THE EFFECT OF EXPOSURE TO *Toxoplasma gondii* PROFILIN ON LEPTIN LEVEL IN *Rattus norvegicus* WISTAR STRAIN RATS GIVEN NORMAL DIET AND HYPERCALORIC DIET

FINAL PROJECT

To Meet the Terms To Obtain a Bachelor Degree in Medicine

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MEDICINE PROGRAMME

FACULTY OF MEDICINE

BRAWIJAYA UNIVERSITY

MALANG

2018

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LIST OF ABBREVIATION

µg / mL	:	Microgram per mililiter
ng/mL	:	Nanogram per mililiter
BMI	:	Body Mass Index
TLR	:	Toll-like Receptor
IL	:	Interleukin
IFN	:	Interferon
NK cell	:	Natural Killer cell
ELISA	:	Enzyme-linked immunosorbent assay

CERTIFICATION PAGE

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THE EFFECT OF EXPOSURE TO *Toxoplasma gondii* PROFILIN ON LEPTIN LEVEL IN *Rattus Norvegicus* WISTAR STRAIN RATS GIVEN NORMAL DIET AND HYPERCALORIC DIET

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Abstract

Obesity is an abnormal accumulation of body fat and has multiple etiologies including an infection. Based on previous study, there is a possible association between *Toxoplasma gondii* (*T. gondii*) infection and obesity. *T.gondii* is classified as zoonotic disease and has a profilin-like protein recognized by toll-like receptor (TLR-11) and stimulates pro-inflammatory cytokines which leads to inflammation of the host cell and possibly associated with leptin level. This research is done to know the effect of exposure to *Toxoplasma gondii* profilin on leptin levels in *Rattus Norvegicus* Wistar Strain rats given normal diet and hypercaloric diet. This experiment was done at Pharmacology and Parasitology Laboratory of Medical Faculty Brawijaya University for rats maintenance, interventions and leptin level measurement. For the positive control groups, the tested concentrations of *T.gondii* profilin was 15µg/ml, 30µg/ml, and 45µg/ml on two group of rats, consuming normal diet and hypercaloric diet. The result was analyzed using one way ANOVA and shows a significant differences between *Toxoplasma gondii* profilin and leptin level ($p=0.001<\alpha$), (alpha=0.05). The Pearson correlation shows that there is a positive direction and a strong enough correlation between *T.gondii* profilin and leptin level in Wistar rats given normal diet ($R=0.557$), with no significant effect ($p=0.087>\alpha$); whereas a negative direction and a strong correlation between *T.gondii* profilin and leptin level in Wistar rats given hypercaloric diet ($R=-0.616$), with a significant effect ($p=0.014<\alpha$). This research shows that the exposure to *Toxoplasma gondii* profilin increase the leptin level in rats given normal diet but decrease in rats given hypercaloric diet.

Key words: *Toxoplasma gondii* (*T.gondii*), Leptin level

PENGARUH PAPARAN PROFILIN *Toxoplasma gondii* PADA TINGKAT LEPTIN PADA TIKUS *Rattus Norvegicus* STRAIN WISTAR YANG DIBERIKAN DIET NORMAL DAN DIET HIPERKALORI

Parveen Anandhan

Abstrak

Obesitas adalah akumulasi lemak tubuh yang abnormal dan memiliki banyak etiologi termasuk infeksi. Berdasarkan penelitian sebelumnya, ada kemungkinan hubungan antara infeksi Toxoplasma gondii (T. gondii) dan obesitas. T.gondii diklasifikasikan sebagai penyakit zoonosis dan memiliki protein seperti profilin yang dikenali oleh receptor seperti-tol (TLR-11) dan merangsang sitokin pro-inflamasi yang menyebabkan radang sel inang dan mungkin terkait dengan tingkat leptin. Penelitian ini dilakukan untuk mengetahui pengaruh paparan profilin Toxoplasma gondii pada kadar leptin pada tikus Rattus Norvegicus Wistar Strain yang diberikan diet normal dan diet hipercalorik. Percobaan ini dilakukan di Laboratorium Farmakologi dan Parasitologi Universitas Kedokteran Universitas Brawijaya untuk pemeliharaan tikus, intervensi dan pengukuran tingkat leptin. Untuk kelompok kontrol positif, konsentrasi profil T.gondii yang diuji adalah 15µg/ml, 30µg/ml, dan 45µg/ml pada dua kelompok tikus, mengkonsumsi makanan normal dan diet hipercalorik. Hasilnya dianalisis dengan menggunakan ANOVA satu arah dan menunjukkan perbedaan yang signifikan antara profilin Toxoplasma gondii dan tingkat leptin ($p = 0{,}001 < \alpha$), (alfa = 0,05). Korelasi Pearson menunjukkan bahwa ada arah positif dan korelasi yang cukup kuat antara tingkat T.gondii profilin dan leptin pada tikus Wistar dengan diet normal ($R = 0{,}557$) tanpa pengaruh yang signifikan ($p = 0{,}087 > \alpha$); sedangkan arah negatif dan korelasi kuat antara T.gondii profilin dan tingkat leptin pada tikus Wistar memberikan diet hiperkalik ($R = -0{,}616$), dengan efek yang signifikan ($p = 0{,}014 < \alpha$). Penelitian ini menunjukkan bahwa paparan profilin Toxoplasma gondii meningkatkan kadar leptin pada tikus yang diberi diet normal namun penurunan pada tikus diberi diet hipercalorik.

Kata kunci: *Toxoplasma gondii (T.gondii)*, tingkat leptin

CHAPTER 1
INTRODUCTION

1.1 Background

Obesity is an abnormal accumulation of body fat which is usually 20% or more over an individual's ideal body weight. It is often associated with increased risk of illness, disability, and death. The World Health Organization (WHO) terms obesity as a worldwide epidemic as it is becoming increasingly prevalent. According to WHO, worldwide obesity has more than doubled since 1980. In 2014, over 600 million, 13% of adults aged 18 years and over, were obese. Most of the world's population live in countries where obesity kills more people than underweight. 41 million children under the age of 5 were obese in 2014 (WHO, 2016).

Obesity has multiple etiologies; while genetic and behavioral components of obesity have been the focus of intense study, an infection as an etiological factor could be an overlooked possibility and has received little attention. The term used for obesity of an infectious origin is 'infectobesity' (Pasarica, 2007).

Based on previous study that had been conducted, the possible association between *Toxoplasma gondii* (*T. gondii*) infection and obesity had been estimated in a sample of 999 psychiatrically healthy adults. Individuals with psychiatric conditions were excluded because of the association between positive serology to *T. gondii* and various forms of serious mental illness that is strongly associated with obesity. In the sample, individuals with positive *T. gondii* serology had twice the odds of being obese compared to seronegative individuals. Latent *T. gondii* infection is common worldwide, so potential public health interventions related to

this parasite could have a high impact on associated health concerns (Reeves et al, 2013).

T.gondii was first discovered by Nicole and Manceaux (1908) on a rodent at North Africa, which is *Ctenodactylus gondi*. That is how the name of *T.gondii* was derived from. In the same year, Splendore found the organism on a guinea pig at Sao Paolo Brazil. Now, this parasit is found worldwide. *T.gondii* is classified as zoonotic disease. It is an intestinal coccidium that parasitizes the cat family as definitive hosts and has a wide range of intermediate hosts. *T.gondii* infection is common in many warm-blooded animals, including humans. In most cases, the infection will be asymptomatic, but devastating disease could occur (Mandell et al, 2006).

T.gondii has a profilin-like protein that is recognized by toll-like receptor (TLR-11) of the natural immune system which leads to inflammation of the host cell. Although profilin does not function in cell growth, it will stimulate gliding motility to invade the host cell and cause virulence. Therefore, profilin is an important element of the two aspects of *T.gondii* infection (Kucera et al, 2010).

A few studies had shown the association of *T.gondii* profilin on lipid metabolism. According to the Asean Pacific Journal of Tropical Disease (Iskandar et al, 2016), a study had been carried out to know the levels of *T.gondii* profilin and adiponectin in obese patients complicated with or without metabolic syndrome as compared to non-obese patients. The results showed that the *T.gondii* profilin level in obese subjects complicated with metabolic syndrome was significantly higher compared to the non-obese subjects. The occurrence of infection by *T.gondii* would increase the expression of profilin, including fat cells. (Iskandar, 2016). This shows that *T.gondii* profilin is strongly correlated with lipid metabolism but the pathogenesis is yet to be understood well. Although *T.gondii*

profilin had been associated with the level of adiponectin and cytokines, there is no study that relates *T.gondii* with the level of leptin.

Leptin, the "satiety hormone" was discovered in 1994. It is a hormone made by adipose cells that helps to regulate energy balance by inhibiting hunger. The actions of the hormone ghrelin, also known as the "hunger hormone" opposes leptin. Both hormones act on receptors in the arcuate nucleus of the hypothalamus to regulate appetite to achieve energy homeostasis. Although the regulation of fat stores is considered to be the primary function of leptin, it also plays a role in other physiological processes, as evidenced by its multiple sites of synthesis other than fat cells, and the multiple cell types beside hypothalamic cells that have leptin receptors. In obesity, a decreased sensitivity to leptin occurs, resulting in an inability to detect satiety despite high energy stores (Brennan and Mantzoros, 2006).

Although few experimental researches had been carried out such as using rat to identify the association between *T.gondii* and body weight (Reeves et al, 2013), yet there is no clear understanding on how the leptin level is affected and which part of the *T.gondii* that is involved on the changes of lipid metabolism. Therefore, a research must be done to know the effect of exposure to *Toxoplasma gondii* profilin on leptin levels in *Rattus Norvegicus* Wistar Strain rats.

1.2 Problem Statement

What is the effect of exposure to *Toxoplasma gondii* profilin on leptin levels in *Rattus Norvegicus* Wistar Strain rats given normal diet and hypercaloric diet?

1.3 Objective of the Research

1.3.1 General Objective

To know the effect of exposure to *Toxoplasma gondii* profilin on leptin levels in *Rattus Norvegicus* Wistar Strain rats given normal diet and hypercaloric diet.

1.3.2 Specific Objectives

1) To know the leptin levels in *Rattus Norvegicus* Wistar Strain rats given normal diet that are exposed to *Toxoplasma gondii* profilin.
2) To know the leptin levels in *Rattus Norvegicus* Wistar Strain rats given hypercaloric diet that are exposed to *Toxoplasma gondii* profilin.

1.4 Significance of the Research

The outcome of the present research are expected to :

1.4.1 Academic Significance

1) Gain more knowledge about other possible etiology of obesity which is infection induced obesity besides the common etiology.
2) Contribute to the medical world about interference in lipid metabolism that could be induced by *Toxoplasma gondii* profilin.

1.4.2 Practical Significance

1) Create awareness on public to get early detection, treat and overcome *Toxoplasma gondii* infection to avoid futher impact on lipid metabolism..
2) Create awareness on public and take preventive steps to protect themselves from the exposure of *Toxoplasma gondii.*

CHAPTER 2
REVIEW OF RELATED LITERATURE

2.1 Obesity

2.1.1 Definition

The term "obesity" refers to body weight that is greater than what is considered healthy for a certain height. It is a complex disorder where a person has accumulated excessive amount of body fat that it might have a negative effect on their health (Ofei, 2005).

2.1.2 Etiology

Obesity usually results from a combination of causes and contributing factors, including genetics. Genes may affect the amount of body fat stored, and where that fat is distributed. Genetics may also play a role in how efficiently the body converts food into energy and how the body burns calories during exercise. Obesity tends to run in families. If one or both of your parents are obese, your risk of being obese is increased. Apart from the genetic factor, family members tend to share similar eating and activity habits (Perusse et al, 2000).

Other factor includes inactivity. With a sedentary lifestyle, you can take in more calories every day than you burn through exercise and routine daily activities. Having medical problems, such as arthritis, can lead to decreased activity, which contributes to weight gain (Schauer, 2011).

Besides, having an unhealthy diet could lead to obesity too. A diet that's high in calories, lacking in fruits and vegetables, full of fast food, and laden with high-calorie beverages contributes to weight gain (Heber, 2010).

For some people, obesity can be traced to a medical cause, such as Prader-Willi syndrome, Cushing's syndrome and other conditions. Medical problems, such as arthritis can lead to decrease in activity, which may result in weight gain (MayoClinic, 2014).

Some medications may lead to weight gain if diet and activities are not compensated. These medications include some antidepressants, anti-seizure medications, diabetes medications, anti-psychotic medications, steroids and beta blockers (Ofei, 2005).

Age is one of the factors leading to obesity. Obesity occurs at any age, even in young children. But as aging, hormonal changes and a less active lifestyle increase your risk of obesity. The amount of muscle in body tends to decrease with age. This lower muscle mass leads to a decrease in metabolism. These changes also reduce calorie needs, and can make it harder to keep off excess weight (Flier, 2012).

Moreover, not getting enough sleep or getting too much sleep can cause changes in hormones that increase your appetite. Craving for foods will futher increase calories and carbohydrates intake, which can contribute to weight gain (Bray, 2015).

Of the several etiological factors, infection, an unusual causative factor, has recently started receiving greater attention. In the last two decades, 10 adipogenic pathogens were reported, including human and non-human viruses, scrapie agents, bacteria, and gut microflora. Some of these pathogens are associated with human obesity, but their causative role in human obesity has not been established (Dhurandhar, 2007). Based on the Journal of Nutrition (Dhurandhar, 2001), six pathogens are reported to cause obesity in animals. Canine distemper

virus was the first virus reported to cause obesity in mice, followed by Rous-associated virus-7, an avian retrovirus, which has been shown to cause stunting, obesity and hyperlipidemia in chickens. Next, the obesity-promoting effect of Borna disease virus was demonstrated in rats. Scrapie agents were reported to induce obesity in mice and hamsters. The final two reports were of SMAM-1, an avian adenovirus, and Ad-36, a human adenovirus that caused obesity in animals. Additionally, an association with human obesity is the unique feature of SMAM-1 and Ad-36. Although the exact mechanism of pathogen-induced obesity is unclear, infection attributable to certain organisms should be included in the long list of potential etiological factors for obesity. In addition, the involvement of some pathogens in etiology of obesity suggests the possibility of a similar role for additional pathogens (Heber, 2010).

2.1.3 Complications

Obesity may cause further health issues and may lead to serious conditions. The complications of obesity are increase in triglycerides and decrease in high-density lipoprotein (HDL) cholesterol; type 2 diabetes; high blood pressure; metabolic syndrome; heart disease; stroke; cancer; breathing disorders, including sleep apnea; gallbladder disease; gynecological problems, such as infertility and irregular periods; erectile dysfunction and sexual health issues and osteoarthritis (Rettner, 2015).

