to micronucleus assay.

Methods and Results

1. **Page 7, lines 23-24: "high risk of bias" repeated twice. Please, correct it. These sentences are confusing to the reader, explain them more.**
   Adjustment: Thank you for your observation. We have changed the first RoB to low risk and changed the reference to be more suitable.

2. **Page 8, line 2: Please, write the full name of "RevMan".**
   Adjustment: Thank you for your observation. Actually it has bee mentioned earlier in page 7; however, we also changed it in the revised manuscript as instructed.

3. **Page 9, lines 46-48: The National Institute for Occupational Safety and Health (NIOSH) (1977) proposed a recommended exposure limit-time-weighted average (RELTWA) of 25 ppm for nitrous oxide (N2O) and 2 ppm for the halogenated anesthetics. However, where the halogenated anesthetics exist in the presence of N2O, the REL-TWA of less than 0.5 ppm is recommended for them. Why did the authors write exposure limit of 50-100 ppm for N2o and 10 ppm for halogenated anesthetics?**
   Adjustment: Thank you for your observation. We have updated it based on the NIOSH recommendation as mentioned above.

4. **"Santovito, et al (2015)[47]" repeated twice in Table 3. Please correct it.**
   Adjustment: Thank you for your observation. We made a mistake on copying the RoB table. It has been corrected (also applies for Rozgaj).

5. **Please, determine how many metaphases cells (100 OR 200), MN frequency (1000 OR 2000), and comet parameters were counted per each individual in original studies listed in figures 2-4.**
   Adjustment: Thank you highlighting this condition. We have added the respective information to the figure legends.

6. **Which values in which studies are modified by the authors. For example, values in Figure 3 especially part A (studies conducted by cakmak, Souza, ….) is different from the original articles.**
   Adjustment: Thank you for highlighting the discrepancy, we will describe it thoroughly below:
   A. Arbitrary unit (2A) → Paes 2014 (derived using WebPlotDigitizer), Wron´ska-Nofer 2009 (derived using WebPlotDigitizer and re-calculated): No change in the picture
   B. Tail length (2B) → no change
   C. %TailDNA (2C) → Souza 2020 (derived using WebPlotDigitizer): No change in the picture
   D. Buccal micronuclei (3B) → Braz 2018 (re-calculated for 1000 cells; for exposed group, the value was merged), Cakmak (corrected), Chandrasekar (re-calculated for 1000 cells), Souza, Silva (Re-calculated with atozmath.com), Braz_2020 (rechecked sample size, re-calculated for 1000 cells), Braz 2020 (corrected): Change in figure (forest and funnel plot)
   E. Lymphocyte micronuclei (3B) → Bilban (re-calculated for 1000 cells), de Araujo (Re-calculated with atozmath.com), Neghab, Rozgaj (re-checked for all components) : Change in figure (forest and funnel plot)
   F. Total chromosomal aberration (4) → Santovito (manually calculated) : No change in the picture

   Although there are some changes above, the overall interpretation remains similar as previous version.

7. **Study of neghab, 2020 should be removed from Figure 3, part A because its results have been written incorrectly.**
   Adjustment: Thank you highlighting this condition. We have added the correct value in the revision.

8. **Values of mean and SD must be checked in all figures according to original articles.**
   Adjustment: Thank you highlighting this condition. We have rechecked all values for meta-analysis (see comment number 6).

Discussion

1. **Possible mechanisms underlying the genotoxic effects of these chemicals on page 10, lines 29-42 must be merged with sentences on page 11, lines 25-40.** Adjustment: Thank you you suggestion. We have merged it as instructed.

2. **Discussion is weak. It consists of mostly isolated sentences without clear logical structure.**
   Adjustment: Thank you for your comment. We have revised the discussion section thoroughly, also by highlighting the suggestion to discuss some controversial findings.

Conclusion

1. **The authors should reach a conclusion about the carcinogenicity of these agents according to different studies. Why are these substances still in the group 3 of carcinogenicity despite all these positive studies?**
   Adjustment: Thank your comment. First of all, we did not analyze the risk of cancer as the primary outcome (we only determine extent of DNA damage) highlighting this condition. We cannot do more interpretations on the IARC classification (actually there are some data on excess cancer-related death for human and no impact on animal based on the explanation). However, an increased rate of surgery worldwide may pose a higher and prolonged exposure to WAGs, which still must be described in the future study with robust methodology. A systematic review on the impact of WAGs and cancer is also a future options to cover what we did not do in the current moment. We already tried to describe it and we hope it is sufficient.

Waste Anesthetic Gases Have a Significant Association with Deoxyribonucleic Acid (DNA) Damage: A Systematic Review and Meta-Analysis of 2,732 Participants

Mayang Indah Lestari[1,2], Krisna Murti[3*], Iche Andriyani Liberty[4], Zen Hafy[1], Violantina Linardi[5], Muhammad Khoirudin[5], Tungki Pratama Umar[5]

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DECLARATIONS

Funding

None

Data Availability Statement

Data Available upon request from the corresponding author

Authors' contributions

MIL, KM, IAL, ZH, VL, TPU: Protocol development; IAL, ZH, TPU: Creation of search strategy; VL, MK, TPU: Study screening, VL, TPU: Data extraction; MIL, TPU: Risk of bias assessment; MIL, VL, MK, TPU: Data analysis and write up; MIL, KM: Data validation; VL, TPU: Visualization; KM, IAL, ZH: Reviewing and editing completed manuscript; MIL, TPU:

Manuscript revision. All authors reviewed the results and approved the final version of the manuscript.

Ethics approval

Not applicable

Acknowledgments

None

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Abstract

Introduction: Operating room workers are at risk of experiencing adverse effects due to occupational exposure to waste anesthetic gases (WAGs). One of the consequences of long-term WAGs exposure is the probability of developing deoxyribonucleic acid (DNA) damage. This systematic review investigated the link between WAGs and DNA damage in operating room workers.

Methods: PubMed, Science Direct, ProQuest, Scopus, and EbscoHost, as well as hand-searching, were used to find literature on the relationship between WAGs and DNA damage. Three independent reviewers independently assessed the study's quality. Meta-analysis was conducted for several DNA damage indicators, such as comet assay (DNA damage score, tail's length, tail's DNA percentage), micronuclei formation, and total chromosomal aberration.

Results: This systematic review included 29 eligible studies (2,732 participants). The majority of the studies used a cross-sectional design. From our meta-analysis, which compared the extent of DNA damage in operating room workers to the unexposed group, operating room workers exposed to WAGs had a significantly higher DNA damage indicator, including DNA damage score, comet tail's length, comet tail's DNA percentage, micronuclei formation, and total chromosomal aberration ($p<0.05$) than non-exposed group.

Conclusion: Waste anesthetic gases have been found to significantly impact DNA damage indicators in operating room personnel, including comet assay, micronuclei development, and chromosomal aberration. To reduce the impact of exposure, hospital and operating room personnel should take preventive measures, such as by adapting scavenger method.

Keywords: waste anesthetic gases, comet assay, micronuclei, chromosomal aberration

Introduction

Waste anesthetic gases (WAGs) are a small amount of anesthetic gas, both nitrous oxide (N_2O) and halogen anesthetics (such as halothane, enflurane, isoflurane, and desflurane), which leak from the patient's breathing circuit into the operating room air during the administration of anesthesia [1]. The WAGs have the potential to endanger health workers in hospitals such as anesthesiology specialists, nurse anesthetists, surgeons, operating room nurses, operating room technicians, and other operating room personnel [2–4]. Its impact can be classified into two categories: short-term (fatigue and lethargy) and long-term exposure (related to many disorders, both for the workers and fetuses) [5,6].

Operating room workers can be exposed to WAGs, even if the scavenging and ventilation systems are properly installed as a result of leaks through anesthetic gas delivery systems during system disconnections, from facemask connections or endotracheal tubes, or during induction of anesthesia [6,7]. Exposure is most common in health facilities that are not equipped with scavenging or ventilation systems or are equipped but in poor condition [1,2]. The United States Regulatory Agency, Occupational Safety and Health Administration (OSHA), estimates as many as 200,000 health workers are at risk of having an occupational disease due to chronic exposure to WAGs [3,8].

