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Ameliorating Effects of Water-Soluble Extract of Goat Milk Yoghurt on Thyroid and Total Thyroxine in Experimental Autoimmune Hypothyroidism Rats

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Abstract : Bioactive peptides derived from Water-soluble Extract (WSE) of goat milk yoghurt had been known having immunomodulatory and antioxidative effect. The therapeutic potential of WSE of goat milk yoghurt for autoimmune thyroiditis (AITD) has not been established. The aim of this study was to investigate the effect of WSE of goat milk yoghurt thyroid histopathology and total thyroxine in AITD rats. Twenty female Wistar rats (*Rattus norvegicus*), were divided into five groups: the control, group of AITD which injected with thyroid protein, and group of AITD treated with varying dose: 300 mg/kg/day, 600 mg/kg/day, and 900 mg/kg/day of WSE of goat milk yoghurt. The thyroid protein in adjuvant were injected subcutaneously on day-0, -14, -28. The supplementation of WSE of goat milk yogurt added to the diet for 28 days on the 35th day after the induction. The results showed that supplementation was induced the improvement of thyroid gland structure and function, as evident by thyroid tissue amelioration and elevated levels of serum total thyroxine. Thyroxine ere measured by ELISA. 600 mg/kg/day WSE was the effective dose increasing level of thyroxine in AITD rats. In conclusion, WSE of goat milk yoghurt can improve the thyroid gland activity in thyroxine production.
Key words: AITD, animal models, goat milk, thyroiditis, thyroxine, water-soluble extract, yoghurt.

Introduction

Autoimmune thyroiditis (AITD) is a common endocrine autoimmune diseases in humans and dogs. The most common disease in humans is hyperthyroid Graves' disease and Hashimoto's (goitrous) thyroiditis, happens in 5% of the human population worldwide. Lymphocytic thyroiditis is an autoimmune disease that often occurs in dogs, similar with HT in humans, which is characterized with lymphocytic-plasmacytic infiltration in the thyroid gland and produces autoantibodies againts triiodothyronine, thyroxine, and thyroglobulin, causing hypothyroidism.

Animal Experimental thyroiditis (EAT) was developed to determine the development and/or pathomechanism of AITD. Induction of AITD on animal model using thyroid extract or thyroglobulin have been done[1,2]. Emulsion of thyroglobulin and complete Freund's adjuvant (CFA) injected into the body of

animal model presenting to T cells by Antigen Presenting Cells (APC) as the Major Histocompatibility Complex (MHC) class II. Expansion of autoreactive T cells is causing the accumulation of activated CD4+ and CD8+ T cells, B cells, plasma cells and macrophages in thyroid gland[3]. Besides inducing the expansion of autoreactive T cells, the immunization of animal model also increases the inflammatory response, and in the subsequent development makes the thyroid follicular cell as an apoptosis target causing hypothyroidism.

Goat milk derives proteins that hypoallergenic, more easily dissolved and absorbed by the body compared to cow milk[4,5]. Goat milk fermentation using lactic acid bacteria has been known as a source of bioactive peptides and amino acids that are beneficial for health. Whey protein in a water-soluble extract (WSE) of goat milk yoghurt derives bioactive peptides include β-lactoglobulin, α-Lactalbumin, immunoglobulins and lactoferrin which is improve the immune response. It makes WSE of goat milk yoghurt can be considered as a supportive therapy in immunocompetent patients.

The beneficial effects of WSE of goat milk yoghurt in autoimmune thyroiditis has not been established. This study was conducted to determine the potential of WSE of goat milk yogurt supplementation on histopathology of thyroid and serum total thyroxine in autoimmune thyroiditis rats immunized with emulsion of 200 µg/mL of thyroid protein and adjuvants.

Material and Methods

Preparation of goat milk yoghurt and water-soluble extract

Raw goat milk yoghurt (Etawa crossbred) for yoghurt preparation was collected from local farm (Batu, Indonesia). The yoghurt starter was purchased from *Yógourmet* (Lyo-SAN INC: 500 Aeroparc, C.P. 589, Lachute, QC. Canada, J8H, 464) containing *L. bulgaricus, S. thermophilus* and *L.acidophilus*.

