ORIGINAL ARTICLE

Nephrolepis exaltata - Hibiscus rosa-sinensis combination herbal mask decreases levels of reactive oxygen species in nasal wash

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ABSTRACT

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BACKGROUND
Poor air and mask quality in the work environment increase the risks of inflammation, reactive oxygen species (ROS) production, ongoing oxidative stress, and superoxide dismutase 3 (SOD3) levels in the airways. The study aimed to investigate the effect of the *Nephrolepis exaltata - Hibiscus rosa-sinensis* (NE-HRS) combination herbal mask on the airways, as measured by ROS and SOD3 levels in the nasal wash of textile industry workers.

METHODS
This was a pre- and post-test randomized controlled trial, consisting of 30 subjects with ≥5-year working time, conducted for eight weeks and monitored every ten days. Subjects were randomized into 3 groups, regular surgical mask group (n=10), NE-HRS combination herbal mask group (n=10) and factory mask group (n=10). SOD3 and ROS levels were determined using ELISA. Data were analyzed with One-way ANOVA and multiple comparison tests.

RESULTS
ROS levels decreased significantly in all groups (p=0.004). Regular surgical masks decreased ROS levels by 67.90 ± 50.66 pg/mL, NE-HRS combination herbal masks by 49.91 ± 45.06 pg/mL, and factory masks by 193.96 ± 128.36 pg/ml. SOD3 levels also decreased but not significantly (p=0.053). Differences in ROS levels between surgical mask and NE-HRS combination herbal mask was not significant (p=0.796).

CONCLUSION
Herbal masks laminated with NE-HRS combination significantly reduced ROS levels among textile industry workers. NE-HRS combination herbal masks have a similar protective effect to the regular surgical mask.

Keywords: Herbal mask, ROS, SOD3, textile industry workers

INTRODUCTION

Poor air condition due to exposure to pollutants can cause serious respiratory problems, especially if the pollutants are reactive chemicals that easily initiate inflammation. The body will respond continuously with a series of dramatic biological processes if the pollutants are inhaled continuously for a long period of time. The initial risk can result from excessive mucus production, damage to the protective epithelium of the respiratory tract, and malignancy.[1,2]

Early prevention of diseases of the respiratory tract consists in the use of good quality masks. Thus, the chance of acute and chronic inflammation in the respiratory tract can be reduced and the inflammatory response including the secretion of pro-inflammatory cytokines, chemokine up-regulation, and other complex transduction signals that lead to proliferation and excessive cell activation can be controlled.[3] Another important focus that attracts attention is the changing balance of reactive oxygen species (ROS) and superoxide dismutase 3 (SOD3) in the body when inflammation occurs. Inhaled air pollutants deposited in the respiratory tract cause chemical reactions in the body that produce ROS (OH^-, O_2^-, HO_2, and H_2O_2) in the respiratory tract. Excessive production of ROS results in oxidative stress which can injure cells and tissues.[4]

Masks with *Nephrolepis exaltata* (NE) herbal laminate are reported to be able to protect the respiratory tract better. The use of NE herbal masks significantly inhibits the production of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) in tests of a limited population of textile industry workers exposed to reactive chemicals. Similar studies also show that NE herbal masks work actively in suppressing high free ROS and result in decreased superoxide dismutase 3 SOD3, leading to an oxidant-antioxidant imbalance.[5] An experimental study showed that the NE herbal mask is better in increasing IgA and improving the pulmonary function in textile workers compared to the regular mask.[6]

The ethanol extract of *Hibiscus rosa-sinensis* (HRS) contains flavonoids, saponins and steroids and might be responsible for significant inhibition of inflammation, which may involve inhibition of prostaglandins,[7] as well as blocking of the oxidative stress.

Our previous study on different subjects recommended the further study of the potency of the NE herbal mask on the sinonasal cellular immune system [6] and the present study added the measurement variables to prove the effect of the NE-HRS combination herbal mask on sinonasal oxidative stress. This study was expected to prove the effectiveness of the NE-HRS combination herbal mask in decreasing the levels of ROS and SOD3 in textile dyeing factory workers.

