



## Original article

# Immunomodulation of tahneeq method in IL-12 and CD8<sup>+</sup> T-Lymphocyte, an in-vivo study in neonatal rats

Susilorini <sup>a,b</sup>, Suradi <sup>c,f</sup>, Dono Indarto <sup>c,e,h</sup>, Brian Wasita <sup>c,g</sup>, Poppy Diah Palupi <sup>d,\*</sup>

<sup>a</sup> Doctorate Student of Medical Sciences, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia

<sup>b</sup> Department of Anatomic Pathology, Faculty of Medicine, Sultan Agung Islamic University, Semarang, Indonesia

<sup>c</sup> Doctorate Program of Medical Sciences, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia

<sup>d</sup> Department of Pharmacology and Clinical Pharmacy, Nusaputera School of Pharmaceutical Sciences, Semarang, Indonesia

<sup>e</sup> Department of Physiology, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia

<sup>f</sup> Department of Pulmonology and Respirology, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia

<sup>g</sup> Department of Anatomic Pathology, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia

<sup>h</sup> Biomedical Laboratory, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia



## ARTICLE INFO

## Article history:

Received 14 February 2020

Revised 27 May 2020

Accepted 28 May 2020

Available online 4 June 2020

## Keywords:

Tahneeq method  
Ajwa palm dates  
IL-12 expression  
CD8<sup>+</sup> lymphocytes  
Immunomodulation

## ABSTRACT

Stimulation of the neonatal immune system is quite important for the proliferation and differentiation of antigen-presenting cells (APCs) and T cells. Tahneeq is a traditional method to manually rub the palatal mucosa of newborn babies with pre-masticated Ajwa palm dates. The present study was to investigate the tahneeq effects on IL-12 expression of dendritic cells (DCs) and blood T lymphocytes expressing CD8<sup>+</sup> in neonatal Wistar rats. The number of 90 healthy neonatal Wistar rats have randomly divided into three groups: control group received breastmilk only, treatment group (T1) receiving breast milk + mild-scratched intensity of tahneeq, and T2 group received breastmilk + strong-scratched intensity of tahneeq on the palatal and gingival mucosa immediately after birth. Seven neonatal Wistar rats in all groups were then sacrificed in three hours after birth and days 1, 5, 7, 13, and 30 treatment. IL-12 expression in the palatal and gingival mucosa was determined using immunohistochemical staining, and blood CD8<sup>+</sup> T-lymphocytes were quantified using a flow cytometer. One way ANOVA was used to analyze the percentage of IL-12 and CD8<sup>+</sup> T-lymphocytes among neonatal Wistar rat groups. The T1 and T2 newborn rat groups had significantly higher IL-12 expression than the control group ( $p < 0.001$ ). The increased IL-12 expression in T2 groups significantly increased ( $p < 0.001$ ) compared to the IL-12 expression in the T1 and control groups. The percentage of CD8<sup>+</sup> T lymphocytes in all neonatal rat groups increased on three hours after birth and day 30 treatment but remained constant on days 5 and 7 treatment and decreased on day 13 treatment. At 5, 13, and 30<sup>th</sup> days treatment, the percentage of CD8<sup>+</sup> T lymphocytes in T1 and T2 neonatal rat groups was significantly higher ( $p < 0.05$ ) than that in the control group. In conclusion, the impact on systemic CD8<sup>+</sup> T cells did not influence by the depth of the scratch. Both mild and strong tahneeq increased the systemic CD8<sup>+</sup> T-lymphocytes in neonatal Wistar rats. The roles of anti-inflammatory cytokines and Treg cells should be further investigated to unravel those different results for the development of mucosal immunity in neonates.

© 2020 The Authors. Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

The neonatal immune system is considered a critical period for immunotolerance during the immune system development (Auray et al., 2013), which is characterized by delayed secretion of interleukin (IL) -12 by dendritic cells (DCs) (Mohr and Siegrist, 2016). Natural protection of a baby's life depends on breast milk that consists of nutrients, microbiomes, and other vital substances such as vitamin A (Brandtzaeg, 2009). Ironically, several studies reveal that many breastfeeding mothers in developing countries suffered

\* Corresponding author.

E-mail address: [poppydiahp@gmail.com](mailto:poppydiahp@gmail.com) (P.D. Palupi).

Peer review under responsibility of King Saud University.



Production and hosting by Elsevier

vitamin A deficiency (Akhtar et al., 2013, Roy et al., 1997). Furthermore, the administration of antibiotics during peripartum affected the diversity of microbiomes in breastmilk and led to microbiome imbalance (dysbiosis) in the infant's alimentary system (Miyoshi et al., 2017). Consequently, dysbiosis often causes various diseases of neonates, including infectious and non-infectious diseases (Levy, 2007), and diminishes vitamin A supply. It, therefore, suggests that infants need vitamin A supplement and complex commensal microbiomes to optimize maturation of their immune system and to prevent dysbiosis in future life.