2.1.4 Diagnosis of Obesity

2.1.4.1 Body Mass Index (BMI)

Body Mass Index (BMI), is used as a screening tool for overweight or obesity. BMI is a statistical measurement derived from height and weight. A

person's weight in kilograms will be divided by the square of height in meters. The criteria for BMI is shown in Table 2.1 and 2.2, (WHO,2016),

Table 2.1 : BMI worldwide

BMI	Nutritional Status
Below 18.5	Underweight
18.5 - 24.9	Normal weight
25.0 - 29.9	Overweight
30.0 - 34.9	Obese class I
35.0 - 39.9	Obese class II
Above 40.0	Obese class III

(WHO, 2016)

Table 2.2 : BMI according to Asian criteria

BMI	Nutritional Status
Below 18.5	Underweight
18.5 - 22.9	Normal weight
23.0 - 24.9	Overweight
25.0 - 29.9	Pre-obese
Above 30.0	Obese

(WHO, 2016)

2.1.4.2 Waist Circumference

Health care professionals also may take waist measurement. This helps to screen for the possible health risks related to overweight and obesity in adults. If there is an abdominal obesity and most of the fat is around waist rather than at hips, the risk for coronary heart disease and type 2 diabetes will increase. The risk goes up with a waist size that's greater than 35 inches for women or greater than 40 inches for men (WHO, 2014).

2.1.5 Treatment and Management

The goal of obesity treatment is to achieve and stay at a healthy range of weight. The initial treatment goal is usually a modest weight loss 3 to 5 percent of your total weight. However, the more weight you lose, the greater the benefits. The first treatment is dietary changes. The key to weight loss is reducing how many calories taken in. Health care providers can review the typical eating and drinking habits to see how many calories are normally consume and where it can be cut back. The typical amount of calories that should be consumed in a day is 1,200 to 1,500 calories for women and 1,500 to 1,800 for men (Sacks et al, 2009).

In addition, the concept of energy density can help you satisfy your hunger with fewer calories. All foods have a certain number of calories within a given amount (volume). Some foods such as desserts, candies, fats and processed foods are high in energy density. This means that a small volume of that food has a large number of calories. In contrast, other foods, such as fruits and vegetables, have lower energy density. These foods provide a larger portion size with a fewer number of calories. By eating larger portions of foods that have fewer calories, the feel of hunger is reduced which contributes to overall satisfaction (Heber et al, 2010).

To make overall diet healthier, eat more plant-based foods, such as fruits, vegetables and whole-grain carbohydrates; also emphasize lean sources of protein such as beans, lentils and soy. Try to include fish twice a week. Limit salt and added sugar. Stick with low-fat dairy products. Eat small amounts of fats, and make sure they come from heart-healthy sources, such as olive, canola and nut oils. Certain diet should be restricted such as drinking sugar-sweetened

beverages because it is a sure way to consume more calories than intended (Hamdy, 2016).

Meal replacement is included in dietary changes too. It suggest that one or two meals is replaced with products such as low-calorie shakes or meal bars and eat healthy snacks and a healthy, balanced third meal that's low in fat and calories. In the short term, this type of diet can help in losing weight (Purnell, 2011).

Apart from diet changes, and increased physical activity or exercise is an essential part of obesity treatment. Most people who are able to maintain their weight loss for more than a year get regular exercise, even simply walking. People who are overweight or obese need to get at least 150 minutes of exercise a week with moderate-intensity physical activity to prevent further weight gain or to maintain the loss of a modest amount of weight. To achieve more-significant weight loss, 300 minutes of exercise has to be done in a week. Gradually increasing the amount of exercise can improve endurance and fitness (Hamdy, 2016).

Other treatment includes behaviour modification, sometimes called behaviour therapy, which include counselling. Therapy or interventions with trained mental health or other professionals can help you address emotional and behavioural issues related to eating. This therapy will teach healthy ways to cope with anxiety, understanding eating triggers, and cope with food cravings. Therapy can take place on both an individual and group basis (Barlow, 2007).

Doctor may recommend weight-loss medication if other methods of weight loss haven't worked and if one of the following criteria is met. Body mass index (BMI) is 30 or greater and also have medical complications of obesity, such as diabetes, high blood pressure or sleep apnea. Before selecting a medication,

doctor will consider health history, as well as possible side effects. Some weight-loss medications can't be used by women who are pregnant, or people who take certain medications or have chronic health conditions. Commonly prescribed weight-loss medications include orlistat (Xenical), lorcaserin (Belviq), phentermine and topiramate (Qsymia), buproprion and naltrexone (Contrave), and liraglutide (Saxenda) (Jensen et al, 2014).

2.2 *Toxoplasma gondii*

2.2.1 Classification

Toxoplasma gondii is an unicellular eukaryotes, called protista. It is an apicomplexa, cells with cluster of organelles known as apical complex; a coccidea, gamonts small and intracellular, form small resistant spores called oocysts. The gametes (*Eimeriida*) develop independently without syzygy; known as coccidian parasites (Allen, 2000).

2.2.2 Family: *Toxoplasmatidae*

This family belongs to the tissue cyst-forming coccidia: heteroxenous (two-host) parasites cycling between predator and prey hosts (transmission to predator via carnivorism of tissue cysts, and to prey via faecal-oral transmission of spores). Parasites undergo sexual reproduction termed gamogony (microgametes fertilize macrogametes) in the gut of the predator (definitive host) resulting in the formation of small spores (oocysts). The oocysts undergo endogenous sporogony (forming sporocysts and sporozoites) and are shed in host faeces. When ingested by prey (intermediate hosts), the parasites multiply by asexual merogony (schizogony) and then form cysts within host tissues (especially striated muscles

and brain). The cysts may remain dormant in the tissues for months or years until eaten by a predator (Manceaux, 1908).

2.2.3 Morphology

Four developmental stages are formed; schizonts, tissue cysts, gamonts and oocysts. Schizonts appear as small basophilic intracellular bodies which divide rapidly to form small collections of tachyzoites (measuring 4-5 x 1-2µm). Tissue cysts (measuring 10-100µm in diameter) are surrounded by a thin primary cyst wall (<0.5m thick) and contain hundreds of basophilic bradyzoites (measuring 3-4 by 1-2µm). Gamonts exhibit sexual differentiation, with microgamonts apparent as multinucleate basophilic stages ultimately shedding small biflagellated microgametes; and macrogamonts evident as uninucleate eosinophilic cells with a single ovoid nucleus. Oocysts are small ovoid stages (10-13 x 9-11m) and contain two round sporocysts, each containing four elongate sporozoites (Ajzenberg, 2011).

2.2.4 Host Range

Infections have been detected worldwide in a diverse range of vertebrate hosts; carnivores, herbivores, insectivores, rodents, pigs, primates (including humans) and occasionally birds. Sexual development and oocyst formation only occurs, however, in feline hosts. Only one parasite species is considered valid due to the lack of intermediate host specificity. Various strains, however, are recognized on the basis of their variable infectivity, growth, virulence and gene expression. *T. gondii* propagates primarily by clonal, asexual or uniparental clonal reproduction, and various strains have been allocated to three clonal lineages (Types I, II and III) on the basis of analyses of multiple independent single-copy

loci as well as microsatellite markers. Type I strains are most often associated with disease in immunocompetent adults and in congenital infections, type II strains with immunocompromised individuals, and type III strains with patients with ocular toxoplasmosis. The prevalence of infections varies according to host populations and geographic location but seroprevalence estimates range from 5-75% in many countries (Flegr, 2014).

2.2.5 Site of Infection

In cats, parasites undergo asexual and sexual multiplication in intestinal epithelial cells culminating in the formation of oocysts 3-5 days after infection. In all other vertebrate hosts, parasites undergo asexual multiplication in a wide range of extra-intestinal locations (cells of the lymphatic and circulatory systems, nervous tissue, skeletal musculature, etc.). During the acute phase of infection, the parasites divide rapidly forming small groups of 8-32 tachyzoites which lyse the host cells. As infections become chronic, the parasites divide more slowly forming large accumulations of bradyzoites particularly within the brain, heart and skeletal muscle. These tissue cysts are surrounded by a thin cyst wall and they persist for months or even years after infection. Cyst formation coincides with the development of host immunity (not sterile immunity but rather a state of premunition) (Webster et al, 2013).

2.2.6 Pathogenesis

Many host species exhibit an age-related resistance to disease therefore most infections in adults and weaned individuals are asymptomatic. In susceptible hosts, symptomatic infections may be acute, subacute or chronic. Acute infections by proliferating tachyzoites cause flu-like symptoms, including lymphadenitis,

fever, headache, muscle pain and anaemia. Symptoms generally subside with the development of immunity, but may sometimes persist producing subacute disease, characterized by extensive lesions in the lung, liver, heart, brain or eyes. Postnatal infections often involve lymphadenitis, myocarditis, central nervous system involvement and retinochoroiditis. Chronic infections by encysted bradyzoites usually cause few clinical signs, although degenerating cysts have been associated with hypersensitive inflammatory reactions, resulting in, for example, encephalitis, myocarditis and/or chorioretinitis. The tissue cysts lay quiescent (dormant) in the tissues for some time, occupying little space and apparently causing few functional deficits, although there is contradictory evidence that infections may be associated with some learning disabilities, slower reflexes and altered behaviour in intermediate hosts. Latent cyst infections may be reactivated in immunocompromised patients (those undergoing immunosuppressive therapy or with acquired immunodeficiencies) resulting in cell lysis, expanding focal lesions, rapid dissemination, encephalopathy and meningoencephalitis (Arantes et al, 2009).

Infections may also be transmitted transplacentally. If the mother contracts infection during pregnancy, parasites may cross the placenta and infect the foetus causing spontaneous abortion, stillbirth or congenital abnormalities, such as hydrocephalus, brain calcification, chorioretinitis and mental retardation. Nonetheless, if the mother/dam is infected prior to pregnancy, her immunity is transferred to her foetus which is consequently protected. Infections in cats by enteric sexual developmental stages are generally subclinical, transient and leave the cat with a solid protective immunity against subsequent oocyst production (Weiss and Kim, 2011).

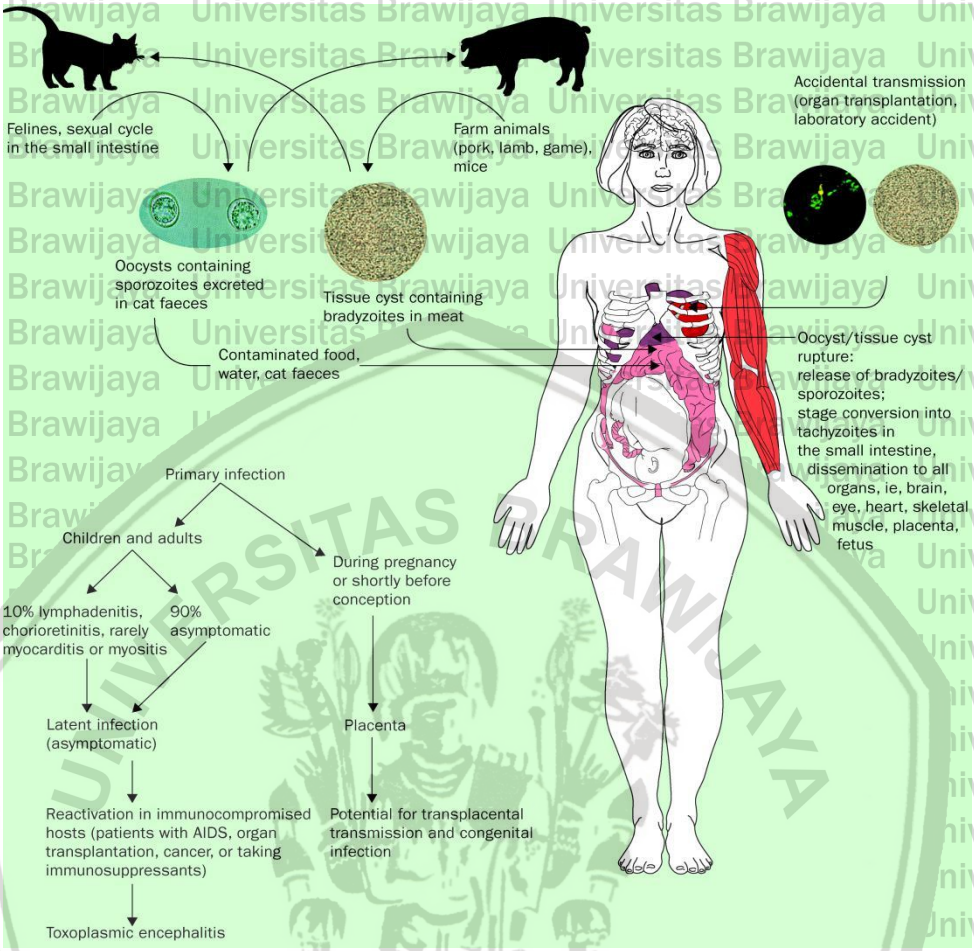

Figure 2.1 Pathogenesis of *Toxoplasma gondii* (Montoya, 2004).

2.2.7 Mode of Transmission

Infections are transmitted horizontally between hosts by the ingestion of oocysts excreted by cats, and vertically between mother and offspring by transplacental or even transmammary transmission of proliferative tachyzoites. Infections may also be transferred between intermediate hosts through the food chain via carnivorism, the ingestion of fresh or undercooked meat containing viable cysts. Bradyzoites released during digestive processes are resistant to enzymatic digestion and revert back to tachyzoite stages which infect the host, multiply, spread and lead to new cyst formation. Infections are more prevalent in human populations which have traditional cultural practices involving the

consumption of raw or partially cooked meat. Oocysts excreted by cats take 1-5 days to sporulate before they become infective and they are resistant to external environmental conditions and may remain viable in contaminated soil and water for some time (Flegr et al, 2014).

2.2.8 Differential Diagnosis

Parasites may be detected in autopsy or biopsy material by histology, immunolabelling or in vivo culture following inoculation into laboratory rodents. Zoites in smears stain well with Giemsa and other Romanowsky stains while cysts in sections have silver-positive walls and the bradyzoites are strongly PAS (periodic acid-Schiff) positive. Monoclonal and polyclonal antibody labels have also been used to detect parasites in tissue sections, and molecular studies using polymerase chain reaction (PCR) amplification techniques have detected parasite DNA in host tissues. Most infections, however, are diagnosed serologically and a range of immunoassays (fluorescence, agglutination and enzyme-based) are commercially available. Recent/acute infection is indicated by a 4-16 fold increase in specific antibody titre over a two-week period, or by the detection of specific IgM antibody titres (Santos et al, 2011).

