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THE ASTAXANTHIN EFFECT ON THE DIFFERENTIAL COUNT OF INFLAMATORY CELL IN NICKEL ALLERGY BALB/C MICE MODEL

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ABSTRACT : Nickel is a material that commonly used in dentistry, but in some individuals, it can cause *type* IV delayed-type hypersensitivity (DTH) reaction. In this case, DTH reaction pathway involves T cell helper 1 (Th1) activation that stimulates macrophages to release pro-inflammatory cytokine. Meanwhile, astaxanthin (AST) is carotenoid from red algae that has an anti-inflammatory effect by inhibiting inflammatory mediators. Hematology evaluation is one of the evaluation methods for allergies. This investigation aimed to determine the effect of astaxanthin supplementation on the differential count of inflammatory cells in nickel allergy of mice model. Sixteen Balb/C mice were randomly divided into 4 groups: normal group (N), without therapy group (NA), Astaxanthin 12 mg treatment group (Ast12) and Astaxanthin 6 mg treatment group (Ast6). All groups were given an injection of Nickel(II) chloride ($NiCl_2$), Complete Freud's Adjuvant (CFA), and Incomplete Freud's Adjuvant (IFA) to obtain the nickel allergy model. Analysis of differential count in an inflammatory cell (neutrophil, lymphocyte, monocyte and eosinophil) performed by utilizing flow cytometry, then, the data were analyzed using Univariate Analysis ($p<0.05$). As the result, there were no any significant differences between groups for neutrophil and lymphocyte showed by One-Way ANOVA test. Kruskal-Wallis test for eosinophil and monocyte also exhibited no significant difference. In sum, astaxanthin supplementation did not have any effect on the differential count of inflammatory cells (neutrophil, lymphocyte, monocyte and eosinophil) in nickel allergy mice model compared to the normal and without therapy ones.

Key words : Delayed-type hypersensitivity, red algae, astaxanthin, anti-inflammatory, inflammatory cells.

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INTRODUCTION

Nickel is a metal that is often used as a mixture of several alloys including dentures, orthodontic wire, and dental implants; however, nickel also potentially cause a metal allergy (Radkevich *et al*, 2013; Saito *et al*, 2016; Goenharto *et al*, 2020a; Goenharto *et al*, 2020b). The metal is considered as a trigger of recurrences in atopic dermatitis and the types of metal that give a high frequency of positive patch test are Nickel, chromium, and cobalt (Widia *et al*, 2017; Sitalaksmi *et al*, 2020). Previous study reported the prevalence of sensitization to nickel, cobalt and chromium were, respectively, 14.5%, 2.1% and 0.8% in the European general population in 2010 (Schuttelaar *et al*, 2018). Other study also reported the same result, from positive metal allergy patients for about 77% had nickel allergy at Dermatology clinic in Brazil between 2003-2015 (Duarte *et al*, 2018). Furthermore, based on Widia *et al* (2017), there was about 17.39% gave a positive result from 23 patients with atrophic dermatitis tested with patch tests of nickel sulfate.

Nickel is commonly found in stainless steel brackets and classified as a chemical carcinogen (Hafez *et al*, 2011). Several factors such as saliva and exposure time can create an effect of nickel corrosion in the oral cavity leading to ion release from nickel resulting in a harmful effect; such as allergic, toxicity, and mutagenicity (Karlina *et al*, 2016; Narmada *et al*, 2018). Moreover, some of the symptoms reported as the allergic reactions to nickel contained in the alloy among others were severely inflamed hyperplastic gingival tissue around the crowns or space maintainers, alveolar bone loss and the edema in the gums, palate, and throat (Kulkarni *et al*, 2016).

