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ORIGINAL ARTICLE

# The Effects of Annatto Tocotrienol on Body Composition and Serum Adiponectin, Leptin and Glucose Level in a Rat Model of Androgen Deficiency Induced by Buserelin

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## *ABSTRAK*

*Terapi ablasi androgen menggunakan hormon pelepasan gonadotrofin agonis dikaitkan dengan keabnormalan metabolik. Annatto tokotrienol (AnTT) dilaporkan mampu mengurangi ekspresi gen yang berkaitan dengan adipogenesis tetapi mekanismanya masih belum difahami. Kajian ini bertujuan untuk menentukan kesan annatto tokotrienol terhadap komposisi badan (jisim lemak dan jisim tanpa lemak), aras serum adiponektin, leptin, dan glukosa dalam tikus jantan yang diaruh dengan buserelin, iaitu agen ablasi testosteron. Tikus jantan Sprague Dawley yang berumur tiga bulan (n=32) dibahagikan secara rawak kepada empat kumpulan. Kawalan normal (n=8) diberikan minyak jagung secara oral setiap hari dan disuntik dengan normal salin secara subkutaneous setiap hari. Kumpulan yang selebihnya disuntik dengan buserelin secara subkutaneous (75 g/kg/hari). Kumpulan buserelin (n = 8) diberi minyak jagung secara oral, manakala kumpulan rawatan diberi AnTT pada 60 dan 100 mg/kg (n = 8/kumpulan) secara oral. Selepas 12 minggu rawatan, semua tikus telah dibunuh. Imbasan Dual-X-ray Absorptiometry dibuat untuk menentukan jisim lemak dan jisim tanpa lemak pada tikus. Darah diambil untuk menentukan aras adiponektin, leptin dan glukosa. Selepas 12 minggu, jisim tanpa lemak, jisim lemak, adiponektin dan leptin untuk semua kumpulan meningkat secara signifikan berbanding aras garis asas kumpulan masing-masing tanpa mengira rawatan mereka (P<0.05). Semua kumpulan kecuali tikus yang menerima AnTT pada 60 mg/kg mengalami peningkatan yang signifikan pada aras glukosa selepas 3 bulan (P<0.05). Ablasi androgen dan AnTT tidak mempengaruhi komposisi badan, aras adiponektin dan leptin pada tikus jantan. Walau bagaimanapun, annatto tokotrienol pada 60 mg/kg mampu memperbaiki metabolisma glukosa.*

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*Kata kunci: androgen, annatto tocotrienol, buserelin, glukosa*


## ABSTRACT

Androgen ablation therapy using gonadotropin-releasing hormone agonists is reported to be associated with metabolic abnormalities. Annatto tocotrienol (AnTT) is reported to reduce the expression of genes related to adipogenesis but the mechanism remains elusive. This study sought to determine the effects of annatto tocotrienol on body composition (lean and fat mass), serum adiponectin, leptin, and glucose levels in male rats treated with buserelin, a testosterone ablation agent. Three-month-old male Sprague Dawley rats (n=32) were randomly divided into four groups. The normal control (n=8) was given corn oil orally daily and normal saline subcutaneously daily. The remaining groups were injected with buserelin subcutaneously (75 g/kg/day). The buserelin group (n=8) was given corn oil orally, while the treatment groups were supplemented orally with AnTT at 60 or 100 mg/kg (n = 8/group). After treatment of 12 weeks, rats were euthanized. Dual-energy x-ray absorptiometry was performed to determine the lean and fat mass of the rats. Blood was collected for the evaluation of adiponectin, leptin and glucose levels. After 12 weeks, the lean mass, fat mass, adiponectin and leptin levels for all groups increased significantly compared to their respective baseline levels irrespective of their treatment ($P<0.05$). All groups, except rats receiving AnTT at 60 mg/kg, experienced a significant increase in glucose level after 3 months ($P<0.05$). Androgen ablation and AnTT do not influence body composition, adiponectin and leptin levels in male rats. However, annatto tocotrienol at 60 mg/kg may improve glucose metabolism.