Chronic exposure to WAGs may harm the genetic composition, including causing deoxyribonucleic acid (DNA) damage [9]. It can elevate the risk of developing chronic illnesses like cancer, liver problem, and kidney disease. Furthermore, congenital defects, preterm deliveries, spontaneous abortions, and infertility can also arise following long-term exposure to WAGs [6]. Nonetheless, volatile anesthetics have been classified as group 3 (not classifiable as carcinogenic) by the International Agency for Research on Cancer (IARC) as long as exposure stays within the permissible range [10]. Assessment of WAGs is still crucial because nearly half of the operating rooms remain functioning without scavenging devices, particularly in less-developed nations, posing excessive and chronic exposure to WAGs that can lead to detrimental effects in humans [9].

Human biomonitoring is needed to evaluate genetic and chromosomal damage in individuals exposed to genotoxic substances [11,12]. Technological developments have made it possible to diagnose genetic disorders down to the molecular level. Comet assays (CA) are recognized for their robustness, sensitivity, and statistical power to evaluate deoxyribonucleic acid (DNA) cleavage [13]. Meanwhile, micronucleus assay examination, especially the assessment in buccal epithelial cells, can detect mutagenicity biomarkers, which are preferred to be used instead of chromosomal aberration tests because they do not require karyotype

analysis and cell cultures, while also fast and inexpensive [14,15]. Due to the potential impact of inhalational anesthetics and genetic problems, we conducted a systematic review to analyze the association between WAGs and DNA damage in operating room workers.

Methods

The researchers conducted a literature search across multiple databases to gather publications on the impact of WAGs exposure to DNA damage. This review was established using the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) 2020 guidelines [16]. This study's protocol has been registered to PROSPERO (CRD42022382476).

Eligibility criteria

This review focused on publications about healthcare workers' exposure to waste anesthetic gases (measured by comet assay, micronuclei formation, and total chromosomal aberration) and their DNA damage asessment in the human operating room landscape. We included observational studies that used a standardized examination method (prospective or retrospective cohort, case-control, or cross-sectional study) and employed study participants aged over 18 years old. They must be consisted of both exposed and non-exposed (control) groups. Conference abstracts, literature reviews, opinion pieces, protocols, case reports, case series, and unretrievable full texts were not considered. Studies conducted in veterinary hospitals were also excluded from our analysis. To ensure data precision, only full-text manuscripts published in English were included.

Search strategy

The electronic search was conducted in five databases: PubMed (29 hits), Science Direct (26 hits), ProQuest (24 hits), Scopus (38 hits), and EbscoHost (29 hits). The search was accomplished on January 8th, 2023, and studies published between 2002 and 2022 were included. Hand-searching was also carried out by manually reviewing the references of the selected papers to locate relevant publications that were not indexed in the previously observed records [17]. The titles and abstracts of the studies found through the database search were assessed, and only those that met the eligibility requirements were contemplated for further analysis. **Table 1** contains a list of the keywords used in the investigation.

Study selection

The retrieved papers were inspected for potential duplication. Two reviewers (VL and MK) used Rayyan QCRI, a semi-automated abstract and title sorting program, to screen the titles and abstracts [18]. Inter-rater disagreements were resolved by careful re-examination and consultation of the paper among reviewers until a consensus was attained. The full texts of potentially eligible studies were acquired and independently evaluated by two reviewers (ZH and TPU) to determine eligibility for inclusion in the final analysis. The full-text screening stage used a similar method of resolving the disagreements among researchers. If no settlement could be actualized, a moderator (MIL) was present to re-evaluate the distinctions and finalize the manuscript inclusion designation.

Data extraction and quality assessment

The primary data extraction was performed by VL, MK, and TPU. The following data were extracted: authorship, country of research, study design, sample size (male/female), participants' age, occupation, body mass index (BMI), exposure period, anesthetic gas description (and concentrations when available), smoking, and alcohol consumption status. Two of the co-authors (KM and IAL) appraised the risk of bias in the included studies autonomously, with discrepancies resolved through mediation among researchers until a decision was attained. The Newcastle-Ottawa Scale (NOS) was used to evaluate every study's methodological quality. There are three sections in the NOS: selection, comparability, and outcome. It is graded using a star system distributed across three domains and then classified based on the level obtained as follows: high (0–3 stars), moderate (4–6 stars), or a low (7–9 stars) risk of bias [19]. For cross-sectional studies, the modified NOS scale follows a slightly different pattern, with low (7–8), moderate (5–6), and high (0–4) risk of bias [20].

Statistical analysis

Following the compilation of all included publications, the data were recorded in Microsoft Excel 2019 (version 2102). The I^2 statistic was used to assess study heterogeneity, with the cut-off $p<0.1$ and $I^2>50\%$ considered as evidence of considerable study heterogeneity [21]. Random-effects and fixed-effects meta-analysis were performed with a 95% confidence interval (CI) using Review Manager (RevMan) Version 5.4.1. (The Cochrane Collaboration). Meta-analysis was performed for each DNA damage indicator (DNA damage score/arbitrary unit, comet tail length, percentage of DNA in comet tail, micronuclei formation, and total chromosomal aberration). To be eligible for inclusion in the meta-analysis, studies had to report mean scores and standard deviations (SDs). However, if the

central tendency was presented as a median or the data distribution was described as an interquartile range (IQR) or range, the calculation from Wan et al. [22] was used to convert it into desirable value. The standard mean difference (SMD) method was applied in the meta-analysis to evaluate the impact of WAGs exposure on DNA damage. We extracted the value from the data presented as a diagram using WebPlotDigitizer version 4.6 (https://automeris.io/WebPlotDigitizer; Pacifica, California, USA).

Publication bias

Publication bias was examined utilising funnel plots and Egger's linear regression test with Review Manager (RevMan) Version 5.4.1 and Comprehensive Meta-Analysis Version 3.3 (Biostat, Englewood, New Jersey). The presence of potential publication bias was indicated by an asymmetric distribution of data points in the funnel plot and a quantified result of $p<0.05$ in the Egger's test. Asymmetry in the funnel plot was caused by factors other than publication bias, including minor study effects, heterogeneity, and chance, particularly in small sample size studies. Sensitivity analysis was performed by discarding each record incrementally to investigate the stability of the outcome. Meta-regression analysis was used to investigate the potential source of heterogeneity if a variable was observed by at least ten studies [23]. In the meta-analysis, all p-values were two-sided, and $p<0.05$ was considered significant.

Results

Study characteristics

The search strategy identified a total of 172 studies (146 from registers and 26 from handsearching. At the final evaluation stage, 29 studies (2,732 participants; 1405 in the exposed group and 1,327 in the non-exposed group) were included (**Figure 1**). Most of the studies were cross-sectional, with only three with case-control design [24–26] and one as the cohort study [4]. Most studies dominated by female, with 13 studies have a >50% proportion of male [24,27–38]. Furthermore, smoking and alcohol consumption was reported in 16 and four studies, respectively, with an overall percentage of 33.92% smoker (326/961) and 36.54% alcohol use (95/260). Maximum exposure period is reported by El-Ebiary, et al. [39], with 19.25 ± 2.36 years. Characteristics of the study population can be seen in **Table 2**.

There are seven types of gases reported across the investigations in the operating room environment, including Isoflurane (20 studies), Sevoflurane (20 studies), Nitrous oxide (19 studies), Halothane (7 studies), Desflurane (5 studies), Enflurane (2 studies), and Sodium

pentothal (1 study). Regrettably ten investigations [9,31,35,37,40–45] found that the regular exposure limit for nitrous oxide (25 ppm time-weighted average/TWA) was exceeded the recommendation from the National Institute for Occupational Safety and Health (NIOSH), and six studies [9,40,41,43,44,46] found that the daily exposure limit for halogenated anesthetics (2 ppm) was breached. Meanwhile, fourteen studies did not report any information on WAGs concentration [4,24–26,28–30,32,33,36,38,39,47,48]. WAGs concentration are listed in **Table 3**.