2.2.9 Treatment and Control

Chemotherapy is successful when pyrimethamine and sulphonamides are given together as they act synergistically. The toxic side-effects of bone marrow depression can be relieved by the administration of folinic acid. Clindamycin and spiramycin have also been reported to be effective. The risk of transmission can be reduced by maintaining high standards of hygiene (particularly where cats are involved), by thoroughly cooking or deep-freezing meat prior to consumption and

washing potentially contaminated foodstuffs. Molecular vaccines are currently being developed for high risk patient groups, and a live vaccine using a low-virulent non-persistent strain has been marketed to protect sheep against toxoplasmosis (Hokelek, 2016).

2.3 *Toxoplasma gondii* Profilin

Apicomplexan parasites stimulate actin-dependent gliding movements that play an important role to invade the host cell. Profilin is a vital contributor in actin polymerization. *T.gondii* has a profilin-like protein that is recognized by toll-like receptor (TLR-11) of the natural immune system which leads to inflammation of the host cell. The damage to the host cell is known to be associated with a gene encoding profilin in *T.gondii* parasites. Although profilin does not function in cell growth, it will stimulate gliding motility to invade the host cell and cause virulence. Besides, the parasites without profilin are unable to induce TLR-11 for the production of interleukin (IL)-12 (cytokine defense of the host cell) both in vivo and in vitro. Therefore, profilin is an important element of the two aspects of *T.gondii* infection. Profilin functions in motility when the microbial host cell recognizes the ligand of the natural immune system (Uliana et al, 2011).

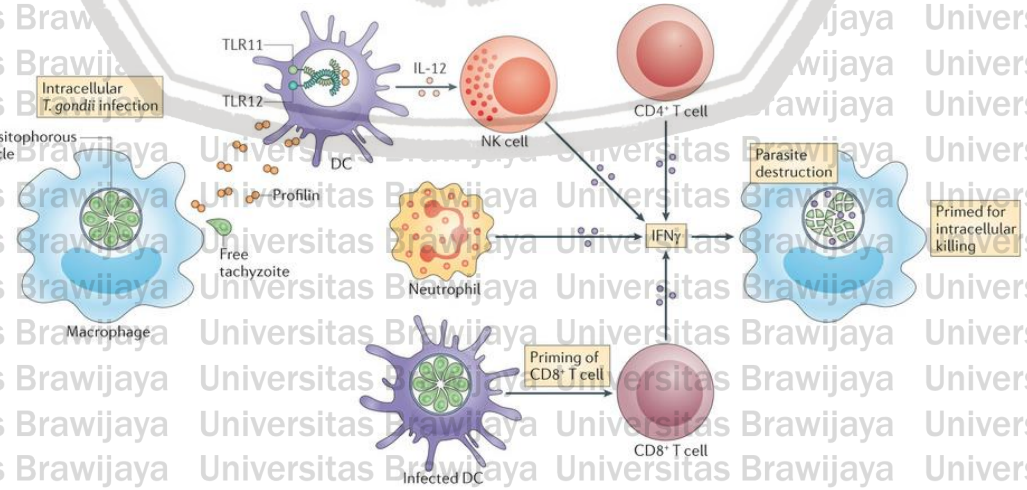

Figure 2.2 Invasion of Toxoplasma gondii profilin (Yarovinsky, 2014).

2.4 Relationship between *Toxoplasma gondii* Profilin and Obesity

A few studies had shown the association of *T.gondii* profilin on lipid metabolism. According to the Asean Pacific Journal of Tropical Disease (Iskandar et al, 2016), a study had been carried out to know the levels of *T.gondii* profilin and adiponectin in obese patients complicated with or without metabolic syndrome as compared to non-obese patients. The results showed that the *T.gondii* profilin level in obese subjects complicated with metabolic syndrome was significantly higher compared to the non-obese subjects, but there was no significant difference in the level of *T.gondii* profilin between the obese subjects complicated with metabolic syndrome and those without metabolic syndrome. This could be due to the excessive fat deposits on the obese subjects. The occurrence of infection by *T.gondii* would increase the expression of profilin, including fat cells. The bond between profilin-like protein and TLR-11 would further increase the expression of pro-inflammatory cytokines which will eventually lead to the increase of inflammation in adipocytes and causing adiposopathy and obesity. This shows that *T.gondii* profilin is strongly correlated with lipid metabolism but the pathogenesis is yet to be understood well. Although *T.gondii* profilin had been associated with the level of adiponectin and cytokines, there is no study that relates *T.gondii* with the level of leptin (Kucera *et al*, 2010).

2.5 Leptin

2.5.1 Definition

A hormone produced mainly by adipocytes (fat cells) that is involved in the regulation of body fat. Leptin interacts with areas of the brain that control hunger and behavior and signals that the body has had enough to eat. A small number of

people have genetic mutations in the leptin gene, leading to a greater demand for food, resulting in obesity (MedicineNet, 2016).

2.5.2 Mechanisms and Actions of Leptin

Leptin acts as a hormone that modulates the size of the adipose tissues in the body. It regulates food intake and body weight. Based on Figure 2.5, leptin also acts on specific receptors in the hypothalamus to inhibit appetite through both counteractive and stimulatory mechanisms (Margeric *et al*, 2002).

Leptin counteracts the effects of a feeding stimulant released in the gut called neuropeptide Y as well as the effects of a cannabinoid neurotransmitter called adandamide which stimulates appetite. Leptin also promotes the synthesis of an appetite suppressant called α-melanocyte-stimulating hormon (Gao *et al,* 2007).

When fat mass decreases, the level of plasma leptin falls so that appetite is stimulated until the fat mass is recovered. There is also a decrease in body temperature and energy expenditure is suppressed. By contrast, when fat mass increases, so do leptin levels and appetite is suppressed until weight loss occurs. In this way leptin regulates energy intake and fat stores so that weight is maintained within a relatively narrow range (Mandal, 2014).

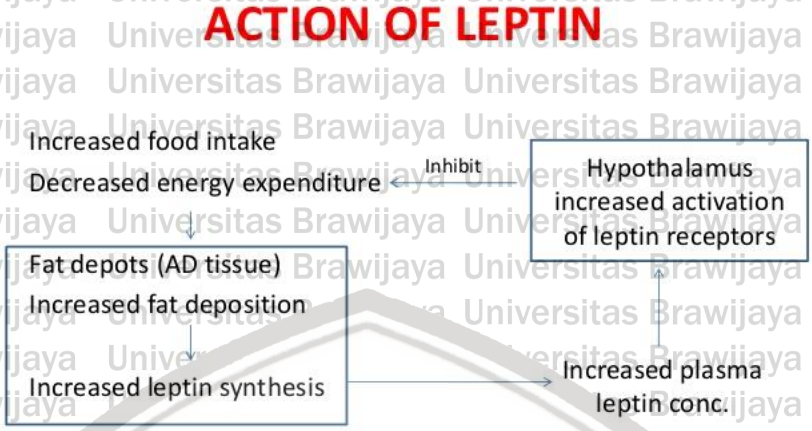

Figure 2.3 Action of Leptin (Saini, 2013).

2.5.3 Leptin Resistance

People who are obese have a lot of body fat in their fat cells. Because fat cells produce leptin in proportion to their size, obese people also have very high levels of leptin. Given the way leptin is supposed to work, people shouldn't be eating and their brain should know that they have plenty of energy stored. However, the problem is that the leptin signal isn't working. There's a whole ton of leptin floating around, but the brain doesn't "see" that it is there. This condition is known as leptin resistance. It is now believed to be the main biological abnormality in human obesity. When the brain doesn't receive the leptin signal, it erroneously thinks that the body is starving, even though it has more than enough energy stored (Brennan and Mantzoros, 2006).

This makes the brain change our physiology and behavior in order to regain the fat that the brain thinks we're missing. The brain thinks that we must eat so that we don't starve to death. The brain also thinks that we need to conserve energy, so it makes us feel lazier and makes us burn fewer calories at rest. For the great majority of people, trying to exert cognitive inhibition (willpower) over the leptin-driven starvation signal is next to impossible (Myers et al, 2010).

2.6 The Role of Leptin in Obesity

Leptin is a neurotransmitter expressed in the brain. This neurotransmitter signals to the brain mainly in the hypothalamus that when a person stops to eat for maintaining hisbody mass index. It has been observed that lab mice have a polymorphism in the leptin gene. Mutations in this gene prevent to manufacture the functional leptin protein. Due to less leptin expression, mice become morbidly obese. Another strain has a mutation or polymorphism in the gene encoding for the Leptin Receptor (LEPR). In this case, signal of the leptin is not received by the brain or the hypothalamus. So due to signal disruption or mutations in the leptin receptor, mice become obese (Wasim, 2015).

Based on Mantzoros, 1999, the dramatic effects of leptin administration to ob/ob mice (obese mouse, a mutant mouse that eats excessively and becomes profoundly obese; also an animal model of type II diabetes) which are leptin deficient because of mutations of the leptin gene, raised expectations that human obesity might also be a leptin-deficient state that could be treated with exogenous leptin administration. Although the first persons with extreme, early-onset obesity due to an inactivating mutation of the leptin gene have been identified and clinically characterized (Montague et al, 1997), several population studies have failed to demonstrate such mutations (Maffel et al, 1996; Morl et al, 1996; Williams et al, 1995). Thus, leptin-deficient persons probably represent only a minority of obese humans. In contrast, most obese humans have increased leptin levels (Considine et al, 1996), indicating that obesity is a leptin-resistant state in most obese persons. However, because one sequence polymorphism and linkage of obesity to regions flanking the leptin gene have been reported in association with

extreme obesity, the leptin gene may prove to be important only in extremely obese persons (Ahima, 2008).

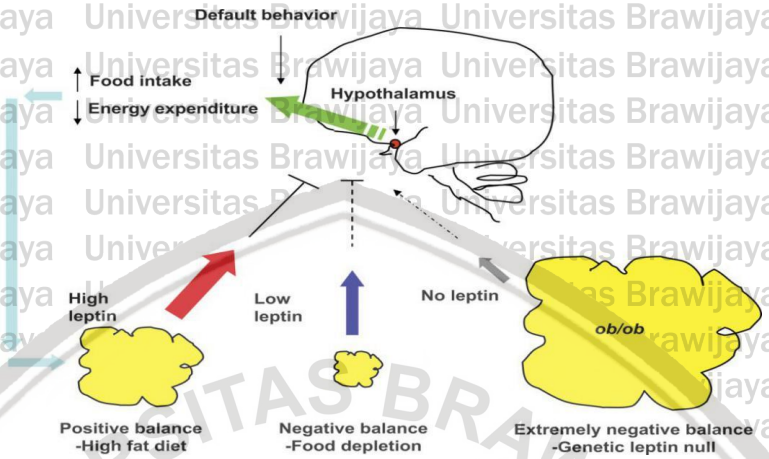

Figure 2.4 Relationship of leptin and adipocytes (Gao and Horvath, 2007).

CHAPTER 3
CONCEPTUAL FRAMEWORK

3.1 Conceptual Framework

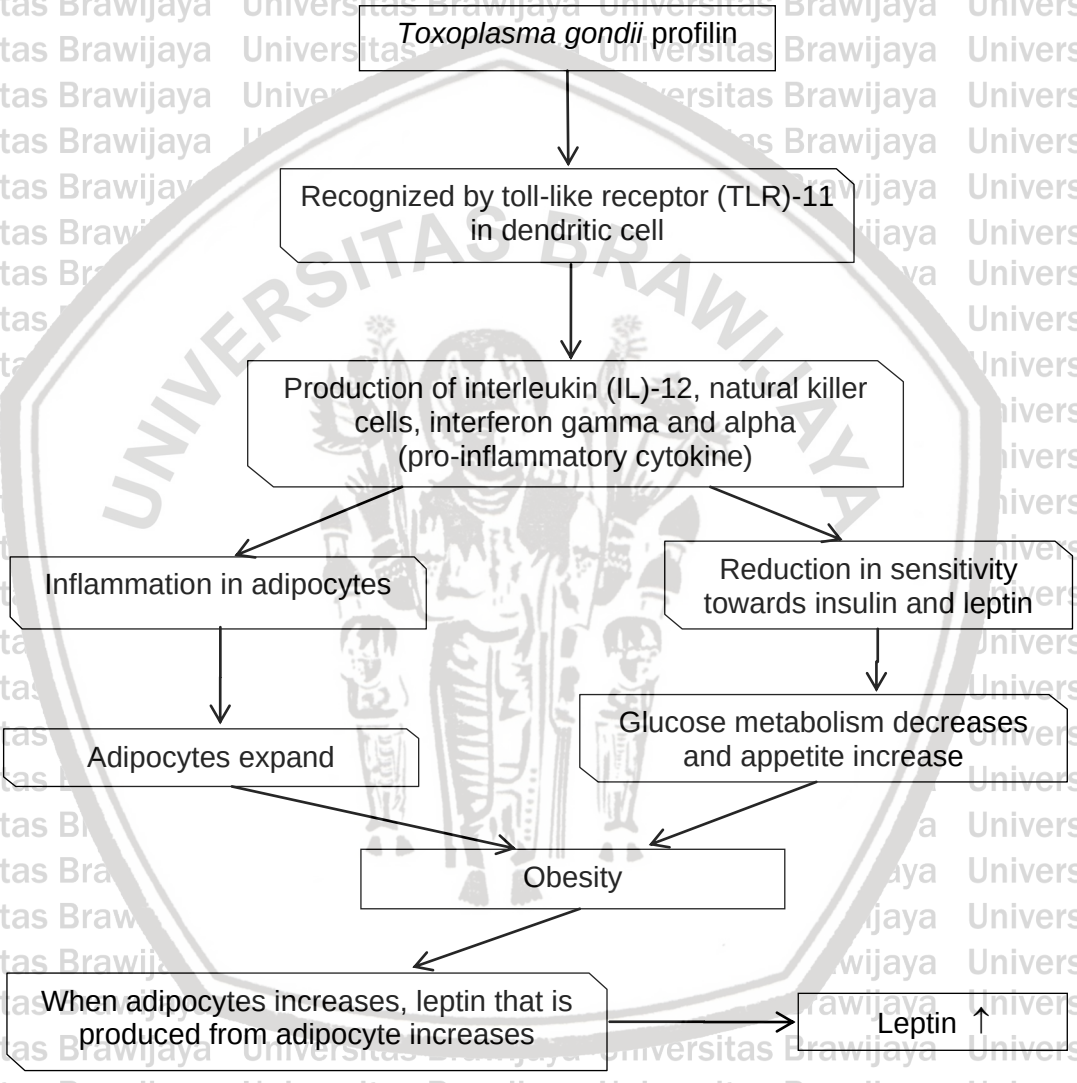

Figure 3.1 Conceptual Framework

Description:

: Studied variable

: Unobserved variable

: Stimulates

Profilin of *toxoplasma gondii* will be inserted into rat and the profilin will stimulate gliding motility to invade the host cell through actin polymerisation and cause virulence. The profilin will be recognized by toll-like receptor (TLR-11) of the natural immune system and leads to the production of interleukin (IL)-12, natural killer cells, interferon gamma and alpha (cytokine defense of the host cell). The production and elevation of pro-inflammatory cytokines causes inflammation in adipocytes. Apart from causing inflammation, the pro-inflammatory cytokines will reduce the sensitivity towards leptin and insulin therefore causing increase in appetite and decrease in glucose metabolism. This will eventually lead to obesity.

