Akt expression and lactose synthesis during heat exposure in Saanen goat

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การแสดงออกของเอเคทีและการสังเคราะห์แลคโตสระหว่างการได้รับความร้อนในแพะนมพันธุ์ซาเนน

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หนึ่งนุช สายปิ่น : การแสดงออกของเอเคทีและการสังเคราะห์แลคโตสระหว่างการได้รับความร้อนในแพะนมพันธุ์ซาเนน. (Akt expression and lactose synthesis during heat exposure in Saanen goat) อ.ที่ปรึกษาหลัก : รศ. น.สพ. ดร.สัมพันธ์ ธรรมเจริญ, อ.ที่ปรึกษาร่วม : อ. น.สพ. ดร. รัฐจักร รังสิวิวัฒน์

ในประเทศเขตร้อน อุณหภูมิแวดล้อมสูง (high ambient temperature, HTa) เป็นปัจจัยพื้นฐานที่ก่อให้เกิดการลดลงการทำหน้าที่ของต่อมน้ำนมในสัตว์ให้นม สัตว์ให้นมที่เลี้ยงภายใต้ HTa มีผลผลิตน้ำนม (milk yield, MY) และองค์ประกอบน้ำนมลดลง การศึกษาปัจจุบันมุ่งเน้นศึกษาผลของ HTa ต่อการลดลงของการสังเคราะห์แลคโตสทั้งในสภาพธรรมชาติและสภาพ HTa ในหลอดทดลอง การทดลองที่หนึ่งดำเนินการเพื่อตรวจสอบผลของ HTa สภาพธรรมชาติ ในแพะพันธุ์ซาเนนช่วงต้นการให้นมสัปดาห์ที่ 2 ถึงสัปดาห์ที่ 4 หลังคลอด (2 PP-4 PP) สัตว์ 5 ตัวถูกศึกษาในฤดูหนาวเป็นสภาพแวดล้อมควบคุม 6 ตัวถูกศึกษาในสภาพธรรมชาติอุณหภูมิแวดล้อมสูงที่ดำเนินการในช่วงฤดูร้อน สภาพแวดล้อม อุณหภูมิกายวัดจากทวารหนัก (rectal temperature, Tr) อัตราการหายใจ (respiratory rate, RR) ปริมาณการกินได้วัตถุแห้ง (dry matter intake, DMI) และ MY ถูกวัดทุกวันตลอดการทดลอง ในวันสุดท้ายของสัปดาห์ที่ 2 และ 3 ตัวอย่างเลือดและน้ำนมถูกเก็บเพื่อวัดระดับน้ำตาลในเลือด สถานะความเครียดออกซิเดชัน และองค์ประกอบน้ำนม นอกจากนี้เซลล์ในน้ำนมถูกแยกออกจากตัวอย่างน้ำนมเพื่อประเมินการแสดงออกของยีน การทดลองที่สองดำเนินการโดยการเพาะเลี้ยงเซลล์เยื่อบุผิวต่อมน้ำนม (mammary epithelial cells, MECs) ที่แยกได้จากน้ำนมแพะเพื่อตรวจสอบผลของ HTa ต่อการสังเคราะห์แลคโตสและสถานะความเครียดออกซิเดชันของการเพาะเลี้ยง MECs โดยเซลล์ถูกแบ่งเป็นสองกลุ่มอุณหภูมิ ซึ่ง 37 °C เป็นกลุ่มควบคุม และ 39 °C เป็นกลุ่ม HTa หลังจากบ่มเซลล์เป็นเวลา 1 และ 48 ชั่วโมง อาหารเลี้ยงเซลล์ถูกเก็บเพื่อตรวจวัดความเข้มข้นของแลคโตสและเก็บ MECs เพื่อตรวจวัดการแสดงออกของยีน ตามลำดับ ในการทดลองที่หนึ่งค่าเฉลี่ยอุณหภูมิแวดล้อม (Ta), ความชื้นสัมพัทธ์ (RH), ค่าดัชนีอุณหภูมิ ความชื้นสัมพัทธ์ (THI), และ RR เพิ่มขึ้นอย่างมีนัยสำคัญในฤดูร้อนเมื่อเทียบกับฤดูหนาว ในทั้งสองฤดู Tr ในตอนบ่ายและเย็นสูงกว่าตอนเช้าอย่างมีนัยสำคัญ อย่างไรก็ตาม Tr ในฤดูร้อนไม่มีความแตกต่างอย่างมีนัยสำคัญกับฤดูหนาว ในขณะที่ DMI และ MY ในกลุ่มฤดูหนาวสูงกว่ากลุ่มฤดูร้อนอย่างมีนัยสำคัญ ในการศึกษาครั้งนี้ระหว่างฤดูร้อนกิจกรรมของ GPx ในพลาสม่าเวลาบ่ายสูงกว่าฤดูหนาวอย่างมีนัยสำคัญ ขณะที่ความเข้มข้นของพลาสม่าคอร์ติซอลในตอนบ่ายสูงกว่าตอนเช้าอย่างมีนัยสำคัญทั้งสองฤดู นอกจากนี้การแสดงออกของยีนอัลฟา-แลคตัลบูมิน (α-LA) และโปรตีนฮีตช็อค 70 (HSP70) เพิ่มขึ้นในฤดูร้อนเมื่อเทียบกับฤดูหนาว อย่างไรก็ตามไม่พบความแตกต่างระหว่างฤดูต่อการแสดงออกของยีนเบต้า-กาแลคโตซิลทรานเฟอเรส (β-GALT1) และเอเคที (Akt) ผลการศึกษาชี้ให้เห็นว่าสภาพแวดล้อมตามธรรมชาติจากการทดลองในปัจจุบันแสดงค่าดัชนีอุณหภูมิและความชื้นสูง โดยเฉพาะอย่างยิ่งในช่วงบ่ายของฤดูหนาวและฤดูร้อน ผลกระทบของ HTa ต่อการสังเคราะห์น้ำนมอาจเกี่ยวข้องกับการเพิ่มความสามารถการต้านอนุมูลอิสระของแพะและการแสดงออกของยีน HSP70 แต่ผลกระทบนี้ไม่เกี่ยวข้องกับการแสดงออกของ Akt ในการทดลองที่สอง ผลการทดลองการแสดงออกของยีน HSP70 เพิ่มขึ้นอย่างมีนัยสำคัญ หลังจากบ่มใน HTa 1 ชั่วโมง เมื่อเทียบกับกลุ่มควบคุม การบ่มเซลล์เป็นเวลา 1 หรือ 48 ชั่วโมง ในสภาพ HTa ไม่ส่งผลกระทบต่อการแสดงออกของยีน β-GALT1 และ α-LA รวมทั้ง Akt นอกจากนี้ไม่พบความแตกต่างความเข้มข้นของแลคโตสระหว่างกลุ่มควบคุมและกลุ่ม HTa ผลการทดลองนี้แสดงว่า HTa ไม่มีอิทธิพลต่อการแสดงออกของยีนสังเคราะห์แลคโตสของ MECs โดยสรุปแล้วผลของ HTa ต่อผลผลิตน้ำนมและการเพาะเลี้ยงเซลล์เยื่อบุผิวจากต่อมน้ำนมนั้นเกี่ยวข้องกับการแสดงออกของยีน HSP70 ที่เพิ่มขึ้นจากการศึกษาทั้งในธรรมชาติและในหลอดทดลอง อย่างไรก็ตาม HTa ไม่ได้ส่งผลกระทบต่อการแสดงออกของยีน β-GALT1, α-LA และ Akt

สาขาวิชา	สรีรวิทยาการสัตว์	ลายมือชื่อนิสิต .....
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Nungnuch Saipin : Akt expression and lactose synthesis during heat exposure in Saanen goat. Advisor: Assoc. Prof. Sumpun Thammacharoen, Ph.D. Co-advisor: Ruttachuk Rungsiwiwat, Ph.D.

In tropical countries, high ambient temperature (HTa) is a fundamental factor that cause a decline in the mammary gland function in dairy animals. Dairy animals fed under HTa decreased milk yield (MY) as well as milk compositions. The current study focuses on the effect of HTa to decrease milk synthesis from natural and *in vitro* HTa condition. The first experiment was performed to investigate the effect of HTa from the natural condition in early lactating Saanen goat, 2-4 weeks of postpartum (2 PP-4 PP). Five animals were investigated during the winter season as control ambient condition. Six animals as the natural HTa group was conducted during summer. The environmental conditions, rectal temperature (Tr), respiratory rate (RR), dry matter intake (DMI) and MY were measured everyday throughout the experiment. At the end of 2 PP and 3 PP, blood and milk samples were collected to measure blood glucose, oxidative stress status, and milk composition. In addition, the milk cells were isolated from milk sample to evaluate gene expression. The second experiment was performed using mammary epithelial cells (MECs) culture isolated from goat milk to investigate the effect of HTa on lactose synthesis and oxidative stress status of MECs culture. The cells were allocated into two temperature groups that were 37 ºC as the control and 39 ºC as the HTa group. After 1- and 48-hour incubation the culture medium was collected for measured lactose concentration and MECs were harvested to determine gene expression, respectively. In the first experiment, the average ambient temperature (Ta), relative humidity (RH), temperature humidity index (THI), and RR were significantly increased in summer when compared with the winter season. In both seasons Tr in the afternoon and evening was significantly higher than that in the morning. However, Tr from summer was not significantly different from winter. Whereas, DMI and MY during the winter group showed significantly higher than that from the summer group. In the present study, during summer, plasma glutathione peroxidase (GPx) activity in the afternoon were significantly higher than that in winter. Whereas, plasma cortisol in the afternoon was significantly higher than that in the morning in both seasons. Moreover, the relative gene expressions of α-lactalbumin (α-LA) and heat shock protein 70 (HSP70) gene were higher in summer when compared with the winter season. However, there were no differences between the season on the relative expression of β-1,4-galactosyltransferase (β-GALT1) and Akt genes. The results suggested that the natural ambient condition from the present experiment showed high temperature and humidity index especially during the afternoon in both winter and summer months. The effect of HTa on milk synthesis might be related to an increase in antioxidative capacity of the goats and expression of HSP70 gene but this effect is not related to Akt expression. In the second experiment, the results of the relative gene expression of HSP70 increased significantly after 1 hour HTa incubation when compared with the control. At 1 or 48 hours incubation, HTa condition could not affect β-GALT1 and α-LA genes expression as well as Akt. In addition, there was no difference in lactose concentration between control and HTa group. These results showed that HTa did not influence lactose synthesis gene expression of MECs. In conclusion, the effect of HTa on MY and MECs culture apparently related with increasing expression of HSP70 from both natural and in vitro study. However, HTa did not affect the expression of β-GALT1, α-LA and Akt genes.

Field of Study:	Animal Physiology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

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Nungnuch Saipin

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1-4 PP	First- Fourth week of postpartum
A_{1-2}	Absorbance 1-2
ACTH	adrenocorticotrophic hormone
Akt	Protein kinase B
AM	Ante Meridiem
alpha-MEM	Minimum Essential Medium Eagle-alpha modification
BHT	Butylated hydroxytoluene
bp	Base pair
BW	Body weight
Ca^{2+}	Calcium
CAT	Catalase
cDNA	Complementary DNA
CK18	Cytokeratin18 proteins
CMT	California mastitis test
CO_2	Carbon dioxide
CRH	Corticotropin releasing hormone
CSN2	β-casein
DAPI	4', 6-diamidino-2-phenylindole
ddH_2O	Double distilled water
dL	Deciliter

DMEM	Dulbecco's modified eagle medium
DMI	Dry matter intake
DMSO	Dimethyl sulfoxide
dT	Deoxy thymine
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal bovine serum
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HPA	hypothalamic-pituitary adrenal axis
HRP	Horseradish peroxidase
H_2SO_4	Sulfuric acid
HSP70	Heat Shock Protein 70
HTa	High ambient temperature
IgG	Immunoglobulin G
kg	Kilogram

NAD^+	Oxidized Nicotinamide adenine dinucleotide
NADPH	Reduced Nicotinamide adenine dinucleotide
nM	Nanomolar
nm	Nanometer
nmol	Nanomole
MDA	Malondialdehyde
Mg^{2+}	Magnesium
MECs	Mammary epithelial cells
ml	Milliliter
mm	Millimeter
mM	Millimolar
mRNA	messenger Ribonucleic acid
MY	Milk yields
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide anion
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PM	Post Meridiem
PRL	Prolactin
RH	Relative humidity
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rpm	Revolutions per minute
RR	Respiratory rate
SNF	Solid not fat
SOD	Superoxide dismutase
Ta	Ambient temperature
TBA	Thiobabituric acid
TBARS	Thiobarbituric acid reactive substances
THI	Temperature humidity index
Tm	Melting temperature
TS	Total solid
Tr	Rectal temperature
UV	Ultraviolet
V/V	Volume by volume
WI	Water intake
α-LA	α-lactalbumin
β-GALT1	β-1, 4-galactosyltransferase
°C	Degree Celsius
µg	Microgram
µl	Microliter

CHAPTER I

INTRODUCTION

In tropical countries, high ambient temperature (HTa) is a fundamental factor that produces a negative effect on the mammary gland function in dairy animals. Dairy animals fed under HTa decreased milk production as well as milk compositions (Rhoads et al., 2009; Shwartz et al., 2009; Mabjeesh et al., 2013; Smith et al., 2013) Although milk synthesis is one of the priority physiological processes during the lactation period, an attempt to maintain body temperature by decreasing heat increment and by the activation of heat dissipation are the major additional physiological processes in dairy animal fed under HTa condition. In Thailand, dairy animals were suffered from HTa throughout the year and the average temperature and humidity index (THI) during morning and afternoon were higher than the normal THI for the dairy animals (Thammacharoen et al., 2014; Nguyen et al., 2018). Acclimation is another adaptive mechanism that has developed in the dairy animal fed under the natural condition of HTa. The decrease in milk yield (MY) during HTa apparently mediates by two major mechanisms. Feed intake (FI) and MY from dairy cows fed under cyclic HTa decreased significantly from control Ta. Importantly, the paired-feeding group had higher MY than that of the cyclic HTa group. This result suggested that the effect of HTa on MY is mediated in part by the reduction of nutrients delivered to the mammary gland and another part by the direct HTa effect on the mammary gland (Rhoads et al., 2009; Baumgard and Rhoads, 2013). This hypothesis was supported by the experiments that have been done in the mammary epithelial cells (MECs). The HTa effect on milk synthesis has also been demonstrated *in vitro*. The HTa decreased numerous proteins related to the biochemical pathway of synthesis and secretion of milk components (Hu et al., 2016; Li et al., 2017).

Besides, heat-shock proteins (HSP) are considered as one part of the stress responses in dairy cattle during HTa or heat stress (Lacetera et al., 2006; Zhang et al., 2014). HTa or heat stress influences the homeostasis of oxidative status. Oxidative stress was induced in animals as well as in dairy animals treated with HTa (Bernabucci et al., 2002; Zhang et al., 2014; Yusuf et al., 2017). In the mammary gland, milk synthesis is associated with an increase in cellular oxidative stress (Castillo et al., 2006). One interesting effect of HTa that has been demonstrated in vitro is the HTa effect to decrease the expression and activities cell signaling enzyme, protein kinase B or Akt (Bang et al., 2000; Galadari et al., 2008). Along with several target cellular functions of Akt (Manning and Cantley, 2007), this serine/threonine kinase has been shown to play a major role in milk and lactose synthesis apparently via the lactose synthase. Lactose synthase is the enzyme derived from the binding of α-lactalbumin (α-LA) and β-1, 4-galactosyltransferase (β-GALT1). In bovine MECs, Akt knockdown decreased the mRNA expression of β-GALT1 (Lin et al., 2016). Therefore, the current study focused on the effect of HTa to decrease milk and lactose synthesis from both natural HTa conditions and in vitro HTa conditions. The relationship of oxidative stress, HSP and Akt on the mammary gland function were investigated.