Correspondingly, the hypersensitivity of an allergic contact to metals is a delayed-type allergy or Type IV

reaction that involves the sensitized T cells and happens once T cells are sensitized to an antigen (Saito *et al*, 2016; Uzzaman and Cho, 2012; Maker *et al*, 2019; Ernawati *et al*, 2017). T cells activated by an exposure to nickel antigens will produce inflammatory cytokines and chemokines at the exposed site which triggers allergic reactions leading to skin lesions progression (Saito *et al*, 2016; Mahdani *et al*, 2020). Meanwhile, Type IV allergic reactions are subcategorized into four groups based on the type of specific T cells involved (Maker *et al*, 2019). Nakasone *et al* (2018) argued that CD8 + T cells involve in the production of high levels of T helper 1 cytokines accumulated in nickel-induced intraoral metal contact allergy murine model

Meanwhile, studies on metal allergy in mice models have been carried out. Previous studies revealed that histological examination carried out on mice ear with nickel mice model exhibited that there was a massive infiltration of inflammatory cells involving mononuclear cells, neutrophils, monocytes, and macrophages. The model of intraoral metal contact allergy is induced by two stages of nickel sensitization; first, the sensitization stages start after nickel exposure to the skin; and second, the elicitation stages, which are characterized by edema and an increase in T cells (Saito *et al*, 2016; Nakasone *et al*, 2018). Based on research conducted by Ashrin *et al* (2014), it displayed that injection of siRNA (Small interfering Ribonucleic acid), TSLP (Thymic Stromal Lymphopoietin) and atelocollagen, as the therapy in mice's ears 3 days before the elicitation or the second injection phase of nickel induction, could have an effect that significantly reduced the thickness of mice's ear compared to mice in the control group. Furthermore, topical corticosteroids or calcineurin inhibitors may be estimated for more rapid control of symptoms for Type IV hypersensitivity therapy, however, some patients have not responded to therapy with steroids due to the contact allergy to gluco-corticosteroids (Maker *et al*, 2019; Kot *et al*, 2017).

Furthermore, astaxanthin (3,32 -dihydroxy-β, β2 -carotene-4,42 -dione) contained in red and green microalgae such as *Haematococcus pluvialis*, *Chlorella zofingiensis* and *Chlorococcum* is a carotenoid xanthophyll that recently has been established to have anti-inflammatory effects and to regulate the expression of inflammatory cytokines (Ambati *et al*, 2014; Yoshihisa *et al*, 2016). Astaxanthin (AST) provided as a nutritional supplement, antioxidant and anticancer agent which can prevents diabetes, cardiovascular diseases, neurodegenerative disorders; enhance the immune response and reduced a DNA oxidative damage biomarker and inflammation (Ambati *et al*, 2014; Park *et al*, 2010). A study discovered anti allergic and anti inflammatory impacts of AST in a dinitrofluorobenzene (DNFB) induced contact dermatitis (CD) mouse model and RBL 2H3 cell lines (Kim *et al*, 2015). Moreover, AST can also be found within various commercial products in the market with effective dosage on human health benefits around 2–12 mg/day (Ambati *et al*, 2014).

Therefore, from the explanation above, this investigation was designed to investigate the impact of astaxanthin supplementation which has an antiallergy and anti-inflammatory impacts using the dosages of 6 mg and 12 mg per day in nickel allergy mice model. To evaluate the effect, this study implemented the differential count of inflammation cell in blood as a parameter.

MATERIALS AND METHODS

This examination employed 16 male *Mus musculus* Balb/C mice (aged around 8-12 weeks old). They were kept in good condition with a specific-pathogen free and temperature-controlled environment and provided with a standard laboratory diet and water. This investigation was an experimental laboratory type with the design of a post-test only control group design. The utilized sampling technique was a simple random sampling technique. Additionally, the feasibility of the research had been approved by the Research Ethics Committee of the Faculty of Dentistry, Universitas Hang Tuah, with the approval number of EC/003/KEPK-FKGUHT/VI/2020.

In the beginning, mice were divided into 4 groups, they were Normal group (N), Nickel allergy non-treatment group (NA), Nickel allergy treated with 12 mg Asthaxantin (Ast12) (Landson, Indonesia) and Nickel allergy treated with 6 mg Asthaxantin (Ast6) (Landson, Bekasi, Indonesia). All groups had been inducted with the Delayed-Type Hypersensitivity using Nickel as described by Ashrin *et al* (2014). The groups of NA, Ast12, and Ast6 implemented a combination of NiCl2 (Sigma Aldrich, St. Louis, MO) and Incomplete Freud's Adjuvant (IFA) (Sigma Aldrich, St. Louis, MO) injection with 125l Nickel(II) chloride ($NiCl_2$) and 125ml Incomplete Freud's Adjuvant (IFA) intraperitoneally on Day 1 (Ashrin *et al*, 2014). Then, NA was the group of nickel allergy mice treated with placebo (Aquadest), while Ast12 and Ast6 groups were treated with 12 mg and 6 mg Asthaxantin orally on days 11-16, respectively.