Keywords: androgen, annatto tocotrienol, buserelin, glucose

## INTRODUCTION

As a major testicular androgen, testosterone plays critical roles in developing and preserving male secondary sexual characteristics and reproductive function. Longitudinal studies have demonstrated a gradual decline in testosterone levels in men associated with age (Feldman et al. 2002). A longitudinal study in Baltimore, USA, reported that 20% men over 60 years suffered from testosterone deficiency and the prevalence rose to 50% in men over 80 years (Harman et al. 2001). Medications like gonadotrophin-releasing hormone (GnRH) agonist commonly used as palliative treatment for prostate cancer can also cause a decline in testosterone level among patients (Crawford et al. 2006). Androgen deprivation therapy (ADT) through GnRH agonist is often achieved by suppressing luteinizing hormone released from the anterior pituitary. Prolonged stimulation of

GnRH receptors on the pituitary glands will cause desensitization, thereby disrupting the hypothalamus-pituitary axis. This will subsequently lead to a reduction in luteinizing hormone and testosterone production.

Androgen also affects protein, carbohydrate, and fat metabolism via actions on androgen receptors in muscle, liver, pancreatic beta-cells and metabolic centres in the hypothalamus (Kohn 2006). Testosterone deficiency invokes multiple metabolic complications, including fat mass accumulation and reduced lean mass, typically encountered by obese men (Dhindsa et al. 2016; Hamilton et al. 2011; Mauras et al. 1998). These features are attributable to dysfunctions in metabolism, such as insulin sensitivity, glycaemic control, energy regulation and lipid metabolism. Several studies have established that a low level of testosterone contributes to the pathogenesis of metabolic diseases (Janjgava et al. 2014; Stellato et al. 2000). Long-term ADT in prostate cancer patients is linked with fat mass accumulation and loss of lean mass. Unfavourable changes in fat mass and lean mass associated with impaired insulin sensitivity were observed in men within 12 weeks of initiating ADT (Smith et al. 2001). On the other hand, testosterone replacement reverses these effects in men (Bhasin et al. 2005). However, withdrawal of physiologic testosterone replacement in men with idiopathic hypogonadotropic hypogonadism leads to a significant decrement in insulin sensitivity within 2 weeks (Yialamas et al. 2007).

The mechanisms by which androgen exerts metabolic effects have not been fully elucidated. Prospective studies suggested that sex steroids might modulate adipokine secretion in men (Smith et al. 2008). Circulating adipokines secreted by adipocytes, such as adiponectin and leptin, appear to be important regulators of insulin sensitivity (Galic et al. 2010). Leptin maintains energy balance by regulating food intake and calorie burn rate and high levels of leptin were reported in obese individuals. Leptin resistance is one of the contributing factors to obesity and testosterone deficiency (Jones 2007). In contrast, adiponectin expression has been associated negatively with adiposity. Plasma adiponectin concentration in obese patients is low (Scherer 2006). This hormone acts as an autocrine and paracrine factor to inhibit the secretion of pro-inflammatory cytokines by adipocytes and macrophages, which in turn, suppress the lipid accumulation and insulin sensitivity (Lara-Castro et al. 2007; Sell et al. 2006).

There is a rapidly growing body of evidence showing that tocotrienol, a subfamily of vitamin E, has diverse effects on the body defense system against oxidative stress (Noor Azliza Wani et al. 2016; Suzana et al. 2009) and metabolic activities. A study by Nakano et al. demonstrated that α-tocotrienol (0.1 g/kg) reduced oxidative damage in diabetic rats but adversely affected the haemoglobin A1c and serum glucose level after 20 weeks (Nakano et al. 2008). Nevertheless, treatment of tocotrienol-rich fraction significantly suppressed the serum level of advanced glycosylation

end-products and lipid peroxidation products in diabetic rats. It also improved glycaemic control, insulin sensitivity and lipid profile in the rats (Budin et al. 2009; Siddiqui et al. 2013; Wan Nazaimoon & Khalid 2002). However, there is still a paucity of data on the effects of annatto tocotrienol on body composition and hormone changes in androgen deficient male rats induced by GnRH agonist (Ima-Nirwana & Suhaniza 2004; Wong et al. 2018; Wong et al. 2018; Wong et al. 2012; Zhao et al. 2015).