Goat milk yoghurt was prepared using the method of Posecion *et al.*(2005) with some modification[6]. Raw milk was pasteurized at 72^{o}C for 5 minutes and inoculated with aliquot of starter culture (3%) when the temperature was cooled down at 43^{o}C, the incubated at 43^{o}C until the required pH of yoghurt 4.5±0.1 was reached. Cooling to 4^{o}C was done to halt further acidification. WSE was prepared by centrifugation of yoghurt at 12,000 rpm for 10 minutes at 5^{o}C[7]. The WSE was freeze-dried and kept at -20^{o}C until used.

Animal Models

All procedures were approved by the Animal Care and Use Committee of Brawijaya University, ethical clearance no. 552-KEP-UB. Twenty of eight weeks old female rats (*Rattus norvegicus*) strain wistar were used. The rats were divided into five groups, i.e. control (A), hypothyroidism-AITD (B), AITD + WSE 300 mg/kg/day (C), AITD + WSE 600 mg/kg/day (D), and AITD + WSE 900 mg/kg/day (E), and were housed in group of four per cage, as a number for replication. Hypothyroidism rats were prepared by subcutaneous injection of protein extracted from dog's thyroid in Complete Freund's Adjuvant (CFA) on day-0, and in Incomplete Freund's Adjuvant (IFA) on day-14 and -28[1]. Extract protein of dog was injected with dose 200 µg/mL, in the 1:1 ratio with the adjuvant. The treated hypothyroidism rats was given by oral administration, using a canula, once a day of 300, 600, and 900 mg/kg body weight dissolved in reverse osmosis water for consecutive weeks. The control group received a normal diet.

Measurement of Total Thyroxine

Total thyroxine (T_4) of serum were measured using ELISA method, using a Rodent T4 Test Kit. The results were read using an ELISA reader.

Histopathology of Thyroid

All rats were euthanized by cervicalis dislocation. Thyroid were fixed in cold 10% buffered formaline, the tissues were then transversally trimmed and submitted to a routine process for paraffin embedding. The thyroid sections were prepared, deparaffinized, and stained with hematoxylin-eosin (HE) for histology analysis.

Statistical Analysis

The result of total thyroxine were expressed as means ± standart deviation (SD). Differences between trial groups were statistically analysed using analysis of variance (ANOVA), followed by the post hoc Tukey test for determining significant difference at $P < 0.05$.

Result

Effect of water-soluble extract on histopathology of thyroid

The thyroid gland consists of round and hollow sac called follicle. Histological analysis of the thyroid gland of the control group (Figure 1A) was showing a normal thyroid follicular cells, where there was no observable cell damages or structural changes of follicle, and there is no infiltration of lymphocytes. Histopathological changes was observed in the architecture of thyroid cells of dog thyroid protein-induced EAT group (Fig 1B), showing a very high degree of damage in thyroid follicular cells. Contrary to control group, the thyroid of EAT group showed necrotic changes of follicular cells with karyolisis of nuclei, destruction of follicle structure and there was infiltration of lymphocytes cell.

Treatment of the EAT rats with 300, 600, and 900 mg/kgBW of water-soluble extract (WSE) of goat milk yoghurt for a period of four consecutive weeks showed features of healing effect of necrotic follicular cells and follicular structure. Fewer necrotic area of follicular cell was showed in the EAT rats treated with 300 mg/kgBW of WSE (fig 1C). follicular injuries were not observed in the EAT rats treated with 600 and 900 mg/kgBW of WSE (fig 1D&E). It means that dose dependant effect was also observed in recovery of thyroid damage by microscopically evident. This result suggested that antioxidative peptide-containing WSE may play important role in repairing structural damage in thyroid.

Effect of water-soluble extract on Total Thyroxine

Serum total thyroxine (TT4) was significantly decreased after the induction of extract protein of dog in adjuvant at the dose of 200 µg/mL (Table 1). The mean TT4 of dog thyroid protein-induced autoimmune thyroiditis rats (AITD) was significantly lower (13.303 ± 2.084 ng/mL) in comparison to control in basal condition (37.755 ± 1.734 ng/mL). The TT4 in AITD group was decreased by 65%. In contrast, the TT4 of AITD rats treated with WSE of goat milk yoghurt significantly elevated after the administration of WSE in a daily dose of 300, 600, and 900 mg/kgbw. The final TT4 at the end of experiment (week 8) were 24.000 ± 0.820 ng/mL, 32.356 ± 1.978 ng/mL, and 30.613 ± 0.846 ng/mL, respectively. Treatment of AITD rats with WSE at the dose 600 mg/kgbw gave the better result. In this study, WSE derived from goat milk yoghurt showed improvement in thyroid gland activity and caused major changes of the TT4 in the treated dog thyroid protein-induce hypothyroidism rats for more than 80%.