Objectives of the study

1. To determine the effects of natural and *in vitro* HTa on heat stress and oxidative stress status in goat and MECs.
2. To investigate the effect of HTa on the milk, lactose concentration and Akt expression in goat and MECs.

Research questions of the study

1. Is MY from goats fed under summer lower than that of the winter season.
2. Is oxidative stress from goats fed under summer higher than that of the winter season.

3. Can HTa affect lactose synthesis, oxidative stress status, and Akt expression in MECs.

Hypothesis of the study

1. MY from goats fed under summer might be lower than that of the winter season.
2. Oxidative stress from goats fed under summer might be higher than that of the winter season.
3. HTa might affect lactose synthesis, oxidative stress status, and Akt expression in MECs.

CHAPTER II

LITERATURE REVIEW

Effects of high ambient temperature in dairy animal

Behavioral and physiological responses

HTa is the major cause leading to the abnormal physiological response. Whenever the animals are exposed to HTa and their body temperature control is imbalance, the animals are in the stage of heat stress (Silanikove, 2000). Normally, the effect of HTa or heat stress can be evaluated from the behavioral and physiological responses such as finding shade area, decreasing motility, decreasing of FI, increasing of water intake (WI), increasing of rectal temperature (Tr), and respiratory rate (RR) and finally decreasing milk yield (Chaiyabutr et al., 2008; Shwartz et al., 2009; Alam et al., 2013; Hamzaoui et al., 2013; Mabjeesh et al., 2013; Smith et al., 2013). Under natural HTa, the behavioral adaptations are preceded the physiological responses. Increasing heat dissipation is the first physiological response. Breathing is the major means that goats use to dissipate heat. The responses of breathing to HTa has been classified into 2 phases. While thermal tachypnea or panting is considered as the first phase, increase tidal volume breathing (hyperpnea) is the second phase when the HTa is maintained at a high level (Hales and Webster, 1967).

Although Tr does not always represent an average of deep body temperature, it has been widely used as the body temperature indicator and the variation of Tr between 39 to 40 °C has been reported in goats fed under HTa (Mehrdad et al., 2012; Nguyen et al., 2018). When the Tr was increased by HTa to the higher level (40-41 °C), the goat was in the stage of extreme heat stress (Alam et al.,

2013; Mabjeesh et al., 2013). Thus, both RR and Tr are among the first important and reliable indicators that have been used to identify heat stress.

THI was used as an indicator of thermal conditions. The values of THI used for dairy cows as less than 68 referring to comfort, 68-72 mild discomfort, 72-75 discomfort, 75-79 alert, 79-84 danger (Habeeb et al., 2018). However, the values of THI used for small dairy animals are higher than that of dairy cows. HTa can lead animals into heat stress conditions. Goat and sheep can be fed without heat stress at THI less than 82. The value of THI between 82 and 84 could induce moderate heat stress. The value of THI between 84 and 86 is the range of severe heat stress. The value of THI greater than 86 is extremes severe heat stress (Silanikove, 2000). THI levels used for goats are higher than that of dairy cows, this supposes due to the small animal high tolerance to heat stress more than cows.

One important stress response that has been well recognized is the activation of adrenal stress hormones. It has been reported that the concentration of plasma cortisol from both cattle and buffaloes increased in non-cooled animals compared with the cooled animals (Chaiyabutr et al., 2008). Cortisol is a steroid hormone in the glucocorticoid hormone class that is a biomarker for the physiological stress responses and determinant of health status in animals. Cortisol is produced by the zona fasciculata of the adrenal cortex in the adrenal gland. The function of cortisol is increase blood glucose through gluconeogenesis and increase in the metabolism of protein, carbohydrate, and fat (Taves et al., 2011).

Normally, cortisol is released in a diurnal cycle, which increases during the morning and decreases in the evening through slow-wave sleep. However, it is released in response to the physiological status of the animals such as late gestation, the metabolic response such as hypoglycemia and stress which is leads to the high levels of plasma cortisol. The regulation of cortisol is the anterior pituitary gland

hormone, adrenocorticotropic hormone (ACTH), which stimulates the cortisol secreting cells. ACTH is controlled by the hypothalamic hormone, corticotropin releasing hormone (CRH), which is under the nervous system activation. Therefore, the regulation of stress hormones involved in the organs as the hypothalamic pituitary adrenal (HPA) axis (Figure 1).

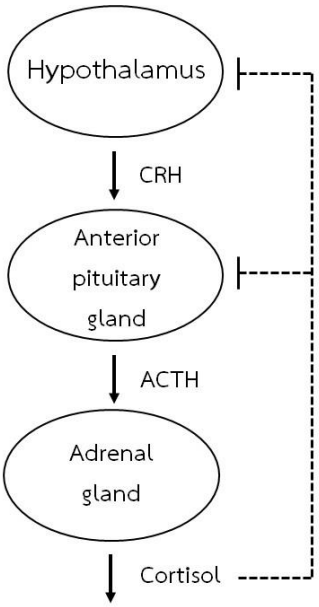


Figure 1 The schematic diagram of the hypothalamic pituitary adrenal (HPA) axis.
The hypothalamic pituitary adrenal (HPA) axis is a direct effect interaction of three organs: the hypothalamus, the anterior pituitary gland, and the adrenal gland. Stimulate: ↓; Inhibit: ⊣

Normally, there is absence of circadian rhythm of cortisol levels. A factor that can affect serum cortisol levels of the goats is the feeding schedule that plasma cortisol levels were fluctuations before feeding times (Eriksson and Teravainen, 1989). In contrast to the goats were provided feeding ad libitum, the cortisol profiles showed monotonous (Alila-Johansson et al., 2003). In addition, the cortisol levels in goats have been shown in transportation stress studies (Kannan et al., 2000). The study suggested the normal goat cortisol concentrations as 14-18 ng/ml and increased to 26-36 ng/ml after transportation. Moreover, it has been shown that long day photoperiod can directly increase cortisol (Leproult et al., 2001). The study of

non-cooled animals tended to be higher than that of cooled animals (Chaiyabutr et al., 2008). This information suggests that plasma cortisol may be used as one indicator of heat stress in ruminants. The variations of cortisol concentration in goats may be associated with heat tolerances and different physiological state such as the plasma cortisol of the Bedouin male goats during summer were 12-17 ng/ml and 8-10 ng/ml in the winter season (Chergui et al., 2017), the Aardi goats showed 30 ng/ml (Al-Badwi et al., 2013) and Saanen kids were 56 ng/ml (Greenwood and Shutt, 1992). The results showed that the goat fed under HTa condition in this study was adapted by acclimatization. Therefore, the data showed that the goats are the best tolerant to elevated ambient temperature and therefore, need not always indicate stress for the goat.

HTa or Heat stress is a powerful factor that affects the cellular functions. Responding to stress signals is activated heat shock factor (HSF) that binds to the heat shock promoter element (HSE) and resulting in leads to the elevated expression of heat shock gene (Figure 2) (Morimoto, 1999).

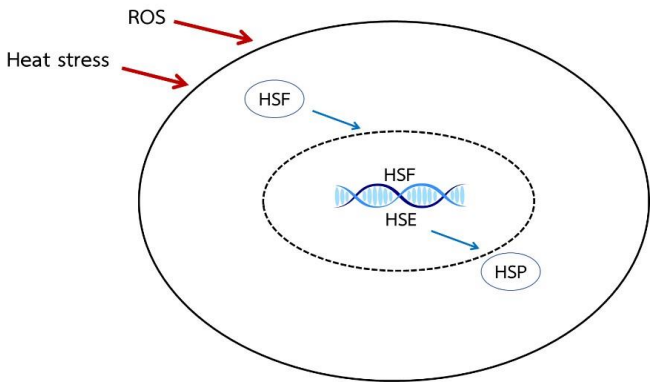


Figure 2 The schematic diagram of heat stress and reactive oxygen species (ROS) induce the heat shock protein.

Heat stress and reactive oxygen species (ROS) induce the heat shock response modified from Morimoto, (1999). The activated HSF is binding to HSE results in up regulation of HSPs expression. Stimulate: ⟶

Heat shock protein (HSP) or known as chaperones are ubiquitous proteins found in mammalian cells and organisms. HSP produced by the cell in response to stressful conditions exposure. Particularly, HSP is important protein to rescue and protect the cell from negative effect during heat exposure (Hu et al., 2016). Hsp70 proteins can act in protecting the cells from thermal or oxidative stress. Normally, the stressor act to damage proteins, causing partial unfolding and possible aggregation. HSP functions are stabilizing new proteins to ensure correct folding or refolding proteins that were damaged by the stressor (Gade et al., 2010; Basiricò et al., 2011).

The family of HSP consists of many proteins which are classified by the range in molecular size from 8 to 150 kilo Dalton (kDa) such as HSP27, HSP60, HSP70, HSP90 and many others (Kregel, 2002). Many of HSP are present continuously and rapidly induced through transcription and translation mechanisms by stressful conditions (Wu, 1995). In dairy cows, HTa exposure increased serum HSP70 and HSP90 (Min et al., 2015). According to the study of bovine MECs, it has been shown the gene expression profile that gene associated with protein repair and stress response was upregulated (Collier et al., 2006). Moreover, HSP70 peaked rapidly within 30 minutes after heat exposure in bovine MECs (Hu et al., 2016). Among the domestic ruminant, goats are tolerant of HTa more than cows (Salama et al., 2014). Among many HSPs, HSP70 was the dominant subtype and can be protected in goat (Luengrattana et al., 2000) and cow (Gaughan et al., 2013; Min et al., 2015).

Plasma HSP70 level may be a good indicator of heat stress responses in ruminant. Therefore, the increased HSP70 chaperone protein during HTa condition has been well researched. In particular, HSP70 is the dominant subtype and can be protected in goats (Luengrattana et al., 2000). For the lactating animal, HTa is a powerful and ubiquitous factor that influence MY and milk compositions. It has been

known that the dairy cow, as well as dairy goat, shows a marked decrease in milk yield during HTa (Rhoads et al., 2009; Hamzaoui et al., 2013; Mabjeesh et al., 2013). Importantly, it has been demonstrated that the effect of HTa on milk yield was mediated in part via the direct mechanism of HTa on mammary gland function. The effect of HTa on milk composition has been well demonstrated as well. Briefly, the concentrations of milk casein (Hu et al., 2016) and lactose (Tao et al., 2011), but not milk fat, during HTa period were decreased compared with the thermal neutral period (Li et al., 2017).

Mammary gland function

The major function of the mammary gland is colostrum and milk synthesis. Colostrum and milk are the biological fluid that provides immune and nutrients for the protection, growth, and development of the newborn animal. Lactose, casein, and triglycerides are the major milk solid compositions. Among these constituents, lactose is carbohydrate. It plays a major role in milk volume regulation, which represents the osmotic composition in milk and draws water into milk (Peaker, 1977; Kuhn et al., 1980; Holt, 1983; Frimawaty and Manalu, 1999).

Lactose is synthesized from 2 monosaccharide components; free glucose and uridine diphosphate (UDP)-galactose. The latter component is also derived from glucose. The lactating mammary gland of ruminants can consume more than 85% of the total circulating glucose (Annison and Jl, 1964). The lactose synthesis pathway is in the compartment of Golgi apparatus. This reaction was catalyzed by an important enzyme; lactose synthase. Lactose synthase is composed of a catalytic component enzyme, β-GALT1, and a regulatory component, α-LA. Then, lactose enters in the secretory vesicle and escapes the Golgi membrane, across the apical membrane, which fuses the plasma membrane and released the lactose into the culture medium by the exocytosis mechanism (Guerin and Loizzi, 1978). According to the

study of the previous report shown that β-GALT1 concentration increased in the milk of dairy cows when the day of lactation progressed (Bleck et al., 2009). The α-LA also showed a positive correlation between milk protein, fat, and lactose concentration. This suggested that the two-compartment of lactose synthase is correlated to milk composition and the lactation stage of the dairy cow. Lactose synthase enzyme activity has been studied for a long time, which revealed that the appropriate temperature for the galactosyltransferase was lower than is usual for enzymes. The maximum activity of this enzyme was at 24 ℃ (Kitchen and Andrews, 1972). However, it is widely known that the appropriate temperature for the lactose synthesis enzyme is 37 ℃ (Watkins and Hassid, 1962). HTa exposure affects bovine mammary cell turnover (Peng et al., 2011; Tao et al., 2011) and mammary function via decreased the mRNA of β-casein (CSN2) level for milk protein synthesis (Hu et al., 2016), α-LA or whey protein concentration (Gellrich et al., 2014), and milk lactose yield (Tao et al., 2011). Thus, the current study investigated the effects of HTa on mammary gland function both in vivo and in vitro study in Saanen goat.

Mammary gland development

The mammary gland is an interesting organ. During pregnancy and lactation, the mammary gland is grown, proliferated, developed and differentiated in varies activity (Linzell and Peaker, 1974; Burd et al., 1978). It is well known that the development of the mammary gland initiated during pregnancy and maintained throughout lactation and involution. The factors influence mammary gland development are hormones (Houdebine et al., 1985; Forsyth, 1986; Macias and Hinck, 2012), nutrient supply (Kjaersgaard, 1968; Chaiyabutr et al., 1980; Chaiyabutr et al., 1997) and MECs capacity (Knight and Peaker, 1982; Wilde et al., 1999; Jedrzejczak and Szatkowska, 2014).

Mammary epithelial cell culture

Mammary epithelial cells (MECs) are widely used as a model to study mammary gland function *in vitro*. An advantage of MECs culture are replacing the animal experiment as an animal model, the ability to control the physiochemical environment such as pH, temperature, osmotic pressure, chemical concentration, etc. Moreover, it can be culture and grow up easily. The source of MECs are tissue biopsy of mammary gland or the establishment of mammary epithelial cell line. Recently, the isolation and culture of the MECs directly from milk (Boutinaud et al., 2015; Saipin et al., 2018) has been reported. Therefore, isolation of MECs from milk might be more advantageous and easier than a tissue biopsy procedure.

The culture medium of MECs culture used for growth and proliferated cells normally consistent with basal media, tissue extracts and body fluids such as fetal bovine serum (Ogorevc and Dovč, 2015; Lin et al., 2016) or chick embryo extract (Maurer et al., 2007; Pajtler et al., 2010). Growth factors are the polypeptide which usually supplemented in the culture medium for propagated the cells at the concentration of 1 to 10 ng/ml range. Nowadays, the extensive growth factors are including epidermal growth factor (EGF) (Hu et al., 2009; Saipin et al., 2018), plated-derived growth factor (PDGF) (Hannink and Donoghue, 1989). The antibiotic (Penicillin, streptomycin, ampicillin, etc.) and essential amino acids (cysteine, cysteine, arginine, glutamine, and tyrosine) are often added in the culture medium as well. Moreover, the lactogenic hormone is required by differentiation process of the MECs culture. It has been known that the combination of lactogenic hormone such as prolactin (PRL), insulin, and hydrocortisone are necessary for MECs differentiation and stimulated the mammary function which secreted lactose into the culture medium (Lin et al., 2016).

