

Conference Paper

Stimulation of the Gut Immune System in Neonatal Wistar Rats Using Tahneeq and or Breastmilk

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Abstract

The neonatal gut plays an important role in the development of the human immune system of which mucosal layer may act as the inductive site for local and systemic immune system. *Tahneeq* is a mechanical procedure that stimulates the palatal mucosa using palm dates (*Phoenix dactylifera*). It has been applied in Arabic neonates for a long time ago but it has not been determined scientifically whether or not it activates the gut immune system whereas breastfeeding has been proven to improve the neonatal immune system. The aim of this study, therefore, was to evaluate the effect of *tahneeq* on intraepithelial lymphocytes (IELs) and Peyer's patch diameter in the small intestine of neonatal Wistar rats. This randomized control trial with the posttest design used 12 neonatal rats and they were randomly divided into two groups: 6 rats in the control group (C) were given breastmilk only and 6 rats in the treatment group (T) were given *tahneeq* and breastmilk for 7 days. Intraepithelial lymphocytes count and Peyer's patch diameter were determined using a light microscope Olympus CX21, equipped with Imaging Software v2.1. Collected data were analyzed using Bland Atman, Shapiro-Wilk and independent t-tests with $\alpha = 0.05$. The mean intraepithelial lymphocytes in the C group was 126.5 ± 1.8708 and lower than that of the T group (346.833 ± 2.4014) with $p < 0.001$. The same pattern was also observed in Peyer's patch diameter (0.1333 ± 0.16 vs. $0.6333 \pm 0.27 \mu\text{m}$ respectively). The difference in Peyer's patch diameter was statistically significant ($p=0.003$). In conclusion, *tahneeq* can stimulate the gut immune system by an increase of IELs count and Peyer's patch diameter in the small intestine of neonatal Wistar rats.

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1. Introduction

Neonatal period is a critical time of the development of the human immune system. Colonization and diversity of microbiome in this period play an important role in the organogenesis and maturation of peripheral lymphoid tissue including Gut Associated Lymphoid Tissue [1]. It also plays an important role in the differentiation of intraepithelial

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lymphocytes. A lack of microbiome give arise to dysbiosis and may increase to the suitability of infectious and non-infectious disease not only in the neonatal period but also later of life [1–3]. It was known that breastmilk was the main source of the microbiome and beneficial biological active components that were important for the development of gut lymphoid tissue (GALT). Unfortunately, the diversity of breastmilk microbiome was influenced by a lot of maternal determinants such as excessive use of antibiotics, unbalanced diet, increasing incidence of cesarean section deliveries, unnecessarily stringent hygiene, and continuous stress influence the maternal microbiome [2, 4, 5].

The recent epidemiological evidence linked the cesarean delivery mode with an increased risk of developing immune diseases such as asthma, allergies, type 1 diabetes, and celiac disease [6]. According to the National Center for Health Statistics since 1970, C-sections have increased from 1 in 20 to 1 in 3 in the U.S [7]. It means that it's important to give neonates microbiome from another source, especially when they delivered by C-section. There were other sources of microbiome that will give impact to the diversity of gut microbiomes such as vaginal discharge [8, 9], early infant feeding practice of the caregiver, probiotic [10] and microbial transfer therapy using healthy adult oral/ fecal microbiome [11, 12]. According to Zhang (2010), premastication in an infant has been practiced a long time ago among a lot of civilizations [13, 14]. Tahneeq is one of caregiving practice in neonatal period which is well known as a religious practice to protect nor to feed infant [12]. Achmad (2013) assumed that tahneeq was an oral microbial transfer method [15]. There was a growing body of evidence that Microbial Transfer Therapy will lead to colonization of the normal microbial ecosystem, and improve dysbiosis [11, 12]. Dysbiosis is a loss of microbial homeostasis that will lead to a lot of diseases [16].

Tahneeq is also an orocutaneous and neuroimmune stimulation method using pre-masticated Ajwa palm dates. Orocutaneous stimulation clinically has been practiced to stimulate the sensory-motor function of neonates [17, 18]. The pre-masticated Ajwa palm dates were rubbed to palatal and gingival mucose by which will stimulate keratinocyte, inductive site of infant NALT, and also sensory-motor neuron of Vagus nerve. It may give rise to activation of lymphocytes in the ileum mucosa and increase the development of GALT in the ileum. Peyer's patches as a part of ileum's GALT were the main site of lymphocyte's proliferation and differentiation. Lymphocytes in the Peyer's patch will be delivered to the effector site including intraepithelial layer. Then, it will lead to an increasing number of IELs. It was believed that tahneeq will prevent a lot of diseases. Karasawa et al (2011) proved that palm dates alone could stimulate the cellular immunity [19], so this belief may be true. There was a little evidence about it, so we conducted this study to explore the effect of tahneeq on the IELs' count and Peyer's patch diameter.