All studies evaluated the association of DNA damage with waste anesthetics gases (WAGs) in operating room workers. DNA damage was analyzed using three methods, comet assay and micronuclei formation assay (buccal and lymphocyte), and total chromosomal aberration. The comet assay was determined as DNA damage score (arbitrary unit) [4,9,26,31–33,44], percentage of DNA in comet tail [24,39,45], and comet tail length [25,33,36,39]. Meanwhile, studies that carried out micronuclei formation assay was divided into two groups, namely buccal [9,25,34,40,43,44,49] and lymphocyte micronuclei [33–35,37,38,41,50,51]. Chromosomal aberrations are also reported in eight studies [25,28–30,42,47,48,50]. Other parameters are γH2AX/β-actin ratio [27] and relative telomere length [45,49].

Meta-analysis on impact of anesthetic gas exposure to comet assay, micronuclei formation, and chromosomal aberration

The pooled mean results and 95% CI of the comet assay, micronuclei formation, and chromosomal aberration are presented in **Figure 2-4**, respectively. All studies have significant heterogeneity (I^2>50%, p<0.1), except for the analysis of buccal micronuclei; thus, random effect size determination was selected (for buccal micronuclei, fixed-effect meta-analysis was conducted). Comet assay examination in exposed individuals showed a significant difference from the non-exposed controls, either using DNA damage score (arbitrary unit) (pooled SMD = 1.15, 95% CI = 0.41-1.89; p=0.002), tail's length (pooled SMD = 1.47, 95% CI = 0.21-2.72; p=0.02), and percentage of DNA in comet tail (pooled SMD = 1.90, 95% CI = 0.89-2.90; p=0.0002). Similar trends were also observed in buccal micronuclei formation (pooled SMD = 0.38, 95% CI = 0.22-0.54; p<0.00001), lymphocyte micronuclei (pooled SMD = 1.25, 95% CI = 0.87-1.63; p<0.00001), and total chromosomal aberration (pooled SMD = 1.50, 95% CI = 0.96-2.05; p<0.00001).

Quality assessment

The Newcastle-Ottawa Scale (NOS) was used to determine the risk of bias. Two case-control and cohort studies received high-quality ratings (7-9), while two others received an intermediate grade (4-6). The NOS instrument was modified to make it more applicable for cross-sectional studies. Ten of the 25 studies (40%) were having low risk of bias (score 7-8), while the others (15/25; 60%) were having a moderate risk of bias. The total rating scores for the included studies ranged from 5 to 8 (mean: 6.36 ± 1.20; cross-sectional) and 4 to 7 (mean: 5.75 ± 1.09; case-control and cohort). **Table 4** summarizes the quality of the included studies.

Publication bias

The funnel plot and Egger's linear regression test were used to demonstrate publication bias. From a visual inspection of the Funnel plot, only buccal micronucleus formation illustrates an asymmetric distribution of the pooled publication, indicating the possibility of publication bias (figure 5). The DNA damage indicators including DNA damage score (comet assay arbitrary unit), comet tail length, %tail DNA, lymphocyte micronuclei, and total chromosomal abbreviation revealed no publication bias ($p>0.05$). However, there is a publication bias ($p=0.002$) for buccal micronuclei based on Egger's test (**Table 5**). Then, we performed a sensitivity analysis based on the comparability and outcome quality assessment. It was demonstrated that there was no significant change, denoting that the finding of the buccal micronuclei meta-analysis was stable. Nonetheless, due to the small sample size (number of included studies) and high heterogeneity across all studies, it is difficult to conclude the existing publication bias based on the above assessments. Despite the significant heterogeneity, we did not conduct meta-regression because all variables were observed in fewer than ten studies.

Discussion

Waste anesthetic gases (WAGs) can have debilitating short- and long-term impacts on the health of individuals. Short-term exposure to WAGs can cause headaches, fatigue, nausea, drowsiness, impaired work productivity, and problems with judgment and coordination. On the other side, long-term exposure to WAGs is associated with an assortment of health issues, including nephrotoxic, neurotoxic, hepatotoxic, immunosuppressive, and reproductive toxicological effects. Additionally, WAG exposure over an extended period may damage DNA [4,5,52].

There are several theories that support the role of oxidizing drug metabolism and anesthetics for generating reactive oxygen species (ROS) and direct damage to genomes in

the cell cycle, nucleic acids, lipids, and proteins. The imbalance between the production of ROS and antioxidants is known as oxidative stress. Oxidative stress can cause damage to macromolecules, including nucleic acids, lipids, and proteins that cause cell damage, as well as various diseases [1,9]. Further understanding of the association between DNA damage and oxidative stress with WAGs is needed to prevent occupational diseases.

Mechanisms of genotoxicity and DNA damage from halogens anesthetics and N_2O are still unclear. There are several hypotheses of DNA damage and one of them is that exposure to N_2O can interfere with the synthesis of nucleic acids and proteins [53]. In addition, a series of stress responses can occur after DNA damage has occurred in cells. This stress response induces a signaling cascade and stops the cell cycle until the damage is repaired. One of the main components of the signaling cascade is histone variant H2AX, which can be phosphorylated when a DNA double-strand break (DSB) occurs and then initiates damage repair mechanisms. H2AX plays a very important role in the identification and repair process of DSB [54,55].

According to our systematic review, WAGs are linked to an array of DNA damage indicators. This connection is most evident in people who have experienced chronic WAG exposure over an average of three to nineteen years. The alteration of the body's endogenous antioxidant framework, which is essential in preventing genotoxicity, may be the cause of this relationship in conjunction with the potential direct genotoxic consequences stated previously. When compared to the non-exposed group, the WAGs-exposed group has increased lipid peroxidation, decreased antioxidant thiol groups and enzyme activity (particularly glutathione peroxidase and superoxide dismutase), and decreased antioxidant capacity [1]. This association was further supported by a study by Wronska-Nofer that showed a substantial correlation between the level of reactive oxygen species (ROS), nitrous oxide concentration, and cumulative DNA damage [32].Furthermore, we also found that approximately half of the included studies have higher than recommended level of WAGs than the guideline announced by the NIOSH, with the recommended daily exposure limit on the concentration of WAGs in the operating room to minimize risk of occupational exposure was 25 ppm for nitrous oxide (N_2O) and 2 ppm for halogen anesthetics such as halothane, enflurane, isoflurane, desflurane, and sevoflurane [56]. The problem of WAGs level were found particularly in N_2O [32] which exceeds the predetermined threshold [9,31,35,37,40–45]. Thus, it is recommended to use a scavenging system in the operating room to reduce levels of anesthetic gas waste and prevent potential health problems [57] However, the application of this system is still difficult in the developing countries, so other preventive

measures must be taken. Regular monitoring of operating room air quality is necessary to determine levels of exposure to WAGs and identify anesthetic gas leaks and anesthetic machine malfunctions are important [58]. In addition, fourteen studies did not record any concentration of WAGs, indicating a potential dearth of workplace WAG surveillance programs, which aim to reduce health risk by assessing work-related exposure to the WAG during operations by reviewing each anesthetic breathing device no less than once every two years [59].

Comet assay (CA), also known as single cell gel electrophoresis or microgel electrophoresis, was introduced to detect DNA damage in eukaryotic cells or decomposing tissues caused by radiation. CA has been used in various studies, such as genetic toxicology, biological monitoring, genotoxicity, molecular epidemiology, nutrigenomics, studies of DNA repair systems, evaluation of the genotoxicity of nanomaterials, evaluation of the DNA integrity of mesenchymal stem cells and spermatozoa [12,15]. Although the majority of studies in our systematic reviews indicate a significant association between WAGs and CA examination (DNA damage score, comet tail length, and the percentage of DNA in the comet tail), there is one research that presents contrasting results. Souza et al.'s research [44] reported no significant changes in the overall DNA damage score. This outcome could be explained by the lymphocytes ability to develop an adaptive response, including memory formation, after prolonged exposure. This adaptive response may enhance the lymphocytes' ability to resist the harmful effects of substances such as anesthetics [33,36].