On day 14, an intradermal injection was carried out in the mice's ears for the groups of N, NA, Ast12 and Ast6 with a combination of $NiCl_2$ and Complete Freud's Adjuvant (CFA) (Sigma Aldrich, St. Louis, MO) solution with a composition of 10ml $NiCl_2$ and 10ml CFA using

26G needle to get DTH. Before intradermal injection in the mice ears, an anesthesia was performed using ketamine 10% at the dose of 0.1 ml/kg BW intramuscularly. Then, on Day 16, all groups were sacrificed by employing euthanasia using ketamine + xylazine. After that, the blood was taken from the heart of the mice and the samples were stored in EDTA tube. These steps were conducted in the laboratory of animal trials at the Faculty of Dentistry, University of Hang Tuah, Surabaya. Meanwhile, the sample analysis was performed using flow cytometry methods (Sysmex XT 2000i Analyzer) conducted in the Regional Health Laboratory Surabaya (Balai Besar Laboratorium Kesehatan Surabaya, Indonesia).

Following those steps, neutrophil and lymphocyte data were tested for normality and homogeneity. If the results were normal, a one-way ANOVA statistical test was then performed. Compared to neutrophil and lymphocyte, eosinophils and monocytes were differently treated, if their data did not show any normally distribution and homogeneous, then the non-parametric Kruskal Wallis test would be performed. Additionally, the statistical analysis was performed using One-Way ANOVA (neutrophil and lymphocyte) and Kruskal Wallis test (eosinophil and monocyte) with p-value of < 0.05 then the result would be considered as statistically significant.

RESULTS

The examination result in the different count of inflammatory cells (neutrophil (%), lymphocyte (%), monocyte (%) and eosinophil (%)) during 3 days after injection of $NiCl_2$, CFA and IFA revealed a change in differential count percentage of inflammation cells. Differential count of eosinophil cell type exhibited that under normal conditions, there was no eosinophil in the blood. However, within the allergic conditions, eosinophil appeared in the blood (systemically). The differential count of eosinophil presented the highest number in Ast12 and the lowest in Ast6 (Fig. 1).

Meanwhile, the differential count for other types of inflammatory cells (neutrophil, lymphocyte and monocyte) exhibited varies values. The highest different count of neutrophil was found in the normal group, while the lowest was presented in the no-treatment group. Furthermore, differential count of lymphocyte discovered the highest number was in no-treatment group, followed by Ast12 and Ast6, while the lowest was displayed in the normal group. Moreover, monocyte observations also differ from 3 previous indicators, where the highest value was located in Ast12, followed by no-treatment control group, Ast6 group and the least one was in the normal group (Fig. 2).

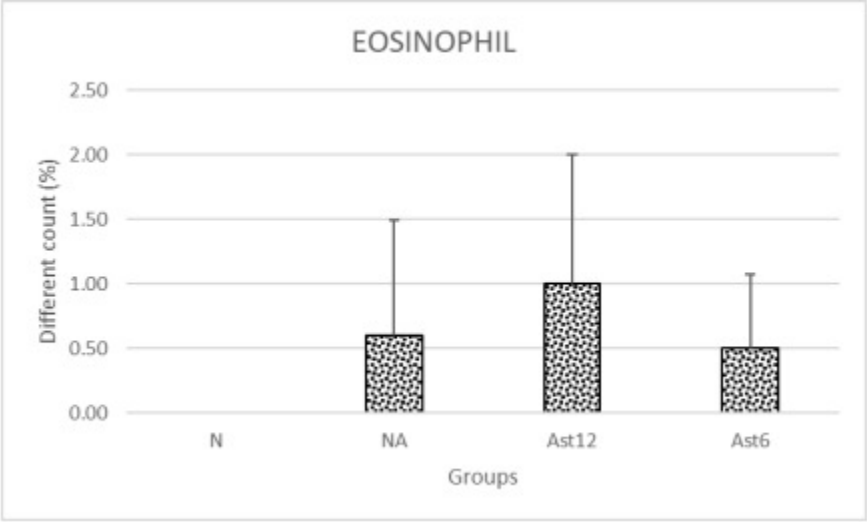

Fig. 1 : Differential count of eosinophil in blood circulation.