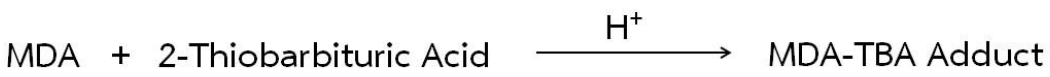
The development of MECs after plated on the culture dishes are started at attached on the plate, followed by cell growth, proliferation, and differentiation. The

success of the mammary function of MECs is up to all of this process. Moreover, the understandings of the growth and proliferation of the MECs culture is necessary. Growth curve analysis of MECs has been demonstrated in bovine mammary gland tissue (Hu et al., 2009) and MECs isolated from goat milk (Saipin et al., 2018). Growth curves showed proliferated doubled in number within 24-72 hours when grown on the cell culture dishes. After 3 days plated, the MECs culture showed normal cell proliferation during 3-7 days.

Oxidative stress

Oxidative stress is the stress that comes mainly from an imbalance of reactive oxygen species production (ROS) and antioxidant capacity of animal cells (Landriscina et al., 2009). ROS are free radicals that the molecules with unpaired electrons producing from normal cell metabolism. However, among the remaining ROS fraction may be harmful to the cells. It has been known that ROS damages all major organelles of the living cell (Barzilai and Yamamoto, 2004; Birben et al., 2012). Thus, oxidative stress is leaded to a negative effect of the cell.

Lipid peroxidation is the reaction of lipid oxidative degradation. Normally, this process leads the cell damage by free radical steal electrons from phospholipid in plasma membrane. Lipid peroxidation is commonly measured as the end product of the reactive aldehydes that malondialdehyde (MDA). Normally, lipid peroxidation measurement is commonly used thiobarbituric acid (TBA) reaction also known as thiobarbituric acid reactive substances (TBARS). Thus, oxidative stress is the one of the reasons lead to an increase in TBARS.



The defense mechanisms against ROS damage in animals generally have 2 groups that enzymatic and non-enzymatic antioxidants. An important of the enzymatic antioxidants are include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are the natural antioxidants system activated intracellular protecting oxidative damage. SOD function is catalyzing the dismutation superoxide and the end-product of this reaction are hydrogen peroxide and oxygen. Whereas, both CAT and GPx act the same way that catalyzes hydrogen peroxide to water and oxygen (Chance et al., 1979).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 + H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$$

Non-enzymatic antioxidants include albumin, protein sulfhydryl groups, ascorbic acid or vitamin C, glutathione, α-tocopherol or vitamin E, retinol or vitamin A, Flavonoids, β-carotene, uric acid, and Phenolic acids. Among this, vitamin A, vitamin E, and vitamin C are classic examples of antioxidants (McDowell and Arthington, 2005).

HTa is one of the causes of oxidative stress in dairy animals. Oxidative stress increases the endogenous free radical (Chaiyabutr et al., 2011) and causes a decrease in an antioxidant capacity of dairy animals such as the activity of GPx and SOD. According to the previous study (Bernabucci et al., 2002), GPx and SOD activity of dairy cows increased significantly during summer.

The study of effects of high temperature on the oxidative status has been performed in the chicken embryonic fibroblasts (Ibtisham et al., 2018). It is reported that the content of MDA from chicken embryonic fibroblasts cell line incubated at 40 ℃ for 6, 12 and 24 hours shows no significant difference when compares with 37 ℃. Moreover, the study of heat exposure (42 ℃) on oxidative stress and apoptosis in bovine MECs (Li et al., 2019) shows MDA content increased significantly when compares with 37 ℃. In addition, the studies of heat stress induce the mitochondrial apoptotic pathway reveal that a high temperature of more than 43-44 ℃ affects MDA concentration of human umbilical vein endothelial cells (HUVECs).

However, oxidative stress could affect varies intracellular signaling pathway, including the PI3K/Akt pathways (Qin et al., 2000; Qin and Chock, 2003; Lahair et al., 2006; Zhang and Yang, 2013). According to the previous study (Lahair et al., 2006), it was suggested that oxidative stress could induce phosphorylation of Akt in the human T-lymphocyte cell line. Moreover, this study report that hydrogen peroxide (H_2O_2) could be induced the phosphorylation of both phosphorylation sites Ser473 in the C-terminal domain and Thr308 in the kinase domain. Moreover, high glucose promoted ROS production leading to increased alkaline phosphatase activity by activated the PI3K/Akt pathway in primary rat osteoblasts (Zhang and Yang, 2013). The relationship of oxidative stress and Akt signaling pathway in bovine MECs have been investigated (Jin et al., 2016). According to this study, H_2O_2 treatment induced stress responses of the endoplasmic reticulum and increased cell apoptosis. The phosphorylated Akt protein level was significantly increased in H_2O_2 treatment group when compared with the non-treated group. This data showed that H_2O_2 treatment induced oxidative stress by activated phosphorylated Akt in bovine MECs.

Akt or Protein kinase B

Akt or Protein kinase B is a serine/threonine protein kinase enzyme. Akt enzyme exists three isoforms in mammals (Green et al., 2013). Akt1 has a wide tissue distribution (Haslinger et al., 2013), Akt2 is found in muscle and fat cells (Cheng et al., 1992), and Akt3 is expressed in testes and brain (Masure et al., 1999). Akt is an effector in signal transduction pathways affect regulates cellular metabolism, cell survival, and cell proliferation. In addition, during pregnancy and lactation, Akt1 is upregulated in the mammary gland (Chodosh et al., 2000; Boxer et al., 2006; Chen et al., 2012).

The growth and development of the mammary gland are regulated by several factors including hormones, growth factors, and their plasma membrane receptors. In MECs culture, it has been demonstrated that mammary function can be stimulated by hormones such as prolactin (PRL), insulin, and glucocorticoid (Doppler et al., 1990; Stocklin et al., 1996). PRL is able to stimulate the Jak-Stat pathway and other signaling pathways. PRL stimulates PI3-kinase/Akt pathway which results in phosphatidylinositol 3-kinase (PI3K) activation and production of phosphatidylinositol (3,4,5)-triphosphate (PIP3) as a second messenger. Then, a translocation of Akt to the plasma membrane is recruited by PIP3. Akt is then phosphorylated by pyruvate dehydrogenase kinase and activation of several targets by binding and regulating many downstream effectors stimulate several cell metabolisms such as cell survival, cell proliferation, angiogenesis, protein synthesis, and glucose metabolism (Osaki et al., 2004; Manning and Cantley, 2007).

Glucose metabolism plays an important role in living cells. Akt signaling pathway is directly correlated with increased rates of glucose metabolism (Rathmell et al., 2003). Akt involve in glucose metabolism by regulating the localization of the glucose transporter 4 (GLUT4) to plasma membrane and regulate hexokinase

expression (Kakinuma et al., 2008). Moreover, Akt stimulates glycogen synthesis via glycogen synthase kinase-3 phosphorylation (Nicholson and Anderson, 2002). According to the study of the investigation of Akt activated glucose metabolism and lactose synthesis in bovine MECs (Lin et al., 2016). This study examines the effect of Akt1 knockdown on the expression of genes involved glucose transportation and lactose synthesis. The result showed that GLUT1, hexokinase 2, and β-GALT1 gene expression in Akt knockdown MECs were significantly decreased. It was concluded that Akt acts as a regulator mediated the effects of glucose concentration on lactose synthesis in bovine MECs.

Furthermore, it has been shown that heat stress or oxidative stress results in increasing Akt phosphorylation. Heat treatment could activate Akt rapidly within minutes in a pheochromocytoma of the rat adrenal medulla cell line (PC12). However, this phosphorylation decreased within 1 hour and returned to control levels by 3 hours (Mearow et al., 2002). In the previous experiments, heat stress decreased Akt protein expression in mouse cell line (L929 cells) and turn to cell death (Galadari et al., 2008). Moreover, the phosphorylated Akt was failed to activate Akt within long-time heat treatment. This resulted in growth arrest and apoptosis (Bang et al., 2000).

CHAPTER III

MATERIALS AND METHODS

The first part of the experiment was carried out at the Veterinary student training center, Faculty of Veterinary Science, Chulalongkorn University, Nakhon Pathom Province. The second part of the experiment was carried out at the Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, Bangkok. The procedures of this experiment were performed according to the guidelines and approved by the Animals Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University (#1831012).

Experiment 1: The effect of seasons on lactation performance and oxidative stress status in Saanen goat

Animals

Eleven multiparous purebred Saanen lactating goats, age between 3 and 6 years old, average body weight 38-42 kg were used in this study. The animals were divided into two groups. Five goats gave birth during winter, in period from November 20 to December 10 were used as control group. The control experimental period was investigated during winter season from November to January. Another group were 6 goats gave birth during summer, in period from March 10 to April 20 were used as HTa group. The natural HTa period of the experiment was conducted from March to May. The goats from both groups were similar for parity.

All goats were kept indoor in the individual pens. After parturition, the experiment was started during the first week and the duration of the experiment was conducted in early lactation (4 weeks). The experimental procedure is presented in Figure 3. The goats were provided the concentrate feed and Pangola hay as roughage according to the national research council (NRC) recommendation (1981). Goats were

fed concentrate diet twice a day (5:00 AM and 3:00 PM). Pangola hay, mineral blocks and water were freely available. At the end of the experiment, each feed samples were analyzed according to AOAC (2016). The chemical compositions of feed are presented in table 1 Milking was done twice a day (6:00 AM and 4:00 PM).

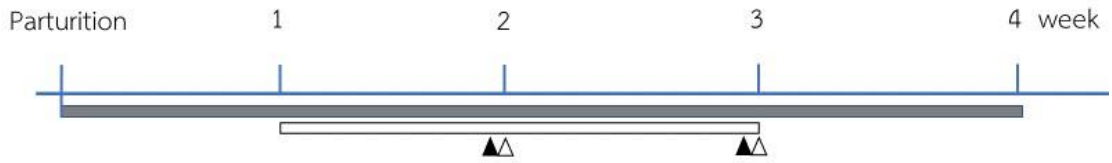


Figure 3 The schematic protocol of the experiment 1 and sample collection.

The experiment was done in Saanen goats during summer and the winter season. The solid black line represents the experimental duration. The environmental condition including ambient temperature (Ta) and relative humidity (RH) were measured and recorded at 6:00 AM, 1:00 PM, and 6:00 PM daily for 4 PP. The solid white line represents the duration of Rectal temperatures (Tr) and respiratory rates (RR) were registered daily at 2 PP and 3 PP at 6:00 AM, 1:00 PM, and 6:00 PM. Milk sample from morning milking (6:00 AM) was collected for compositions measurement and isolation of goat milk cells for β-GALT1, α-LA, Akt, and HSP70 gene expression (black arrowhead). Blood sample was collected at 7:00 AM and 1:00 PM at 2 PP and 3 PP for measured blood glucose and oxidative stress status (white arrowhead). Blood sample was collected at 2 PP for measured cortisol concentration.

Table 1 Chemical compositions of feed components (% on dry matter basis)

Composition (%)	Concentrate	Roughage
Dry matter	90.21 ± 0.75	87.12 ± 0.82
Crude protein	19.79 ± 0.40	7.62 ± 2.22
Crude fiber	21.02 ± 0.66	39.66 ± 0.51
Fat	2.00 ± 0.28	0.23 ± 0.07
Ash	4.95 ± 0.33	4.66 ± 0.14

The temperature and humidity index (THI)

Ambient temperature (Ta) and relative humidity (RH) at goat barns were recorded using a Temperature, Humidity and Dew Point data logger (EL-USB-2-LCD, Lascar electronics, Kwun Tong, Hong Kong). Ta and RH were measured every day during the experimental period at 6:00 AM, 1:00 PM and 6:00 PM. THI was determined according to NRC (1971) as follow:

$$THI = ((1.8 \times Ta) + 32) - [(0.55 - (0.0055 \times RH)) \times ((1.8 \times Ta) - 26.8)]$$

Where: Ta = ambient temperature and is expressed as ℃

RH = relative humidity

Data collection and measurement in Saanen goat

Feed samples were collected daily and divided into two parts. The first part was immediately dried at 105 ℃ overnight in the oven to determine dry matter (DM). The second part of feed samples was kept frozen at –20 ℃ until chemical analysis. Proximate analysis was performed by thawed all feed samples and mixed thoroughly, and the samples were dried at 65 ℃ overnight (approximately 12 hours) for proximate analysis according to AOAC (2016). Daily intake was measured daily from feed offer and refusal. Daily dry matter intake (DMI) was calculated by the following formula:

Daily feed intake = feed offer – feed refusals (Dry matter basis)

Daily MY was recorded daily from day 1 of postpartum throughout the experiment. Milk samples were collected at before the end of the second and third week postpartum (days 13 and 20) and divided into two parts. One part (30 ml) was kept frozen at –20 ℃ until milk compositions analysis. The remaining sample (250 ml) was collected into the plastic sterile tubes and transferred immediately to icebox for goat milk cells isolation and gene expression determination.

Breathing from each goat was counted from the movement of the flank to represent the RR and recorded by a digital camera. Then, Tr was measured by a digital clinical thermometer (digital clinical thermometer C202, Terumo, Tokyo, Japan). RR and Tr were measured at 6:00 AM, 1:00 PM, and 6:00 PM daily from the second and third weeks of postpartum (2 PP and 3 PP).

Blood sample was obtained from the jugular vein, after milking (7:00 AM) and afternoon (1:00 PM) at the end of the second and third weeks postpartum (2 PP and 3 PP). Blood glucose was measured using glucometer immediately (Accu-Chek Adv II, Roche diagnostic GmbH, Manheim, Germany). Blood sample was placed in the EDTA tube, kept in crushed ice and transferred to the laboratory. Blood sample was centrifuged at 4,000 rpm, 4 ℃ for 10 minutes. Then, plasma samples were collected and stored at –80 ℃ until the analysis of oxidative stress status. Plasma samples at 2 PP was stored at –20 ℃ until the analysis of cortisol concentration.

Determination of milk compositions

Milk secretion from morning milking (6:00 AM) was corrected by hand milking. The udder and teats were cleaned with chloroxylenol solution and the first strips of milk were discarded before the milking procedure. 30 ml of milk sample was collected from each animal and kept at –20 ℃ until analysis. Milk compositions were determined at the day at the end of 2 PP and 3 PP during the experimental period. Milk sample was analyzed for protein, lactose, fat, solid not fat (SNF), and total solids (TS) using Milkoscan (FT2; Foss, Hilleroed, Denmark).

Goat milk cells isolation and collection for gene expression determination

The somatic cells were obtained by cells collected from raw milk. Raw goat milk (250 ml) was centrifuged at 1,200 rpm for 5 minutes. The aqueous part was decanted and the cell pellets were suspended with sterile phosphate buffered saline (PBS; Thermo Fisher Scientific, Logan, Utah, USA) and centrifuged at 1,200 rpm for 5

minutes. The washing step was repeated twice by discharge the supernatant and the somatic cell pellets were suspended with 12 ml PBS.