Inflammation precede the development of obesity by the expansion of the adipocytes. In other hand, there could be a possibility that if the inflammation takes place in the brain (specifically the hypothalamus), it might causes leptin resistance. Leptin is a hormone that regulates appetite and metabolism. It does this through its effect on the hypothalamus. When the hypothalamus becomes resistant to leptin, fat metabolism are impaired and weight will be gained. Leptin is produced mainly by adipocytes (fat cells) that is involved in the regulation of body fat.

If the rat is obese, it indicates that the amount adipocytes is increased and therefore the amount of leptin will be increased too.

3.2 Hypothesis of the Research

The effect of exposure to *Toxoplasma gondii* profilin is increase in the level of leptin in *Rattus norvegicus* Wistar strain rats given normal diet and hypercaloric diet.

CHAPTER 4
RESEARCH METHODS

4.1 Research Design

This study is an experimental study, in vivo on experimental animals of Wistar rats (*Rattus norvegicus*) by using the *post test control group* design. With this design, it will allow the researcher to measure the effect of intervention in the experimental group by comparing the experimental group and control group.

4.2 Sample and population

4.2.1 Population of the Research

Population of the research is male, white *Rattus Norvegicus* Wistar strain rats that are obtained from Pharmacology Laboratory of Medical Faculty, Brawijaya University.

4.2.2 Sample Selection

4.2.2.1 Inclusion criteria

1. Male rats.
2. Weight of the rats are approximately 50-100 gram.
3. Age of 3-5 months.
4. Rats that are healthy, active and without disability.

Gender of subjects used in experiment will be male that are healthy instead of female because female rats have reproductive cycles and hormone fluctuations that would confound the results of experiment. They have estrogen hormone that influences the metabolism of lipid dan cholesterol, while male rats don't have hormonal changes (Marcotte, 2014).

Rats are chosen as sample because they are classified as benign animal, easy to be housed and maintained. Scientists and researchers rely on rats for several reasons as below (Melina, 2010):

a. Small in size.

b. High sensitivity towards interventions.

c. More standardized compared to other experimental animals.

d. Can be bred to guarantee the authenticity and uniformity of strain.

e. Rats cannot vomit because they do not have vomiting centre.

This research divides the sample into seven groups which are :

Table 4.1 Groups of the Research

Group	*Toxoplasma gondii* profilin	Hypercaloric diet
K	-	-
D1	15µg/ml	-
D2	30µg/ml	-
D3	45µg/ml	-
D4	15µg/ml	+
D5	30µg/ml	+
D6	45µg/ml	+

4.2.3 Number of Samples

The amount of replication that is used for each group using the formula:

$(t-1)(r-1) \geq 15$ (Hanafiah, 2005),

t = number of groups

r = number of replication

15 = constant value

In this research, the number of groups are 7 (t = 7), therefore the number of replication would be:

$$(7-1)(r-1) \geq 15$$

$$6r \geq 21$$

$$r \geq 21 : 6$$

$$r \geq 3.5 \approx 4$$

So, the number of replication needed for each group will be a minimum of 4 with an addition of 1 for each group. The total number of sample that is used for this research will be 35 rats for 7 groups of intervention and control since 5 rats are needed for each group.

4.3 Variables of the Research

4.3.1 Manipulative Variable (Independent)

The manipulative or independent variable in this research is the exposure of *Toxoplasma gondii* profilin with three different dosage on *Rattus Norvegicus* Wistar strain rats.

4.3.2 Responding Variable (Dependent)

The responding or dependent variable in this research is the level of leptin in *Rattus Norvegicus* Wistar strain rats.

4.3.3 External Variable

1. Gender of rats : Male
2. Age : 3-5 months
3. Weight : 50-100 gram
4. Environmental factor in the laboratory where the rats were housed and the leptin levels were measured.

4.4 Location and Time of the Research

This research was executed in Pharmacology Laboratory of Medical Faculty Brawijaya University for experiments and maintenance of the rats while Parasitology Laboratory of Medical Faculty Brawijaya University for leptin level measurement. The duration of the research was from February to July 2017.

4.5 Materials and Equipment / Instrument of the Research

4.5.1 Equipments

4.5.1.1 Equipments for the Maintenance of Wistar Rats

a) Rat cage along with the chaff.

b) Cage cover made of woven wire.

c) Water bottles and feeding spot.

4.5.1.2 Equipment for the Intervention on Wistar Rats

a) Syringe 1 cc for intraperitoneal injection of *Toxoplasma gondii* profilin.

4.5.1.3 Equipments for Serum Intake from Wistar Rats

a) Syringe 5cc to take blood sample of rats.

b) Medical gloves.

c) Test tubes.

d) Vacutainer

e) Eppendorf

f) Centrifuge

4.5.1.4 Equipment for the Measurement of Leptin Level

a) ELISA (Enzyme-Linked Immunosorbent Assay) Kit

b) Incubator

c) Micropipette

d) Yellow tip

e) Eppendorf

f) Microplate reader with 450 ± 10nm wavelength filter

4.5.2 Materials

4.5.2.1 Experimental animal

Experimental animal used for this research is *Rattus Norvegicus* Wistar strain rats that are male and healthy with the age of 3-5 months and weigh 50-100 gram.

4.5.2.2 Materials for the Maintenance of Wistar Rats

a) Rat diet according to standards of Pharmacology Laboratory of Medical Faculty Brawijaya University.

b) Rat drinks according to standards of Pharmacology Laboratory of Medical Faculty Brawijaya University.

c) Specific to particular groups : Hypercaloric diet according to standards of Pharmacology Laboratory of Medical Faculty Brawijaya University.

4.5.2.3 Material for Intervention on Wistar Rats

a) *Toxoplasma gondii* profilin

b) Ketamine 50mg/ml

4.5.2.4 Materials for the Measurement of Leptin Level

a) Streptavidin-Peroxidase Conjugate

b) Diluent N Concentrate

c) Wash Buffer Concentrate

d) Biotinylated Mouse Leptin Antibody

e) Chromogen Substrate

f) Leptin Microplate

g) Leptin Standard

h) Sealing Tapes

i) Stop Solution

4.6 Operational Definition

1. Obese models are made by providing a hypercaloric diet according to Nascimento et al., 2008.
2. The recombinant *Toxoplasma gondii* profilin taken from host *Escherichia coli* was imported from Adipogen Corp., San Diego, USA on 28th March 2017 by storage using Blue Ice -20°C, in phosphate-buffered saline.
3. *Toxoplasma gondii* profilin had been injected on rats intraperitoneally, twice with time span of 11 weeks and 4 days between the first injection on 30th March 2017 and the second injection on 20th June 2017.
4. Measurement of leptin levels was done at week 15 using Rat Leptin (LEP) Elisa Kit E0561Ra.

4.7 Data Collection

4.7.1 Division of the Group

35 rats were divided into 7 groups using randomization method to obtain negative control group and positive control groups. Each groups differs in intervention:

K - Normal diet without profilin

D1 - Normal diet with 15 µg/mL profilin

D2 - Normal diet with 30 µg/mL profilin

D3 - Normal diet with 45 µg/mL profilin

D4 - Hypercaloric diet with 15 µg/mL profilin

D5 - Hypercaloric diet with 30 µg/mL profilin

D6 - Hypercaloric diet with 45 µg/mL profilin

4.7.2 Procedure of the Research

4.7.2.1 Procedure to Obtain Profilin

Toxoplasma gondii profilin were provided by Parasitology Laboratory of Medical Faculty Brawijaya University.

4.7.2.2 Procedure of Intervention on the Rats

The rats are left to get through adaptation for 2 weeks at Parasitology Laboratory of Medical Faculty Brawijaya University. After 2 weeks, the positive control groups are injected with different dosage of profilin (15,30 and 45µg/ml) according to groups by intraperitoneal. The profilin takes approximately 6 weeks to react on the rats. In addition to profilin, hypercaloric diet is also given to 3 of the positive control group.

4.7.2.3 Procedure of Leptin Level Measurement

The serum of the rats are first obtained by withdrawal of blood sample from the rats. The test tubes are placed in centrifuge machine and centrifugated with the speed of 3000 rpm for 5-10 minutes. The serum is taken with pipette and moved to eppendorf. The sample is then tested using ELISA (Enzyme-Linked Immunosorbent Assay) kit for the quantitative measurement of leptin levels.

A Leptin specific antibody is precoated onto 96-well plates and blocked. Standards or test samples are added to the wells and subsequently a Leptin specific biotinylated detection antibody is added and then followed by washing with wash buffer. Streptavidin-Peroxidase Conjugate is added and unbound conjugates are washed away with wash buffer. Tetramethylbenzidine (TMB) is

then used to visualize Streptavidin-Peroxidase enzymatic reaction. TMB is catalyzed by Streptavidin-Peroxidase to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the amount of Leptin captured in plate.

4.8 Data Analysis

The tests used in the processing of the data are One-Way Annova Test, Correlation Test (Pearson) and Linear Regression Analysis Test.

4.9 Procedure of Study

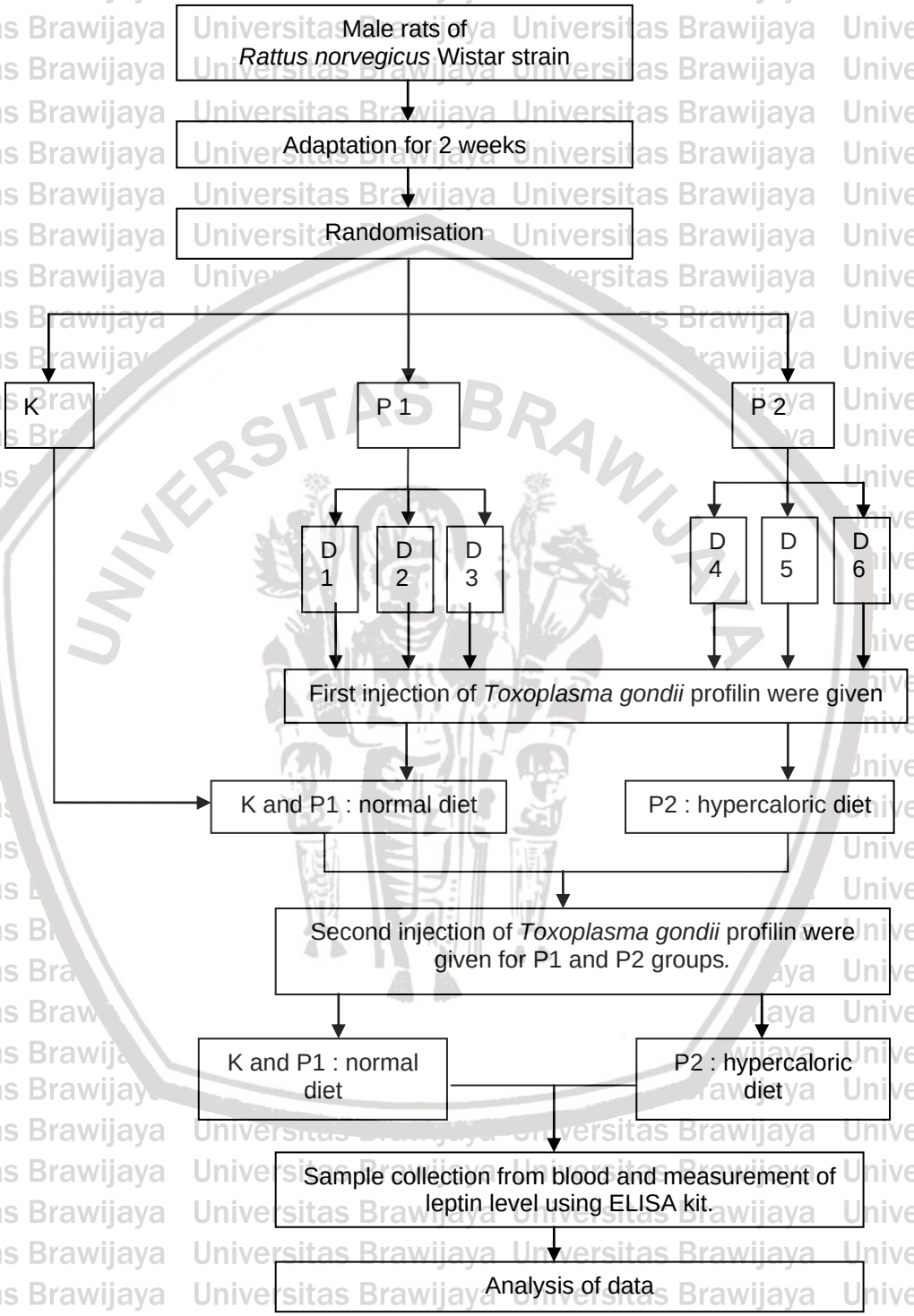

Figure 4.1 Procedure of Study

CHAPTER 5
RESULTS AND DATA ANALYSIS

5.1 Research Results

5.1.1 Collecting Samples

After the intervention has been completed, the rats were dissected to obtain the blood plasma samples. With the initial step of anaesthesia using 50 mg of Ketamine as much as 0.4 to 0.5 ml. After the blood plasma has been taken, it was centrifuged with a speed of 3000 rpm for 10 minutes. The serum from the sample that has been centrifuged was taken to be inserted into eppendorf. It was then stored in the freezer for -2 to -8 degree Celsius.