Tahneeq is a traditional method that involves mechanical, physical, and biological activities to transfer oral microbiomes from a male adult to his neonates for the development of T helper cells (Dzidic et al., 2018; Yu et al., 2018). The tahneeq procedure stimulates the palatal mucosa using pre-masticated Ajwa palm dates and has been applied for Arabic neonates for a long time ago. However, very limited studies have scientifically examined the tahneeq that can activate the immune system. The Ajwa palm dates (*Phoenix dactylifera*) are recommended to use in the tahneeq method because they contain essential nutrients such as vitamin A for the regulation of immune cells in the intestinal mucosa, and polyphenols including chlorogenic acid and caffeic acid for stimulation of the cellular immune system and increase of interferon (IFN)- $\gamma$  levels and CD4<sup>+</sup> cells number (Karasawa et al., 2011; Rahmani et al., 2014). *In-vitro* and *in-vivo* studies have revealed that water and ethanol extracts of palm dates increased IFN- $\gamma$  and IL-12 levels (Karasawa et al., 2011). Recently, the tahneeq method has slightly been modified by scratching the palatal mucosa (Ahmad, 2013) to raise cell damage, resulting in the release of DAMP molecules to produce IL-1 and initiation of the next immunity process (Chen et al., 2018). Therefore, the aim of this investigation evaluated the tahneeq effects on modulation of DCs and T cells in neonatal Wistar rats.

## 2. Materials and methods

### 2.1. Materials and chemicals

Fruit palm dates used in this study were Royal Ajwa Al Madinah purchased from PT. Demuria Agung (Semarang, Indonesia). Pilocarpine eye drop (Cendocarpine 2%) was procured from Cendo (Jakarta, Indonesia). Xylocaine solution for injection (AstraZeneca, 2016) was purchased from AstraZeneca (Cambridge, UK), ketamine 10% injection was obtained from Kepro B.V (Deventer, NL). Monoclonal Antibody Anti CD8<sup>+</sup> and IL-12 were purchased from Bio Legend (California, USA).

### 2.2. Experimental animals and groups

Pregnant female rats were acclimatized for 24 hours and maintained at a 25–26°C temperature in the Animal Laboratory, Gadjah Mada University, Yogyakarta, Indonesia. According to the Institutional Animal Care and Use Committee (OLAW, 2002), each experiment has to minimize the number of animal models. Therefore, the sample size was calculated using *Federer's formula* (1966) =  $(T-1)(N-1) > 15$ , T= group number, N= rat number and we got at least 7 neonatal rats/group. Ninety healthy post-natal Wistar rats, which weighed 5–6 g and aged less than 24 hours, were kept together with their mothers in 15 different plastic cages with husks bedding, which were adjusted into appropriate ecological conditions and ventilation, freely accessed food and water for the rat mothers, and provided breastfeeding for the neonatal rats. Before initiating this study, the research protocol was approved by The Bioethics Committee, Faculty of Medicine, Sultan Agung Islamic University (number: 404/XII/2018/Komisi Bioetik). The

neonatal rats were randomly distributed into three groups: Group I (control) given breastmilk only, Group II (T1) given breastmilk and mild-scratched intensity of tahneeq, and Group III (T2) given breastmilk and strong-scratched intensity of tahneeq. After that, seven neonatal rats in all groups were sacrificed in serial times as follow: after three hours (1), the first day (2), the fifth day (3), the seventh day (4), the 13<sup>th</sup> day (4), and the 30<sup>th</sup> day (5).

### 2.3. Rat saliva collections

Adult male rats of Wistar strain were given 0.5 mL of 2% pilocarpine intramuscular injection, and 3–5 min later injected intramuscularly with 0.05 mL of ketamine and 0.05 mL of xylocaine. The rats were allowed to stand for 10 minutes until hypersalivation, and then rats saliva was aspirated using a 1cc syringe and collected into a cup (Varghese et al., 2018). The collected saliva was kept at room temperature for uses in the next experiment.

### 2.4. Palm dates dose calculation

The palm dates dose for human neonates is one fruit/day or equivalent to 7 g/day. That dose was then converted into the dose of rat neonates using the formula of body surface area ratio Crawford-Terry Rourke (1950). Ultimately, we got a 0.056 g dose of palm dates for rat neonates.

### 2.5. Tahneeq methods

According to a hadith narrated by Al-Bukhari and Muslim, the tahneeq method was conducted by rubbing a softened date on the palate of a newborn baby. However, we got a technical problem because it was difficult to rub the rat palate using our finger. Therefore, we developed a stainless steel device to rub the palatal and gingival mucosa of rat neonates, so it called scratch. The ajwa fruit palm dates were crushed and mixed with rat saliva on the petri dish. The mixed palm dates were then weighed and smeared on the palatal mucosa of rat neonates by using a sterile ose with different scratch intensities: 3–5 mm/s for group II and 7–9 mm/s for group III. The scratch intensity was measured using the vibration meter, and tahneeq was applied immediately after birth.

### 2.6. Blood sampling

Blood samples in rat groups I–III, which were terminated three hours after birth, were taken via jugular vein, was previously given an anesthetic cream (Parasuraman et al., 2010). Meanwhile, blood samples of all neonatal rat groups in the following times were taken by cardiac puncture, and the blood samples were collected into Eppendorf tubes containing EDTA (Kumar et al., 2017).