Goat milk cells were harvested from the somatic cells by density gradient centrifugation with Ficoll (GE Healthcare Chicago, Illinois, USA) as described by the previous report (Nonnecke and Kehrli, 1985). The mixture of somatic cells pellets sample with PBS and Ficoll (12:3, V/V) was centrifuged at 1,500 rpm for 40 minutes. The upper aqueous supernatant part with white blood cells (WBC) was decanted and the cell pellets were resuspended with PBS and centrifuged at 1,200 rpm for 5 minutes. The cells were washed once. The cells from this step were used for the determination of β-GALT1, α-LA, Akt, and HSP70 genes expression by PCR.

Determination of plasma cortisol concentration

Plasma cortisol concentration was determined using the goat cortisol ELISA kit (CBS-E18048G, CUSABIO, Houston, USA) according to the manufacturer's instruction. Briefly, 50 µl of standard and plasma samples were added to the wells. Then, the antibody (50 µl) was added to each well immediately and incubated at 37 °C for 40 minutes. All wells were washed with 200 µl wash buffer and let it stand for 2 minutes and complete removal of liquid. The washing step was repeated three times.

HRP-conjugate (100 µl) was added to each well immediately. The plate was covered by the adhesive strip and incubated at 37 °C for 30 minutes. All wells were aspirated and washed five times. 90 µl of TMB substrate was added to each well and incubated at 37 °C for 20 minutes. The stop solution 50 (µl) was added to each well. Determination of the optical density of each well within 5 minutes, using a microplate reader set to 450 nm.

Determination of plasma oxidative stress status

Oxidative stress status of the animals was determined from both plasma malondialdehyde (MDA) as the lipid peroxidation products and plasma glutathione peroxidase activity as the antioxidant defense. Plasma concentration of MDA was analyzed using the lipid peroxidation (MDA) assay (Colorimetric/Fluorometric) (ab118970, Abcam, Oregon, USA). Plasma glutathione peroxidase activity was measured by the glutathione peroxidase activity assay (Colorimetric/Fluorometric) (ab102530, Abcam, Oregon, USA).

Plasma MDA was obtained using lipid peroxidation determinations by the lipid peroxidation (MDA) assay as manually instructed has been described. In brief, the plasma sample (20 µl) was mixed with 500 µl of 42 nM H_2SO_4 in the microcentrifuge tube. 125 µl of the phosphotungstic acid solution was added and mixed by vortexing. The mixture was incubated at room temperature for 5 minutes and centrifuged at 10,000 rpm for 3 minutes. Collected the pellet and resuspended with 100 µl ddH_2O (with 2 µl BHT (100x)). Then, adjusted the final volume to 200 µl with ddH_2O.

The generation of MDA-TBA adduct was performed by added TBA reagent (600 µl) into each microtube containing 200 µl standard and 200 µl of a prepared plasma sample. The mixture was incubated at 95°C with dry bath (AccuBlock, digital dry Bath, Labnet International, Inc., New York, USA) for 60 minutes and cooled to room temperature in an ice bath for 10 minutes. The upper layer of the mixture (200 µl) was placed into the microplate on a microplate reader (SpectraMax M3, Molecular Devices, San Jose, California, USA) and measured at 532 nm.

Plasma glutathione peroxidase activity was measured by the glutathione peroxidase activity assay. Briefly, plasma samples (50 µl) were added into the reaction wells. The reaction mixture was then added (40 µl; 33 µl assay buffer, 3 µl

of 40 mM NADPH solution, 2 µl glutathione reductase solution, 2 µl glutathione solution). The mixture was mixed and incubated at room temperature for 15 minutes. The cumene hydroperoxide solution (10 µl) was added to each well. The reaction mixture was measured output (A_1) on a microplate reader at 340 nm. After incubated at 25 °C for 5 minutes, measured output (A_2) at 340 nm.

Extrapolate sample readings from the standard curve plotted was calculated as follows equation:

ΔA 340 nm = ((Sample A_1 – Sample A_2) –

(Reagent control A_1 – Reagent control A_2))

The next step was to apply the ΔA 340 nm to the NAPDH standard curve to get NADPH amount B using the following equation:

B = (ΔA 340 nm – intercept slope)

The result of GPx concentration in the test samples is calculated as (nmol/minute/ml) using the following equation:

GPx activity = ([B / (T_1 – T_2)] x V) x D

Where:

B = NADPH amount that was decreased between T_1 and T_2 (in nmol)

T_1 = Time of the first reading (A_1) (minutes)

T_2 = Time of second reading (A_2) (minutes)

V = Pretreated sample volume added into the reaction well (ml)

D = Sample dilution factor

Experiment 2: The effect of in vitro HTa on lactose synthesis and oxidative stress status of MECs

This experiment was divided into two part. The first part was conducted for the characterization and determination of mammary function of MECs isolated from

goat milk and investigated MECs function by comparing the supplementation with or without lactogenic hormone. The second part was investigated the effect of HTa on lactose synthesis and Akt expression of MECs culture

I. Characterization and determination of mammary function of MECs culture

Goat MECs collection and isolation from goat milk

Goat milk sample was collected by hand milking from 3 early lactating Saanen goats. All goats were determined to be free of clinical mastitis by physical examination and California Mastitis Test (CMT) solution. The udder and teats were cleaned with chloroxylenol solution and the first strips of milk were discarded before the milking procedure. Milk sample was collected 50 ml into the plastic sterile tube, and kept immediately on ice and transferred to the laboratory.

The somatic cells from each goat milk sample were isolated by centrifugation as described by the previous report (Saipin et al., 2018). Raw milk was diluted 1:1 in the sterile Dulbecco's modified eagle medium (DMEM; Thermo Fisher Scientific, Logan, Utah, USA) and centrifuged at 1,200 rpm for 10 minutes. The aqueous part was decanted and the cell pellets were resuspended with PBS and centrifuged at 1,200 rpm for 10 minutes. Washing step was repeated twice by discharge of the supernatant. 1 ml culture medium was resuspended into the cell pellets tube. The cells derived from this step were used for MECs culture.

Culture of MECs

The somatic cells were plated in the 60 mm plate. The culture medium used for the growth of the cells was contained sterile alpha-MEM (Thermo Fisher Scientific, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Logan, Utah, USA), 1% Glutamax (Gibco, Grand Island, New York, USA), 1% penicillin-streptomycin (Gibco, Grand Island, New York, USA), and 10 ng/ml epidermal growth factor (EGF; R&D Systems, Minneapolis, Minnesota, USA). The cells

were allowed to grow in the incubator at 37 ℃ in humidified 5% CO_2. The floating cells which are non-epithelium somatic cells were removed after the first day of culture. The attached cells were washed with PBS without Ca^{2+} and Mg^{2+} (Thermo Fisher Scientific, Logan, Utah, USA) and the fresh culture medium was added to the culture plate. The culture medium was changed every 3 days.

After the cells reached 80% confluences, the adherent cells sample were harvested from the plates for subculturing of primary cell culture. In brief, the cells were detached by trypsinization using 0.25% trypsin-EDTA (Thermo Fisher Scientific, Logan, Utah, USA), the cell suspension was added alpha-MEM contained 10% FBS (1:2, V/V) for stop trypsin reaction. MECs were isolated from the cell suspension by centrifugation at 1,200 rpm for 5 minutes. The supernatant was decanted and the cell pellets were resuspended with alpha-MEM (1 ml). The cell number was evaluated. MECs were divided into 2 portions. The first portion was frozen in freezing medium consisting of 80% culture medium, 10% FBS and 10% DMSO (Sigma- Aldrich, St Louis, Missouri, USA) for future experiment. The second portion was used for routine culture. MECs were cultured in the second passage. This passage was used for further experiments.

MECs from the second passage that has been described in the culture of MECs part were harvested and allocated into two sets. The first set was used for epithelial cells characterization and the second set was used for determination of the mammary function.

Immunocytochemistry

MECs isolated from goat milk at the second passage was used for epithelium characterization by immunocytochemistry. The cells were allowed to grow in 4-well dishes until reaching 60-80% of confluency. The cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich, St Louis, Missouri, USA) for 15

minutes. The cells were permeabilized using 0.2 % Triton X-100 (Sigma-Aldrich, St Louis, Missouri, USA) in PBS for 20 minutes. Follow by blocked with 5% FBS in PBS and incubated with 1:100 cytokeratin (CK) 18 primary antibody (R&D Systems, Minneapolis, Minnesota, USA) overnight at 4 °C. The cells were washed three times with PBS, followed by incubated with 1:200 dilutions of appropriate conjugated Anti-Mouse IgG secondary antibody (R&D Systems, Minneapolis, Minnesota, USA) for 1 hour at room temperature. Finally, the cells were washed with PBS for three times and the nuclear were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, Missouri, USA). The CK 18 positive cells were observed a red fluorescent signal under the fluorescence phase-contrast microscope (Nikon Eclipse, Tokyo, Japan).

To determine the function of isolated cells, MECs were plated at the density of 5 x 10^5 cells in 35 mm plate and allowed to grow in growth medium. After the cells reached 80% confluency, MECs were allocated into two groups. The first group as the control was culture in growth medium without EGF. The second group was culture in growth medium without EGF and additionally lactogenic hormone contain 5 µg/ml Insulin-Transferrin-Selenite (Gibco, Grand Island, New York, USA), 1 µg/ml prolactin (Gibco, Grand Island, New York, USA) and 1 µg/ml hydrocortisone (Sigma-Aldrich, St Louis, Missouri, USA). MECs were allowed to grow at 37 °C in humidified 5% CO_2 and the culture medium was changed every 3 days. Each group was carried out in duplicate.

The culture medium was collected and stored at –20 °C until analysis. Lactose concentration was determined using the Lactose/D-Glucose (Rapid) Assay (K-LACGAR, Megazyme, Ireland, UK). The culture medium from days 6, 9, and 12 were collected for lactose concentration determination. On day 12 after incubation, the adherent cells sample were harvested from the plates by trypsinization as has been

described. MECs were collected for the determination of β-GALT1, α-LA, and CNS2 gene expression by PCR. The experimental procedure is presented in Figure 4.

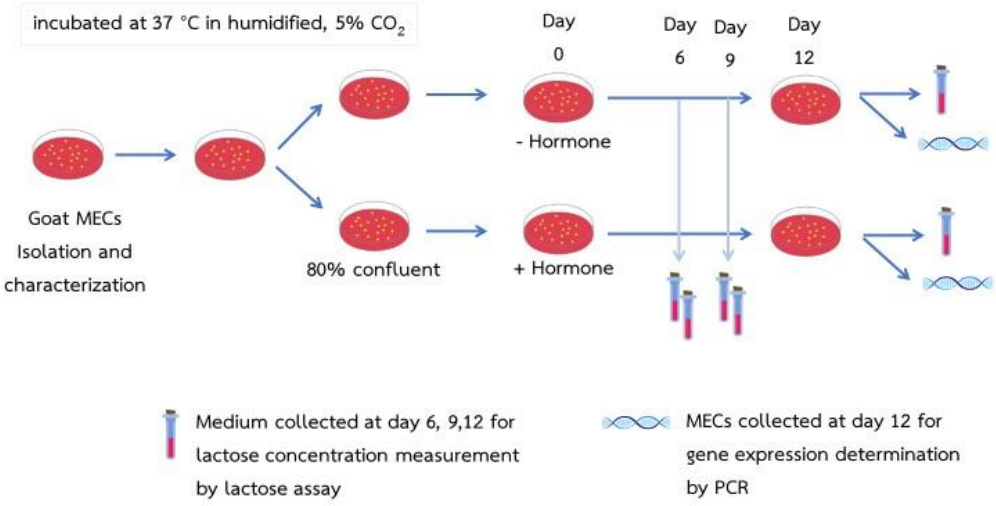


Figure 4 The schematic protocol of the first part of experiment 2.

The first part of experiment 2 is isolation, characterization, and determination of MECs function. The experimental design and samples collected were done in MECs culture incubated with or without hormone supplementation at 37 °C in humidified 5% CO_2 for 12 days. At day 6,9, and 12 the culture medium sample was collected for determination of lactose concentrations and after 12 days of incubation, MECs were harvested for β-GALT1, α-LA, and CSN2 gene expression determination.

Determination of lactose concentration of culture medium

Lactose concentration from the medium culture was measured using the Lactose/D-Galactose (Rapid) Assay. In brief, the culture medium (200 µl) was added to the UV cuvette and 200 µl of the β-galactosidase solution, mixed the contents by gentle swirling, the cuvettes were covered and incubated at 25 °C for 2 hours. 200 µl assay buffer and 100 µl of NAD^+ solution were added, followed by adjusted the volume to 2,700 µl with ddH_2O. Mixed well and read the absorbances of the solutions (A_1) at 340 nm using a UV spectrophotometer (Evolution 60 UV

spectrophotometer, Thermo Scientific, Wisconsin, USA). After 3 minutes, start the reaction by addition of 20 µl β-Galactose dehydrogenase plus galactose mutarotase suspension. Mixed well and read the absorbance of the solutions (A_2) at the end of the reaction.

To determine the absorbance differences ($A_2 - A_1$) of blanks and samples was calculated as follows equation:

Determination of D-galactose:

ΔA D-galactose = ($A_2 - A_1$) galactose sample – ($A_2 - A_1$) galactose blank

Determination of lactose + D-galactose:

ΔA lactose + D-galactose = ($A_2 - A_1$) lactose sample – ($A_2 - A_1$) lactose blank

Determination of lactose:

ΔA lactose = ΔA lactose + D-galactose – ΔA D-galactose

The concentration of D-galactose and lactose can be calculated as follows:

The concentration = [(V x MW) / (ε x d x v)] x ΔA

Where: V = final volume (ml)

MW = molecular weight of the substance assayed (g/mol)

ε = extinction coefficient of NADH at 340 nm

d = light path (cm)

v = sample volume (ml)

II. Investigation of the effect of HTa on lactose synthesis and Akt expression of MECs culture

MECs from the second passage and the culture condition for MECs propagation was followed the result of part I of the experiment 2. After the cells reached 80% confluency, MECs were used for study the effect of HTa on lactose synthesis was evaluated. MECs culture were change with the fresh alpha-MEM culture medium contained 10% FBS, 1% Glutamax, 1% penicillin-streptomycin supplemented with 5 µg/ml Insulin-Transferrin-Selenite, 1 µg/ml prolactin and 1 µg/ml hydrocortisone were added to the culture plate. MECs were incubated at 37 ℃ in humidified 5% CO_2 for 6 days and then the cells were changed the culture medium followed by continuous incubated at 37 ℃ for 1 day. At days 7, MECs were allocated into two groups. The first group were transferred to incubate at 37 ℃ as control group and the second group as HTa incubated at 39 ℃ in humidified 5% CO_2.

MECs culture were prepared for collection of the culture medium and the cells were harvested from the plates after 1 and 48 hours incubated at the treatment temperature. The culture medium collection was carried out in duplicate. The culture medium was collected and stored in the refrigerator at –20 ℃ until analysis of lactose concentration determination using the Lactose/D-Galactose (Rapid) Assay that has been described in part I of the experiment 2.