Micronuclei (MN) are small chromatin-containing spherical bodies that are visible in the cytoplasm of the cell. MN forming is caused by DNA damage or genomic instability. MN can occur as a result of natural processes, such as metabolism or aging or it can be caused by many different environmental factors, harmful habits, and diseases. The micronucleus examination that is often carried out is the buccal micronucleus cytome assay and lymphocyte [14]. From this examination, it was found that the frequency of micronuclei in the exposed group was higher than in the unexposed group and statistically significant. In our systematic review, we discovered a single investigation that gave a distinct conclusion from the majority of the included studies. This particular study revealed no difference in micronucleus (MN) development between the group exposed to WAGs and the non-exposed group [51]. However, it is crucial to highlight that the study used volatile anesthetic doses that were considerably lower than the suggested limit (0.2 ppm). It has been established that MN accumulates due to prolonged high-level WAG exposure, not low-level exposure [60].

This is a real concern since increased micronuclei formation may be associated with early carcinogenic events [61].

Other parameters such as chromosomal aberrations showed significant differences between the exposed and unexposed groups. These events are associated with late stages of apoptosis and cell death, respectively, although the exact mechanism is unknown [62]. In addition, basal cells in the exposed group were lower than in the unexposed group. The proportion of basal cells and cells undergoing cell death in the buccal mucosa is an indication of the regenerative capacity of the tissue. If the proportion is low, the regenerative capacity of the tissue is also low so that it can cause accelerated aging [63]. In this specific parameter, all of the included studies showed similar pattern, with positive difference on the extent of chromosomal aberration.

This systematic review has several limitations. Most of the included study designs were cross-sectional, indicating a lack of evidence. Furthermore, several studies only used a low sample size (<30 in each group). More studies with prospective cohort designs and large sample sizes are expected in the future. Meanwhile, our study strengths include large research inclusion, more variable description, and exclusively doing the meta-analysis of observational studies as compared with a previous systematic review (without the meta-analysis) [64].

Conclusion

There is a clear association between exposure to WAGs and DNA damage. Although the pathway of WAGs-induced DNA damage is uncertain, precautionary measures should be implemented. Some preventive measures include assembling a sufficient scavenging system in the operating room, using low fresh gas flow, increasing intravenous anesthetics administration, and limiting or avoiding nitrous oxide use. Furthermore, antioxidant supplementation can be carried out by operating room personnel.

We did not examine the risk of cancer as the primary outcome (we simply looked at the extent of DNA damage). Furthermore, we are unable to offer any additional interpretations of the IARC classification (the explanation cites some evidence of an increase in human cancer-related deaths/incidence but no effect on animal populations with low-level of exposure). However, an increased rate of surgery worldwide may represent a higher and longer exposure to WAGs, which must be described in a future study using rigorous methods. Moreover, additional research can be directed to other possible causes of DNA deterioration in operating room personnel, such as ionizing radiation from surgical techniques like the spine and endovascular surgery that may have synergistic implications for genotoxicity.

Ultimately, a systematic review of the relationship between WAGs and cancer is another option for the future to address the things we have not done yet.

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Table 1. Search Strategy

Search	Query	Results
EbscoHost	(“Anesthetic Gases” OR “waste anesthetic gases”) AND (“Anesthesiologists” OR “Operating Room Nurse” OR “Anesthesiology resident” OR “Operating room worker”) AND (“DNA Damage” OR “DNA Injury” OR “genetic damage” OR “genetic instability”)	29
ProQuest	(“Anesthetic Gases” OR “waste anesthetic gases”) AND (“Anesthesiologists” OR “Operating Room Nurse” OR “Anesthesiology resident” OR “Operating room worker”) AND (“DNA Damage” OR “DNA Injury” OR “genetic damage” OR “genetic instability”)	24
Pubmed	(“Anesthetic Gases” OR “waste anesthetic gases” OR “Nitrous Oxide” OR “halogen anesthetics” OR “halogen” OR “sevoflurane” OR “isoflurane” OR “desflurane”) AND (“Anesthetists” OR “Anesthesiologists” OR “Operating Room Nurse” OR “Anesthesiology resident” OR “Anaesthetic Trainee” OR “Operating room personnel” OR “Operating room worker”) AND (“DNA Damage” OR “DNA Injury” OR “genetic damage” OR “genetic instability”)	29
Science Direct	(“Anesthetic Gases” OR “waste anesthetic gases”) AND (“Anesthesiologists” OR “Operating Room Nurse” OR “Anesthesiology resident” OR “Operating room worker”) AND (“DNA Damage”)	26
Scopus	(“Anesthetic Gases” OR “waste anesthetic gases” OR “Nitrous Oxide” OR “halogen anesthetics” OR “halogen” OR “sevoflurane” OR “isoflurane” OR “desflurane”) AND (“Anesthetists” OR “Anesthesiologists” OR “Operating Room Nurse” OR “Anesthesiology resident” OR “Anaesthetic Trainee” OR “Operating room personnel” OR “Operating room worker”) AND (“DNA Damage” OR “DNA Injury” OR “genetic damage” OR “genetic instability”)	38

Table 2. Population's Characteristics

ID	Author	Study Design	Country	Population (Exposed/ Control)	Physician proportion (Exposed)	Age (Exposed)[a]	Exposure period (year) [a]	Gas Type	BMI	Gender (Exposed, Male/Total)	Smoking (Exposed, Yes/Total)	Alcohol (Exposed, Yes/Total)
1.	Aldrieny et al. 2013[47]	Cross-sectional	Egypt	26/13	NA	31.19 ± 3.06	10.89 ± 1.93	H, I	NA	15/26	2/26	NA
2.	Baysal, et al. (2009)[26]	Case-control	Turkey	30/30	NA	33 ± 5	7 ± 4	D, H, I, N, S	25 ± 5	19/30	NA	NA
3.	Bilban, et al. (2005)[50]	Cross-sectional	Slovenia	153/153	153/153	NA	12.94 ± 6.52	H, I, N	NA	153/153	99/153	NA
4.	Borayek et al. (2018)[28]	Cross-sectional	Egypt	32/32	0/32	34.9 ± 6.5	17.75 ± 5.3	I	NA	0/32	NA	NA
5.	Braz, et al. (2018)[40]	Cross-sectional	Brazil	30/30	30/30	28.5267 ± 1.61	3.06 ± 0.47	I, N, S	24.62 ±	18/30	NA	NA
6.	Braz, et al. (2020)[9]	Cross-sectional	Brazil	31/32	NA	28.7 ± 1.9	3	I, N, S	24.6 ± 3.8	20/32	NA	NA
7.	Braz, et al (2020)[49]	Cross-sectional	Brazil	40/40	40/40	39 ± 14.3	3.5	I,S	25.5 ± 3.2	26/40	NA	NA
8.	Cakmak, et al. (2019)[34]	Cross-sectional	Turkey	46/21	13/46	32.4 ± 5.7	NA	S	23.5 ± 3.2	9/46	21/46	4/46
9.	Chandrasekhar, et al. (2006)[25]	Case-control	India	45/45	19/45	38.76 ± 8.66	10.468 ± 4.70	D, E, H, I, N, S, SP	NA	25/46	20/46	15/46
10	de Araujo et al. (2013)[38]	Cross-sectional	Brazil	30/30	30/30	40.97 ± 11.25	13.83 ± 10.93	E, H, I, N, S,	NA	14/30	NA	NA
11	El-Ebiary, et al. (2013)[39]	Cross-sectional	Egypt	40/20	23/40	39.6 ± 6.32	19.25 ± 2.36	H, I, N, S	NA	25/40	14/40	NA
12	Hua, et al. (2021)[27]	Cross-sectional	China	68/82	NA	31.56 ±	8.29 ± 5.15	S	21.28 ±	19/68	4/68	6/68
13	Izdes, et al. (2010)[24]	Case-control	Turkey	40/40	0/40	36.8 ± 5.7	14.5 ± 6.6	D, I, N, S	NA	9/40	22/40	NA
14	Kargar-Shouroki, et al. (2019)[42]	Cross-sectional	Iran	60/60	10/60	36.17 ± 7.36	10.95 ± 5.58	I, N, S	20.75 ± 2.8	30/60	NA	NA