The objective of this study was to further determine the effects of annatto tocotrienol on body composition, particularly fat mass and lean mass, glucose level and metabolic hormones (leptin and adiponectin) in male rats with androgen deficiency induced by buserelin. Other data from this study were published in an earlier paper (Mohamad et al. 2018). It was hypothesized that annatto tocotrienol could prevent unfavourable metabolic changes, including hormones and body composition, induced by ADT.

## MATERIALS AND METHODS

### Animals and Treatment

Three-month-old male Sprague-Dawley rats (n=32) were sourced from the Laboratory Animal Resource Unit of Universiti Kebangsaan Malaysia (Kuala Lumpur, Malaysia). The rats were housed in plastic cages at the vivarium of Department of Pharmacology, Faculty of Medicine, University Kebangsaan Malaysia (Kuala Lumpur, Malaysia) under standard conditions (27°C, natural dark-light cycle) and given free access to standard rat chow (Gold Coin Holdings, Kuala Lumpur, Malaysia) and treated tap water. After seven days of acclimatization, the rats were randomly assigned into four groups (n=8/group). The normal control group (NC) received the vehicle for tocotrienol, i.e. corn oil orally daily and the vehicle for buserelin, i.e. normal saline subcutaneously daily. The buserelin control (BuC) received corn oil orally daily and subcutaneous buserelin injection (75 µg/kg/day). Annatto tocotrienol was given to the remaining rats orally at either 60 or 100 mg/kg/day in addition to subcutaneous buserelin injection (75 µg/kg/day). The dose of buserelin used was determined by a pilot study, as the results showed a significant decline in serum testosterone level and deterioration in bone microstructure after 12 weeks in male rats (Mohamad et al. 2018). Upon termination of the study after 12 weeks of treatment, all animals were sacrificed humanely through cardiac puncture under anaesthesia. Universiti Kebangsaan Malaysia Animal Ethics Committee had reviewed and approved the protocol of this study (Approval Code: FP/FAR/2015/CHIN/29-JULY/698-JULY-2015-MAY-2017).

### Body Composition Measurements

Fat mass and lean mass of the rats was measured using dual-energy X-ray absorptiometry (DXA) (Hologic QDR-1000 System, Bedford, USA) at week 0 and 12. DXA scans were processed using the small animal analysis

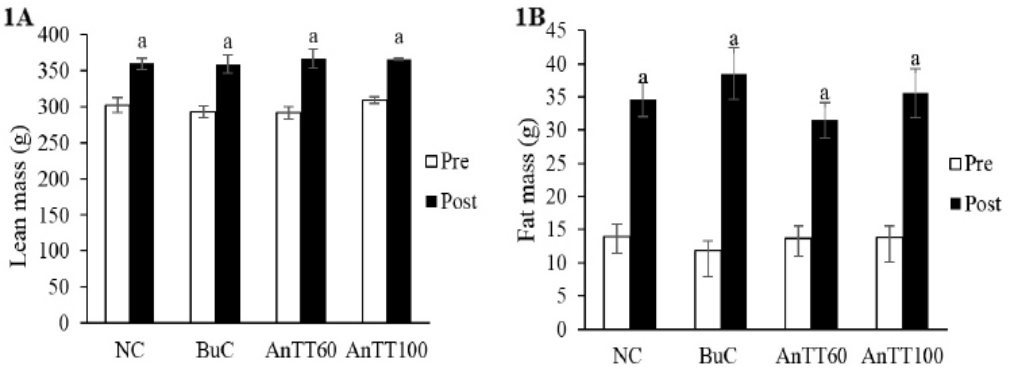


Figure 1: Body composition in rats before and after the 12-week treatment i.e lean mass (1A) and fat mass (1B). The data are shown as mean ± standard error of the mean. Abbreviation: NC= normal control group; BuC=buserelin control group; AnTT60=annatto-tocotrienol 60 mg/kg group; AnTT100=annatto-tocotrienol 100 mg/kg group. Notes: a=significantly different versus pre-treatment. The statistical significance was set as $p<0.05$.

program provided by the manufacturer (Hologic QDR-1000 System, Bedford, USA).