### 2.7. Expression of IL-12

Three hours after birth and giving tahneeq for rats in groups II and III, the palatal and gingival mucosa of all neonatal rats was cut and immunologically stained with the anti-IL-12 monoclonal antibody as routinely performed for histopathological analysis. A pathologist then examined the immunohistochemical-stained tissues under an Olympus CX-21 microscope (Devon, UK) with 400x magnification. The IL-12 expression was determined using Image J1.5R2 software and was then calculated the percentage of fraction areas that represented a brownish color on the palate and gingiva mucosa. The slide observations were performed twice with a one-week interval using the protocols from Biologend (2020).

## 2.8. CD8 + T-lymphocyte examination

The analysis of CD8<sup>+</sup> T-lymphocytes in all blood samples of neonatal rats was carried out using a flow cytometer, as described in the previous publication (Skrajnar et al., 2009). Briefly, 50 µL of each blood sample was transferred into a falcon tube and then added with a five µL anti-CD8<sup>+</sup> PE-A antibody. The mixed sample was incubated for 15–30 min in an airtight container, room temperature, and then added with 450 µL FACS Lysing solution. All lysed samples were re-incubated for 15–30 min at room temperature and then centrifuged 3,000 rpm for 5 min. Cell pellets were added with 450 µL FACS perm wash solution and incubated for 15 minutes. After 5 min centrifugation, cell pellets were added with five µL of Fox P3 anti-antibody APC-A 7 and were incubated for 15–30 min in the darkroom at room temperature. The incubated solution was washed with 450 µL perm wash buffer and was then resuspended in 200 µL perm wash solution. The fluorescence signals of T-lymphocytes were analyzed using a FACStar flow cytometer (Becton Dickinson, Mount View, CA) (WHO, 2007). Finally, the percentage was used to determine the number of T-lymphocytes expressing CD8<sup>+</sup> from total lymphocytes (Franch et al., 1993).

## 2.9. Statistical analysis

Data of immunostaining and flow cytometry analysis were presented as Mean ± Standard Error of Mean (SEM). Significant differences among the groups were determined using the One-way ANOVA test and followed by LSD multiple comparisons *post-hoc* analysis. All data analyses used SPSS 22 software (SPSS Inc., Chicago, IL, USA), and  $p < 0.05$  was considered a significant difference.

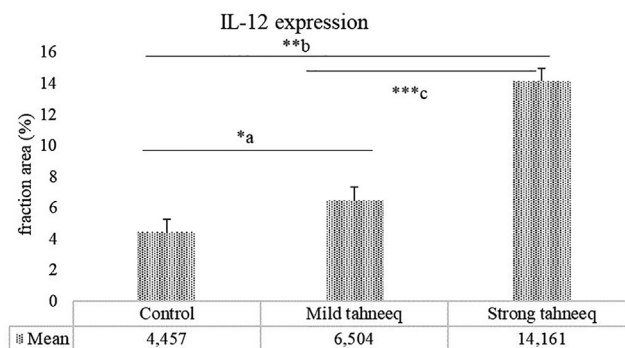
## 3. Results and discussion

### 3.1. Effect of Tahneeq method in IL-12 expression

The present study indicates that the tahneeq method increases IL-12 expression in the palatal and gingival mucosa neonates of Wistar rats after 3 hours (See Fig. 1). Based on the assessment of brown-colored in the cytoplasm of the stained tissues (See Fig. 2), our findings showed that IL-12 expression significantly increased more than three times on the palatal and gingival mucosa of neonatal rats with strong tahneeq intensity, compared with the control group ( $p < 0.001$ ), and a lesser extent of increment showed in the mild-tahneeq intensity group. Moreover, a significant higher of IL-12 expression was observed in the T2 rat group with strong tahneeq intensity, compared with the T1 rat group with mild tahneeq intensity ( $p < 0.001$ ).

Previous studies showed that the neonatal period is a critical time for DC maturation that provides a molecular signal for Th<sub>1</sub> cells expressing alternative IL-4 receptors. This condition leads to DCs more sensitive to apoptotic stimulation, triggered by IL-4, and shifts Th cells from Th<sub>1</sub> to Th<sub>2</sub>. In the normal state, several DCs that express CD11c<sup>+</sup> and CD8α<sup>+</sup> will produce IL-12, resulting in resuming Th1 cells after the sixth day of birth (Kim and Harty, 2014). The failure of IL-12 expression showed a debilitated response of CD8<sup>+</sup> T cells that were susceptible to *Listeria monocytogenes* infection (Pearce and Shen, 2007).