The adherent cells sample were harvested from the plates by trypsin-EDTA and separated for 3 set. The first and second sets of the total cells were used for oxidative stress status determination. Cell lysate preparation with assay buffer according to the manufacturer's protocol. The determination of oxidative stress status was measured both MDA concentration from MECs was analyzed using the lipid peroxidation (MDA) assay and Glutathione peroxidase activity was measured by the glutathione peroxidase activity assay. The third set of the cells was used for the

determination of β-GALT1, α-LA, Akt, and HSP70 genes expression by PCR. The experimental procedure is presented in Figure 5.

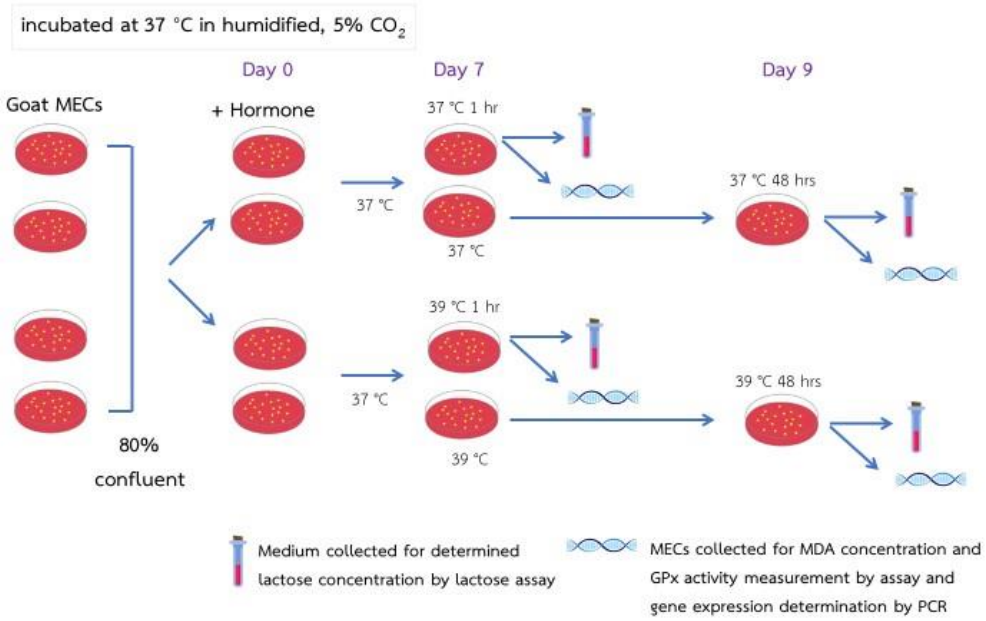


Figure 5 The schematic protocol of the second part of experiment 2.

The second part of experiment 2 is an investigation of the effect of HTa on lactose synthesis and Akt expression of MECs culture. The experimental design and samples collected from MECs 7 days old incubated at 37 ℃ compare with 39 ℃ in humidified 5% CO_2. After 1 or 48 hours of incubation, medium sample was collected for lactose concentration measurement and MECs was harvested for oxidative stress status (MDA concentration and GPx activity) measurement, and determination of β-GALT1, α-LA, Akt and HSP70 gene expression.

Determination of oxidative stress status of MECs culture

Cell lysate were collected from MECs for investigation of oxidative stress status. Oxidative stress status was carried out by detection of lipid peroxidation products, Malondialdehyde (MDA) using lipid peroxidation assay and glutathione peroxidase activity by glutathione peroxidase activity assay. Each experiment was

carried out in duplicate. MECs were determined MDA concentration using lipid peroxidation assay. The cells were added in 303 µl lysis solution (300 µl of the MDA lysis buffer with 3 µl BHT) and centrifuged (Thermo Scientific centrifuge, Legend Micro 21 and 21R, Nussloch, Germany) at 10,000 rpm at 4 ℃ for 10 minutes to remove insoluble material. The supernatant was collected in a new clean microtube and stored at –80 ℃ until analysis.

MDA concentration from MECs samples was performed by the lipid peroxidation (MDA) assay as manually instructed has been described. In brief, TBA reagent (600 µl) was added into each microtube containing 200 µl standard and 200 µl of MECs supernatant prepared the sample. The mixture was incubated at 95 ℃ for 60 minutes and cooled to room temperature in an ice bath for 10 minutes. The upper layer of the mixture (200 µl) was placed into the clear microplate and measured at 532 nm.

The determination of glutathione peroxidase activity from MECs were performed by the glutathione peroxidase activity assay. The cells were resuspended in 200 µl of ice-cold assay buffer. The cells were homogenized quickly by pipetting up and down a few times. The cell suspension was centrifuged at 8,000 rpm at 4 ℃ for 15 minutes in a cold microcentrifuge to remove any insoluble material.

The supernatant (50 µl) was added into the reaction wells of the UV microplate. Follow by the reaction mixture (40 µl; 33 µl assay buffer, 3 µl of 40 mM NADPH solution, 2 µl glutathione reductase solution, 2 µl glutathione solution) was then added. The mixture was mixed and incubated at room temperature for 15 minutes. The cumene hydroperoxide solution (10 µl) was added to each well. The reaction mixture was immediately measured output (A_1) on a microplate reader at 340 nm. After incubating at 25 ℃ for 5 minutes, measured output (A_2) at 340 nm. The calculation of GPx activity has been described in experiment 1.

Reverse Transcription PCR

Total RNA from MECs was extracted by using GeneJET RNA purification kit (Fermentas, Thermo Fisher Scientific, GmbH, Schwerte, Germany) according to the manufacturer's instructions. The quantity of extracted RNA was determined using a spectrophotometer (Nanodrop ND-2000, Wilmington, Delaware, USA). The reverse transcription was used 1 µg of total RNA and oligo (dT) primers to perform first-strand synthesis by using a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific, GmbH, Schwerte, Germany) according to the manufacturer's instructions.

PCR was performed with PCR Super Mix (Quanta BioSciences, Bevery, Massachusettes, USA). In order to reduce the error, the PCR reaction was used the same cDNA concentration of the samples in each primer set. The target-specific primer for β-GALT1, α-LA, Akt and HSP70 genes was designed base on the type of the goat (*Capra hircus*) used as a prototype for PCR experiments. Primers were designed using the Primer-BLAST program of NCBI. PCR conditions and primers of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CSN2 were used as described in the previous reports (Ogorevc and Dovč, 2015). PCR was performed using mRNA specific primers in the PCR system (ARKTIK Thermal Cycler, Thermo Fisher Scientific, Ratastie, Vantaa, Finland). The mRNA specific primers are shown in Table 2.

The amplification reaction was contained PCR super mix, primers, DNA template, and nuclease-free water in a total volume of 25 µl. The PCR amplification condition of GAPDH and β-GALT1 gene were conducted as the following order: initial denaturing at 95 ℃ for 2 minutes, 35 cycles of denaturation at 94 ℃ for 30 seconds, annealing at 58 ℃ for 30 seconds, and extension at 72 ℃ for 30 seconds.

The PCR amplification condition of α-LA, Akt, and HSP70 gene were conducted as the following order: initial denaturing at 95 ℃ for 2 minutes, 35 cycles of denaturation at 94 ℃ for 30 seconds, annealing at 55 ℃ for 30 seconds, and extension at 72 ℃ for 30 seconds. Once the PCR program had finished the samples were stored at 4℃ until analysis by agarose gel electrophoresis.

Table 2 Primer sequence were used for PCR.

Gene	Primer sequence (5'-3')	Tm/℃	Product size (bp)	References
Akt	F: CCAAGTCCTTGCTCTCGGG	60.08	166	This dissertation
	R: GACGTGACCTGAGGCTTGAA	59.97		
α-LA	F: TGGAGATTGGTTGGAGAGCC	59.38	231	This dissertation
	R: GAGTGAGGGTTCTGGTCGTC	59.76		
β-GALT1	F: AAGGTGAAGTTGGGTGGTCG	60.18	141	This dissertation
	R: CTGTTGACGCTGCAGGATTG	59.83		
CSN2	F: ACAGCCTCCCACAAAACATC	58.38	206	Ogorevc and Dovc, 2015
	R: AGGAAGGTGCAGCTTTTCAA	57.93		
HSP70	F: ATGGCGAAAAACACGGCTAT	58.54	143	This dissertation
	R: TCGGTATCGGTGAAAGCCAC	60.11		
GAPDH	F: CATGTTTGTGATGGGCGTGAACCA	64.36	230	Ogorevc and Dovc, 2015
	R: TAAGTCCCTCCACGATGCCAAAGT	63.92		

PCR products were visualized by electrophoresis on 2 % agarose gel with RedSafe; nucleic acid staining solution (10x) (RedSafe, iNtRON Biotechnology, Gyeonggi-do, South Korea)

PCR products 3 µl was loaded into the wells in the gel. The 100-bp band from the molecular weight DNA marker was used for comparison. Electrophoresis was performed for approximately 30 minutes at 100 volts until the dye front migrated sufficiently to visualize bands under UV illumination. Gel electrophoresis images were captured and measured band intensities using gel documentation system (Gel Doc XR, BioRad, California, USA). The relative semi-quantitative PCR was performed to measure gene expression of the target gene. The relative gene expression was calculated by the quantity for each sample was normalized to GAPDH (Barre et al., 2000; Valiellahi et al., 2009; Martiansyah et al., 2018).

Statistical analysis

The data were shown as a mean±standard error. The data of the environmental conditions, Tr, RR, DMI, MY, and plasma cortisol were analyzed with the repeated two-way analysis of variance (ANOVA). Significance of main effects was performed by Bonferroni *t*-test. The data for body weight, milk compositions, blood glucose, plasma MDA concentration, plasma GPx activity and the relative gene expression of the animals between seasons were compared by unpaired *t*-test. The data for lactose concentration of MECs culture with or without hormone supplementation were analyzed with the repeated two-way analysis of variance (ANOVA). The data for lactose concentration, the relative gene expression, MDA concentration, and GPx activity of MECs culture incubated at 37 ℃ and 39 ℃ groups were compared by unpaired *t*-test. The p-value was considered less than 0.05 as a significant difference.

CHAPTER IV

RESULTS

Experiment 1: The effect of seasons on lactation performance and oxidative stress status in Saanen goat

The environmental conditions and the effect of HTa on rectal temperature and respiratory rate of the goats during summer and the winter season

During summer, the average Ta, RH and THI were significantly higher than in the winter throughout the experimental period. The average Ta, RH and THI at 6:00 AM, 1:00 PM and 6:00 PM were 25.24±0.07, 32.99±0.10, and 30.13±0.08 ℃, 79.99±1.10, 54.95±0.39, and 64.60±0.47%, 75.3±0.12, 83.0±0.01, and 80.6±0.10, respectively. ($P<0.05$; Table 3).

During the experimental period, there was no significant differences between season on Tr in this study ($P>0.05$). The average Tr during summer and winter at 6:00 AM, 1:00 PM and 6:00 PM were 39.34±0.07, 39.62±0.09, 39.66±0.12 and 39.02±0.19, 39.18±0.19, 39.42±0.19 ℃, respectively. In addition, when Tr was analyzed by time, Tr at 1:00 PM and 6:00 PM was significantly higher than that at 6:00 AM ($P<0.05$, Figure 6).

During summer, the average RR was significantly higher than in the winter throughout the experimental period. The average RR during summer and winter at 6:00 AM, 1:00 PM and 6:00 PM were 57±6.0, 99±12.7, 95±9.0 and 37±4.9, 60±7.5, 70±6.6 ℃, respectively. Moreover, when RR was analyzed by time, the average RR at 1:00 PM and 6:00 PM was significantly higher than that at 6:00 AM ($P<0.05$; Figure 7).

Table 3 The environmental conditions of the experiment between season during the daytime

Parameter[1]	Daytime			P-value[1]
	06:00 AM	01:00 PM	06:00 PM	
Ta (°C) *, #				
Winter	23.56±0.17	30.21±0.33	28.38±0.22	<0.001
Summer	26.95±0.24**	35.36±0.18**	31.75±0.32**	<0.001
P-value		<0.001		
RH (%) *, #				
Winter	80.5±0.73	59.1±0.14	64.9±0.37	<0.001
Summer	79.4±1.27	51.4±0.81**	64.7±0.69	<0.001
P-value		<0.001		
THI *, #				
Winter	72.7±0.30	80.1±0.45	78.4±0.36	<0.001
Summer	78.0±0.24**	86.0±0.29**	83.1±0.35**	<0.001
P-value		<0.001		

[1] Ta; Ambient temperature, RH; Relative humidity, THI; Temperature humidity index.

*Significant season effect (P<0.05), #Significant time effect (P<0.05), **Significant between season at the same time point (P<0.05).

P-values from the measurement of differences values between the seasons.

P-values[1] from the measurement of differences values among the period of the daytime in the same group.

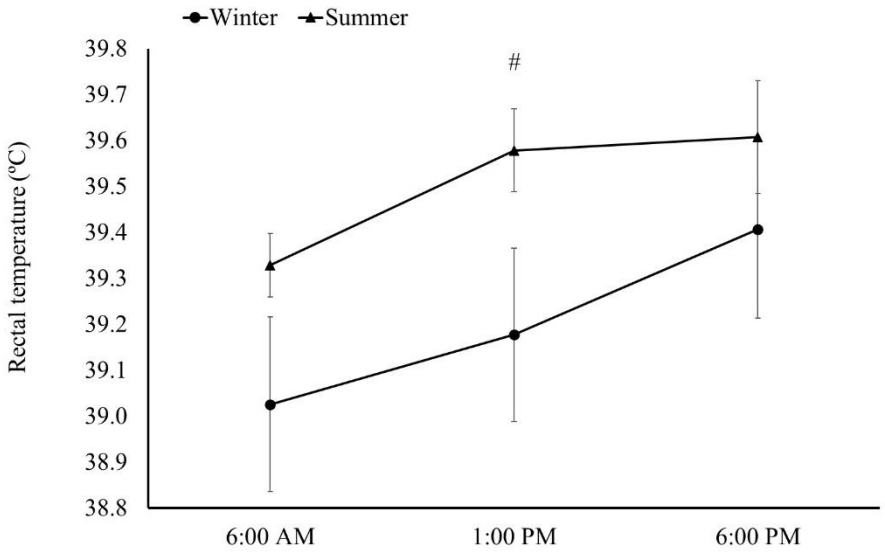


Figure 6 Effect of HTa on rectal temperature (Tr) of the goats between season.

No significant difference of Tr is noticed between winter and summer groups. The average Tr is analyzed by time during the experimental period. The average Tr at 1:00 PM and 6:00 PM show significantly higher than that at 6:00 AM in both winter and summer groups ($^{\#}P<0.05$). $^{\#}$Significant time effect.