### Biochemical Analysis

Animal blood was collected in the morning from the tail vein of the rats before and after the 12-week treatment. The rats were not fasted prior to blood withdrawal. The blood was collected in plain tubes and centrifuged to separate the serum (3000 rpm for 10 minutes). It was then stored at -70°C until analysis. Serum level of adiponectin (Catalog number: E-EL-R0329; Elabscience Biotechnology Co., Ltd, China) and leptin (Catalog number: 27295; Immuno-Biological Laboratories Co., Ltd, Japan) were quantified using enzyme-linked immunosorbent assay kits as per manufacturer's instructions. The level of glucose was determined using a quantitative colourimetric glucose determination kit (QuantiChrom™ Glucose Assay Kit, BioAssay Systems, USA) according to the manufacturer's instructions.

### Statistical Analysis

Distribution of the data was presents using the Shapiro-Wilk test and all data were found to be normally distributed. The changes in fat mass, lean mass, adiponectin, leptin and glucose level were compared using mixed design one-way analysis of variance (ANOVA), followed by small effects analysis. The data was presented in mean ± standard error of the mean. Statistical Package for Social Sciences (SPSS) version 23.0 (IBM, Armonk, USA) was used for the analysis. A p-value <0.05 was considered statistically significant.

## RESULTS

### Body Composition Measurements

There were no significant differences in the pre-treatment lean and fat mass parameters among all the groups ($p>0.05$). After the 12-week treatment,

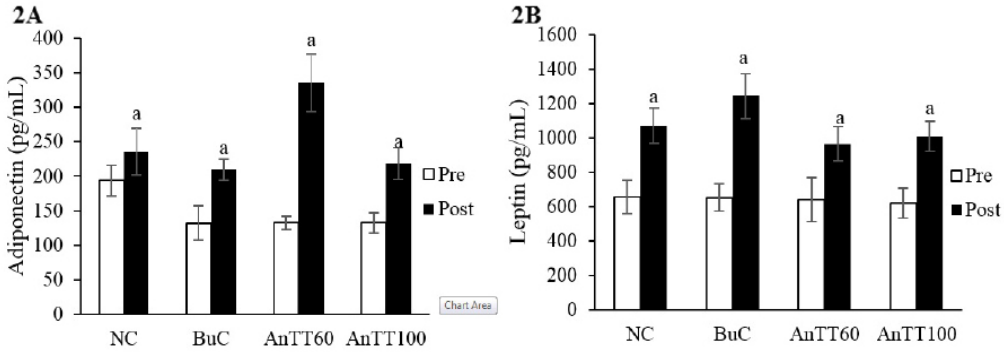


Figure 2: Hormone levels in rats before and after treatment i.e adiponectin (2A) and leptin (2B). The data are shown as mean ± standard error of the mean. Abbreviation: NC=normal control group; BuC=buserelin control group; AnTT60=annatto-tocotrienol 60 mg/kg group; AnTT100=annatto-tocotrienol 100 mg/kg group. Notes: a=significantly different versus pre-treatment. The statistical significance was set as $p<0.05$.

there was a significant increase in both parameters for all groups compared to their respective baseline ($p<0.05$). However, differences in lean and fat mass were not statistically significant among all the groups across all time points ($p>0.05$)(Figure 1A & 1B).

## Adiponectin and Leptin Level

There were no significant differences in the pre-treatment adiponectin and leptin parameters among all the groups ($p>0.05$). At the end of the experiment, there was a significant increase in both parameters for all groups compared to their respective baseline ($p<0.05$). However, differences in adiponectin and leptin were not statistically significant among all the groups across time points ($p>0.05$)(Figure 2A & 2B).