The finding of our study is in line with Karasawa's study that the tahneeq stimulates IL-12 expression in the rat keratinocytes and DCs mucosa. They mechanically stimulated rat keratinocytes by scratching the palatal and gingival mucosa with the mixture of rat saliva and palm dates. Polyphenols in palm dates increase IFN-γ and IL-12 mRNA expression either *in vitro* or *in vivo* study (Karasawa et al., 2011). In contrast, the combination of polyphenols and lipopolysaccharides of oral microbiomes triggers DCs maturation and increases secretion of pro-inflammatory cytokines such as IFN-γ and IL-12 (del Cornò et al., 2016). Besides, the



**Fig. 1.** The IL-12 expression on the palatal and gingival mucosa in neonatal Wistar rats with and without tahneeq. Three hours after birth, all rats were sacrificed, and then the palatal and gingival mucosa was taken for immunohistochemical staining. Data of IL-12 expression were presented as mean ± SEM, and each bar represented seven independent neonatal Wistar rats/group. Significant differences among groups were designated as \*a:  $p < 0.001$ , \*\*b:  $p < 0.001$ , and \*\*\*c:  $p < 0.001$ .

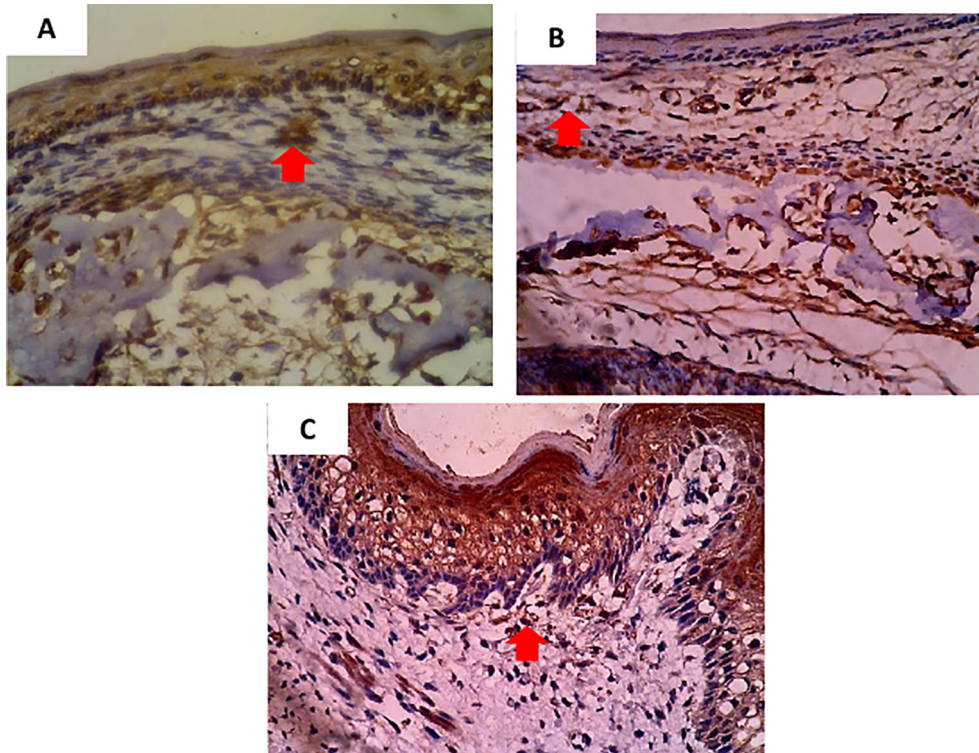
scratches on the palatal and gingival mucosa using palm dates increase cytokines release for the development of effector and memory cells.

IL-12 is one of the essential cytokines which affect NK cells stimulating T cells to differentiate into Th1 cells (Vacaflores et al., 2016), boosts the T-lymphocyte CD8<sup>+</sup> that recently developed as a vaccine adjuvant, and excites the formation of CD8<sup>+</sup> T memory cells, IFN γ, IL-6, and TGFβ (Kim and Harty, 2014; Pearce and Shen, 2007). Therefore, tahneeq may increase the release of other cytokines that affect the differentiation of lymphocytes in the blood circulation.

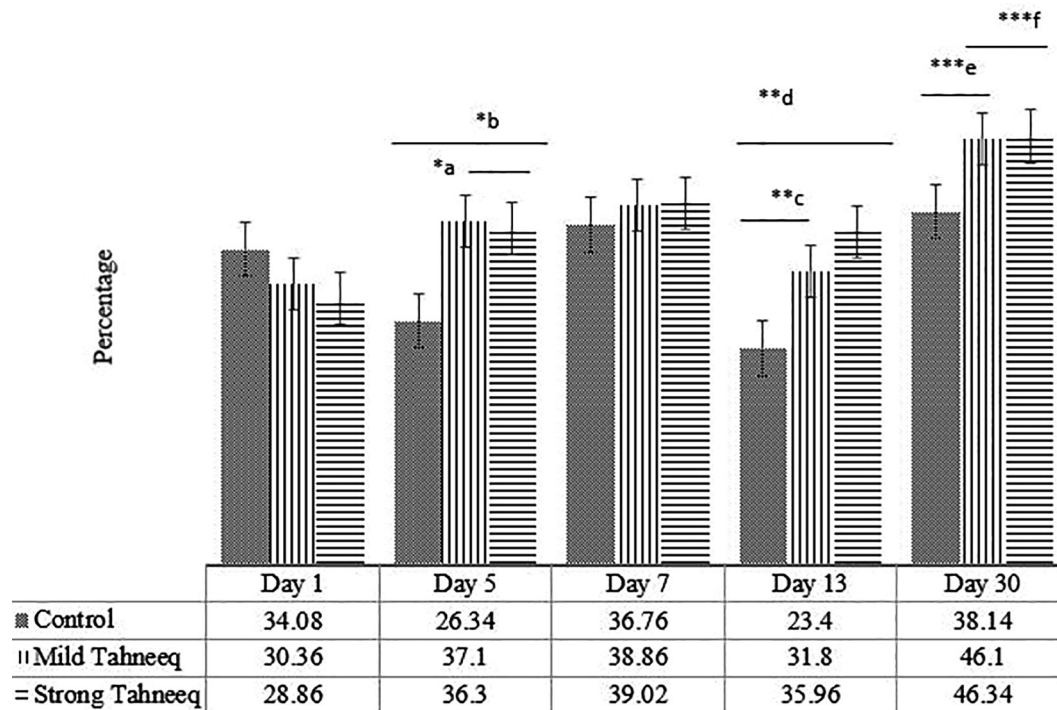
### 3.2. Effect of tahneeq method in T-lymphocyte CD8 + expression