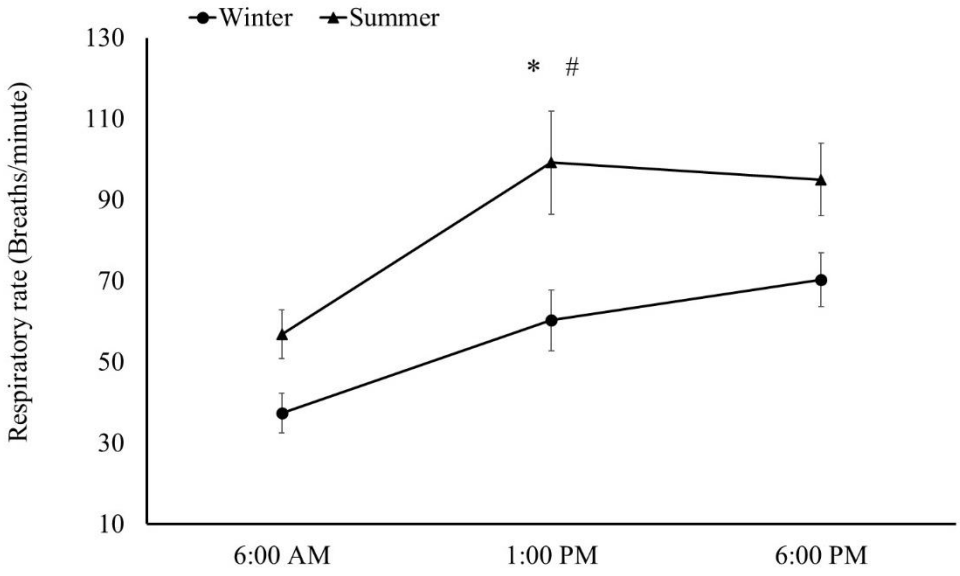


Figure 7 Effect of HTa on respiratory rate (RR) of the goats between season.

During summer RR is significantly higher than in the winter (*$P<0.05$). The average Tr is analyzed by time during the experimental period. The average RR at 1:00 PM and 6:00 PM is significantly higher than that at 6:00 AM ($^{\#}P<0.05$). *Significant season effect, $^{\#}$Significant time effect.

The effect of HTa on body weight, dry matter intake, milk yield, milk compositions, and feed efficiency for milk of the goats during summer and the winter season

There were no significant differences between the two groups with regard to the average percent change in body weight of the goats from the beginning and the end of the experiment (P>0.05) in winter (6.10±0.95 %) as compared with summer group (5.86±2.07 %).

The effect of HTa on DMI of the goats during summer and the winter season is shown in Figure 8a-c. There was HTa effect on DMI between seasons in this study. The average total DMI at 1 PP-4 PP were 33.12±0.98, 37.75±1.59, 42.01±2.28, and 38.72±1.79 g/kgBW, respectively. The average roughage DMI at 1 PP-4 PP were 9.01±0.75, 8.94±1.11, 9.40±0.73, and 9.82±1.24 g/kgBW, respectively.

During summer, the average total DMI (Figure 8a), and roughage DMI (Figure 8b) were significantly lower than in the winter (P<0.05). During the experimental period, there was no significant differences between season on concentrate DMI in this study (P>0.05). In addition, when concentrate DMI was analyzed by week, concentrate DMI at 2 PP (28.81±0.87 g/BW), 3 PP (31.56±1.31 g/BW), and 4 PP (28.90±2.89 g/BW), were significantly higher than that at 1 PP (23.69±0.47 g/BW) (P<0.05; Figure 8c).

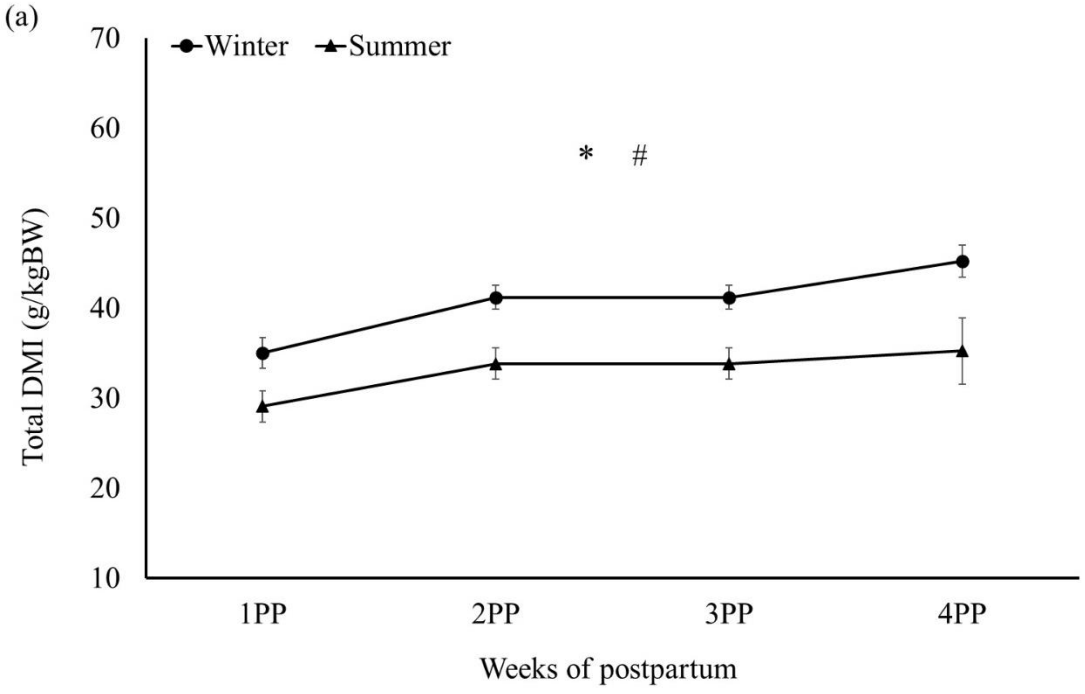
The effect of HTa on MY of milk of the goats during summer and the winter season is shown in Figure 9. After parturition, the average MY in 1 PP-4 PP from the goats were 1.58±0.08, 1.98±0.14, 2.26±0.16, and 2.21±0.11, respectively. During summer, the average MY was significantly lower than the goats from winter season (P<0.05). The effect of HTa on milk compositions of the goats during summer and the winter season is shown in Table 4. During the experimental period, there were no differences between the two groups when it came to the compositions of milk (P>0.05).

The average feed efficiency for milk in 1 PP-4 PP from the goats were 1.35±0.10, 1.39±0.08, 1.44±0.08 and 1.56±0.17 kg of milk/kg of total DMI, respectively. During the experimental period, there was no significant differences between season and week on feed efficiency for milk in this study (P>0.05, Figure 10).

Table 4 The effect of HTa on milk compositions during winter and summer

Item	Milk compositions		P-value
	Winter	Summer	
Fat (g%)	3.66±0.25	3.72±0.21	0.778
Protein (g%)	2.89±0.12	3.12±0.14	0.247
Lactose (g%)	4.57±0.05	4.68±0.03	0.771
TS (g%)	11.96±0.41	12.46±0.26	0.283
SNF (g%)	8.39±0.16	8.59±0.22	0.314

P-values from the measurement of differences values between the seasons.



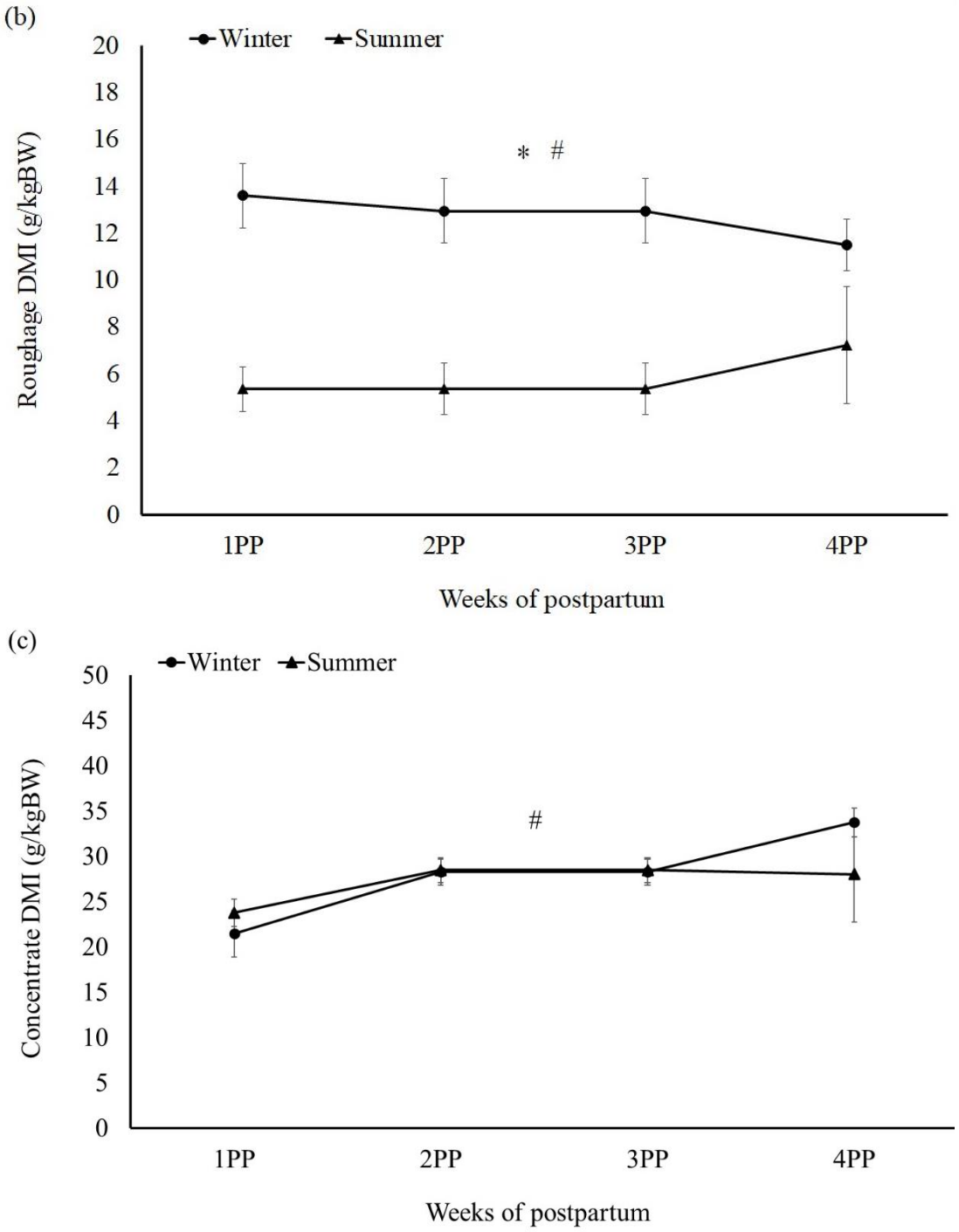


Figure 8 Effect of HTa on total, roughage, and concentrate dry matter intake (DMI) per body weight of the goats between season.

Total DMI (a) and roughage DMI (b) of the goats during winter are significantly higher than that in summer (*P<0.05). Concentrate DMI (c) of both seasons are analyzed by week, which at 2 PP-4 PP is significantly higher than at 1 PP ($^{\#}$P<0.05). *Significant season effect, $^{\#}$Significant time effect.

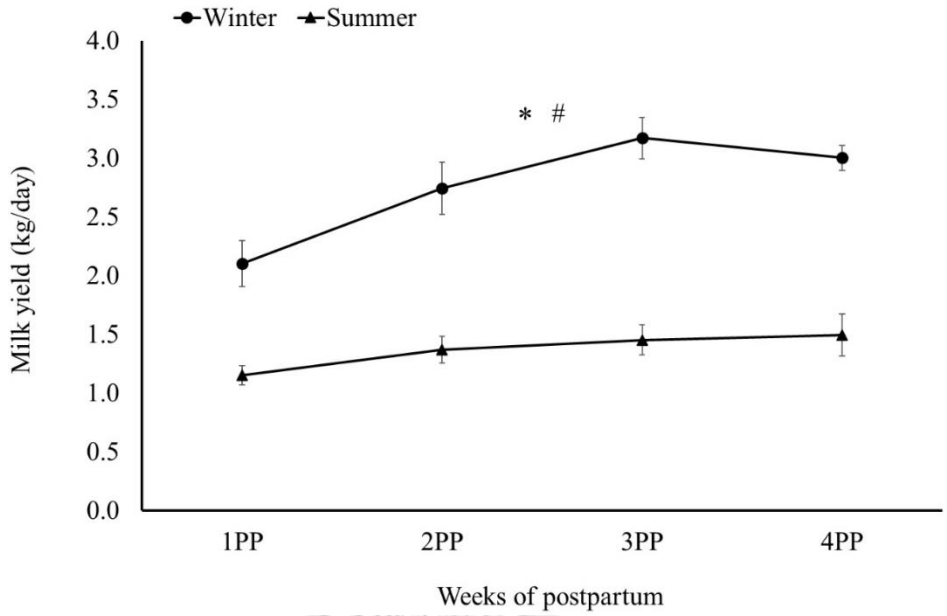


Figure 9 Effect of HTa on milk yield of the goats between season.

The average MY of the goats during winter is significantly higher than that in summer (*P<0.05). MY of both seasons are analyzed by week, which at 2 PP-4 PP is significantly higher than at 1 PP ($^{\#}$P<0.05). *Significant season effect, $^{\#}$Significant time effect.

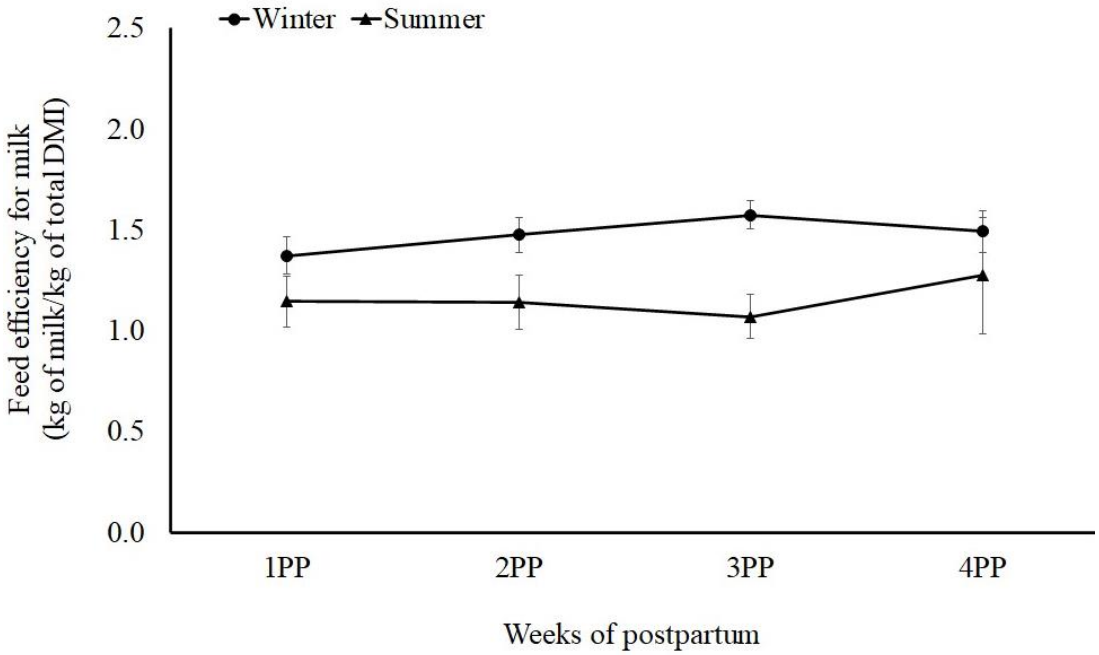


Figure 10 Effect of HTa on feed efficiency for milk of the goats between season.