## Glucose Level

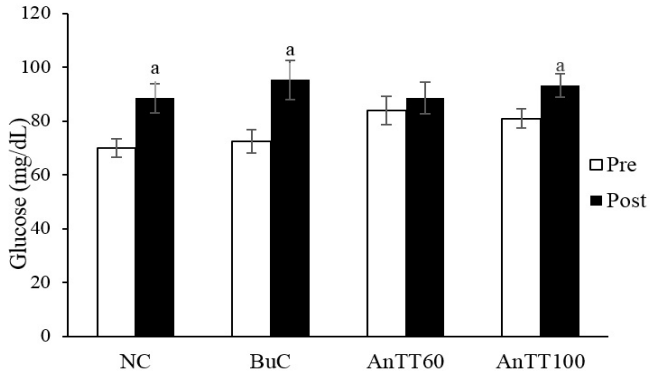


Figure 3: The glucose level in rats before and after the 12-week treatment. The data are shown as mean ± standard error of the mean. Abbreviation: NC=normal control group; BuC=buserelin control group; AnTT60=annatto-tocotrienol 60 mg/kg group; AnTT100=annatto-tocotrienol 100 mg/kg group. Notes: a=significantly different versus pre-treatment. The statistical significance was set as $p<0.05$.

There was no significant difference in the pre-treatment glucose level among all the groups ($p>0.05$). After 12 weeks, there was a significant increase in all groups compared to their respective baseline ($p<0.05$), except AnTT 60 ($p>0.05$). However, the difference in glucose level was not statistically significant among all the groups across time points ($p>0.05$)(Figure 3).

## DISCUSSION

This study was a part of the continuous project to investigate the effects of tocotrienol on musculoskeletal health in rats receiving androgen ablation therapy. The results published earlier showed that buserelin injection at 75 µg/kg/day for 12 weeks in male rats significantly decreased serum testosterone to a level comparable to orchidectomy (Mohamad et al. 2018). In the present study, all rats, regardless of treatment, experienced an increase in lean mass, fat mass, adiponectin, leptin and glucose levels compared to their respective baseline after 3 months ($p<0.05$). Administration of annatto tocotrienol at 60 mg/kg and 100 mg/kg did not alter lean mass, fat mass, adiponectin, leptin and glucose levels ($p>0.05$). Despite this, annatto tocotrienol at 60 mg/kg was able to prevent a significant increase in glucose level in the rats after 3 months of treatment.

Androgen has been proven to influence body composition, including lean mass and fat mass (Ng Tang Fui et al. 2016; Mauras et al. 1998). Notably, ADT used in prostate cancer treatment has been associated with an increased risk of diabetes and obesity in several large epidemiological studies (Muraleedharan et al. 2013; Smith et al. 2001; Wang et al. 2011). Since androgens preferentially preserve lean body mass over fat mass, androgen deprivation, therefore, can lead to adverse changes in body habitus (Lee et al. 2005; van Londen et al. 2008; Vermeulen et al. 1999). A previous study among healthy men receiving GnRH agonist resulted in increased fat mass and decreased lean mass (Berruti et al. 2002; Smith 2004; Smith et al. 2002). Men with acquired hypogonadism developed increased fat mass (Basaria et al. 2002), and testosterone replacement in hypogonadal men eventually reduced fat mass (Frederiksen et al. 2012; Mårin 1995). In the current study, the changes in body composition between the normal control and buserelin group were comparable. The relatively short experimental length could have contributed to the lack of significant changes in these compartments, despite the evidence from previous studies showing that it was sufficient to reduce bone mass (Wang et al. 2017).

A study by earlier researchers demonstrated that eight-week γ-tocotrienol (60 mg/kg/day) supplementation caused significant body fat reduction in an adrenalectomized, dexamethasone-induced osteoporotic rat model (Ima-Nirwana & Suhaniza 2004). In contrast, supplementation of annatto tocotrienol (60 and 100 mg/kg of body weight/day) in rats fed with high carbohydrate high fat failed to reverse the changes in body composition parameters after 3 months of treatment (Wong et al.

2018). Consistent with these findings, our results also displayed no significant changes in fat mass and lean mass for both doses of annatto tocotrienol (60 and 100 mg/kg of body weight/day). The discrepancy between these studies suggested that pure γ-tocotrienol isomer may have higher metabolic effects compared to the natural tocotrienol mixture. It should be noted that the overall concentration of γ-tocotrienol in annatto tocotrienol mixture is low (84% δ-tocotrienol and 16% γ-tocotrienol), thereby contributing to the lack of significant results in this study.