15	Kargar-Shouroki, et al. (2022)[35]	Cross-sectional	Iran	45/45	45/45	37.73 ± 6.91	12.36 ± 6.3	N	NA	19/45	5/45	NA
16	Lewinska et al. 2005[37]	Cross-sectional	Poland	46/28	0/46	42.9 ± 8.6	17.7 ± 10.1	I, N, S	NA	0/46	21/46	NA
17	Musak et al. (2009)[29]	Cross-sectional	Czech Republic	76/76	41/76	36.89 ± 8.75	11.75 ± 9.35	NA	NA	15/76	23/76	NA
18	Neghab, et al. (2020)[41]	Cross-sectional	Iran	60/60	NA	36.17 ± 7.36	10.95 ± 5.58 [b]	I, N, S	NA	30/60	NA	NA
19	Paes, et al. (2014)[4]	Cohort	Brazil	15/15	NA	27.9 ± 2.3	NA	I, N, S	25.5 ± 3.8	14/15	NA	NA
20	Rozgaj, et al (2009)[33]	Cross-sectional	Croatia	50/50	20/50	38.88 ± 7.59	12.96 ± 8.96	NA	NA ± NA	12/50	16/50	NA
21	Santovito, et al (2015)[48]	Cross-sectional	Italy	21/21	21/21	35.524	8.619 ± 4.364	NA	NA	15/21	NA	NA
22	Shaker, et al. (2011)[30]	Cross-sectional	Egypt	27/18	0/27	33.7 ± 7	15 ± 6.7	D, I, N, S	NA	0/27	0/27	NA
23	Silva, et al. (2022)[43]	Cross-sectional	Brazil	100/93	NA	34.2 ± 11.8	NA	I, N, S	25.5 ± 4.3	55/100	8/100	70/100
24	Souza, et al (2016)[44]	Cross-sectional	Brazil	30/30	30/30	42 ± 15.9	NA	D, I, N, S	26.1 ± 3.3	20/30	NA	NA
25	Souza, et al. (2021)[45]	Cross-sectional	Brazil	30/30	30/30	NA	NA	H, N	26 ± 3	20/30	NA	NA
26	Szyfter, et al. (2016)[36]	Cross-sectional	Poland	100/100	26/100	NA	NA	NA	NA	15/100	24/100	NA
27	Wiesner, et al. (2008)[51]	Cross-sectional	Germany	14/14	14/14	32 ± 5	NA	S	NA	8/14	4/14	NA
28	Wron´ska-Nofer, et al. (2009)[31]	Cross-sectional	Poland	84/83	29/84	40.73	15.77	I, N, S	NA	29/84	39/84	NA
29	Wronska-Nofer, et al. (2012)[32]	Cross-sectional	Poland	36/36	0/36	NA	NA	I, N, S	NA	0/36	NA	NA

Results presented in mean ± standard deviation or mean (range).

Notes: *NA= Data Not Available, D = Desflurane, E = Enflurane, H = Halothane, I = Isoflurane, N = Nitrous oxide, S = Sevoflurane, SP = Sodium pentothal

Table 3. Concentrations (ppm) of WAGs in operating rooms

	N_2O (ppm)	Isoflurane (ppm)	Sevoflurane (ppm)	Desflurane (ppm)	Halothane
Bilban et al. (2005)[50]	0-100[b]	0-10[b]	-	-	0-10
Braz et al. (2018)[40]	155 ± 138	5.1 ± 4.2	9.8 ± 9.0	-	-
Braz et al. (2020)[9]	180 (61-350)[a]	5.3 (0.3-17.8)[a]	9.7 (1.0-34.1)[a]	-	-
Braz et al. (2020)[49]	-	1.25 ± 0.61[a]	1.74 ± 0.73[a]	-	-
Cakmak et al. (2019)[34]	-	-	0.427 (0.32-0.58)[a]	-	-
Hua et al. (2021)[27]	-	-	1.11 ± 0.65	-	-
Lewinska et al. (2005)[37]*	7.78-1282.13	-	-	-	-
Neghab et al. (2020)[41] and Kargar-Shouroki et al. (2019)[42]	850.92 (10–3895)[a]	2.4 (0.49–4.15)[a]	0.18 (0.01–0.59)[a]	-	-
Kargar-Shouroki et al. (2022)[35]	450.27 ± 327.44[a]	-	-	-	-
Silva et al. (2022)[43]	165 ± 15	7 ± 5	9 ± 7	-	-
Souza et al. (2016)[44]	150.3 ± 135.7	5.5 ± 4.4	7.7 ± 8.7	16.4 ± 6.0	-
Souza et al. (2021)[45]	150 ± 136	-	-	-	10 ± 6.4
Wiesner et al. (2008)[51]	-	-	0.2 (0.08-2.24)[c]	-	-
Wron´ska-Nofer et al. (2009)[31]*	244.43 (19.89-834.39)[a]	0.689 (0.066-1.855)[a]	0.574 (0.05-1.83)[a]	-	-
Wron´ska-Nofer et al. (2012)[32]*	102.77-834.39[b]	0.053-1.988[b]	0.061-1.711[b]	-	-

Note: *value presented as the conversion from mg/m^3 using the formula: Concentration (ppm)= $\frac{24.45 \text{ x concentration (mg/m3)}}{\text{molecular weight}}$.

Data was presented in mean ± standard deviation except stated otherwise ([a] Mean (range) [b] Range, [c] Median (range). Data was compiled only from studies which stated the gas concentration explicitly

Table 4. Risk of Bias Analysis

CROSS SECTIONAL	Selection	Comparability	Outcome	Total Score	Interpretation (Risk of Bias)
Aldrieny et al. 2013**[47]**	***	0	**	5	Moderate
Bilban, et al. (2005)**[50]**	****	0	*	5	Moderate
Borayek et al. (2018)**[28]**	****	0	*	5	Moderate
Braz, et al. (2018)**[40]**	****	0	**	6	Moderate
Braz, et al. (2020)**[9]**	****	0	**	6	Moderate
Braz, et al (2020)**[49]**	****	*	**	7	Low
Cakmak, et al. (2019)**[34]**	****	**	**	8	Low
de Araujo et al. (2013)**[38]**	****	*	*	6	Moderate
El-Ebiary, et al. (2013)**[39]**	****	0	*	5	Moderate
Hua, et al. (2021)**[27]**	****	0	*	5	Moderate
Kargar-Shourouki, et al. (2019) **[42]**	****	**	**	8	Low
Kargar-Shourouki, et al. (2022) **[35]**	****	*	*	6	Moderate
Lewinska et al. 2005**[37]**	****	**	*	7	Low
Musak et al. (2009)**[29]**	****	0	*	5	Moderate
Neghab, et al. (2020)**[41]**	****	**	**	8	Low
Rozgaj, et al (2009)**[33]**	****	0	**	6	Moderate
Santovito, et al (2015)**[48]**	****	0	**	6	Moderate
Shaker, et al. (2011)**[30]**	****	0	*	5	Moderate
Silva, et al. (2022)**[43]**	****	**	**	8	Low
Souza, et al (2016)**[44]**	****	*	*	6	Moderate
Souza, et al. (2021)**[45]**	****	**	**	8	Low
Szyfter, et al. (2016)**[36]**	****	**	**	8	Low
Wiesner, et al. (2008)**[51]**	****	**	**	8	Low
Wron´ska-Nofer, et al. (2009)**[31]**	****	0	*	5	Moderate
Wronska-Nofer, et al. (2012)**[32]**	***	**	**	7	Low
CASE CONTROL/ COHORT	**Selection**	**Comparability**	**Outcome**	**Total Score**	**Interpretation (Risk of Bias)**
Baysal, et al. (2009)**[26]**	**	0	**	4	High
Chandrasekhar, et al. (2006)**[25]**	**	**	***	7	Low
Izdes, et al. (2010)**[24]**	**	*	***	6	Moderate
Paes, et al. (2014)**[4]**	***	0	***	6	Moderate