METHODS

Research design

This study used a non-blinded experimental design with pre-and post-test randomized controlled trial and was conducted in PT. APAC Inti Corpora, Bawen, Semarang, between September-October 2019.

Research subjects

The study recruited 36 subjects that fulfilled the inclusion criteria, but only 30 subjects were included (2 subjects not meeting the inclusion criteria and 4 subjects declining to participate). The study sample consisted of employees of a garment factory in Bawen, Semarang, who had been exposed to occupational chemicals in the dyeing process section. In total, 30 employees were selected based on the inclusion criteria i.e., 25–50 years old, healthy, and willing to be a research subject; and the exclusion criteria i.e., having a history of alcohol consumption, smoking, liver disease, autoimmune disease, cancer, pulmonary and heart disease and/or being pregnant;. The sample was not similar to that of our previous study.[6]

The sample comprised 30 male textile factory workers, 25-50 years old, and working

in the dyeing and sizing areas. The sample size determination was based on the total number of male workers in the dangerously polluted area of the factory (dyeing area). The inclusion criteria also included subjects having complaints of mucus or watery discharge out of the nose and a working time (in a textile factory) of ≥5 years. The allocation of the groups was done using the randomized block method with block size=6 (using randomly generated numbers). The subjects were randomized into the groups wearing i) regular medical/surgical masks (n=10), ii) NE-HRS combination herbal masks (regular medical/surgical masks that were laminated with NE and HRS extracts) (n=10) and iii) factory masks (single-ply cloth masks) (n=10). To the subjects it was explained how to use the masks correctly. The researchers gave the masks to the supervisor of the subjects every 10 days, then the supervisor distributed the masks to the subjects and ensured the compliance of mask wearing. The masks were used in the work area and were replaced daily for 8 weeks. The duration of wearing masks per day was around 6-7 hours on work days. Compliance in using the masks was ensured by daily supervision using monitoring cards and follow-up by the researchers was done every 10 days to evaluate the usage of masks and the health condition of the research subjects. After 8 weeks, a second measurement of ROS and SOD3 was done. The present study determined the protective effect of the masks from the ROS and SOD3 levels, but did not report on the evaluation of the usage of masks and health conditions of the subjects.

Preparation of plant extracts and herbal masks

The process of making extracts of *Nephrolepis exaltata* and *Hibiscus rosa-sinensis* was carried out at the Laboratory of Science and Mathematics, Diponegoro University, Semarang, while the herbal masks as well as the regular surgical masks were made by CV. Beauty Kasatama, Surabaya. The herbal masks were prepared by a lamination technique (three-ply surgical masks were laminated with NE-HRS extracts) to maximize the quality and comfort of the mask design. The single-ply cloth masks were produced by the factory itself. Details of the procedures are as specified in Indonesian simple patents nos. S00201909409 and S00201909412, both published on January 21, 2020.

Laboratory analysis

For examination of SOD3 and ROS levels nasal wash samples were used. Subjects were instructed to sit with the head extended at 45°, then to take a deep breath and hold their breath. A syringe filled with 5 ml of isotonic solution at 37°C was inserted into one nostril (with the other nostril closed). The subjects were to hold their position for a few seconds, then look down and slowly drain the liquid into a container. The procedure was to be repeated for other nostril and the whole procedure done in duplicate. All nasal wash fluid samples were stored at 4°C. Each sample was processed for testing by the ELISA method. The ELISA test for SOD3 levels used human SOD3 (Extracellular superoxide dismutase [Cu-Zn]) ELISA kit) while for ROS levels the human reactive oxygen species (ROS) ELISA kit was used.

Statistical analysis

The data were analyzed with SPSS for Windows version 25. Primary data on SOD3 and ROS were analyzed by the Shapiro-Wilk test to determine the data distribution, which turned out to be normal. The One-way ANOVA test was used to analyze the data. If significant the analysis was continued using multiple comparison tests.