5.2 Average Weight of *Rattus Norvegicus* Wistar Strain Rats Based on The Exposure To *Toxoplasma gondii* Profilin

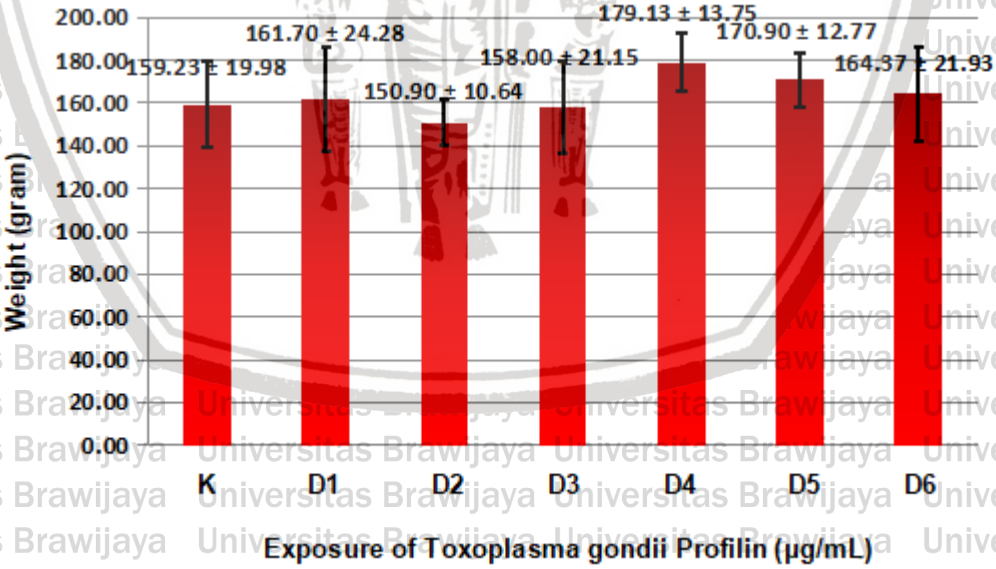

Figure 5.1 Average Weight of Rattus Norvegicus Wistar Strain Rats Based on Exposure To Toxoplasma gondii Profilin

Description :
K - Normal diet without profilin
D1 - Normal diet with 15 µg/mL profilin

D2 - Normal diet with 30 µg/mL profilin
D3 - Normal diet with 45 µg/mL profilin
D4 - Hypercaloric diet with 15 µg/mL profilin
D5 - Hypercaloric diet with 30 µg/mL profilin
D6 - Hypercaloric diet with 45 µg/mL profilin

Based on the descriptive analysis of the seven treatments it was found that the group given D2 had the lowest average weight, while the group given D4 had the highest average weight.

5.3 Average Leptin Levels in *Rattus Norvegicus* Wistar Strain Rats Based on Exposure to *Toxoplasma gondii* (T.gondii) Profilin

The serum was collected according to the group of intervention and measured using Elisa Kit.

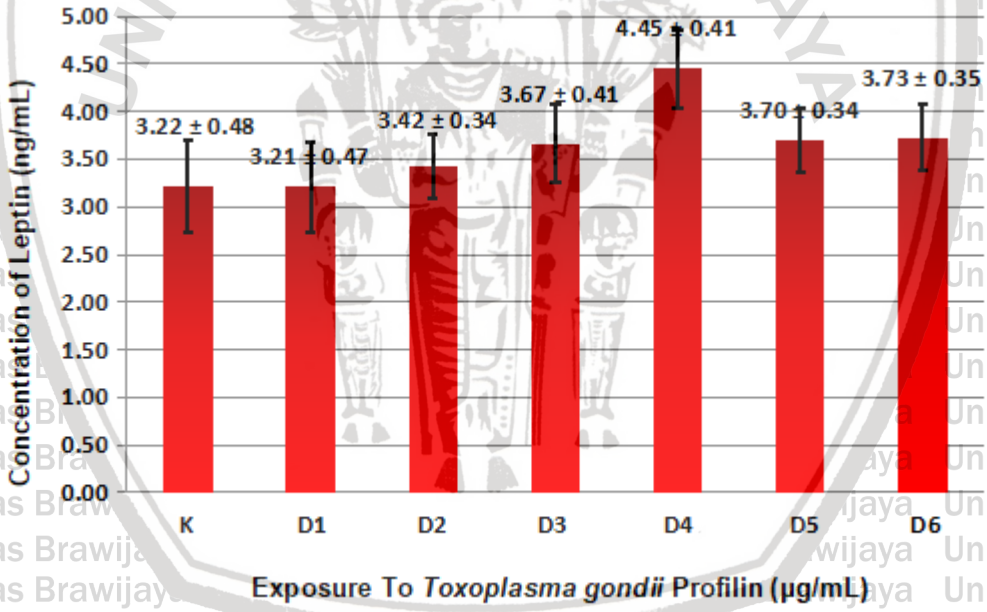

Figure 5.2 Concentration of Leptin in Rattus Norvegicus Wistar Strain Rats Based on Exposure to Toxoplasma gondii Profilin

Description :
K - Normal diet without profilin
D1 - Normal diet with 15 µg/mL profilin
D2 - Normal diet with 30 µg/mL profilin
D3 - Normal diet with 45 µg/mL profilin
D4 - Hypercaloric diet with 15 µg/mL profilin
D5 - Hypercaloric diet with 30 µg/mL profilin
D6 - Hypercaloric diet with 45 µg/mL profilin

Based on the descriptive analysis of the seven groups, it was found that the group given D1 had an average leptin level in the lowest, whereas the group given D4 had an average leptin level in the highest.

5.4 Normality Test for The Effect of *Toxoplasma gondii* Profilin Exposure on Leptin Level in *Rattus Norvegicus* Wistar Strain Rats

Residual normality test of the effect of T.gondii profilin exposure on leptin level in the rats aim to determine the normal or absence of residuals resulting from the effect of T.gondii profilin exposure on leptin levels in rats. Residual normality test is performed using Kolmogorov-Smirnov, with criterion if probability value > level of significance (alpha = 5%), then the residual is stated normal. The result of residual normality test can be seen through the following table:

Table 5.1. Normality Test of Data between *T.gondii* Profilin and Leptin Level

Kolmogorov- Smirnov	0.111
Probability	0.200

Based on the above table it can be seen that testing of residual normality of the effect of T.gondii profilin exposure on leptin level in the rats yield Kolmogorov-Smirnov statistic of 0.111, with probability equal to 0.200. It can be seen that the residual normality test yields probability > alpha (5%), so the residual is stated normal.

5.5 Homogeneity Test for The Effect of Exposure to *Toxoplasma gondii* Profilin on Leptin Level in *Rattus Norvegicus* Wistar Strain Rats

Tests of residual homogeneity of the effect of T.gondii profilin exposure on leptin level in rats aim to determine whether residuals have homogeneous variety or not. The residual homogeneity test was performed using Levene Test, with criterion if probability value > level of significance (alpha = 5%), then the residual

is homogeneous. The result of residual homogeneity test can be seen through the following table:

Table 5.2. Homogeneity Test of Data between *T.gondii* Profilin and Leptin Level

Levene Statistic	0.318
Probability	0.922

Based on the above table it can be seen that residual homogeneity testing of the effect of T.gondii profilin exposure on leptin level in the rats yield Levene statistics of 0.318 with a probability of 0.922. It can be seen that residual testing yields probability > alpha (5%), so the residual is expressed as homogenous.

5.6 Testing Differences of The Effect of *Toxoplasma gondii* Profilin Exposure on Leptin Level in *Rattus Norvegicus* Wistar Strain Rats using Anova

Testing of the effect of T.gondii profilin exposure on leptin level in the rats was performed using Anova with the following hypothesis:

H0 : There was no significant difference in the effect of *Toxoplasma gondii* profilin exposure on leptin levels in *Rattus Norvegicus* Wistar Strain rats.

H1 : At least one pair of the effect of *Toxoplasma gondii* profilin exposure on leptin levels in *Rattus Norvegicus* Wistar strains rats are significantly different.

The test criteria stated that if the probability ≤ level of significance (alpha = 5%), then H0 is rejected and it can be stated that there is at least one pair of T.gondii profilin exposure which affect leptin levels in the rats are significantly different.

The result can be seen through the following table:

Table 5.3. Anova Test between *T.gondii* Profilin and Leptin Level

Anova	
F Statistic	5.520
Probability	0.001

The above table shows that the test yielded F statistic test statistic of 5.520 with probability of 0.001. It is known that probability < alpha (5%), so H0 is rejected. Therefore, it can be stated that there is at least one pair of T.gondii profilin exposure group with leptin level that is significantly different.

5.7 Post Hoc Test

Tukey's Honest Significant Difference (Tukey HSD) test is performed to determine whether the effect of T.gondii profilin exposure on leptin level was significantly different or not; with the criteria that if one pair of T.gondii profilin exposure group resulted in probability ≤ level of significance (alpha = 5%), it can be stated that there is a difference in the influence of exposure to Toxoplasma gondii profilin on leptin levels in rats. The result of Tukey HSD analysis can be known through the following table:

Table 5.4. Post Hoc Test between *T.gondii* Profilin and Leptin Level

Concentr-ation	Average	Probability							Notat-ion
		D1	K	D2	D3	D5	D6	D4	
D1	3.21		1.000	0.979	0.555	0.477	0.412	0.001	a
K	3.22	1.000		0.985	0.589	0.511	0.444	0.001	a
D2	3.42	0.979	0.985		0.956	0.923	0.885	0.006	a
D3	3.67	0.555	0.589	0.956		1.000	1.000	0.064	ab
D5	3.70	0.477	0.511	0.923	1.000		1.000	0.083	ab
D6	3.73	0.412	0.444	0.885	1.000	1.000		0.105	ab
D4	4.45	0.001	0.001	0.006	0.064	0.083	0.105		b

The results of the above analysis informed that the group D4 had the highest leptin level and significantly different with the groups D1, D2 and K; but did not differ significantly with the group D3, D5 and D6. While the group D1 had the

lowest leptin level and significantly different with group D4; but did not differ significantly with the other groups.

5.8 Testing The Relationship of *Toxoplasma gondii* Profilin Exposure with Normal Diet and Average Weight of *Rattus Norvegicus* Wistar Strain Rats

5.8.1 Data Normality Test of *Toxoplasma gondii* Profilin Exposure with Normal Diet and Average Weight of *Rattus Norvegicus* Wistar Strain Rats

The normality test of data is done using Kolmogorov-Smirnov, with criterion if probability value> level of significance (alpha = 5%) then the data is stated normal. The results can be seen through the following table:

Table 5.5. Normality Test of Data between *T.gondii* Profilin and Weight of Rats given Normal Diet

	Kolmogorov- Smirnov	Probability
Toxoplasma Gondii Profilin with Normal Diet	0.215	0.061
Average body weight	0.157	0.200

Based on the above table it can be seen that *T.gondii* profilin exposure with normal diet and body weight of rats' Kolmogorov-Smirnov yield statistics each of 0.215 and 0.157 with probabilities of 0.061 and 0.200. It can be seen that the normality test produce probability> alpha (5%), so the data is normal.

5.8.2 Analyse The Correlation of *Toxoplasma gondii* Profilin Exposure with Normal Diet and Average Weight of *Rattus Norvegicus* Wistar Strain Rats

Analysis was performed using Pearson correlation with the following hypothesis:

H0 : There is no significant relationship of *Toxoplasma gondii* profilin exposure with normal diet and average body weight of *Rattus Norvegicus* Wistar Strain rats

H1 : There is a significant association of *Toxoplasma gondii* profilin Exposure with normal diet and average body weight of *Rattus Norvegicus* Wistar Strain rats

The test criteria states if the probability of ≤ level of significance (alpha = 5%) then H0 is rejected. The result can be seen through the following table:

Table 5.6. Pearson Correlation between *T.gondii* Profilin and Weight of Rats given Normal Diet

Correlation Coefficient	Probability
-0.084	0.767

The above table shows that the association yields a probability of 0.767. It is known that probability> alpha (5%), so H0 is accepted. Therefore, it can be stated that there is no significant association of *T.gondii* profilin exposure with normal diet and average body weight of the rats.

The correlation coefficient of -0.084 indicates that there is a negative (opposite) and very weak relationship. This means the average weight decreases as the exposure of *T.gondii* profilin with normal diet increases and vice versa.

5.9 Testing The Relationship of *Toxoplasma gondii* Profilin Exposure with Hypercaloric Diet and Average Weight of *Rattus Norvegicus* Wistar Strain Rats

5.9.1 Data Normality Test of *Toxoplasma gondii* Profilin Exposure with Hypercaloric Diet and Average Weight of *Rattus Norvegicus* Wistar Strain Rats

Testing of data normality is done using Kolmogorov-Smirnov, with criterion if probability value> level of significance (alpha = 5%) then the data is stated normal. The results can be seen through the following table:

Table 5.7. Normality Test between *T.gondii* Profilin and Weight of Rats given Hypercaloric Diet

	Kolmogorov- Smirnov	Probability
Toxoplasma gondii Profilin with Hypercaloric Diet	0.215	0.061
Average weight	0.126	0.200

Based on the above table it can be seen that Kolmogorov-Smirnov of 0.215 and 0.126 with probabilities of 0.061 and 0.200. It can be seen that the probability> alpha (5%), so data is normal.

5.9.2 Analyse The Correlation of *Toxoplasma gondii* Profilin Exposure with Hypercaloric Diet and Average Weight of *Rattus Norvegicus* Wistar Strain Rats

Analysis was performed using Pearson correlation with the following hypothesis:

H0 : There is no significant association of *Toxoplasma gondii* profilin exposure with hypercaloric diet and average weight of the rats.

H1 : There is a significant association of *Toxoplasma gondii* profilin exposure with hypercaloric diet and average weight of the rats.

The test criteria states if the probability of ≤ level of significance (alpha = 5%) then H0 is rejected. The results can be seen through the following table:

Table 5.8. Pearson Correlation between *T.gondii* Profilin and Weight of Rats given Hypercaloric Diet

Correlation Coefficient	Probability
-0.375	0.169

The table above informs that the association yields a probability of 0.169. It is known that probability> alpha (5%), so H0 is accepted. Therefore, it can be stated that there is no significant association of *T.gondii* profilin with hypercaloric diet and average body weight of the rats.

The correlation coefficient of -0.375 indicates that there is a negative (opposite) and weak relationship. This means that This means that the average weight decreases as the exposure of *T.gondii* with hypercaloric diet increases and vice versa.

5.10 Analysis of The Effect of *Toxoplasma gondii* Profilin Exposure with Normal Diet on Leptin Levels in *Rattus Norvegicus* Wistar Strain Rats

Analysis of the effect of T.gondii profilin exposure with normal diet on leptin levels in the rats was performed by using simple linear regression.

5.10.1 Estimation Result of The Effect of *Toxoplasma gondii* Profilin Exposure with normal diet on Leptin Levels

The results of the influence of T.gondii profilin exposure with normal diet on leptin levels can be seen through the following table:

Table 5.9. Pearson Correlation between *T.gondii* Profilin and Leptin Level in Rats given Normal Diet

Variable	**Coefficient**	**T *statistic***	**Probability**
Constant	2.742	7.096	0.000
T.gondii profilin with normal diet	0.230	1.851	0.087
R-squared = 0.310	R = 0.557		

5.10.1.1 Coefficient of Determination Test

The amount of contribution of T.gondii profilin exposure with normal diet on leptin levels can be known through the coefficient of determination (R2) that is 0.310. It means the diversity of leptin levels can be explained 31.0% by the exposure of T.gondii profilin with normal diet or in other words the contribution of T.gondii profilin exposure with normal diet to leptin level is 31.0%. While the rest of 69.0% is a contribution from other variables that are not discussed in this study.