2. Method

This was a randomized control trial with the posttest design only. Twelve neonatal Wistar rats (*Rattus norvegicus*) between the weight of 5 and 6 gram were randomly divided into two groups: 6 rats in the control group (C) were given breastmilk only and 6 rats in the treatment group (T) were given tahneeq and breastmilk for 7 days. Tahneeq was performed by rubbing 100 mg of premasticated Ajwa palm dates into the palatal, gingival and buccal mucosa. After that the neonatal Wistar rats were housed with their mother, so the mother could breastfeed their baby.

Necropsy to collect ileum specimens was performed during euthanasia at the 8th day. Histopathological materials of ileum was processed using Leica tissue processor. Parafin block was stained with Hematoxylin-Eosin. Intraepithelial lymphocytes count and Peyer's patch diameter were determined using a light microscope Olympus CX21 by two observers, equipped with Imaging Software v2.1. Statistical analyzing were performed by using Bland Atman, Shapiro-Wilk and independent T-tests with $\alpha = 0.05$. All animal procedures were approved by the Institutional Animal Care and Use Committee and were performed strictly in accordance with the Helsinki guidelines.

3. Result

According to the results of Shapiro Wilk test, normality analysis test and the Levene test, the mean of Peyer's patch diameter and The IELs count were normally distributed and homogenous ($p > 0.05$). The results of independent T-test of the Peyer's patch showed a significant difference between the two groups ($p = 0.003$). The results of the statistical analysis of Peyer's patch's diameter was displayed in Table 1.

TABLE 1: Statistical analysis output of Peyer's patch diameter and IELs count.

Variable	Groups	Mean	Std	Min	Max	p-value
Peyer's patch diameter	Breastmilk only	0.1333	0.16330	0.00	0.40	0.003
	Tahneeq and breastmilk	0.6333	0.26583	0.30	0.90	
IELs count	Breastmilk only	126.500	1.8078	124	129	0.0001
	Tahneeq and breastmilk	346.833	2.4014	343	349	

Histopathological staining using Hematoxylin-Eosin (HE) was performed, and the results are displayed in Figure 1.

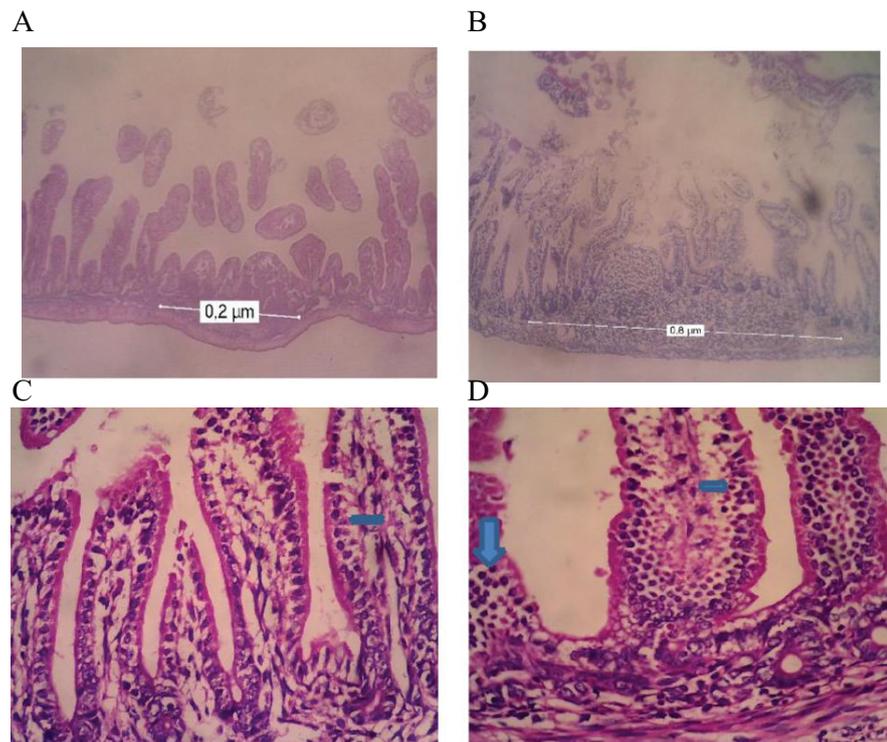


Figure 1: HE staining. The histopathology of *Peyer's Patch* in the control group: A. breastmilk only group; B. treatment group (Tahneeq and breastmilk group), C. IELS in control group, blue arrow; D. IELS in the treatment group, blue arrow.