Ethical clearance

This study was approved by the health research ethics commission of the Faculty of Medicine, UNDIP and Dr. Kariadi Hospital, Semarang, under No. 194/EC/KEPK/FK UNDIP/V/2019.
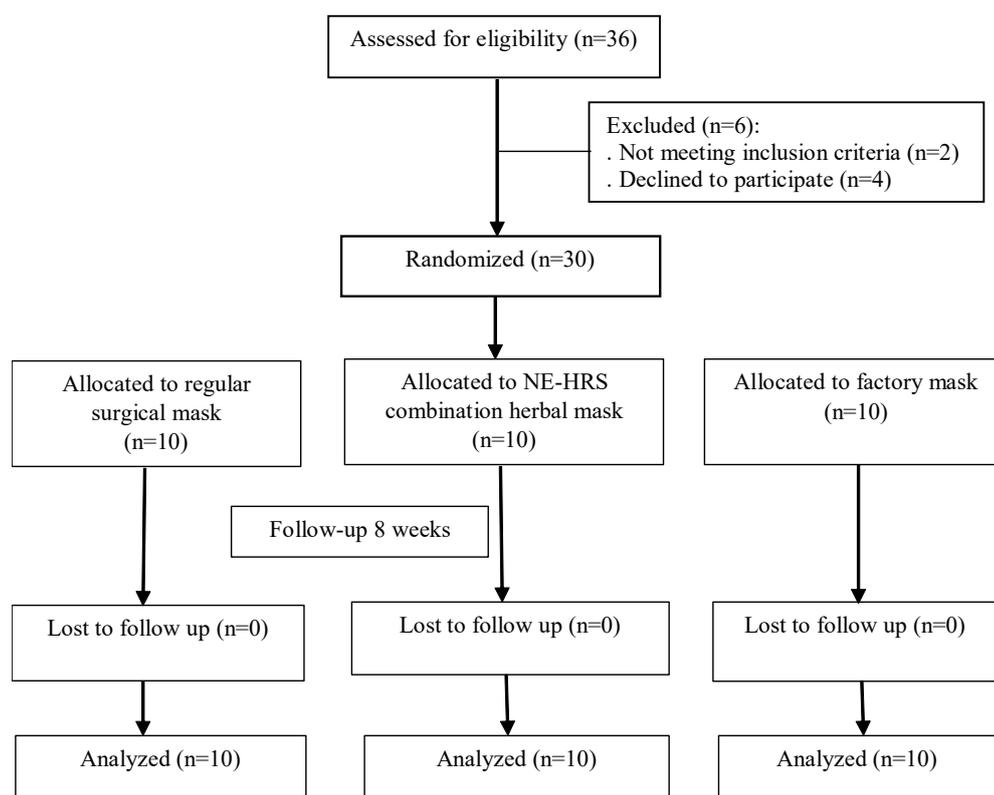
Figure 1. Flow diagram of the participants

RESULTS

The study included 36 subjects that fulfilled the inclusion criteria, but only 30 subjects were included (2 subjects not meeting the inclusion criteria and 4 subjects declining to participate). During the study period, 30 male subjects were randomized into three groups. Ten subjects were in the regular surgical mask group, 10 subjects in the NE-HRS combination herbal mask group and 10 subjects in the factory mask group. They were followed-up for 8 weeks (Figure 1).

At base line, there were no significant differences in age, work duration, and SOD3 level, respectively, between the three groups (p=0.318;

p=0.626; p=0.29), but there was a significant difference in ROS level between the three groups (Table 1). This means that the randomization process was unable to equally distribute all variables among the three groups. After exposure for 8 weeks, it appears that ROS showed significant results (Table 2).

The ROS levels between groups were significantly different, in that the pre- and post-test multiple comparison showed significant results between regular surgical mask, NE-HRS combination herbal mask, and factory mask (p<0.05), but no significant difference between regular surgical mask and NE-HRS combination herbal mask (Table 3). This indicates that the

Table 1. Demographic characteristics, ROS and SOD3 by treatment group at base-line

Variables	Regular surgical mask (n=10)	NE-HRS herbal mask (n=10)	Factory mask (n=10)	p value
Age (yrs)	38.25 ± 6.76	38.13 ± 7.81	41.63 ± 8.57	0.318
Length of work (yrs)	15.00 ± 9.74	16.25 ± 9.74	19.25 ± 7.65	0.626
ROS (pg /mL)	147.00 ± 79.89	134.66 ± 73.33	329.44 ± 122.28	0.001
SOD3 (ng /mL)	4.02 ± 2.18	4.26 ± 1.62	3.27 ± 0.96	0.239