Table 5. Tests for publication bias

DNA damage indicator	Egger's test		
	t-value	95% CI	P-value
Comet assay (arbitrary unit)	1.787	-3.192 ─ 17.762	0.134
Tail length (μm)	0.653	-39.209 ─ 53.248	0.581
%Tail DNA	1.172	-234.494 ─ 282.149	0.450
Buccal micronuclei	5.489	4.465 ─ 12.332	0.002
Lymphocyte micronuclei	0.551	-27.657 ─ 42.742	0.605
Total chromosomal aberration	0.239	-10.445─ 12.704	0.819

Figure Legends

Figure 1. Study selection

Figure 2. Effect of WAG exposure to (A) Comet's assay arbitrary unit, (B) Comet's tail length, (C) %Tail DNA. The arbitrary unit was displayed as a weight-averaged degree of DNA breakage (between 0-400), tail length was determined in micrometers (μm), and %tail DNA was examined utilizing a computerized image evaluation system.

Figure 3. Effect of WAG exposure to (A) Micronuclei (buccal), (B) Micronuclei (lymphocyte). Data was presented per 1000 cells.

Figure 4. Effect of WAG exposure to total chromosomal aberration. Data was counted per 100 metaphases cells.

Figure 5. Funnel plot for the (A) comet tail length, (B) comet assay/DNA damage score (arbitrary unit), (C) %tail DNA, (D) buccal micronuclei, (E) lymphocyte micronuclei, (F) total chromosomal aberration. Y-axis (SE(SMD)) is Standard Error of Standardized Mean Difference, while X-axis is SMD.
Abbreviation: a.u. = arbitrary unit

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RESEARCH ARTICLE

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NFATc1 is Suppressed in Tumor Microenvironment of Hodgkin Lymphoma

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Abstract

Objective: The aims of this research are to evaluate the expression and distribution of *NFATc1* in tumor microenvironment of Hodgkin lymphoma. **Methods:** Twenty-eight cases of Hodgkin lymphoma were selected. Clinicopathological data of age, gender, location and subtypes were obtained. Immunohistochemistry was performed to the all cases by using anti-CD163, anti-NFATc1 and anti-PD-L1 antibodies. All protein expression was calculated by using Image J software. **Results:** Nuclear expression of *NFATc1* was not observed in Hodgkin cells neither in TAM nor in small lymphocytes surrounding Hodgkin cells in all the samples, this meant that *NFATc1* showed negative nuclear expression in almost all these cells. Cytoplasmic expression of *NFATc1* was observed in small lymphocytes surrounding tumor cells. While there were only few small lymphocytes which were located far from tumor cells showed nuclear expression of *NFATc1*. Meanwhile, 57.14% samples showed high density of TAMs CD163+, and 50% tumor cells as well as 50% TAMs exhibited positive *PD-L1* expression. In addition, all macrophages did not have *NFATc1* expression both in their nuclei and in their cytoplasm. **Conclusion:** *NFATc1* was suppressed both in Hodgkin cells and inflammatory cells surrounding the tumor cells. This condition may contribute to progressivity and aggressiveness of the diseases. Therefore, certain mechanisms to reactivate functional *NFATc1* in HL tumor microenvironment may be necessary; hence, the tumor cells are able to be eradicated by patient's immune mechanisms.

Keywords: Hodgkin lymphoma- tumor microenvironment- *NFATc1- CD163- PD-L1*

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Introduction

From early development to tumor progression and then metastasis, tumor cells are counter acted by numerous types of tumor microenvironment (TME) elements i.e., stromal factors and immune cells. Therefore, beside the other factors, TME is also an essential component in determinating tumor behavior and prognosis (Kim and Bae, 2016). Numerous markers and therapeutic strategies were developed based on TME context.

In 2018, Hodgkin lymphoma (HL) incidence of new cases were around 79,990 with number of deaths were circa 26,167 (Bray et al., 2018). The incidence of HL varies considerably by age, sex, ethnicity, geographic location and socioeconomic status, and its rates are higher among males and in developed countries, but lower in Asian population. Meanwhile, mortality rates were lower in underdeveloped and higher developing regions (Zhou et al., 2019; Salati e al., 2014). Indonesia ranks 25th in incidence of HL (Ferlay, 2013). Young population at ages 15 to 25 years are mostly affecting by HL with higher incidence (Bigenwald et al., 2017). Despite its relatively low incidence and its low lifetime risk, HL comprises 15% of all cancers in young adults with a high impact on quality of life (Salati et al., 2014).

HL is a curable disease; more than 90% cure rate for patients with early disease and in more than 70% patients with advanced disease (Shanbhag and Ambinder, 2018). The crucial point is to recognize high-risk patients who will relapse after initial therapy. Therefore, identifying these high risks patients by characterization of pathobiological and clinical prognostic factors then followed by designing properly novel treatment strategies with minimal treatment toxicities is demanding.

Morphologic characteristic of HL is heavily infiltrating inflammatory cells surrounding tumor cells as its tumor microenvironment (Calabretta et al., 2019). In classical HL (cHL) cells NF-κB is constitutively activated (Weniger and Küppers, 2016), however the exact factors regulate its microenvironment is still unclear. Latest findings revealed that abundant component cellular and humoral generated by interaction of Hodgkin cells with their environment, which might contribute to the characteristic background inflammatory cells (Calabretta et al., 2019).

Macrophages are the other types of inflammatory cells observed heavily infiltrate the background of Hodgkin

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cells. Unlike *PD-L1* expressed on tumor cells, *PD-L1* expressed on macrophages is able to protect macrophages from destruction by T cells (Singhal et al., 2019). In addition to this, other studies showed that *PD-L1* in macrophages inducing T cell anergy and M2 polarization (Lu et al., 2019).

Known as an essential transcription factor in many physiologic systems comprising immune cells (Vaeth and Feske, 2018), including in regulation of PD-1 activation (Oestreich et al., 2008), nuclear factor of activated T cell (*NFATc1*) has roles in tumor microenvironment (Li et al., 2018; Gholami et al., 2017). *NFATc1* may contribute to the molecular pathways entailed in tumor microenvironment of HL, which, then both promote to HL progression and worsen prognosis.

The aims of this research are to evaluate the expression and distribution of *NFATc1* in tumor microenvironment of Hodgkin lymphoma. Together our results may identify *NFATc1* as promising target for alternative novel marker of prognostic and or predictive factors of Hodgkin lymphoma.

Materials and Methods

Patient data

Initially, we collected 44 cases of Hodgkin lymphoma diagnosed based on the 2016 World Health Organization classification (Swerdlow et al., 2017) from January 2014 to November 2019 at Department of Anatomic Pathology, Faculty of Medicine University of Sriwijaya, Dr. Mohammad Hoesin Hospital, Palembang, Indonesia. After careful selection based on quality of fixation and processing which can be assessed by carefully examined the HE and IHC slides, 28 cases were obtained as samples. Clinicopathological parameters i.e., age, gender of patients, subtypes, and location of tumors were attained from patient's pathology records. Ethical committee approval from Faculty of Medicine University of Sriwijaya was also attained.