Table 1 : Data analyses of differential count in inflammatory cells (eosinophil, neutrophil, lymphocyte, monocyte) in blood circulation.

Groups	Eosinophil		Neutrophil		Lymphocyte		Monocyte	
	X	SD	X	SD	X	SD	X	SD
N	-	-	46.75	35.34	49.50	32.64	3.75	3.10
NA	0.60	0.89	13.60	6.84	80.00	8.34	5.60	4.22
Ast12	1.00	1.00	18.80	14.75	72.00	12.90	8.00	10.67
Ast6	0.50	0.58	29.75	6.85	64.00	7.75	4.75	2.22
Univariate analysis	0.306[2]		0.090[1]		0.111[1]		0.783[2]	

Notes : 1 = one way ANOVA test, 2 = Kruskall-Wallis test.

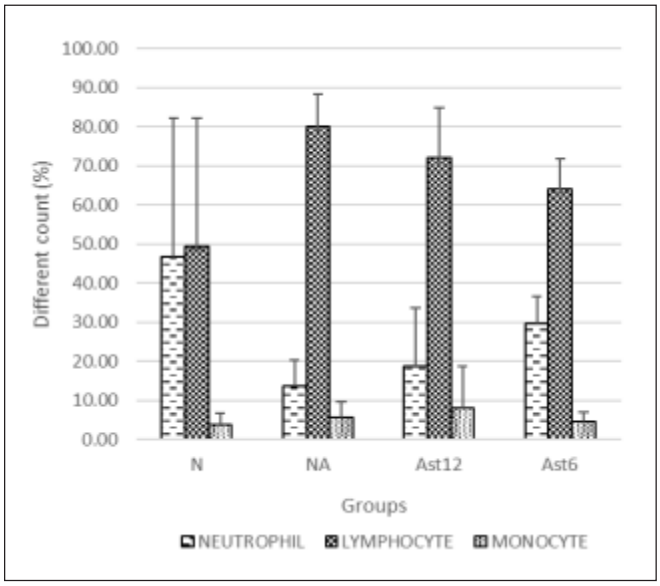

Fig. 2 : Differential count of neutrophil, lymphocyte and monocyte in blood circulation.

Despite the observations demonstrated some differences in the differential count of each inflammatory cell, however, the statistical analysis (univariate) showed there was no any significant difference, both through the Kruskal-Wallis test (for eosinophil and monocyte) and the one-way ANOVA (for neutrophil and lymphocyte) (Table 1).

DISCUSSION

The most effective and reliable method for diagnosing of Type IV hypersensitivity is by employing the patch test (Brandão *et al*, 2012). As astaxanthin itself has been tested and proven to have anti-inflammatory, immunomodulatory, and anti-allergic effects, this study performed the differential count of inflammation cell analysis to evaluate the consequence of astaxanthin therapy on the nickel allergy systemically.

Furthermore, the number of eosinophils, neutrophil, lymphocyte and monocyte in the blood in this study comparing the allergic nickel groups with the normal groups demonstrated that there was no any significant difference. Similar results were found in the earlier study conducted by Pazzini *et al* (2011), in patients using fixed orthodontics, which also revealed that there was no any difference in the amount of eosinophil, neutrophil, lymphocyte and monocyte in the blood of patients both allergic or non-allergic to nickel. However, the number of bands which stimulated by inflammation significantly increased in allergic patients compared to the non-allergic subjects. This study also exhibited that the fragment had stratified hyperplastic squamous epithelium, with chronic inflammatory cells and newly formed blood vessels, suggesting the existence of gingival hyperplasia in biopsy results (Pazzini *et al*, 2011; Larasputri *et al*, 2018). It is indicated that blood tests for nickel allergy should be performed in examining the number of bands in the blood and histopathological examination needs to be done as well.