5.10.1.2 Correlation Coefficient

The correlation coefficient is used to determine the level of closeness of the relationship and the direction of the relationship between the exposure to T.gondii profilin with normal diet and leptin levels according to table below (Sarwono, 2006):

Table 5.10. Relationship Strength on The Correlation Test

Statistical Correlation Value	Relationship Strength
0 - 0.05	No correlation between the two variable
0.05 – 0.19	Very weak correlation
0.20 – 0.39	Weak correlation
0.40 – 0.59	Correlation is partially strong
0.60 – 0.79	Strong correlation
0.80 - 0.99	Very strong correlation
1	Perfect correlation

The result of the correlation coefficient of 0.557 shows there is a partially strong relationship between exposure to Toxoplasma gondii profilin with normal

diet and leptin level with positive direction (unidirectional). This means that the higher the exposure to Toxoplasma gondii profilin with normal diet, the higher the leptin level.

5.10.1.3 Hypothesis Testing

Hypothesis testing is used to determine the presence or absence of in the effect of T.gondii profilin exposure with normal diet on leptin levels. The test criteria states that if probability < level of significance (= 5%), there is a significant effect of exposure to T.gondii with normal diet on leptin levels.

a. Hypothesis Test of The Effect of *Toxoplasma gondii* Profilin Exposure with Normal Diet on Leptin Levels

Testing of the hypothesis yields t value count equal 1.851 with probability equal to 0.087. The test results show probability > level of significance (= 5%). This shows that there is no significant effect of exposure to T.gondii profilin with normal diet on leptin level.

b. Hypothesis Test between Constants and Leptin Levels

Testing the hypothesis of the constant yields a t value of 7.069 with a probability of 0.000. The test results show the probability < level of significance (= 5%). It means there is a partial significant effect of the constant on leptin levels.

5.10.1.4 Empirical Model of Simple Linear Regression

The equation of the result from simple linear regression analysis is:

Y = 2.742 – 0.230 X

This equation shows the following:

1. The constant of 2.742 indicates that if exposure to T.gondii profilin with normal diet is constant (unchanged), then average leptin level change is 2.742 ng/mL.
2. Coefficient of T.gondii profilin exposure with normal diet of 0.230 indicates that exposure to T.gondii profilin with normal diet has a positive effect on leptin level. This means that any increase of 1 µg/mL in exposure to T.gondii profilin with normal diet will increase leptin level by 0.230 ng / mL.

5.11 Analysis of The Effect of *Toxoplasma gondii* Profilin Exposure with Hypercaloric Diet on Leptin Level in *Rattus Norvegicus* Wistar Strain Rats

Analysis of the effect of T.gondii profilin exposure with hypercaloric diet on leptin levels in the rats was performed by using simple linear regression analysis.

5.11.1 Estimation Results in The Effect of *Toxoplasma gondii* Profilin Exposure with Hypercaloric Diet on Leptin Levels

The results of the effect of exposure to T.gondii profilin with hypercaloric diet on leptin levels can be seen through the following table:

Table 5.11. Pearson Correlation between *T.gondii* Profilin and Leptin Level in Rats given Hypercaloric Diet

Variable	**Coefficient**	**T *statistic***	**Probability**
Constant	6.119	7.920	0.000
Profilin with hypercaloric diet	-0.360	-2.822	0.014
R-squared = 0.380	R = -0.616		

5.11.1.1 Coefficient of Determination Test

The amount of contribution of exposure to T.gondii profilin with hypercaloric diet on leptin levels can be known through the coefficient of determination (R2) that is equal to 0.380. This means that the diversity of leptin levels can be

explained by exposure to T.gondii profilin with a hypercaloric diet of 38.0% or in other words the contribution of T.gondii profilin exposure to hypercaloric diet on leptin levels is 38.0%. While the rest of 62.0% is a contribution from other variables that are not discussed in this study.

5.11.1.2 Correlation Coefficient

The correlation coefficient was used to determine the level of closeness of the relationship and the direction of the relationship between the exposure to T.gondii profilin with hypercaloric diet and leptin levels. The result of correlation coefficient of -0.616 shows there is a strong correlation between exposure to T.gondii profilin with hypercaloric diet and leptin level with negative direction (opposite). This means that the higher exposure to T.gondii profilin with hypercaloric diet decreases the leptin levels, and lower exposure to T.gondii profilin with hypercaloric diet increases the leptin levels.

5.11.1.3 Hypothesis Test

Hypothesis testing is used to determine the presence or absence of the effect of T.gondii profilin exposure with hypercaloric diet on leptin levels. The test criteria states that if probability < level of significance (= 5%), then there is a significant effect of exposure to T.gondii profilin with hypercaloric diet on leptin levels.

a. Hypothesis Test of The Effect of *Toxoplasma gondii* Profilin Exposure with Hypercaloric Diet on Leptin Levels

Testing of hypothesis yield t value count equal to -2.822 with a probability of 0.014. The test results show the probability < level of significance (= 5%). This means that there is a significant effect of exposure to T.gondii profilin with hypercaloric diet on leptin levels.

b. Hypothesis Test between Constant and Leptin Levels

Testing the hypothesis of the constant yields a t value of 7.920 with a probability of 0.000. The test results show the probability < level of significance (= 5%). This means there is a partially significant effect of the constant on leptin levels.

5.11.1.4 Empirical Models of Simple Linear Regression

The equation of the result from simple linear regression analysis is:

Y = 6.119 - 0.360 X

This equation shows the following:

1. Constant of 6.119 indicates that if exposure to T.gondii profilin with hypercaloric diet is constant (average), then average leptin level is 6.119 ng/mL.
2. Coefficient of -0.360 indicates that exposure to T.gondii profilin with hypercaloric diet has a negative effect on leptin levels. This means that any increase of 1 µg/mL in exposure to T.gondii profilin with hypercaloric diet will decrease leptin level by 0.360 ng/mL.

5.12 Testing Average Weight and Leptin Level of *Rattus Norvegicus* Wistar Strain Rats

5.12.1 Data Normality Test of Average Weight and Leptin Level of *Rattus Norvegicus* Wistar Strain Rats

Normality test of average body weight and leptin level of the rats is intended to determine the normality of data. The test is done using Kolmogorov-Smirnov, with criterion if probability value> level of significance (alpha = 5%) then the data is stated normal. The results can be seen through the following table:

Table 5.12. Normality Test between Weight and Leptin Level in Rats

	Kolmogorov- Smirnov	Probability
Average weight	0.100	0.200
Leptin Level	0.070	0.200

Based on the above table it can be seen that average weight and leptin level yield Kolmogorov-Smirnov statistics of 0.100 and 0.070 respectively with probabilities of both 0.200. It can be seen that probability> alpha (5%), so the data of average weight and leptin level are normal.

5.12.2 Analyse The Correlation of Average Weight and Leptin Level in Rattus Norvegicus Wistar Strain Rats

The analysis was performed using Pearson with the following hypothesis:

H0 : There is no significant relationship average weight and leptin level

H1 : There is a significant correlation of average weight and leptin level

The test criteria states if the probability of ≤ level of significance (alpha = 5%) then H0 is rejected. The result can be seen through the following table:

Table 5.13. Pearson Correlation between Weight and Leptin Level in Rats

Correlation Coefficient	Probability
0.159	0.363

The table above shows that the testing of the relationship yields a probability of 0.363. It is known that probability> alpha (5%), so H0 is accepted. Therefore, it can be stated that there is no significant correlation of average body weight and leptin level of the rats.

The correlation coefficient of 0.159 indicates that there is a positive (unidirectional) and weak relationship. This means that the leptin level gets lower as the average weight decreases whereas the level of leptin gets higher as the average weight increases.

5.12.3 Correlation of Body Weight Delta and Leptin Level

The analysis was performed using Spearman correlation with the following hypothesis:

H0: There is no significant relationship of weight delta and level of leptin

H1: There is a significant correlation of weight delta and level of leptin

The result can be seen through the following table:

Table 5.14. Correlation of Body Weight Delta and Leptin Level

Correlation coefficient	Probability
0.750	0.052

The table above informs that the correlation of weight delta and leptin level yields a probability of 0.052. It is known that probability> alpha (5%), so H0 is accepted. Therefore, it can be stated that there is no significant correlation of weight delta and leptin level in Rattus Norvegicus Wistar Strain rats.

The correlation coefficient of 0.750 indicates that there is a positive direction and a strong relationship. This means that the higher the weight delta, the higher the leptin level, and conversely the lower the weight delta, the lower the leptin level.

CHAPTER 6
DISCUSSION

6.1 Discussion

The purpose of this study was to determine the increase in leptin levels in *Rattus Norvegicus* wistar strains rats exposed to *Toxoplasma gondii* profilin. The intervention was performed by intraperitoneally injecting *T.gondii* profilin in the rats according to the dosage of 15µg/mL, 30µg/mL, and 45µg/mL. At week 15 after injection, surgery had been done on rats to obtain blood. The serum was obtained after centrifugation and it was used to measure leptin level. Leptin level was calculated using ELISA kit.

T.gondii is an intracellular pathogenic parasite that has a heteroxenous life cycle and can infect all warm-blooded animals (mammals and birds) and humans. Profilin used in this experiment is an extract from *Toxoplasma gondii* tachyzoite with a particular strain whose RNA profilin is broken down (Yuan, et al., 2015). The process had been carried out in the United States and the results of its recombinant profilin was imported into Indonesia. This molecular extraction approach is used because the reaction to antibodies have been demonstrated in rabbit and gene specimens gene-coded profilin *Toxoplasma gondii* in order to support a stronger hypothesis. *T gondii* profilin molecule had been associated with infection of host cells through activation of toll-like receptor (TLR) (Iskandar et al, 2011). *T. gondii* profilin binds to TLR-11 to enhance the expression of interleukin-12 (IL-12). IL-12, a cytokine largely produced by phagocytic cells in response to intracellular bacteria and also parasites. IL-12 will activate NK and T cells. Furthermore, interferon gamma (IFN ɣ) will be formed which activates phagocytic cells and inflammatory cells (Yarovinsky, 2014).

According to Asean Pacific Journal of Tropical Disease (Iskandar et al, 2016), the occurrence of infection by T.gondii would increase the expression of profilin, including fat cells. The bond between profilin-like protein and TLR-11 would further increase the expression of pro-inflammatory cytokines which will eventually lead to the increase of inflammation in adipocytes. This shows that *T.gondii* profilin is strongly correlated with lipid metabolism but the pathogenesis is yet to be understood well.

Leptin is a hormone produced mainly by adipocytes (fat cells) that is involved in the regulation of body fat. It regulates food intake and body weight by acting on specific receptors in the hypothalamus to inhibit appetite through both counteractive and stimulatory mechanisms (Mandal, 2014). Leptin is a neurotransmitter expressed in the brain.

Transcription of the leptin gene expressed primarily in adipose tissues, but few of the studies confirmed that some other tissues also express leptin, including placenta, ovaries, skeletal muscle and stomach. Moreover, leptin circulates in the blood as protein. In humans, leptin is encoded by a gene located in human chromosome and is similar to that in rodents (Margetic et al, 2002).

People who are overweight have increased body fat. Because fat cells produce leptin in proportion to their size, overweight people also have increased level of leptin. Given the way leptin supposed to work, people shouldn't be eating and their brain should know that they have plenty of energy stored. However, the problem is that the leptin signal isn't working. There's a whole ton of leptin in the fat cells, but they are not transmitted to the brain. So, the brain does not get the signal of leptin. This condition is known as leptin resistance. It is now believed to be the main biological abnormality in human obesity. When the brain doesn't

receive the leptin signal, it erroneously thinks that the body is starving and so the person eats more (Myers et al, 2010).

6.1.1 Effect of exposure to *Toxoplasma gondii* on average weight of *Rattus Norvegicus* wistar strain rats

The weight of the rats had been correlated with *T.gondii* profilin to analyse the relationship between both variables. The results showed that the weight of the rats had negative (opposite) effect and a weak relationship for both major group of rats given normal diet and hypercaloric diet. It means the average weight decreases as the exposure of *T.gondii* profilin increases. In support to this finding, a study shows that anorexia, which is seen in the course of infections, is believed to be the host's acute phase response to infection. Bacterial or viral products stimulate the production of proinflammatory cytokines. Cytokines, in turn, increase leptin expression in adipose tissue (Baltaci and Mogulkoc, 2012). Increased amount of leptin will increase the feel of satisfaction and therefore the rats reduce their food intake (Benoit, 2002). Reduced food intake will decrease the weight which supports the results of this experiment.

Apart from that, another study proves that *T.gondii* infected mice presented a reduction in the serum VLDL cholesterol fraction. Specifically, a decrease in serum cholesterol was also described by (Milovanovi et al, 2009) who fed infected with *T.cruzi* a high-fat diet. Further, systemic chronic infections and inflammations are associated with low cholesterolemia and HDL cholesterol. However, in the examples provided above, the reduction of total cholesterol reflects an unspecific reduction of all lipoprotein fractions. The reduction of total cholesterol leads to reduction of weight.

6.1.2 Effect of exposure to *Toxoplasma gondii* on leptin level in *Rattus Norvegicus* wistar strain rats

Based on the measurement data of leptin, it can be seen that the leptin level has increased gradually with the dosage of profilin 15, 30 and 45µg/ml in groups of rat given normal diet. The results partially supports the hypothesis that exposure to profilin has the effect of increasing leptin levels in rats. Leptin is known to have a substantial part in natural and acquired immunity. As leptin levels are elevated during infection/ inflammation, it has been argued that leptin is a crucial factor in the host's response to inflammation (Ziylan et al, 2009). Elevated leptin levels were established in rats with induced T.gondii infection and may be seen as an expected result (Baltaci and Mogulkoc, 2012).

However, the group of rats given hypercaloric diet had decreasing level of leptin as the *T.gondii* profilin exposure increases. As explained above, T.gondii profilin reduces the lipoprotein fractions and the weight of rats. As fat cells produce leptin in proportion to their sizes, the level of leptin reduces as the weight reduces (Ahima, 2008). The hypercaloric diet given could be a factor of interference in hormonal feedback that takes place in central nervous system, causing changes in the level of leptin. Diets that are high in fat and simple carbohydrates is often taken as and evidence that homeostatic (energy demand-driven) systems are being overridden by non-homeostatic (palatability, pleasure or reward-based) and the interaction between these systems may underlie the tendency to ignore feedback repletion signals such as leptin and insulin to over-consumption of calories (Figlewicz and Benoit, 2009). There is an extensive motivational circuitry involved in the regulation of food intake within the limbic system of the central nervous system. Both circulating glucose and fatty acids decrease food intake via a central mechanism involving the hypothalamus and brain stem (Rijnsburger *et al*, 2016). Leptin is an external hormonal signal that can influence

non-homeostatic behaviour in addition to its role in energy homeostasis in the hypothalamus. Leptin receptors are expressed throughout the limbic forebrain, for example, in dopaminergic neurons, suggesting that these neurons are directly targeted by the leptin hormone (Figlewicz, 2003). The effect of leptin on these neurons is based on the behaviours influenced. Food restriction enhances the rewarding properties of a variety of stimuli including food, and such states of negative energy balance are accompanied by reduced levels of leptin (Joost, 2012). This would explain why leptin level decrease in wistar rats given hypercaloric diet.

