Original article

Immunomodulation of tahneeq method in IL-12 and CD8+ T-Lymphocyte, an in-vivo study in neonatal rats

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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 premasticated 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+ 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+ T-lymphocytes were quantified using a flow cytometer. One way ANOVA was used to analyze the percentage of IL-12 and CD8+ 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+ 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 30th days treatment, the percentage of CD8+ 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+ T cells did not influence by the depth of the scratch. Both mild and strong tah-neeq increased the systemic CD8+ 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.

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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

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Tahneeq is a traditional method that involves mechanical, physical, and biological activities to transfer oral microorganisms 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 premasticated 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 has been applied for Arabic neonates for a long time ago. However, very limited studies have scientifically examined 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)-γ levels and CD4+ cells (Karasa et al., 2011; Rahmani & Ahmad, 2014). In-vitro and in-vivo studies have revealed that water and ethanol extracts of palm dates increased IFN-γ and IL-12 levels (Karasa 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+ 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): 

\[ n = \frac{\left( T - 1 \right) \left( N - 1 \right)}{T \times N} \]

where 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 13th day (4), and the 30th 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 Biologic (2020).
2.8. CD8 + T-lymphocyte examination

The analysis of CD8+ T-lymphocytes in all blood samples of neonatal rats was carried out using a flow cytometer, as described in the previous publication (Skrjanić 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+ 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+ 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 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 Th1 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 Th1 to Th2. In the normal state, several DCs that express CD11c+ and CD80+ 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+ 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 microorganisms triggers DCs maturation and increases secretion of pro-inflammatory cytokines such as IFN-γ and IL-12 (del Cormo et al., 2016). Besides, the 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+ that recently developed as a vaccine adjuvant, and excites the formation of CD8+ T memory cells, IFN-γ, IL-6, and TGF-β (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+ T-lymphocytes in all neonatal groups tends to increase during their lives except on day 13 (See Fig. 3). The CD8+ 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 30th days treatment, the CD8+ T-lymphocyte 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+ T-lymphocytes in neonatal rat groups with mild tahneeq did not differ from the percentage of CD8+ T-lymphocytes in newborn rat groups with strong tahneeq.

Figures 3 and 4 indicate that the tahneeq increases the number of CD8+ T-lymphocytes in the 5, 13, and 30th 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+ T-lymphocytes in neonatal rats with strong tahneeq are not different from activated CD8+ 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+ 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 (Achmad, 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⁺ 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⁺ T-lymphocytes were presented as mean ± SEM, and each bar represented seven independent neonatal Wistar rats/group. Significant differences among groups were designated as *p=0.004, *b p=0.006, **c p=0.023, **d p=0.02, ***e p=0.023, ***f p=0.020.
number of CD8+ 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+ 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

| Day | Control | Soft tahneeq | Strong tahneeq |
|-----|---------|--------------|----------------|
| 0   | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| 5   | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| 7   | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| 13  | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| 30  | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |

Fig. 4. Flow cytometry images of CD8+ 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.
communities from an adult’s saliva will lead to the up-regulation of CD8+ 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+ 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+ 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+ 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 scraping 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.

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