Immunohistochemical analysis

The paraffin blocks of selected HL cases were retrieved from the archives. Immunohistochemical staining was conducted using manual system according to standard immunohistochemical protocol of our lab. The analyses were validated using appropriate negative and positive controls by using several tissue blocks consisting of tonsil, appendix, melanoma and breast cancer tissues. After sectioning, the blocks were dried in a lab heating and drying followed by deparaffinization and rehydration. Then antigen retrieval was performed by treating the slides in a microwave in citrate buffer. After blocking step the tissues were incubated for 60 minutes with primary antibody *NFATc1* (clone 7A6, dilution 1:200, BD Pharmingen, Franklin Lakes, New Jersey), CD163 (clone 10D6, rabbit, monoclonal, dilution 1:100, thermo fisher, USA) and *PD-L1* (clone SP142, dilution 1:100, Abcam, Cambridge, MA). Lastly, the slides were covered with mounting medium and coverslips. Stained tissues and all pictures were analyzed and captured using Olympus BX41 (Tokyo, Japan) couple with camera (12MP1/1.7" Sony Exmor CMOS Sensor, Beta Industrial Digital Camera, China) at a ×400 magnification.

Expression of NFATc1, CD163, and PD-L1

The positive expression of all antibodies was determined disregard staining intensity, since the later was most likely influenced by inconsistency of tissue fixation and processing. *NFATc1* positive expression was determined in nuclei of tumor cells as well as in lymphocytes and macrophages surrounding tumors. Positive expression of CD163 was calculated in membrane and or cytoplasm of macrophages around tumor cells. In addition, positive expression of *PD-L1* was counted in membrane of Hodgkin tumor cells and macrophages around tumor cells. Image J was used to quantify the numbers of protein expression of *NFATc1*, *CD163*, and *PD-L1*.

Density of NFATc1, CD163, and PD-L1

Reactivity of every antibody was differentiated into high and low density based on cut-off point obtained from median value. At the beginning the most concentrated five locations containing brown staining either *NFATc1*, or *CD163* or *PD-L1* were selected under low power field (100x). Then among these areas, the five most densest focuses were carefully chosen and photographed under high magnification (400x). By using image J software, the all cells expressed either *NAFTc1*, or *CD163*, or *PD-L1* were calculated and noted. Of these five areas, the average was counting by using excel. The median of all samples of each antibody was considered as a cut-off point for differentiation of *NAFTc1*, or *CD163*, or *PD-L1* expression into high or low density.

Statistical Analysis

Since *NFATc1* expression was negative in the evaluated area of all the samples, the statistical analysis was not performed.

Results

Patients Characteristics

Among 28 total samples, our data only have one case of NLPHL and 27 cHLs. The age was differentiated into five groups i.e., under 20 years (10.7%), between 20 to 29 years (25%), between 30 to 39 years (10.7%), between 40 to 49 years (28.6%) and after 50 years (25%). More patients in the ages of 40 to 49 years suffer from HL. Males suffer from HL more than that in females (57.1%). Tumor masses were mostly found in head and neck (78.6%). Lymphocyte-rich cHL was the subtype which mostly observed (57.1%) among others (Table 1).

Immunohistochemistry

NFATc1, CD163 and PD-L1

Nuclear expression of *NFATc1* was not observed in Hodgkin cells neither in TAM nor in small lymphocytes surrounding Hodgkin cells in all samples (Table 2), this meant that *NFATc1* showed negative expression in almost all these cells. There were only few small lymphocytes showed nuclear expression of *NFATc1* in some patients

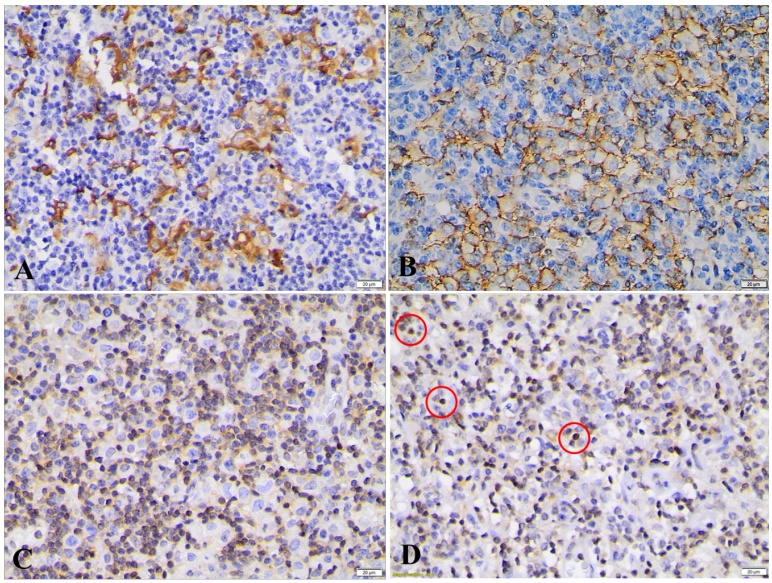

Figure 1. The Immunoreactivity of CD163, PD-L1 and NFATc1 Proteins of Patient #1. A. Showed various positive cytoplasmic expression of TAMs CD163+. B. Immunoreactivity of PD-L1 in membrane of Hodgkin cells and TAMs. C. Demonstrated various negative nuclear expression of NFATc1 protein in all cells; the only finding was cytoplasmic expression of NFATc1, particularly in small lymphocytes surrounding the Hodgkin cells. D. It can be seen few small lymphocytes showed nuclear expression but far from Hodgkin tumor cells (red circles). Original magnifications ×400.

Table 1. Patient Characteristics

Clinical features	N (28)	%
Age (years)		
<20 years	3	10.7
20-29	7	25.0
30-39	3	10.7
40-49	8	28.6
≥50 years	7	25.0
Gender		
Male	16	57.1
Female	12	42.9
Location		
Head-neck	22	78.6
Body	2	7.1
Extremities	4	14.3
Subtypes and Variant		
NLPHL	1	3.6
CHL		
NSCHL	4	14.3
LRCHL	16	57.1
MCCHL	7	25.0
LDCHL	0	0.0

(Figure 1). These cells were located far from tumor cells, while small lymphocytes surrounding tumor cells have only cytoplasmic expression of *NFATc1* (Figure 1 and Figure 2). Approximately 57.14% samples showed high density of TAMs CD163+. In addition, all macrophages did not have *NFATc1* expression both in their nuclei and in their cytoplasm's. The expression of *PD-L1* was observed in tumor cells and in TAMs surrounding tumor cells, with similar percentage (50%) both in high and low density in those two types of cells (Table 2).

Discussion

Recent studies have identified the impact of non-neoplastic cells on disease pathobiology, particularly immunohistochemical studies of cells in the tumor microenvironment. As a result, some biomarkers have identified and translated into clinical practice. The transcription factors NF-κB and NFAT are known as essential factors in activation of B cell lymphocytes (Muhammad K et all., 2014). However, in Hodgkin cells *NFATc1* is not expressed caused by epigenetic silenced mechanism (Akimzhanov et al., 2008), while NF-κB is constitutively active in these tumor cells (Weniger and Kuffer, 2016). Our finding confirmed the results of previous studies (Akimzhanov et al., 2008; Marafioti et al.,

Table 2. The Expression of NFATc1, CD163 and PD-L1

Antibodies	Lymphocytes		MΦ		Tumor cells	
	H	L	H	L	H	L
NFATc1	0	0 (0%)	0	0	0	0
	0%		0%	0%	0%	0%
CD163	-	-	16 (57.14%)	12 (42.86%)	-	-
PD-L1	-	-	14 (50%)	14 (50%)	14 (50%)	14 (50%)