From Figure 3, the percentage of CD8<sup>+</sup>Tlymphocytes in all neonatal groups tends to increase during their lives except on day 13 (See Fig. 3). The CD8<sup>+</sup> percentages increase on three hours after birth (data were not shown), remain constant on days 5 and 7 treatment, decrease on day 13, and begin to rise on day 30. At 5, 13, and 30<sup>th</sup> days treatment, the CD8<sup>+</sup> rate rose significantly ( $p < 0.05$ ) in neonatal rat groups with mild and strong tahneeq, respectively, compared to that in the control group. However, the percentage of CD8<sup>+</sup>Tlymphocytes in neonatal rat groups with mild tahneeq did not differ from the percentage of CD8<sup>+</sup>Tlymphocytes in newborn rat groups with strong tahneeq.

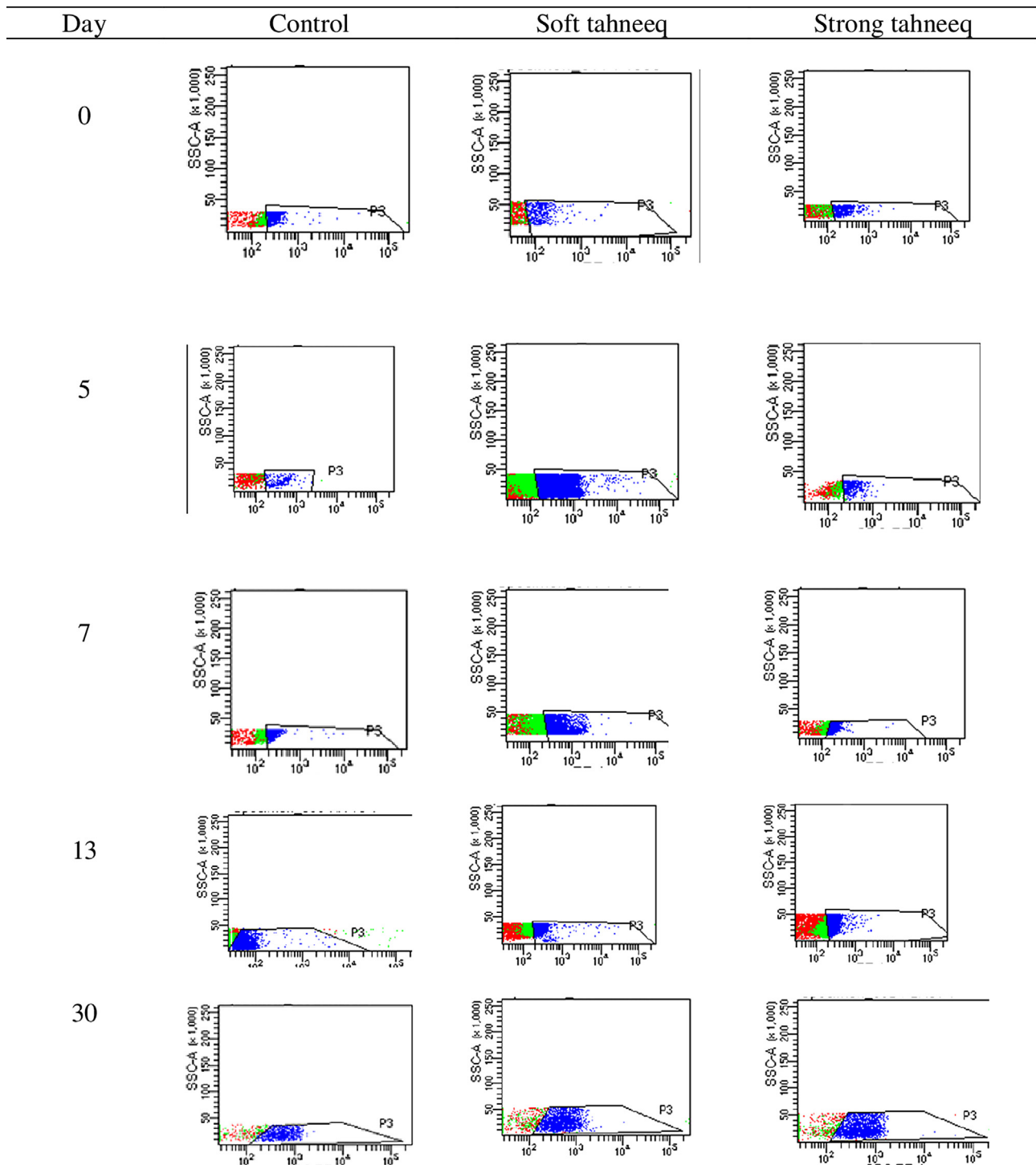
Figures 3 and 4 indicate that the tahneeq increases the number of CD8<sup>+</sup> T-lymphocytes in the 5, 13, and 30<sup>th</sup> days (See Figs. 3 and 4). In this present study, we have first demonstrated that tahneeq may induce proliferation and differentiation of systemic naïve T cells in response to local inflammation. On the other hand, activated CD8<sup>+</sup> T-lymphocytes in neonatal rats with strong tahneeq are not different from activated CD8<sup>+</sup> T-lymphocytes in neonatal rats with mild tahneeq. Therefore, we speculated that the activation of T-lymphocytes effector requires not only the exposure of antigen and pro-inflammatory cytokines but also the presence of anti-inflammatory cytokines and other Th cells. The presence of APCs such as DCs and keratinocytes is essential for cell signaling, which will provide fragmented peptides captured by the T cell receptor (TCR). Moreover, antigen-activated innate immune cells can cause secretion of pro-inflammatory cytokines, which promotes differentiation of CD8<sup>+</sup> effector T-lymphocytes (Kim and Harty, 2014). Achmad, in his study, stated that tahneeq is a method of adult's good bacterial transfer to the newborn (Ahmad, 2013). In the present investigation revealed that tahneeq using adult's Wistar rat saliva with strong intensity would lead to the increasing of IL-12 expression in the palatal and gingival mucosa and the