There is no effect of HTa on the average feed efficiency for milk of the goats between season and weeks postpartum during the experimental period (P>0.05).

The effect of HTa on plasma cortisol, blood glucose, plasma MDA concentration, and plasma GPx activity of the goats during summer and the winter season

Plasma cortisol of the goats during summer at 7:00 AM and 1:00 PM were 56.6±3.33 and 85.1±12.40 ng/ml. During winter plasma cortisol of the goats were 53.0±16.44 and 83.3±15.30 ng/ml, respectively. There were no significant differences between season on plasma cortisol in this study. In addition, the effect of HTa on plasma cortisol was analyzed by time, plasma cortisol at 1:00 PM (84±10.49 ng/ml) was significantly higher than that at 7:00 AM (54.80±9.61 ng/ml) ($P<0.05$, Figure 11).

The effect of HTa on blood glucose, oxidative stress status both plasma MDA concentration and plasma GPx activity of the goats during summer and winter season of the experimental period are shown in Table 5.

There was no difference between the goats in winter and summer group at 1:00 PM on blood glucose. However, during summer, the average blood glucose level of the goats at 07:00 AM (79±2.9 mg/dL) was significantly higher than that in winter group (69±10.8 mg/dL) ($P<0.05$).

During the experimental period, the average plasma MDA concentration of the goats was analyzed by the average of both 2 PP and 3 PP. There was no difference on plasma MDA concentration at 07:00 AM and 1:00 PM (Figure 12) between the goats in winter (1.12±0.25 and 1.07±0.23 nmol/ml) and summer groups (1.44±0.12 and 1.48±0.07 nmol/ml) ($P>0.05$, Table 5). Plasma GPx activity of the goats was analyzed by the average of 2 PP and 3 PP. There was no difference on plasma GPx activity at 07:00 AM between the goats in winter (450.2±89.0 nmol/ml) and summer groups (527.9±81.7 nmol/ml) ($P>0.05$). However, plasma GPx activity during summer at 1:00 PM (541.18±34.38 nmol/ml) was significantly higher than that in winter (405.00±27.43) ($P<0.05$, Table 5 and Figure 13).

Table 5 Effect of HTa on blood glucose, plasma MDA concentration, and plasma GPx activity of the goats between season during the daytime

Parameters[1]	7:00 AM	1:00 PM
Blood glucose (mg/dL)		
Winter	69±10.8	76±2.7
Summer	79±2.9	79±2.9
P-value	0.033*	0.412
MDA concentration (nmol/ml)		
Winter	1.12±0.25	1.07±0.23
Summer	1.44±0.12	1.48±0.07
P-value	0.181	0.159
GPx activity (nmol/ml)		
Winter	450.2±89.0	405.0±27.4
Summer	527.9±81.7	541.2±34.4
P-value	0.447	0.013*

[1] MDA; Malondialdehyde, GPx; Glutathione peroxidase

*Significant differences between season (P<0.05).

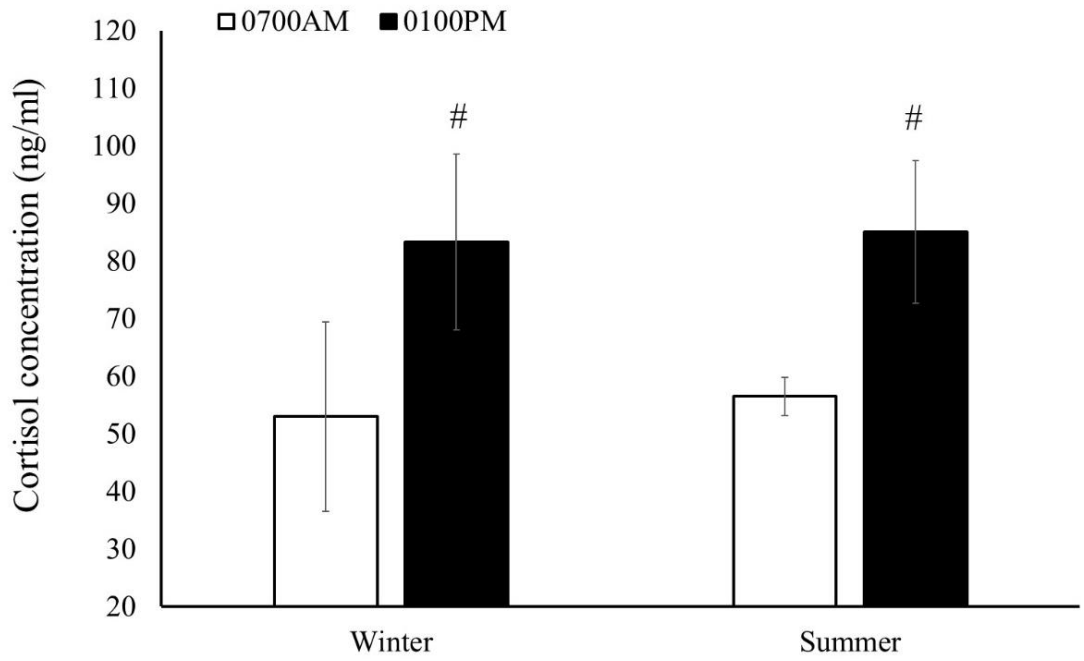


Figure 11 Effect of HTa on plasma cortisol of the goats between season.

Plasma cortisol is analyzed by time, at 1:00 PM, plasma cortisol is significantly higher than that at 7:00 AM during the experimental period ($^{\#}$P<0.05). $^{\#}$Significant time effect.

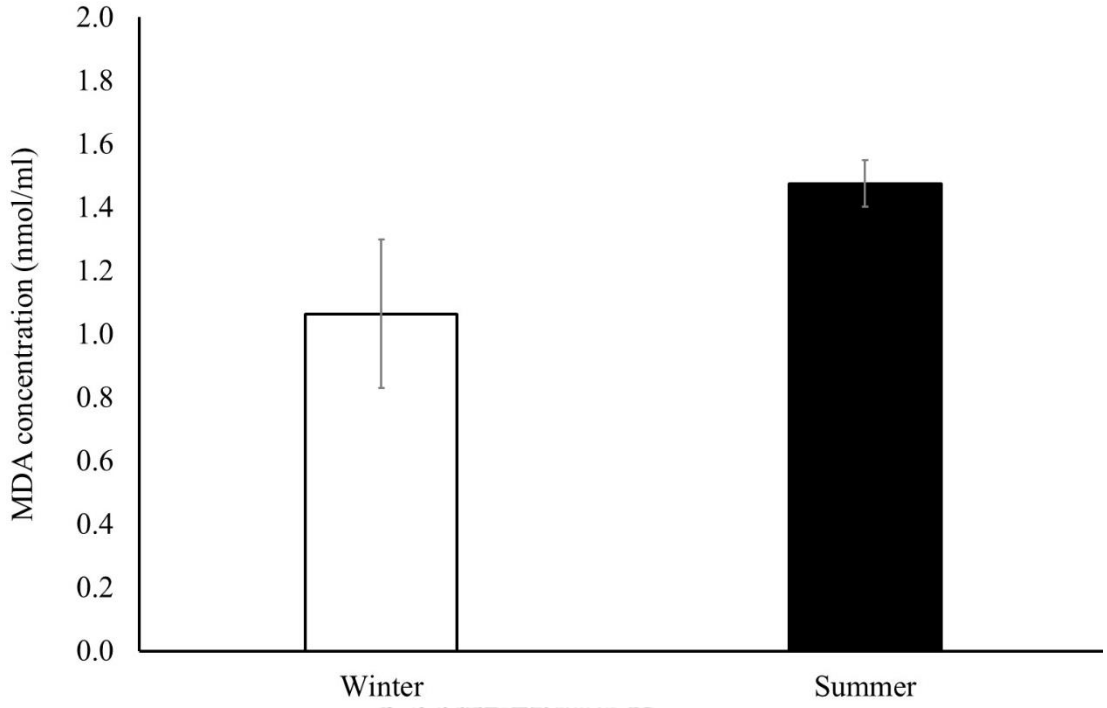


Figure 12 Effect of HTa on plasma MDA concentration of the goats between season.

Plasma MDA concentration at 1:00 PM during summer, is no differences between season (P>0.05).

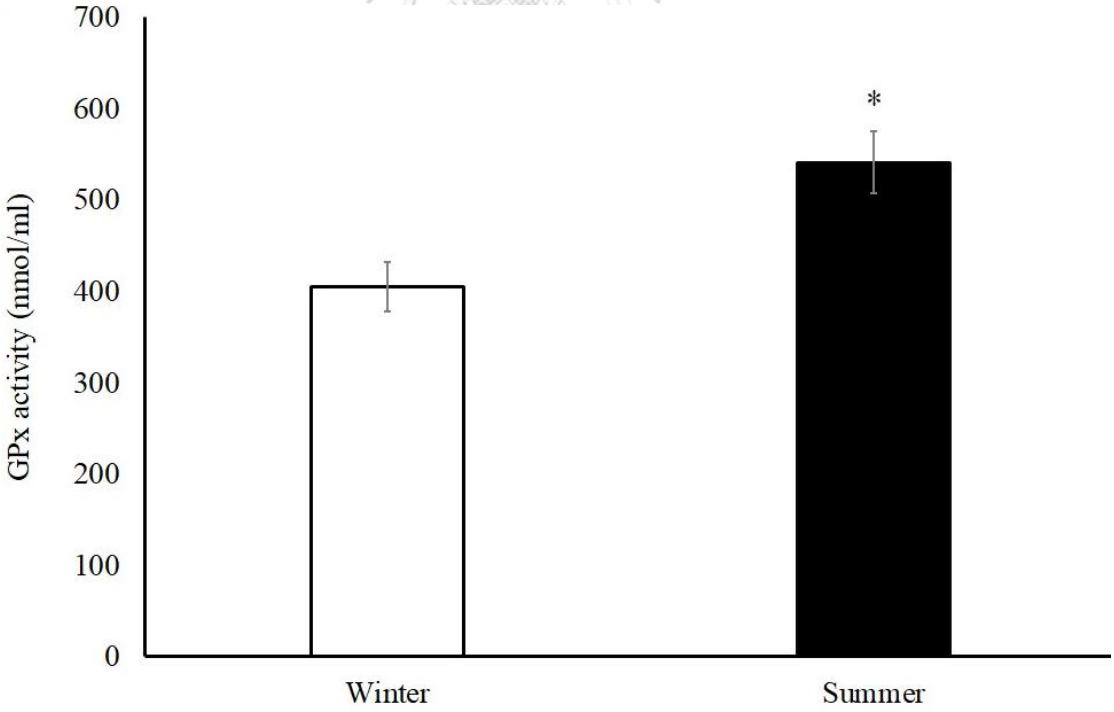


Figure 13 Effect of HTa on plasma GPx activity of the goats between season.

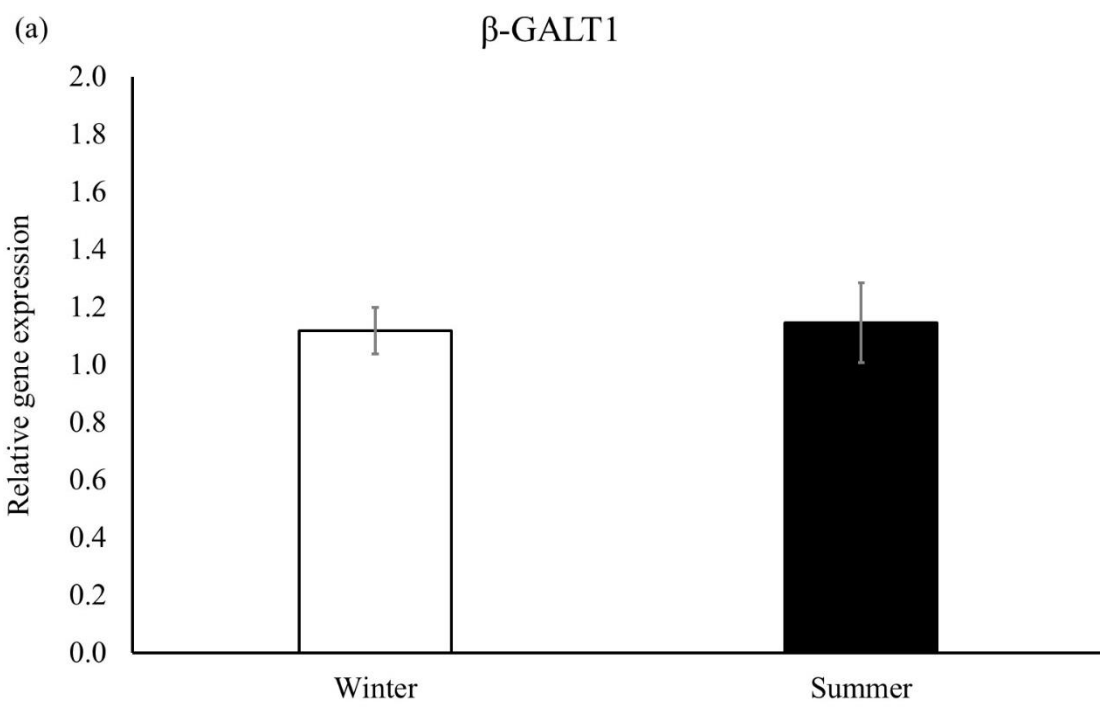
Plasma GPx activity at 1:00 PM during summer, is significantly higher than that the winter season (*P<0.05). *Significant differences between season.