N, 28; MΦ, macrophages; H, high; L, low

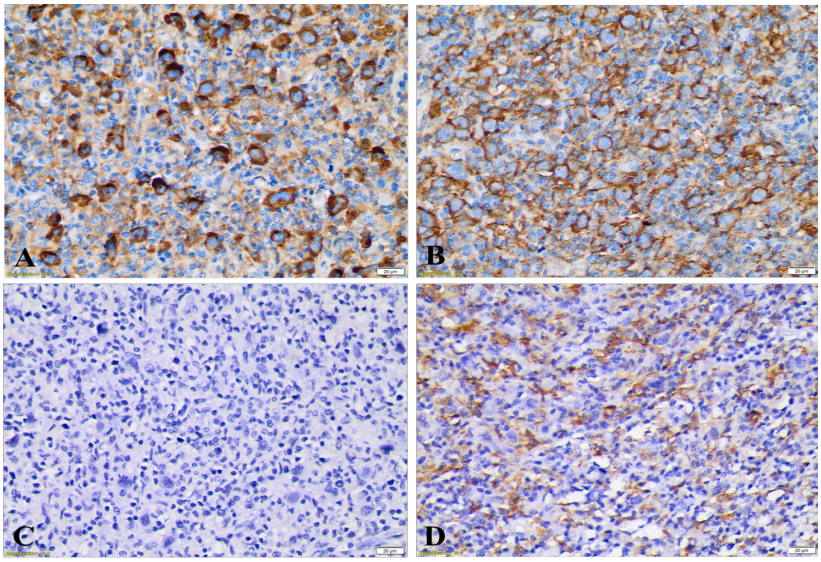

Figure 2. The Immunoreactivity of CD163, PD-L1 and NFATc1 Proteins of Patient #2. A. Varied immunoreactivity of PD-L1 in membrane of Hodgkin cells and TAMs. B. Positive cytoplasmic expression of TAMs CD163+. C. Negative nuclear expression of NFATc1 protein in all cells. D. Membrane expression of CD163. Original magnifications ×400.

2005) that *NFATc1* was not expressed in Hodgkin cells. However, *NFATc1* expression in tumor microenvironment was not discussed in earlier experiments. Our data showed that there were only few small lymphocytes expressed nuclear *NFATc1*, but these cells were situated far from tumor cells. While small lymphocytes which located closed to Hodgkin cells only showed cytoplasmic *NFATc1* expression, none of them have *NFATc1* nuclear expression.

It is known that T cell lymphocytes surrounding Hodgkin cells exhibited unusual phenotypic and functional characteristics may be due to impairment of their regulation (Fozza and Longinotti, 2011). Initially, the lymphocytes were most likely activated and induced to come to tumor microenvironment, as can be seen from Figure 1 that few lymphocytes located far from tumor cells which showed nuclear expression of *NFATc1* suggesting that *NFATc1* is essential for T and B lymphocytes activation, homeostasis and differentiation (Vaeth and Feske, 2018). Most Hodgkin tumor cells were surrounded by T-lymphocytes expressing PD-1 (Ilcus et al., 2017). The expression of PD-1 receptor driving in decreased activation of *NFATc1* (Sharpe and Pauken, 2018), thereby, this mechanism is one factor that was most likely led to down regulation of *NFATc1* in lymphocytes surrounding tumor cells in our samples, yet the exact mechanism is still unclear. This mechanism benefits for survival of tumor cells since TILs expressing PD-1 impaired their effector functions by displaying exhausted phenotype (Thommen

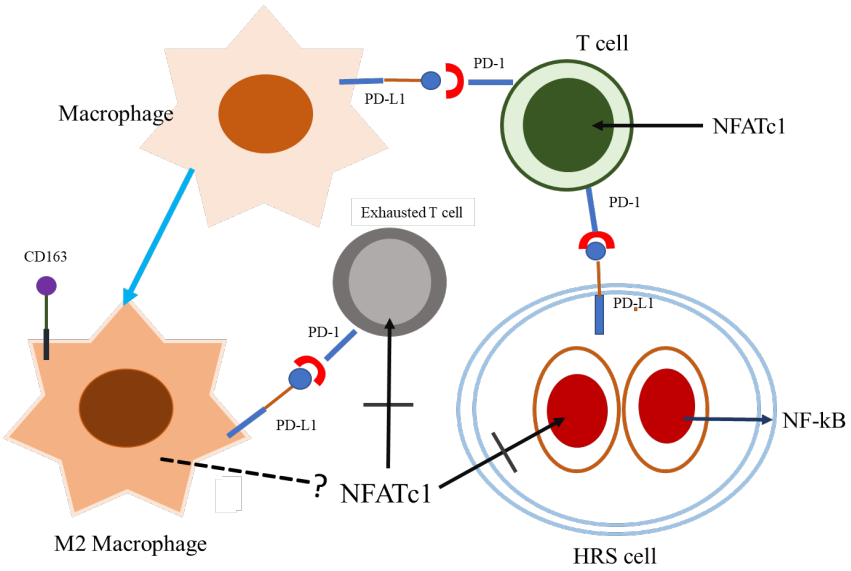

Figure 3. NFATc1 Loss in Hodgkin Cells and CTLs may Contribute to the Molecular Pathways Entailed in Tumor Microenvironment of HL. Macrophages and T cells are heavily infiltrate the background of HL. PD-L1 in macrophages inducing T cell anergy and M2 polarization. While NFATc1 is not expressed in Hodgkin cells caused by epigenetic silenced mechanism, NF-κB is constitutively activated in these cells. The exact regulation of this microenvironment is still unclear and need to be elucidated.

and Schumacher, 2018; Ilcus et al., 2017). Future study is needed to unravel how the precise mechanisms control the silencing of *NFATc1* in tumor microenvironment of HL.

Increased TAMs CD163+ was correlated to unfavorable outcomes (Guo et al., 2016). We did not have any data of patient survival; therefore, we were unable to correlate the presence of TAMs to our patient outcomes. However, here we would like to know whether *NFATc1* may have roles in activation of TAMs CD163+ in tumor microenvironment of HL. In fact, both the nuclear and cytoplasmic *NFATc1* expression in TAMs CD163+ were not observed. Down regulation of *NFATc1* in TAMs and Hodgkin cells may result in T cells anergy, thus, promotes tumor progression. The exact role of *NFATc1* in recruitment and or activation of TAM in tumor milieu is unclear

In our samples, half patients showed high density of *PD-L1* in tumor cells and the same percentage as in macrophages around tumor cells. Patients with high density of tumor cells expressing *PD-L1*, also showed high density of TAMs CD163+ with *PD-L1* expression. This suggests TAMs have important roles in microenvironment of Hodgkin lymphoma. However, we have no information about survival data, hence, we cannot correlate the expression of *PD-L1* in those cells with patient survival, thus, patient prognosis. The expression of *PD-L1* in Hodgkin cells usually correlated to worse prognosis (Jalali et al., 2019). While the expression of *PD-L1* in macrophages could lead to T cell anergy and M2 polarization, indicating that high levels of *PD-L1* expression in macrophages were in accordance with an immunosuppressive tumor environment and decreased anti-tumor immunity (Lu et al., 2019: Jalali et al., 2019; Gordon et al., 2017). Together the expression of *PD-L1* in Hodgkin tumor cells and TAMs lead to worse prognosis of Hodgkin lymphoma patients (Karihtala et al., 2020). It was possible that silencing of *NFATc1* expression may contribute to HRS cells to become immortal and correlated to inferior outcomes. This hypothesis should be investigated by further experiments. Understanding the exact mechanism of *NFATc1* regulation in TME could lead to development of therapeutic pathway by restoring antitumor immunity.

In conclusion *NFATc1* was suppressed both in Hodgkin tumor cells and inflammatory cells surrounding the tumor cells. This condition may contribute to progressivity and aggressiveness of the diseases (Figure 3). Therefore, certain mechanisms to reactivate functional *NFATc1* in cHL tumor microenvironment may be necessary; hence, the tumor cells are able to be eradicated by patient's immune mechanisms.

Author Contribution Statement

All authors read, critically reviewed and approved the final manuscript. KM designed the study, analyzed all data, drafted the manuscript, conducted pathologic interpretation and lymphoma diagnosis also edited the final manuscript text. NN and NFK assisted the experimental process. IK contributed to lymphoma diagnosis. RPR and ZH contributed to the preparation of the manuscript, editing and review. This manuscript is a part of an approved student thesis

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Ethics Statement

The study which has involved paraffin blocks of human tissues, was reviewed and approved by Health Research Review Committee of Mohammad Hoesin Central General Hospital and Faculty of Medicine University of Sriwijaya with Ethical Approval Certificate No. 325/kepkrsmhfkunsri/2019

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