Table 1. Effect of water-soluble extract derived from goat milk yoghurt on the concentration of Total Thyroxine of dog thyroid protein-induced autoimmune thyroiditis rats (mean±SD) (n=4)

Groups	Mean of Total Thyroxine concentration (ng/mL)	Reduce in total thyroxine consentration (%) compared to control	Increase in total thyroxine consentration (%) compared to AITD rats
Control	37,755±1,734[d]	-	-
AITD	13,303±2,084[a]	65	-
AITD + WSE 300	24,000±0,820[b]	-	80
AITD + WSE 600	32,356±1,978[c]	-	143
AITD + WSE 900	30,613±0,846[c]	-	130

Values not sharing a common superscript differ significantly at $P<0.05$.

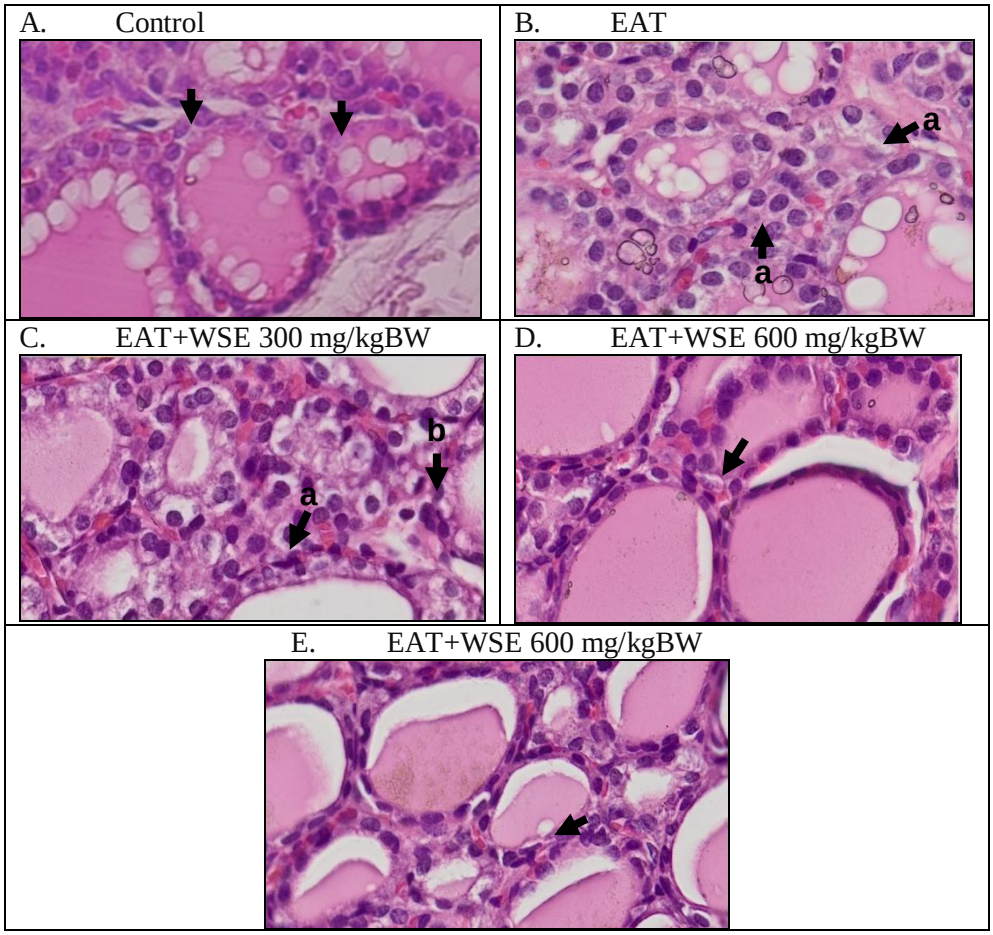

Figure 1. Histopathological changes in the thyroid (H&E 400x). A: control showing normal follicle structure and cells; B:dog thyroid protein-induced experimental animal thyroiditis (EAT) showing a high degree of follicular structure injury and karyolysis of the nuclei; C: dog thyroid protein + WSE (300 mg/kg bw) treated thyroid showing fewer damage of follicle and lymphocyte infiltration; D: dog thyroid protein + WSE (600 mg/kg bw) treated thyroid showing near normal architecture of follicle; E: dog thyroid protein + WSE (900 mg/kg bw) treated thyroid showing near normal follicle structure and cells; (a) karyolisis; (b) lymphocyte infiltration.