**Fig. 2.** Immunohistochemical staining of the palatal and gingival mucosa in neonatal Wistar rats 3 hours after birth with and without tahneeq. All tissue sections were observed by a pathologist using a light microscope with 400x magnification. Each image represented seven independent rats in the control group (A), mild-tahneeq intensity (B), and strong-tahneeq intensity (C). Brown color indicated dendritic cells expressing IL-12.



**Fig. 3.** The CD8<sup>+</sup> T-lymphocyte expression in the blood circulation of neonatal Wistar rats with and without tahneeq. Venous blood samples were withdrawn from neonatal Wistar rats in all groups from days 1, 5, 7, 13, and 30, and total lymphocytes were immunologically quantified using a flow cytometer. Percentage of CD8<sup>+</sup> T- lymphocytes were presented as mean ± SEM, and each bar represented seven independent neonatal Wistar rats/group. Significant differences among groups were designated as \*a p=0.004, \*b p=0.006, \*\*c p=0.023, \*\*d p=0.02, \*\*\*e p=0.023, \*\*\*f p=0.020.



**Fig. 4.** Flow cytometry images of CD8<sup>+</sup> T-lymphocyte profile in the blood circulation of neonatal Wistar rats with and without tahneeq. Venous blood samples were withdrawn from neonatal Wistar rats in all groups from days 1, 5, 7, 13, and 30, and total lymphocytes were immunologically quantified using a flow cytometer. Each image represented seven independent neonatal Wistar rats.

number of CD8<sup>+</sup> T cells in the blood. Both control and mild intensity tahneeq had the lowest IL-12 expression. Although the IL-12 expression of the mild intensity group was smaller than the strong ones, the number of CD8<sup>+</sup> T cells expression in the blood is similar. It showed that the intensity of scratch did not influence the immunomodulatory effects of tahneeq but likely related to other mechanisms such as the colonization of the adult microbial community. The previous study in the *in-vitro* experimental approach

showed the combination of breastmilk and saliva reduces the pathogenic commensal bacteria in neonates, then followed by the growth of good commensal bacteria (Sweeney et al., 2018). The colonization of good commensal bacteria such as *Lactobacillus* and *Bacteroides* is vital to stimulate both effector and regulator T cells lineages, and influence the differentiation of T cells, especially induced Treg (i-Treg) (Nutsch and Hsieh, 2012). This present study assumes that the colonization of diverse commensal microbial

communities from an adult's saliva will lead to the up-regulation of CD8<sup>+</sup> T cells and i-Treg. A previous study (Jiao et al., 2009) in HIV-1 infected individuals, the decrease of T reg cells will lead to excessive activation and apoptosis of CD8<sup>+</sup>T cells. This condition is likely to be seen in the neonates period as its immune immaturity will lead to a physiologically immunocompromised immune system. The lowest expressions of IL-12 and the CD8<sup>+</sup> T cell in the control group showed the weakest immune response and correlated with the less microbial diversity of the control group. In conclusion, both mild and strong tahneeq methods could stimulate DCs maturation, but the strong tahneeq method has a more effective, which is indicated by the up-regulation of IL-12 expression. The activated DCs in the upper gastrointestinal system can induce proliferation and differentiation of systemic CD8<sup>+</sup>T cells lymphocytes regardless of the use of either mild or strong tahneeq method. Besides that, both mild and strong tahneeq methods probably lead to neonatal immune tolerance by transferring the adult's microbiome into the neonatal gut to generate commensal bacteria colonization. The intensity of scratching did not affect the immunomodulatory effect. Further research is required for the investigation of the roles of anti-inflammatory cytokines and Treg cells in these homeostasis responses, and their correlation with adult's microbial colonization.