Note: ROS: reactive oxygen species; SOD3: superoxide dismutase 3

Table 2. ROS and SOD3 levels after 8 weeks of exposure, by treatment group

Characteristics	Regular surgical mask (n=10)	NE-HRS herbal mask (n=10)	Factory mask (n=10)	p value
ROS (pg/mL)	79.10 ± 32.89	84.76 ± 40.99	135.48 ± 20.98	0.004*
SOD3 (ng /mL)	0.81 ± 0.12	0.67 ± 0.10	0.77 ± 0.12	0.053

Note: ROS: reactive oxygen species; SOD3: superoxide dismutase 3

protective effect of the NE-HRS combination herbal mask was similar to that of the regular surgical mask.

DISCUSSION

The study showed the effectiveness of the NE-HRS combination herbal mask in reducing the levels of ROS significantly, but failed to prove a significant decreasing effect on SOD3 level. All treatment groups showed a decrease in SOD3 levels with different protective powers. Statistically, SOD3 levels after wearing the masks did not show any significant differences, although the greatest decline was found in the herbal mask group.

It has been known that volatile organic compound (VOC), azo dyes, anthraquinone derivatives, CH3COOH, HCOOH, NaOH, NaHS, and formaldehyde are some of the ingredients that are often involved in the coloring process in the textile industry. The heating process at high temperatures (60°-79°C) to strengthen covalent bonds with fabric fibers causes the reactive dyes to evaporate and emit a sharp odor.[8] The fumes released into the air during the coloring process contains H_2S and NO which are easily inhaled by workers and become inflammatory agents in the respiratory tract.[9] Microscopic observations show that airway inflammation due to exposure to formaldehyde (H_2CO) and VOC complexes with NOx in the air is characterized by erosion of airway epithelial cells, goblet cell metaplasia and hyperplasia of flat epithelial cells.[10,11]

Inhaled pollutants deposited in the respiratory tract cause inflammation and chemical reactions in the body and produce reactive oxygen species (ROS, OH-, O_2^-, HO_2, and H_2O_2) that cover the respiratory tract. Some studies have shown that excessive ROS production in oxidative stress can injure cells and tissues in the respiratory tract. This condition then disturbs the oxidant-antioxidant balance in the body. *N. exaltata* extract was chosen as a coating on herbal masks because of its strong antioxidant content, inhibition of human bronchial epithelial carcinoma (BEAS-2B) cells and counteracting of oxidative stress due to inhaled VOCs.[12] In this study, NE herbs were enriched with the addition of HRS to maximize its protective function in suppressing inflammation and oxidative stress due to an imbalance between ROS and antioxidants.[13-15]

The analysis of ROS levels of the workers showed that all three types of masks used were protective. All study groups (regular, herbal mask or factory mask groups) were capable of reducing workers' ROS levels significantly. At base-line, the distribution of all variables between groups was homogenous, except for the ROS level. After 8 weeks exposure the factory mask group surprisingly showed an extreme decrease in ROS level. Theoretically, the reactive oxygen species levels in the factory mask group should have been the highest compared to the other two groups. This is because in factory masks, the filter

Table 3. Multiple comparison of pre- and post-test of ROS levels

Treatment groups	Mean differences	p value
Regular surgical mask NE-HRS combination herbal mask	12.34 ± 47.18	0.796
Factory mask	-182.44 ± 47.18	0.001
NE-HRS combination herbal mask Factory mask	-194.78 ± 47.18	0.000

The mean difference was significant at the 0.05 level

density used tends to be more lax and more likely to pass inhaled pollutants into the respiratory tract. Exogenous agents (reactive chemicals, ozone, nitrogen dioxide, cigarette smoke, pollutants, radiation, drugs, nutritional intake) and endogenous agents (inflammation, organelle damage, peroxisomes, mitochondria and cell metabolism) are the components that are involved in causing the levels of ROS in the body.[14]