Discussion

Hypothyroidism is a chronic disease as a result of inhibition in thyroid function as a thyroid hormones producer. Autoimmune response induce activation and proliferation of autoreactive T lymphocytes cells. CD4+ T cells differentiate into Thelper-1 (Th1), Th2 or Th17 and infiltrate the thyroid, secrete pro-inflammatory cytokines, causing a chronic inflammation and follicular cell death in animal models of autoimmune. Pro-inflammatory cytokines secreted by CD4+ T cells not only induce damage of the epithelial cells of the thyroid follicular, but also recruits other cells, such as CD8+ T cells, B cells, plasma cells and macrophages into the thyroid. This triggers the expansion of autoreactive T cells and increases the inflammatory response, and in the subsequent development making follicular cells as a target of apoptosis causing hypothyroidism[8].

Tomer and Huber (2009) explains that the mechanism of cell damage in AITD involves several things, such as antibody dependent cell-mediated citotoxicity (ADCC), apoptosis thyroid cells mediated by Fas/FasL, a cytotoxic effect of CD4+ and CD8+, and granule-exocytosis pathway[9]. The damage of follicular cell causes thyroid gland failure to produce thyroxine and triiodothyronine in sufficient amount causing hypothyroidism[10]. Inhibition of thyroid hormone synthesis can be induced by pro-inflammatory cytokines exposure, such as IFN-γ and TNF-α. produced by infiltrating lymphocytes infiltrating in the thyroid[11].

Kemppainen et al. (2006) reported that the use of different methods for measuring concentrations of total thyroxine can cause different results[12]. ELISA is a common method used in veterinary practice. In this

study, level of total thyroxine in the control is 37.755 ± 1.734 ng/mL. This situation is similar to a study conducted by Corrêa da Costa *et al.* (2001) that the normal level of total T4 female rats aged 3-5 months ranged from 33.2 to 37.4 ng/mL[13].

The increasing level of thyroxine showed on AITD rats treated with dose of 600 mg/kg bw per day WSE of goat milk yoghurt was the highest, even though dose 300 mg/kg bw per day have shown improvement when compared with AITD rats. The result at dose 900 mg/kg bw per day was not significantly different ($P > 0.05$) from the dose of 600 mg/kg bw per day. Those were making treatment with WSE dose of 600 mg/kg bw per day was the most effective dose in increasing level of thyroxine in AITD rats.

The decrease in T4 concentration in serum of rat in this study is caused by the damage of thyroid gland due to the condition of autoimmunity resulted from immunization of crude thyroid protein containing thyroglobulin (Tg). Induction of experimental animal thyroiditis using thyroid extract or thyroglobulin has caused lesion in the thyroid[1]. AITD groups of rats treated with water-soluble extract (WSE) goat milk yogurt with varying doses showed an elevation levels of thyroxine and amelioration of thyroid follicular cell, compared to AITD rats. This is likely due to improvement in the structure of follicular cells of thyroid.

The therapeutic potential of WSE of goat milk yoghurt for autoimmune thyroiditis has not been established. WSE is the liquid remaining after milk has been curdled and strained. WSE contains whey protein which has been known to have antimicrobial activity, modulation of the immune system, increasing muscle strength, and preventing cardiovascular disease and osteoporosis[14]. Whey consist of β-lactoglobulin (β-Lg) and α-lactalbumin (α-La), bovine serum albumin (BSA), immunoglobulins, lactoferrin, lactoperoxidase enzyme, glicomacropeptide, lactose and mineral[15].

Lactoferin contained in whey has an ablility to reduce inflammation by regulating TNF-α and IL-6 in mice[16]. Whey contains high concentrations of branched-chain amino acids, e.g. leusine, isoleusine, and valine. Leusine is an important factor in growth and repair of the tissue. Whey also contains cysteine and methionine which have potential antioxidant activity that may improve immune function. Cysteine-containing whey plays role in the synthesis of glutathione (GSH) as a potential intracellular antioxidant[15]. Cysteine is a reducing agent in preventing oxidation and tissue damage.

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