6.1.3 Effect of weight on leptin level in *Rattus Norvegicus* wistar strain rats

From the result of analysis, it is stated that there is no significant correlation of average body weight and leptin level of the rats. The correlation indicates that there is a positive (unidirectional) effect but a weak relationship. This means that the leptin level gets lower as the average weight decreases whereas the level of leptin gets higher as the average weight increases. When fat mass decreases, the level of plasma leptin falls so that appetite is stimulated until the fat mass is recovered. There is also a decrease in body temperature and energy expenditure is suppressed. By contrast, when fat mass increases, so do leptin levels and appetite is suppressed until weight loss occurs (Mandal, 2014). This could explain the result that leptin level is directly proportional to fat cells and weight.

6.2 Implications for the field of Medicine

This study is expected to provide knowledge about the lipid metabolism induced by *Toxoplasma gondii* infection by measuring the leptin levels. Increased exposure to *T.gondii* infection could interfere lipid metabolism and respark

inflammation that leads to increase in leptin levels and further reduces weight, provided that there is no presence of leptin-resistance state (Baltaci and Mogulkoc, 2012).

6.3 Limitations of the study

This study only used serum samples as a single indicator of measurement of leptin levels. Leptin hormone is primarily produced in adipose tissues, therefore, increased leptin positively correlated only in measurement of adipose, whereas serum results were not significant. Suggested subsequent research to use more than one indicator to be able to see the difference in leptin levels more significantly (Margetic et al, 2002).

Moreover, this study had only one control group but two major groups given normal diet and hypercaloric diet were intervented with *T.gondii* profilin. This causes the bias of the incidence between the two major groups. This would not provide a conformation on difference in leptin level caused by T. gondii profilin infection or the hypercaloric diet. So further research has to be carried out by adding a control group given hypercaloric diet alone without injecting *T.gondii* profilin to confirm the effect of *T.gondii* profilin exposure on leptin level.

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CHAPTER 7
CONCLUSION

7.1 Conclusions

1. The effect of exposure to *Toxoplasma gondii* profilin is increase in leptin levels in *Rattus norvegicus* wistar strain rats given normal diet.
2. The effect of exposure to *Toxoplasma gondii* profilin is decrease in leptin levels in *Rattus norvegicus* wistar strain rats given hypercaloric diet.

7.2 Suggestions

1. Further research should be performed using more than one measurement indicator of leptin in order to see the difference in serum and tissue leptin levels more significantly.
2. An intervention group of hypercaloric diet without *T.gondii* profilin should be added to confirm the effect of *T.gondii* profilin exposure with hypercaloric diet on leptin level.
3. Inoculation *T.gondii* profilin should be conducted with complete components in accordance with the facilities and infrastructure that compact.

REFERENCES

Ahima RS.. Revisiting Leptin's Role in Obesity and Weight Loss. *The Journal of Clinical Investigation*. 2008; 118(7): 2380-3.

Ajzenberg D., Devillard S., Demar MP., de Thoisy B., Bonnabau H., Mercier A.. Human impact on genetic diversity of Toxoplasma gondii: example of the anthropized environment from French Guiana. *Infections, Genetics and Evolutions*. 2011 Aug;11(6):1378-87.

Allen., Heckeroth AR., Weiss LM.. Toxoplasma gondii: from animals to humans. *International Journal of Parasitology*. 2000 Nov; 30(12-13): 1217-58.

Anonymous. *BMI Classification: World Health Organization*. Online. 2016. http://apps.who.int/bmi/index.jsp?introPage=intro_3.html [Accessed on 10 October 2016].

Anonymous. Symptoms and Causes of Obesity. *MayoClinic*. Online. 2014. https://www.mayoclinic.org/diseases-conditions/obesity/symptoms-causes/syc-20375742 [Accessed on 10 October 2016].

Arantes TP., Lopes WD., Ferreira RM., Pieroni JS., Pinto VM., Sakamoto CA.. Toxoplasma gondii: Evidence for the transmission in dogs. *Experiments of Parasitology*. 2009 Oct;123(2):190-4.

Baltaci AK., Mogulkoc R., Plasma Leptin Levels in Rats with Induced *Toxoplasma gondii* Infection. *Bratislavske Lekarske Listy*. 2012; 113(2): 67-69.

Barlow SE.. Expert committee recommendations regarding the prevention, assessment, and treatment of child and adolescent overweight and obesity: summary report. *Pediatrics*. 2007;120(4): 164-92.

Benoit SC., Air EL., Clegg DJ., Seeley RJ., Woods SC.. Insulin and Leptin Combine Additively to Reduce Food Intake and Body Weight in Rats. *Endocrinology*. 2002; 143(6): 2449-52.

Brennan AM., Mantzoros CS.. Drug Insight: the role of leptin in human physiology and pathophysiology-emerging clinical applications. *International Journal of Pediatric Obesity*. 2006; 6: 419-427.

Considine RV., Caro JF., Sinha MK., Kolaczynski JW., Zhang PL.. Leptin: the tale of an obesity gene. *Diabetes*. 1996 Nov;45(11):1455-62.

Figlewicz DP., Benoit SC.. Insulin, leptin, and food reward. American Journal of Physiology, Regulatory, Integrative and Comparative Physiology. 2009; 296(1): R9-R19.

Figlewicz DP.. Expression of receptors for insulin and leptin in the ventral tegmental area/substantia nigra (VTA/SN) of the rat. Brain Research. 2003 Feb 21; 964(1): 107-15.

Flegr J., Klapilova K., Kankova S.. Toxoplasmosis can be a sexually transmitted infection with serious clinical consequences. *Medical Hypotheses*. 2014; 83: 286–289.

Flier J.S.. Obesity wars: molecular progress confronts an expanding epidemic. *Cell.* (2012);116: 337–350.

Gao S., Horvath, Kinzig KP., Aja S., Scott KA., Keung W.. Leptin activates hypothalamic acetyl-CoA carboxylase to inhibit food intake. *Proceedings of the Natlonal Academy of Science*. 2007 Oct 30;104(44):17358-63.

Hamdy O.. Overweight and Obese Type 2 Patients Show Significant Improvements with Structured Nutrition Therapy. *Joslin Diabetes Center*. Online. 2016. www.joslin.org/news/overweight-obese-type-2-improvements-with-structured-nutrition-therapy.html [Accessed on 29 October 2016].

Heber D.. An integrative view of obesity. *The American Journal of Clinical Nutrition*. 2010 Jan; 91(1): 280-283.

Iskandar A., Indra MR., Satuman.. Profilin as an adipocyte dysfunction biomarker (Study of the relationship of adipocyte dysfunction to *Toxoplasma gondii* infection in obese individuals). [Abstract]. 2011.

Iskandar A., Indra MR., Satuman., Firani NK., Wihastuti TA.. The levels of *Toxoplasma gondii* profilin and adiponectin in obese patients complicated with or without metabolic syndrome as compared to non-obese patients. *Asian Pacific Journal of Tropical Disease.* 2016; 6(4): 265-268.

Jensen MD.. Guideline for the management of overweight and obesity in adults. *Journal of the American College of Cardiology*. 2014; 63(1): 2985-3023.

Joost HG.. Appetite Control. German Institute of Human Nutrition. 2012:209:3-14.

Kucera K., Koblansky AA., Saunders LP., Frederick KB., Cruz EM., Ghosh S. Structure-based analysis of *Toxoplasma gondii* profilin: a parasite-specific motif is required for recognition by Toll-like receptor 11. *Journal of Molecular Biology*. 2010; 40(4): 16-29.

Mandal A. Leptin Mechanism. *News of Medical and Life Science.* Online. Jan; 2014. https://www.news-medical.net/health/Leptin-Mechanism.aspx [Accessed on 4 August 2017].

Mandell, Bennett, Dolin. *Toxoplasmosis: History of Discovery*. Online. May; 2006. https://web.stanford.edu/group/parasites/ParaSites2006/Toxoplasmosis/history.html [Accessed on 10 October 2016].

Margetic S., Gazzola C., Pegg GG., Hill RA.. Leptin: a review of its peripheral actions and interactions. *International Journal of Obesity Relating Metabolic Disorder*. 2002; 26(11): 1407-33.

Mantzoros CS.. The role of leptin in human obesity and disease: a review of current evidence. *Annals of Internal Medicine*. 1999 Apr 20;130(8):671-80.

Milovanovi I., Vujani M., KlunII I., Bobi I., Nikoli A., Ivovi V., *et al*. *Toxoplasma gondii* Infection Induces Lipid Metabolism Alterations in the Host. *Memórias do Instituto Oswaldo Cruz.* 2009; 46(2): 459-469.

Montoya JG., Liesenfeld O.. Toxoplasmosis. [Abstract]. *Lancet*. London. June 2004; 364(9434): 579.

Myers MG., Leibel RL., Seeley RJ., Schwartz MW.. Obesity and leptin resistance: Distinguishing cause from effect. *Trends of Endocrinology Metabolism*. 2010; 21(11): 643-51.

Ofei F.. Obesity-A Preventable Disease. *Ghana Medical Journal*. 2005 Sep; 39(3): 98–101.

Pasarica M., Dhurandhar NV.. Infectobesity: obesity of infectious origin. [Abstract] *Advance in Food and Nutrition Research.* United States, 2007;52:61-102.

Perusse L., Rice T., Chagnon YC., Despres JP., Lemieux S., Roy S., *et al*. A genome-wide scan for abdominal fat assessed by computed tomography in the Quebec Family Study. *Metabolism* 2000; 49:203–207.

Purnell JQ.. Brain functional magnetic resonance imaging response to glucose and fructose infusions in humans. *Diabetes, Obesity and Metabolism*. 2011;13: 229–234.

Reeves GM., Mazaheri S., Snitker S., Langenberg P., Giegling I., Hartmann AM., *et al*. A Positive Association between *T. gondii* Seropositivity and Obesity. *Frontiers in Public Health*. 2013; 1:73.

Rettner R.. Obesity: Causes, Complications & Treatments. *Live Science*. Online. 2015. https://www.livescience.com/34787-obesity-high-bmi-causes-diabetes-heart-disease.html [Accessed on 10 October 2016]

Rijnsburger M., Belegri E., Eggels L., Unmehopa UA., Boelen A., Serlie MJ., et al. The effect of diet interventions on hypothalamic nutrient sensing pathways in rodents. Physiology & Behavior. 2016; 162(1): 61-68.

Sacks G., Swinburn B., Lawrence M.. Obesity Policy Action framework and analysis grids for a comprehensive policy approach to reducing obesity. *Obesity Review*. 2009 Jan;10(1): 76-86.

Saini V, Yadav A., Kataria MA.. Role ofleptin and adiponectin in insulin resistance. *Clinica Chimca Acta*. 2013 Feb 18;417:80-4.

Santos DV., Dodds EM., Orefice F.. Review for disease of the year: differential diagnosis of ocular toxoplasmosis. *Ocular Immunology and Inflammation*. 2011; 19(3):171-9.

Wasim M., Awan FR., Najam SS., Khan AR., Khan HN.. Role of Leptin Deficiency, Inefficiency, and Leptin Receptors in Obesity. *Biochemical Genetics*. 2016 Oct; 54(5): 565-72.

Webster, Joanne P., Maya K., Greg C., Bristow, McConkey AG.. *Toxoplasma gondii* infection, from predation to schizophrenia: can animal behaviour help us understand human behaviour. *Journal of Experimental Biology*. 2013 Jan 1; 216(1): 99–112.

Yarovinsky F., Romero S., Didry D., Carlier MF., Sher A., Soldati-Favre D.. *Toxoplasma gondii* profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. [Abstract] *Cell Host & Microbe.* United States. Feb. 2008; 3(2): 77-87.

Yuan F., Liu ZZ., Zhang B., Cao JP., Zheng KY., Wang DG.. Prokaryotic Expression and Immunoreactivity Analysis on Profilin of *Toxoplasma gondii*. [Abstract] *Chinese Journal of Parasitology*. China, Feb. 2015; 33(1): 4-21.

Ziylan YZ., Baltaci AK., Mogulkoc R.. Leptin transport in the central nervous system. Cell BiochemicalFunction. 2009; 27(2): 63-70.

APPENDICES

Appendix 1. Analysis of Differences in Effect of *Toxoplasma gondii* Profilin Exposure on Leptin Level in *Rattus Norvegicus* Wistar Strain Rats

Descriptive Statistics

Dependent Variable: Konsentrasi Leptin

Serum	Mean	Std. Deviation	N
Normal diet without Profilin	3.5620	.58781	5
Normal diet + Profilin 15 µg/mL	3.7000	.57131	5
Normal diet + Profilin 30 µg/mL	3.7860	.30550	5
Normal diet + ProfiliN 45 µg/mL	3.6060	.66131	5
Hypercaloric diet + Profilin 15 µg/mL	4.4480	.42008	5
Hypercaloric diet + Profilin 30 µg/mL	3.7000	.33638	5
Hypercaloric diet + Profilin 45 µg/mL	3.7280	.34666	5
Total	3.7900	.51859	35

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for Y	.081	35	.200*	.988	35	.964

*. This is a lower bound of the true significance.
a. Lilliefors Significance Correction

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Konsentrasi Leptin

F	df1	df2	Sig.
.879	6	28	.523

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a. Design: Intercept + X

ANOVA

Tests of Between-Subjects Effects

Dependent Variable: Konsentrasi Leptin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.694[a]	6	.449	1.950	.107
Intercept	502.743	1	502.743	2182.628	.000
X	2.694	6	.449	1.950	.107
Error	6.449	28	.230		
Total	511.887	35			
Corrected Total	9.144	34			

a. R Squared = .295 (Adjusted R Squared = .144)

(Post Hoc) – Tukey's HSD

Multiple Comparisons

Dependent Variable: Konsentrasi Leptin

Tukey HSD

(I) Serum	(J) Serum	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Normal diet without Profilin	Normal diet + Profilin 15 µg/mL	-.1380	.30354	.999	-1.1009	.8249
	Normal diet + Profilin 30 µg/mL	-.2240	.30354	.989	-1.1869	.7389
	Normal diet + ProfiliN 45 µg/mL	-.0440	.30354	1.000	-1.0069	.9189
	Hypercaloric diet + Profilin 15 µg/mL	-.8860	.30354	.087	-1.8489	.0769
	Hypercaloric diet + Profilin 30 µg/mL	-.1380	.30354	.999	-1.1009	.8249
	Hypercaloric diet + Profilin 45 µg/mL	-.1660	.30354	.998	-1.1289	.7969
Normal diet + Profilin 15 µg/mL	Normal diet without Profilin	.1380	.30354	.999	-.8249	1.1009
	Normal diet + Profilin 30 µg/mL	-.0860	.30354	1.000	-1.0489	.8769
	Normal diet + ProfiliN 45 µg/mL	.0940	.30354	1.000	-.8689	1.0569
	Hypercaloric diet + Profilin 15 µg/mL	-.7480	.30354	.211	-1.7109	.2149
	Hypercaloric diet + Profilin 30 µg/mL	.0000	.30354	1.000	-.9629	.9629
	Hypercaloric diet + Profilin 45 µg/mL	-.0280	.30354	1.000	-.9909	.9349