The mechanisms of interaction of pollutants and ROS components with the body structure are complex, but one hypothesis is the involvement of reduction-oxidation (redox) reaction cycle in the epithelial lining of the respiratory tract.[4] This process is initiated by the transfer of electrons from antioxidants to quinone transition metal ions to form reduced metal ions or semiquinones. This process is further rearranged by the reaction with O_2 to form the oxygen free radical which is then converted to H_2O_2 which is the center of the cycle of radical reactions and oxidative stress in the respiratory tract.[15] Basically, oxidant production is neutralized by several antioxidants through cellular mechanisms in the respiratory tract. Intracellular antioxidants in the respiratory tract include catalase, SOD, and glutathione, formed by the enzymes γ-glutamyl cysteine synthetase and glutathione synthetase.

The SOD3 test results of the workers after using a mask showed better data (because the improving of SOD3 levels) and these indicated protective functions; however, the differences among groups were not significant. All treatment groups showed a decrease in SOD3 levels with a different protective power. Statistically, the difference in the SOD3 levels after wearing the masks did not show any significant difference, but the greatest decline was found in the herbal mask group. One possible reason for this finding is that the herbal mask gives double protection to the factory workers, as antioxidant as well as anti-inflammatory agent, to prevent pulmonary edema.[16]

This study found that using masks prevented the decrease in the level of SOD3 and the increase of ROS, although the results were not significant.

This is in line with a previous study, that found that using a mask protected the respiratory system from harmfull pollutants.[17] These results also represent the concept of an oxidant-antioxidant balance, where good health status allows one to be able to adapt to external stimuli and maintain the immune system, so the ups and downs of SOD3 levels are closely related to the deposition of high and low ROS in the body.[18] This result was also in line with a previous study stating that SOD3 levels in the extracellular matrix decreased with the alveolar injury condition of animals which gradually improved.[19]

Previously, observations of the level of the cytokine TNF-α as inflammatory marker showed a significant decrease in levels after routine wearing of masks by the workers.[20] This condition turned out to be synergistic with SOD3's position as an antioxidant agent that plays a role in reducing the migration of inflammatory cells with regulation of adhesion molecules such as tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL6, macrophage inflammatory protein (MIP)-2, monocyte chemoattractant protein (MCP)-1, vascular adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), P-selectin, and E-selectin in inflammatory tissues. The balance between antioxidants and ROS is also supported by research showing that antioxidant levels increase with increasing ROS levels in the healthy control group. Conversely, antioxidants drop when ROS levels are reduced. This will be different in pathological conditions in which ROS accumulate in many cells and tissues. The addition of exogenous antioxidants to a certain extent actually stimulates the production of higher levels of endogenous antioxidants because it balances excessive ROS levels in the body.[21]

Some factors influenced the results of this study, both internal and external factors. The dyeing area was chosen as the location of the study because it involved the greatest number of chemicals in the coloring process. The risk of inhalation of these chemicals is due to the low degree of awareness of the workers who lack discipline in using personal protective

equipment. In fact, the coloring process takes place at high temperatures and emits strong-smelling hot steam. In addition to the location and discipline of workers, smoking habits, good nutritional intake, age and sex are thought to have participated in influencing ROS and SOD levels throughout the study. Further study is needed about the use of the NE-HRS combination herbal mask in other industries, the prolonged use of the NE-HRS combination herbal mask, and other combinations of herbal mask composition.

The limitation of this study was that the time for data collection could not be equated to all samples, and may have been aûected by the physical and mental conditions of the subjects. The limited population of subjects was also suggested to be another limitation of the study, and may be attributed to differences in the conditions of the work environment. The clinical implication of the study is the importance of wearing a mask, especially in polluted areas, that should become a daily habit. The further studies should be directed to prove the potency of the NE-HRS combination herbal mask on the cellular immune system as well as lung functions in acute exposure, compared to the N95 mask.

CONCLUSION

This study demonstrated that the NE-HRS combination herbal mask was capable of reducing the ROS levels among industrial textile workers. The protective effect of the NE-HRS combination herbal mask was similar to that of the regular surgical mask.

CONFLICT OF INTEREST

The authors declare that they have no conûicts of interest concerning this article.

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