In the same manner, based on the results of this study, it was discovered that the number of eosinophils in the blood increased in the groups of nickel allergy without therapy and astaxanthin therapies, however, any significant difference was not seen in both groups of without or with astaxanthin therapy (6 mg and 12 mg). In contrast, a research by Yoshihisa *et al* (2016) about the efficacy of astaxanthin for the treatment of Atopic Dermatitis in a Murine Model argued that there was the decreased number of eosinophils in the skin of AST-treated mice (dose 100mg/kg, three times a week for 26 days). The difference results might be determined by the duration of administration and the dosage, and also the methods for analysis in evaluating eosinophil in which the eosinophil number was counted using histological analysis while this study applied a differential count of the blood. Moreover, the skin inflammation observed was the cases of Atrophic dermatitis, which was also one of Type IV hypersensitivity allergy characterized the increased numbers of eosinophils histopathologically (Yoshihisa *et al*, 2016).

The differential count results from neutrophils in the nickel allergy groups compared to the astaxanthin treatment groups (6 mg and 12mg) proved the non-existence of difference which indicates that astaxanthin therapy has no impact in elevating or suppressing the number of the blood neutrophil. On the contrary, a research performed by Kim *et al* (2015) presented different result in which AST topical therapy (0.1 mg/ml) may contribute to alleviate the ear swelling and hyperplasia due to its suppression of inflammatory reactions including the infiltration of neutrophils, activation of keratinocytes and proliferation of fibroblasts on dinitrofluorobenzene-induced contact dermatitis in mice. The difference of this result might be caused by the different methods of administration and the dosage of astaxanthin along with the different methods for analysis in evaluating neutrophil in which the neutrophil associated with mast cell was calculated using histological analysis, while this study employed a differential count of blood. Furthermore, neutrophils contribute greatly in the inflammatory process, but the involvement of astaxanthin does not have a systemic effect due to the nickel allergy model does not involve any systemic inflammatory cells.

Additionally, topical application of AST (0.1 mg/ml) was reported effectively reducing the inflammatory

hyperplasia, epidermal spongiosis, edema and mononuclear cell infiltration (monocytes and lymphocytes) (Kim *et al*, 2015). In this examination, the differential count results from lymphocytes and monocytes of the nickel allergy group compared to the astaxanthin treatment groups (6 mg and 12 mg) displayed the same value. It presented that astaxanthin has no effect both in lymphocyte and monocytes number. In the atrophic dermatitis case, the skin inflammation observed characterized by the infiltration of T lymphocytes and monocytes histopathologically (Yoshihisa *et al*, 2016). Furthermore, a histological examination of mononuclear cells in the astaxanthin topical treatment on cutaneous wound expressed markedly decrease compared to the control group (Meephansan *et al*, 2017). The difference of these results might be led by the distinctive methods of administration and the dosage of astaxanthin along with the incomparable methods for the analysis of both mononuclear cell (monocytes and lymphocytes) evaluation. Moreover, the immunomodulatory consequences of astaxanthin might also play a role in the results of this study.

Moreover, AST has immunomodulatory impacts by increasing proinflammatory cytokine or anti-inflammatory cytokine production without inducing cytotoxicity effects in primary cultured lymphocytes (Lin *et al*, 2016). AST also protects lymphocyte and neutrophils towards the oxidant stresses determined by the actions of certain white blood cells without reducing the white blood cells' killing effect itself (Bolin *et al*, 2012). The impacts of astaxanthin in accelerating healing was facilitated either by suppression of the level of inflammation or by accelerating the inflammatory phase which can be observed on the first day after injury during the inflammatory phase (Meephansan *et al*, 2017).

In this study, the consequence of systemic astaxanthin by analyzing the differential count of inflammatory cells may not be apparent, due to many factors apart from not having the significant differences in the differential count in the normal group compared to the allergic nickel groups; the administration duration and method of astaxanthin along with the examination method also affect the results.

CONCLUSION

This examination results indicate that astaxanthin supplementation did not have any effect in the results of inflammation cells' (neutrophil, lymphocyte, monocyte and eosinophil) differential count in nickel allergy mice model compares to the normal and without therapy groups. Further histological examination and specific marker examinations are needed to further confirm the impacts of astaxanthin therapy on the exposed site of this nickel allergy model.

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