Normal diet + Profilin 30 µg/mL	Normal diet without Profilin	.2240	.30354	.989	-.7389	1.1869
	Normal diet + Profilin 15 µg/mL	.0860	.30354	1.000	-.8769	1.0489
	Normal diet + ProfiliN 45 µg/mL	.1800	.30354	.997	-.7829	1.1429
	Hypercaloric diet + Profilin 15 µg/mL	-.6620	.30354	.337	-1.6249	.3009
	Hypercaloric diet + Profilin 30 µg/mL	.0860	.30354	1.000	-.8769	1.0489
	Hypercaloric diet + Profilin 45 µg/mL	.0580	.30354	1.000	-.9049	1.0209
Normal diet + ProfiliN 45 µg/mL	Normal diet without Profilin	.0440	.30354	1.000	-.9189	1.0069
	Normal diet + Profilin 15 µg/mL	-.0940	.30354	1.000	-1.0569	.8689
	Normal diet + Profilin 30 µg/mL	-.1800	.30354	.997	-1.1429	.7829
	Hypercaloric diet + Profilin 15 µg/mL	-.8420	.30354	.117	-1.8049	.1209
	Hypercaloric diet + Profilin 30 µg/mL	-.0940	.30354	1.000	-1.0569	.8689
	Hypercaloric diet + Profilin 45 µg/mL	-.1220	.30354	1.000	-1.0849	.8409
Hypercaloric diet + Profilin 15 µg/mL	Normal diet without Profilin	.8860	.30354	.087	-.0769	1.8489
	Normal diet + Profilin 15 µg/mL	.7480	.30354	.211	-.2149	1.7109
	Normal diet + Profilin 30 µg/mL	.6620	.30354	.337	-.3009	1.6249
	Normal diet + ProfiliN 45 µg/mL	.8420	.30354	.117	-.1209	1.8049
	Hypercaloric diet + Profilin 30 µg/mL	.7480	.30354	.211	-.2149	1.7109
	Hypercaloric diet + Profilin 45 µg/mL	.7200	.30354	.247	-.2429	1.6829
Hypercaloric diet + Profilin 30 µg/mL	Normal diet without Profilin	.1380	.30354	.999	-.8249	1.1009
	Normal diet + Profilin 15 µg/mL	.0000	.30354	1.000	-.9629	.9629
	Normal diet + Profilin 30 µg/mL	-.0860	.30354	1.000	-1.0489	.8769
	Normal diet + ProfiliN 45 µg/mL	.0940	.30354	1.000	-.8689	1.0569
	Hypercaloric diet + Profilin 15 µg/mL	-.7480	.30354	.211	-1.7109	.2149
	Hypercaloric diet + Profilin 45 µg/mL	-.0280	.30354	1.000	-.9909	.9349

Hypercaloric diet + Profilin 45 µg/mL	Normal diet without Profilin	.1660	.30354	.998	-.7969	1.1289
	Normal diet + Profilin 15 µg/mL	.0280	.30354	1.000	-.9349	.9909
	Normal diet + Profilin 30 µg/mL	-.0580	.30354	1.000	-1.0209	.9049
	Normal diet + ProfiliN 45 µg/mL	.1220	.30354	1.000	-.8409	1.0849
	Hypercaloric diet + Profilin 15 µg/mL	-.7200	.30354	.247	-1.6829	.2429
	Hypercaloric diet + Profilin 30 µg/mL	.0280	.30354	1.000	-.9349	.9909

Based on observed means.

The error term is Mean Square(Error) = .230.

Konsentrasi Leptin

Tukey HSD[a,b]

Serum	N	Subset 1
Normal diet without Profilin	5	3.5620
Normal diet + ProfiliN 45 µg/mL	5	3.6060
Hypercaloric diet + Profilin 30 µg/mL	5	3.7000
Normal diet + Profilin 15 µg/mL	5	3.7000
Hypercaloric diet + Profilin 45 µg/mL	5	3.7280
Normal diet + Profilin 30 µg/mL	5	3.7860
Hypercaloric diet + Profilin 15 µg/mL	5	4.4480
Sig.		.087

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .230.

a. Uses Harmonic Mean Sample Size = 5.000.

b. Alpha = .05.

Appendix 2. Simple Linear Regression Analysis of Effect of Profilin Exposure given Normal Diet on Leptin Levels

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.079[a]	.006	-.070	.51793

a. Predictors: (Constant), Profilin + Normal diet

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.022	1	.022	.082	.779[b]
	Residual	3.487	13	.268		
	Total	3.509	14			

a. Dependent Variable: Leptin

b. Predictors: (Constant), Profilin + Normal diet

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.791	.354		10.716	.000
	Profilin + Normal diet	-.003	.011	-.079	-.287	.779

a. Dependent Variable: Leptin

Appendix 3. Simple Linear Regression Analysis of Effect of Profilin Exposure given Hypercaloric Diet on Leptin Levels

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.614[a]	.377	.329	.40582

a. Predictors: (Constant), Profilin +Hypercaloric diet

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1.296	1	1.296	7.869	.015[b]
	Residual	2.141	13	.165		
	Total	3.437	14			

a. Dependent Variable: Leptin

b. Predictors: (Constant), Profilin +Hypercaloric diet

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.679	.277		16.877	.000
	Profilin +Hypercaloric diet	-.024	.009	-.614	-2.805	.015

a. Dependent Variable: Leptin

Appendix 4. Analysis of Relationships between *Toxoplasma gondii* given Normal Diet and Average Weight of *Rattus Norvegicus* Wistar Strain Rats

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Leptin	.070	35	.200*	.989	35	.978
Weight	.100	35	.200*	.982	35	.829

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Pearson

Correlations

		Leptin	Weight
Leptin	Pearson Correlation	1	.159
	Sig. (2-tailed)		.363
	N	35	35
Weight	Pearson Correlation	.159	1
	Sig. (2-tailed)	.363	
	N	35	35

Appendix 5. Analysis of Relationships between *Toxoplasma gondii* Profilin given Hypercaloric Diet and Average Weight of *Rattus Norvegicus* Wistar Strain Rats

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Toxoplasma	.215	15	.061	.805	15	.004
Weight	.157	15	.200*	.950	15	.525

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Pearson

Correlations

		Toxoplasma	Weight
Toxoplasma	Pearson Correlation	1	-.084
	Sig. (2-tailed)		.767
	N	15	15
Weight	Pearson Correlation	-.084	1
	Sig. (2-tailed)	.767	
	N	15	15

Appendix 6. Analysis of Relationships between Average and Delta Weight and Leptin Level of *Rattus Norvegicus* Wistar Strain Rats

A) Average weight

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Toxoplasma	.215	15	.061	.805	15	.004
Weight	.126	15	.200*	.977	15	.940

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Pearson

Correlations

		Toxoplasma	Weight
Toxoplasma	Pearson Correlation	1	-.375
	Sig. (2-tailed)		.169
	N	15	15
Weight	Pearson Correlation	-.375	1
	Sig. (2-tailed)	.169	
	N	15	15

B) Weight delta - Rank Spearman

Correlations

			D_BB	Leptin
Spearman's rho	D_BB	Correlation Coefficient	1.000	.750
		Sig. (2-tailed)	.	.052
		N	7	7
	Leptin	Correlation Coefficient	.750	1.000
		Sig. (2-tailed)	.052	.
		N	7	7

Appendix 7. Ethic Form

KEMENTERIAN RISET, TEKNOLOGI, DAN PENDIDIKAN TIN
UNIVERSITAS BRAWIJAYA
FAKULTAS KEDOKTERAN
KOMISI ETIK PENELITIAN KESEHATAN
Jalan Veteran Malang - 65145, Jawa Timur - Indonesia
Telp. (62) (0341) 551611 Ext. 168; 569117; 567192 - Fax. (62) (0341) 564755
http://www.fk.ub.ac.id e-mail : kep.fk@ub.ac.id

KETERANGAN KELAIKAN ETIK
("ETHICAL CLEARANCE")

No. 134 / EC / KEPK / 04 / 2017

KOMISI ETIK PENELITIAN KESEHATAN FAKULTAS KEDOKTERAN UNIVERSITAS BRAWIJAYA, SETELAH MEMPELAJARI DENGAN SEKSAMA RANCANGAN PENELITIAN YANG DIUSULKAN, DENGAN INI MENYATAKAN BAHWA PENELITIAN DENGAN

JUDUL	:	**Efek Paparan Profilin *Toxoplasma gondii* terhadap Profil Lipid, Aktivitas Radikal Bebas, dan Kadar Adipositokin pada Tikus Rattus Norvegicus Strain Wistar yang Diberi Diet Tinggi Kalori.**
PENELITI UTAMA	:	**dr. Agustin Iskandar, M.Kes.,Sp.PK**
ANGGOTA	:	**1. M. Kaviyarasan** **2. Agung Nurwahyudi** **3. Dio Tri Agysta Putra** **4. Zulkifar Ramadhan** **5. Fathi Nabila Alim** **6. Lanisa Hapsari** **7. Florentina R. Eka R.** **8. Mira Raissa Santosa** **9. Jivanathan A/L Baskaren** **10. Parveen Anandhan** **11. Ahmad Adib**
UNIT / LEMBAGA	:	**Fakultas Kedokteran - Universitas Brawijaya Malang**
TEMPAT PENELITIAN	:	**Laboratorium Parasitologi Fakultas Kedokteran - Universitas Brawijaya Malang**

DINYATAKAN LAIK ETIK.

Malang, 08 APR 2017
Ketua,
Komisi Etik Penelitian Kesehatan

Prof. Dr.dr. Moch. Istiadjid ES, SpS, SpBS (K), M.Hum
NIK. 160746683

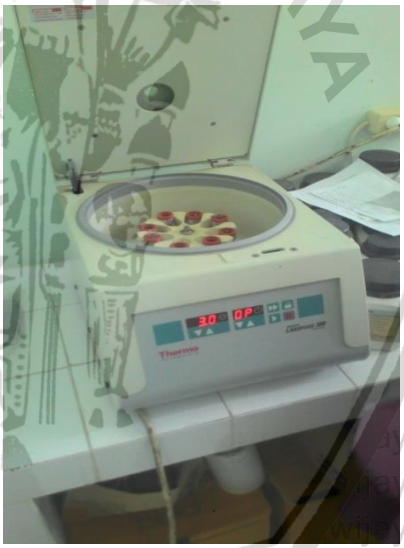
Catatan :
Keterangan Laik Etik Ini Berlaku 1 (Satu) Tahun Sejak Tanggal Dikeluarkan
Pada Akhir Penelitian, Laporan Pelaksanaan Penelitian Harus Diserahkan Kepada KEPK-FKUB Dalam Bentuk Soft Copy. Jika Ada Perubahan Protokol Dan / Atau Perpanjangan Penelitian, Harus Mengajukan Kembali Permohonan Kajian Etik Penelitian (Amandemen Protokol)

Appendix 8. Documentation of Project

(a)

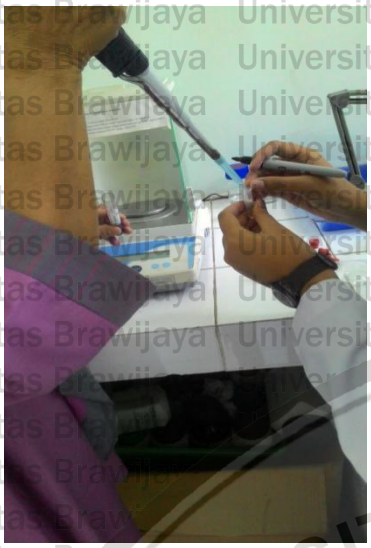
(b)

(c)

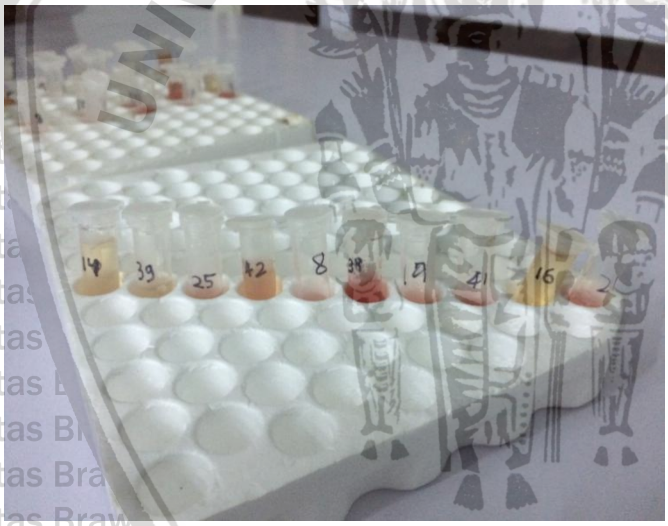


(d)

Figure 1 Surgery Process of Rats: a. Rats were drugged first using 50 mg ketamine as much as 0.4 to 0.5 ml; b. Surgery of mice; c. Rat blood withdrawal; d. The blood samples were then centrifuged for serum.



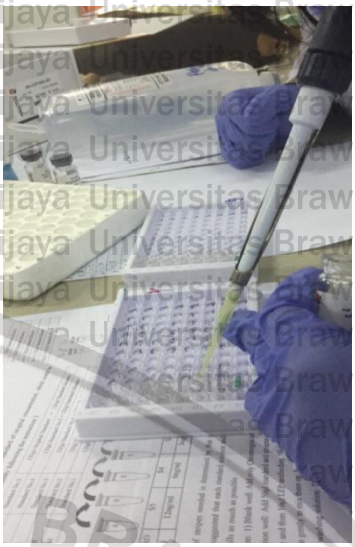
(f)



(g)

Figure 2 Sample collection; e. The process of taking the serum after centrifugation; f. The end result of serum and were stored in the freezer

(a)



(b)

(c)

(d)

Figure 3 Leptin Level Measurement; a. Preparation of standard solutions; b. Addition of leptin specific antibody and serum; c. Making of Washing Buffer; d. Washing 5 times

Solution A and *Solution B* were added

Stop Solution were added

Figure 3 Leptin Level Measurement ; e. *Solution A* and *Solution B* added; **f.** *Stop Solution* added.