#### Declaration of Competing Interest

All authors declare that they do not have any conflict of interest in the present study.

#### Acknowledgment

The authors would like to thank The Dean of Faculty of Medicine and The Rector of Sultan Agung Islamic University, The Dean of Faculty of Medicine and The Rector of Universitas Sebelas Maret, Dr. dr. Indra Widjaya, Sp.P.A (K), dr. Sumarno, M.Si, Med, Sp.P.A., for support during the study. This study was funded by the Sultan Agung Islamic University Semarang and Universitas Sebelas Maret Surakarta, Indonesia.

#### References

- Ahmad, 2013. Sang Bayi: Kusambut Kelahiranmu dengan Sunah-sunah Nabimu, 1. Kiswah Media, Solo.
- AstraZeneca, 2016. Xylocaine injection solution: Safety data sheet [Online] [Accessed 26th May 2020] Available from World Wide Web <https://www.astrazeneca.com/media-centre/press-releases/2016/astrazeneca-enters-commercialisation-agreement-with-aspen-for-anaesthetic-medicines-portfolio-08062016.html#>
- Akhtar, S, Ahmed, A, Randhawa, M, Atukoral, S, Arlappa, N, Ismail, T, Ali, Z, 2013. Prevalence of vitamin A deficiency in South Asia: causes, outcomes, and possible remedies. *Journal of health, population, and nutrition* 31 (4), 413–423. <https://doi.org/10.3329/jhpn.v31i4.19975>.
- Auray, G., Facci, M.R., van Kessel, J., Buchanan, R., Babiuk, L.A., Gerdt, V., 2013. Porcine Neonatal Blood Dendritic Cells, but Not Monocytes, Are More Responsive to TLRs Stimulation than Their Adult Counterparts. *PLoS One* 8, 1–12. <https://doi.org/10.1371/journal.pone.0059629>.
- Biologend, 2020. Immunohistochemistry Protocol for Paraffin-Embedded Sections [Online] [Accessed 26th May 2020] Available from World Wide Web <https://www.biologend.com/en-us/protocols/immunohistochemistry-protocol-for-paraffin-embedded-sections>.
- Brandtzaeg, P., 2009. Mucosal Immunity: Induction, Dissemination, and Effector Functions. *Scand. J. Immunol.* 70, 505–515. <https://doi.org/10.1111/j.1365-3083.2009.02319.x>.
- Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., Zhao, L., 2018. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget* 9, 7204–7218 <https://doi.org/10.18632/oncotarget.23208>.
- Crawford, J.D., Terry, M.E., Rourke, G.M., 1950. Simplification of drug dosage calculation by application of the surface area principle. *Pediatrics* 5, 783–790.
- Del Cornò, M., Scazzocchio, B., Masella, R., Gessani, S., 2016. Regulation of Dendritic Cell Function by Dietary Polyphenols. *Crit. Rev. Food Sci. Nutr.* 56, 737–747. <https://doi.org/10.1080/10408398.2012.713046>.
- Dzidic, M., Boix-Amorós, A., Selma-Royo, M., Mira, A., Collado, M.C., 2018. Gut Microbiota and Mucosal Immunity in the Neonate. *Med. Sci. (Basel, Switzerland)* 6. <https://doi.org/10.3390/medsci6030056>.
- Federer, W.T., 1966. Randomization and sample size in experimentation [Online] [Accessed 26th May 2020] Available from. World Wide Web.
- Franch, A., Castellote, C., Pelegrí, C., Tolosa, E., Castell, M., 1993. Blood B, T, CD4+, and CD8+ lymphocytes in female Wistar rats. *Ann. Hematol.* 67, 115–118. <https://doi.org/10.1007/BF01701732>.
- Jiao, Y, Fu, J, Xing, S, Fu, B, Zhang, Z, Shi, M, Wang, X, Zhang, J, Jin, L, Kang, F, Wu, H, Wang, F, 2009. The decrease of regulatory T cells correlates with excessive activation and apoptosis of CD8 + T cells in HIV-1-infected typical progressors, but not in long-term non-progressors. *Immunology* 128 (1pt2), 366–375. <https://doi.org/10.1111/j.1365-2567.2008.02978.x>.
- Karasawa, K., Uzuhashi, Y., Hirota, M., Otani, H., 2011. A Matured Fruit Extract of Date Palm Tree (*Phoenix dactylifera* L.) Stimulates the Cellular Immune System in Mice. *J. Agric. Food Chem.* 59, 11287–11293. <https://doi.org/10.1021/jf2029225>.