The association between diabetes, metabolic syndrome and hypogonadism are interdependent. Testosterone deficiency is one of the major risk factors for diabetes and metabolic syndrome because it affects adiposity and cellular glucose transport (Muraleedharan & Jones 2010). However, the causal relationship between diabetes and testosterone deficiency is also disputable as high blood glucose levels can suppress the production of luteinizing hormones by the pituitary gland and subsequently testosterone (Jones et al. 2011; Pitteloud et al. 2005; Pitteloud et al. 2005). In this study, the glucose level in rats receiving buserelin was similar to the normal control. We suggested that the short treatment period and the collection of non-fasting blood samples might have caused the effects of androgen ablation on glucose level to be not significant. The results of this study also showed a progressively increased blood glucose level in the rats with or without buserelin treatment. It is postulated that the sedentary lifestyle of the rats within the enclosure of the cage and unlimited food supply could be the contributing factors for this phenomenon.

Tocotrienol has been studied extensively for its anti-diabetic properties. Oral tocotrienol-rich fraction from palm oil (200 mg/kg of body weight/day) and rice bran oil (400 mg/kg of body weight/day) has been shown to reduce fasting serum glucose level in the streptozotocin-induced diabetic rat by significantly increasing glucose metabolism after 8 and 16 weeks of administration (Budin et al. 2009; Siddiqui et al. 2013). In this study, the glucose level in rats fed with annatto tocotrienol at 60 mg/kg did not increase compared with their baseline, which hinted at the potential hypoglycaemic effects of annatto tocotrienol. However, it should be noted that further tests to determine the effects of annatto tocotrienol on glucose metabolism were not performed. Furthermore postprandial, not fasting, glucose level was measured.

Increase in leptin level has been observed in men with hypogonadism and decreased serum leptin observed after testosterone replacement (Jockenhovel et al. 1997; Kalinchenko et al. 2010). This effect might be the link between the frequent associations of low serum testosterone with visceral obesity. Notably, increased testosterone level appears to directly cause suppression of adipocyte leptin production probably by an androgen-receptor-mediated pathway (Zitzmann et al. 2003). Contrary to expectations,

other adipocytokines of adiponectin were found to be decreased in obesity despite being produced by adipose tissues (Sheriff et al. 2011). An interplay between testosterone and adiponectin has been hypothesized and a previous study demonstrated that testosterone therapy in combination with diet and exercise in type 2 diabetic men increased adiponectin levels (Hotta et al. 2000). Conversely, adiponectin levels were found to be markedly higher in hypogonadal men than eugonadal men following testosterone therapy (Lanfranco et al. 2004). *In vivo* data also provide evidence that low testosterone level due to castration increased high-molecular-weight (HMW) adiponectin concentration and the level was decreased by testosterone replacement (Xu et al. 2005).

Administration of annatto tocotrienol at 60 mg/kg and 100 mg/kg had been shown to significantly decrease leptin level but increase adiponectin level in rats suffering from metabolic syndrome induced by high-carbohydrate high-fat diet after 3 months of treatment (Wong et al. 2018). However, in this study, the level of leptin and adiponectin levels in rats treated with annatto tocotrienol did not differ from other groups. We postulated that since leptin and adiponectin are synthesized by adipocytes; thus, the comparable fat mass among the studied groups could contribute to the lack of changes in these hormones.

Several limitations of our study need to be acknowledged. Non-fasting blood glucose level was measured and other more sensitive indicators of glucose metabolism were not determined. The study duration was rather short and the metabolic effects of androgen ablation might not have manifested. Despite these shortcomings, this study is novel because it is the first that characterised metabolic changes, defined by body composition and hormone of energy regulation, caused by short-term buserelin and annatto tocotrienol administration in male rats. It serves as a reference point to future studies examining the metabolic effects of tocotrienol in androgen deficiency. Besides, the apparent normoglycaemic effect of annatto-tocotrienol warrants further investigation.

## CONCLUSION

Androgen deprivation using buserelin and annatto tocotrienol at 60 and 100 mg/kg for 3 months did not influence body composition, glucose, adiponectin and leptin levels in male rats. Annatto tocotrienol at 60 mg/kg showed a tendency to improve glucose metabolism. Further investigations using more sensitive markers of glucose metabolism are warranted to validate the effects of annatto tocotrienol and its glucose-lowering mechanisms.

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