4. Discussion

We have examined that giving tahneeq at an early life followed by breastmilk increased the IELs count and Peyer's patch diameter significantly. Tahneeq as an oral Microbial Transferred Therapy (MTT) supplies healthy oral microbiome from adult microbiome to the oral mucosal inductive site of Nasopharyngeal Lymphoid Associated Tissue (NALT). It was also a plant-based orocutaneous and neuroimmune stimulation method using pre-masticated Ajwa dates. Combination of healthy oral microbiome with pre-masticated Ajwa dates and the mechanical stimulation provide the conducive microenvironment to the colonization of beneficial microbes in the surface epithelia of the ileum. When the neonates got breastmilk, the breastmilk microbiome may colonize the ileum and stimulate the inductive site of the gut and gave rise to the development of Peyer's patch. Breastmilk contains 700 species of microbiota that will shape the neonates' gut microbiome [20].

The human immune system is stimulated with the first exposure to microorganisms during the neonatal period [1]. The gut and lungs are the most affected organs because they are predominant sites of microbial exposure [21]. Further studies suggest that the immune effects of early-life microbial exposure are durable and persist into later life

because they can be associated with prevention of diseases not only infectious diseases but also non-infectious diseases [1, 2, 7, 15, 22]. Colonizations of microbes are among the most important environmental factors influencing specific signals to stimulate immune system development and maturation [1]. A lot of studies highlight the important role of microbes in metabolic, immunological, and microbial programming [1–3]

Premasticated Ajwa dates contain a beneficial bioactive component of saliva which prevents the colonization of pathogen in the oral mucosa and ileum. Saliva is the source of 700 species of oral microbiota [21, 24] and it also contains IgA, histatin, and lactoferrin which act as natural antimicrobial substances [24, 25]. Healthy adult's oral microbiome and breastmilk's microbiome will colonize epithelial surfaces, shaping the infant microbiome to become a stable adult-like problem during 3 years of age [21].

Orocutaneous stimulation by mechanical stretch lead to secretion of keratinocytes cytokines[26], Epidermal Growth Factor (EGF) [26], and maybe Thymic Stromal Lymphopietin (TLSP). It also leads to the expression of damaged molecular antigens patterns (DAMPs) such as HSP70 [28]. The microbial cross-talk with the surface epithelial of ileum especially microfold (M) cells leads to the secretion of a lot of cytokines signal and TSLP [21]. Ajwa dates may contain beta carotene and polyphenol such as chlorogenic acid, caeic acid, pelargonin, and ferulic acid. According to Karasawa et al (2011), palm dates extract stimulated IFN- γ mRNA expression in mouse Peyer's patch cell cultures [18]. Palm dates contain chlorogenic acid and caeic acid which increased the number of IFN- γ +CD4+cells, while some polyphenols increased the number of IFN- γ +CD49b+ and IL-12+CD11b+ cells [18]. Beta-carotene was metabolized by the epithelial cells and immune cells and it provides retinoic acid which was crucial for the expression of cytokines such as IL-10 and TGF β that were needed for activation of the naive lymphocyte cells [28]. It also plays important role in the induction of CCR-9 and integrin $\alpha E\beta 7$ expression CD8, then it will lead to the homing of IELs to the effector site in the surface epithelial of ileum [21, 29]. All of these will lead to proinflammatory signal in which play an important role in the maturation of Peyer's patch. The maturation of the Peyer's patch was signed by the increasing Peyer patch diameter.

On the other hand, keratinocytes of the oral mucosa just like the skin may contribute to the initial transduction process by releasing neuroactive substances [30]. A lot of studies explained that these substances include glutamate ATP, acetylcholine (ACh), epinephrine, CGRP, and neurotrophic growth factors [30]. These also indicate that there is a complex neuroendocrine signaling between the surface epithelium and neural tissues underlie sensory signaling [30]. The oral mucosa is innervated with a lot of sensory afferents that crucial in mediating feeding [31]. Tahneeq as natural stimulation by rubbing

of the palatal and gingival site affect both keratinocytes and sensory-motor neuron. It also stimulates the swallowing to reflect and the afferent of the Vagus nerve. Stimulation of the Vagus nerve prevent the negative side effects such as excessive inflammation by secretion of ACh [32].

5. Conclusions

We concluded that Tahneeq together with breastmilk increased the intraepithelial lymphocytes count dan Peyer's patch's diameter of neonatal Wistar Rat's ileum. Further studies are needed to evaluate the mechanism of tahneeq and breastmilk stimulate the gut immune system, as it is also important to make sure that tahneeq is safe and effective to prevent a lot of diseases. There was a limitation in this study because the breastmilk was given ad libitum.

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