- Kim, M.T., Harty, J.T., 2014. Impact of Inflammatory Cytokines on Effector and Memory CD8+ T Cells. *Front. Immunol.* 5. <https://doi.org/10.3389/fimmu.2014.00295>.
- Kumar, M., Dandapat, Sukumar, Sinha, Manoranjan Prasad, A.K.R., Singh, B., 2017. Different blood collection methods from rats: A review. *Balneo Res. J.* 8, 46–50.
- Levy, O., 2007. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat. Rev. Immunol.* <https://doi.org/10.1038/nri2075>.
- Miyoshi, J., Bobe, A.M., Miyoshi, S., Huang, Y., Hubert, N., Delmont, T.O., Eren, A.M., Leone, V., Chang, E.B., 2017. Peripartum Antibiotics Promote Gut Dysbiosis, Loss of Immune Tolerance, and Inflammatory Bowel Disease in Genetically Prone Offspring. *Cell Rep.* 20, 491–504. <https://doi.org/10.1016/j.celrep.2017.06.060>.
- Mohr, E., Siegrist, C.A., 2016. Vaccination in early life: Standing up to the challenges. *Curr. Opin. Immunol.* <https://doi.org/10.1016/j.coi.2016.04.004>.
- OLAW, 2002. Institutional Animal Care and Use Committee Guidebook: Office of Laboratory Animal Welfare [Online] [Accessed 26th May 2020] Available from World Wide Web [https://www.who.int/zoonoses/resources/lab\\_animals/en/](https://www.who.int/zoonoses/resources/lab_animals/en/).
- Nutsch, Katherine M., Hsieh, Chyi Song, 2012. T cell tolerance and immunity to commensal bacteria. *Current Opinion in Immunology* 24 (4), 385–391. <https://doi.org/10.1016/j.coi.2012.04.009>.
- Parasuraman, S., Raveendran, R., Kesavan, R., 2010. Blood sample collection in small laboratory animals. *J. Pharmacol. Pharmacother.* 1, 87–93. <https://doi.org/10.4103/0976-500X.72350>.
- Pearce, E.L., Shen, H., 2007. Generation of CD8 T Cell Memory Is Regulated by IL-12. *J. Immunol.* 179, 2074–2081. <https://doi.org/10.4049/jimmunol.179.4.2074>.
- Rahmani, A.H., Aly, S.M., Ali, H., Babiker, A.Y., Srikar, S., Khan, A.A., 2014. Therapeutic effects of date fruits (*Phoenix dactylifera*) in the prevention of diseases via modulation of anti-inflammatory, anti-oxidant, and anti-tumor activity. *Int. J. Clin. Exp. Med.* 7, 483–491.
- Roy, S.K., Islam, A., Molla, A., Akramuzzaman, S.M., Jahan, F., Fuchs, G., 1997. Impact of a single megadose of vitamin A at delivery on breastmilk of mothers and morbidity of their infants. *Eur. J. Clin. Nutr.* 51, 302–307. <https://doi.org/10.1038/sj.ejcn.1600398>.
- Skrajnar, S., Anzur Lasnik, M., Bedina Zavec, A., 2009. A flow cytometric method for determination of the blood neutrophil fraction in rats. *J. Am. Assoc. Lab. Anim. Sci.* 48, 152–156.
- Sweeney, E L, Al-Shehri, S S, Cowley, D M, Liley, H G, Bansal, N, Charles, B G, Shaw, P N, Duley, J A, Knox, C L, 2018. The effect of breastmilk and saliva combinations on the in vitro growth of oral pathogenic and commensal microorganisms. *Scientific Reports* 8 (1), 1–9. <https://doi.org/10.1038/s41598-018-33519-3>.
- Vacaflares, A., Chapman, N.M., Harty, J.T., Richer, M.J., Houtman, J.C.D., 2016. Exposure of Human CD4 T Cells to IL-12 Results in Enhanced TCR-Induced Cytokine Production, Altered TCR Signaling, and Increased Oxidative Metabolism. *PLoS One* 11. <https://doi.org/10.1371/journal.pone.0157175>.
- Varghese, J.J., Schmale, I.L., Hansen, M.E., Newlands, S.D., Benoit, D.S.W., Ovitt, C.E., 2018. Murine Salivary Functional Assessment via Pilocarpine Stimulation Following Fractionated Radiation. *J. Vis. Exp.* <https://doi.org/10.3791/57522>.
- WHO, 2007. Laboratory Guidelines for enumerating CD4 T Lymphocytes in the context of HIV/AIDS. WHO Regional Office for South-East Asia, New Delhi.
- Yu, J.C., Khodadadi, H., Malik, A., Davidson, B., Salles, É.Da.S.L., Bhatia, J., Hale, V.L., Baban, B., 2018. Innate Immunity of Neonates and Infants. *Front. Immunol.* 9, 1759. <https://doi.org/10.3389/fimmu.2018.01759>.