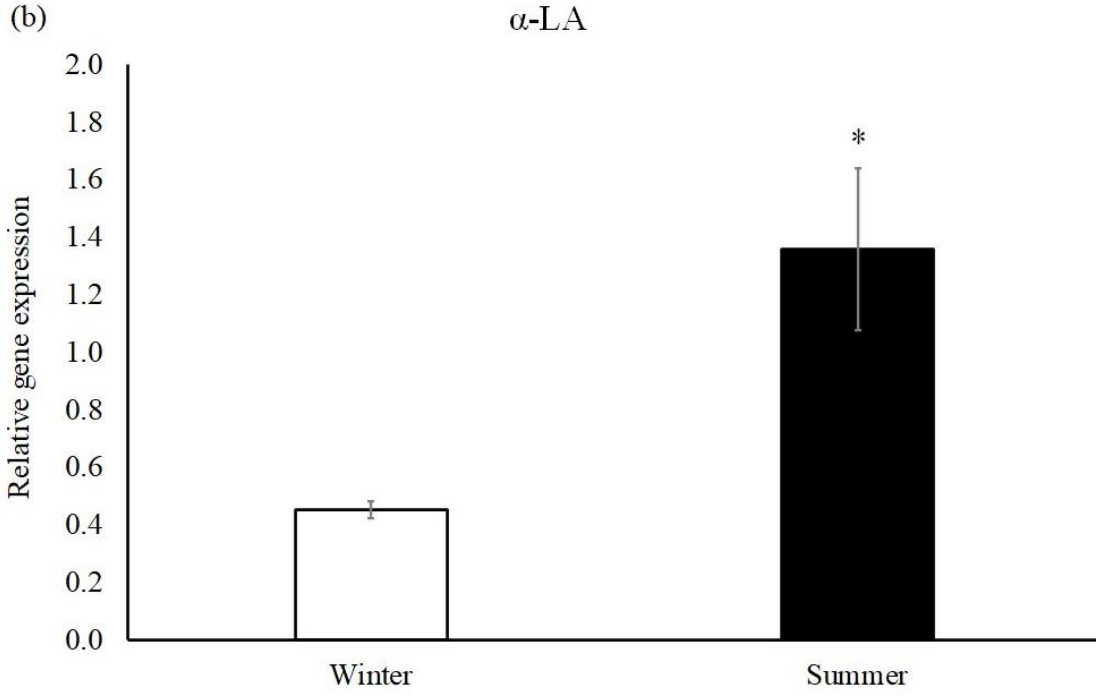
The effect of HTa on relative gene expression of the goats during summer and the winter season

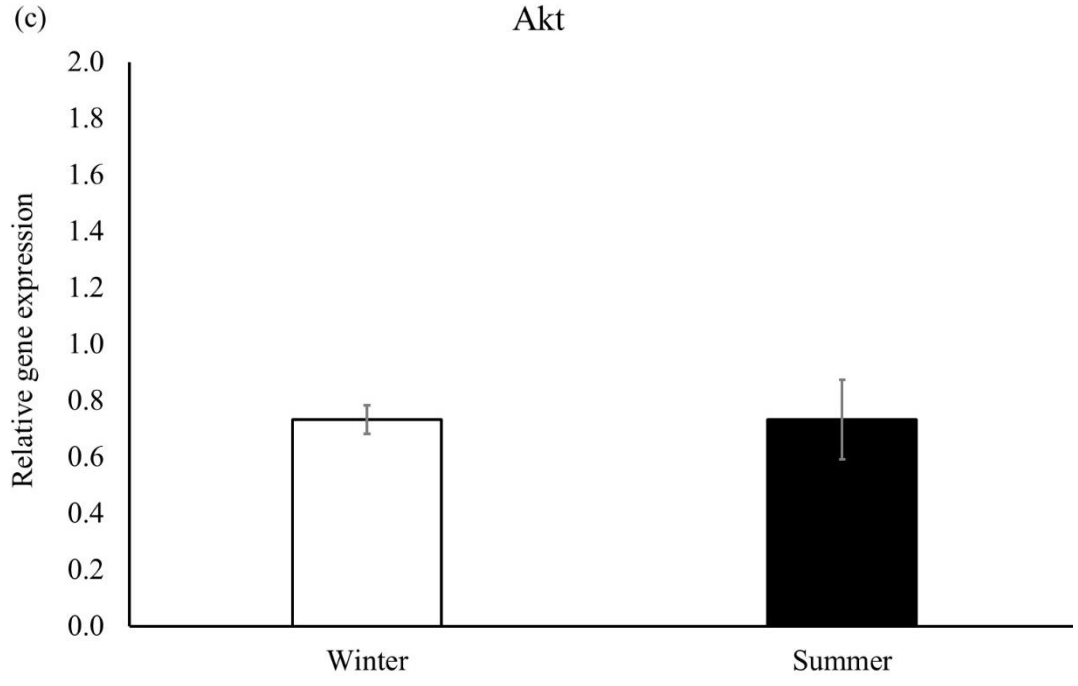
The relative gene expression of the goat milk cells isolated from milk of the goats between season from the experiments are shown in Figure 14a-d. There were no significant differences in the relative expression of β-GALT1 (Figure 14a) and Akt genes (Figure 14c) between both groups (P>0.05).

During summer, α-LA gene was up-regulated under HTa conditions during summer as compared with the winter season. The relative expression of α-LA gene in summer (1.36±0.28) was significantly higher than that winter season (0.45±0.03) (P<0.05, Figure 14b).

Similar to α-LA gene, the relative expression of HSP70 gene was significantly higher than that in summer (0.95±0.12) when compared with winter season (0.51±0.07) (P<0.05, Figure 14d).




(b)
α-LA
Relative gene expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Winter
Summer


(c)
Akt
Relative gene expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Winter
Summer

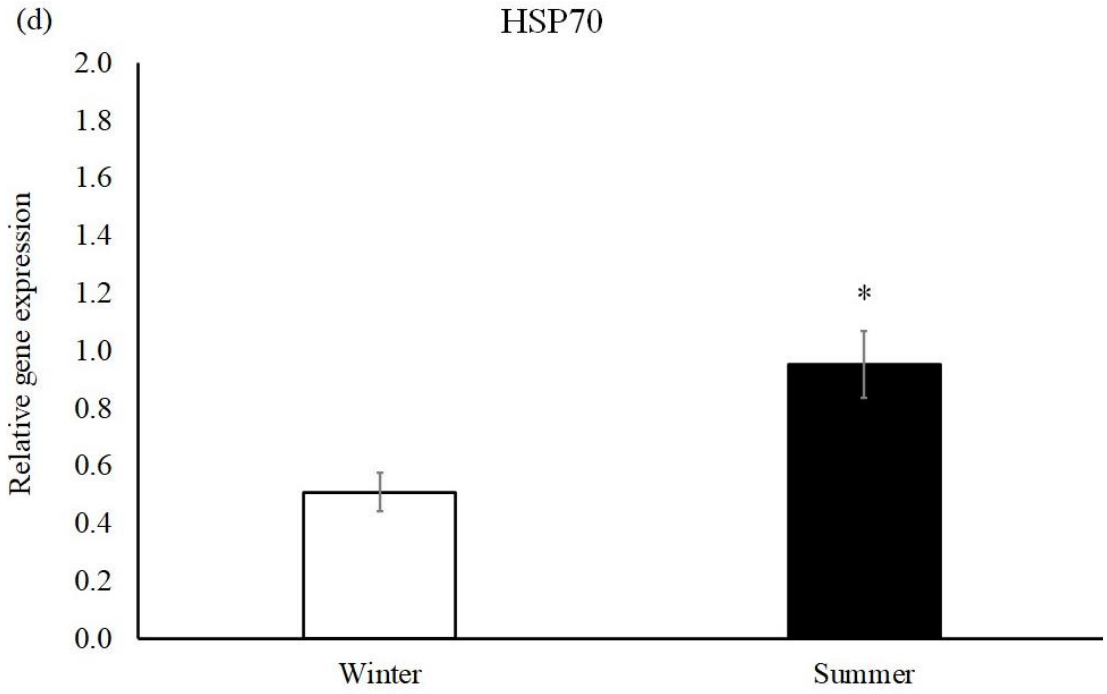


Figure 14 Effect of HTa on relative gene expression of the goat milk cells isolated from milk. The relative gene expression of β-GALT1 (a), α-LA (b), Akt (c), and HSP70 (d) of of the experiment. There were no significant differences in the relative expression of β-GALT1 and Akt genes between both groups (P>0.05). α-LA gene and HSP70 gene is up-regulated under HTa conditions during summer higher than that as compared with winter season (*P<0.05). *Significant differences between season.

Experiment 2: The effect of in vitro HTa on lactation synthesis and oxidative stress status of MECs

I: Characterization and determination of mammary function of MECs culture

Isolation and culture of mammary epithelial cells from goat milk

MECs in the present experiment are shown in Figure 15a-b. MECs culture revealed the cobblestone morphology and it was contained prominent nuclei. Moreover, the cells were displayed in monolayer and formed alveoli-like structures after being plated for 6 days. The characterization of MECs was examined by CK 18

and DAPI. MECs was positive for CK 18 protein. The immunostaining results are shown in Figure 16a-c.

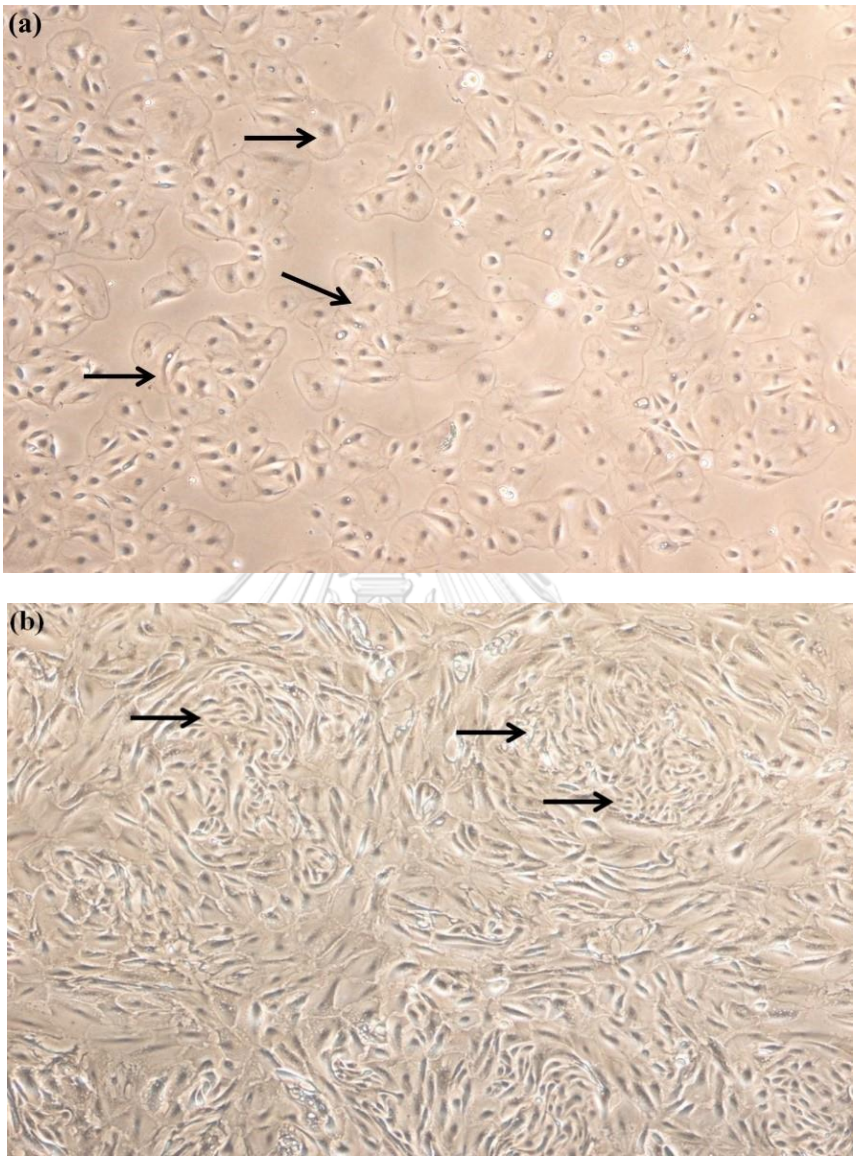


Figure 15 Characterization of MECs isolated from goat milk by morphological appearances. The primary mammary epithelial cells culture was characterized by morphological appearances. Three days old of the isolated cells grow as monolayer colonies, contained prominent nuclei (a, arrow), after 12 days plated the cells reached 80% confluency and formed alveoli like structure (b, arrow). There were no differences in cells isolated from milk of all milk samples in this study. (Scale bars 100 μm).

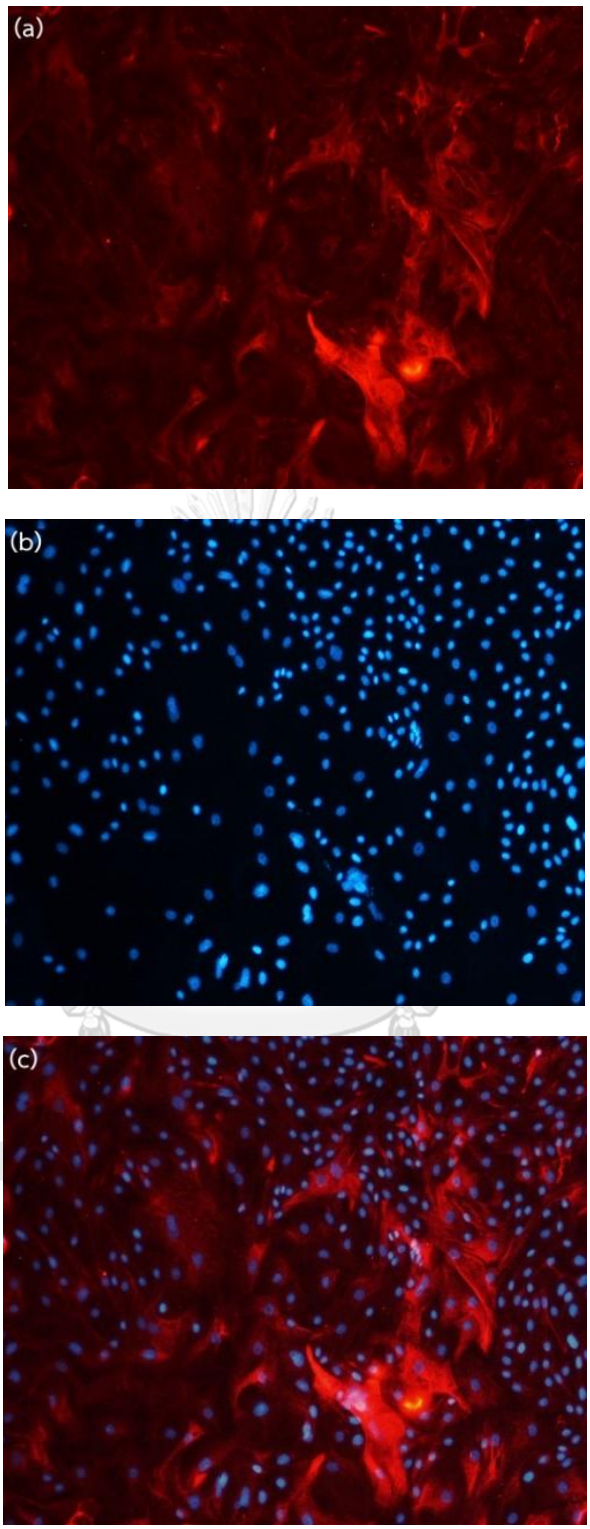


Figure 16 Characterization of MECs culture by immunocytochemistry.

The characteristics of MECs isolated from goat milk were acquired using the fluorescence phase-contrast microscope. The cells are positive for CK 18 (red) (a). Nuclei are counterstained with 4′,6-diamidino-2-phenylindole; DAPI (blue) (b) and merge (c). (Scale bars 100 μm).

The effect of lactogenic hormone supplementation on relative gene expression and lactose concentration of MECs culture

After day 12, the relative CSN2 gene expression of MECs culture supplemented with lactogenic hormone (0.731 ± 0.053) was significantly higher than in comparison with the group without hormone supplementation (0.603 ± 0.033) ($P<0.05$, Figure 17). However, the relative β-GALT1 gene expression showed no difference between MECs supplementation with lactogenic hormone (0.792±0.035) and no supplementation (0.748±0.040) group. Similarly, there was no significant differences between MECs culture supplemented with and without lactogenic hormone (0.984±0.054 and 0.943±0.034) groups on the relative expression of α-LA gene ($P>0.05$).

During the experiment, lactose concentration at day 6, 9, and 12 from MECs cultured with lactogenic hormone supplementation group (2.48±0.37, 4.30±0.35 and 2.58±0.57 µg/ml) were significantly higher than that no hormone supplementation group (1.23±0.57, 2.58±0.57 and 1.35±0.45 µg/ml) ($P<0.05$; Figure 18).

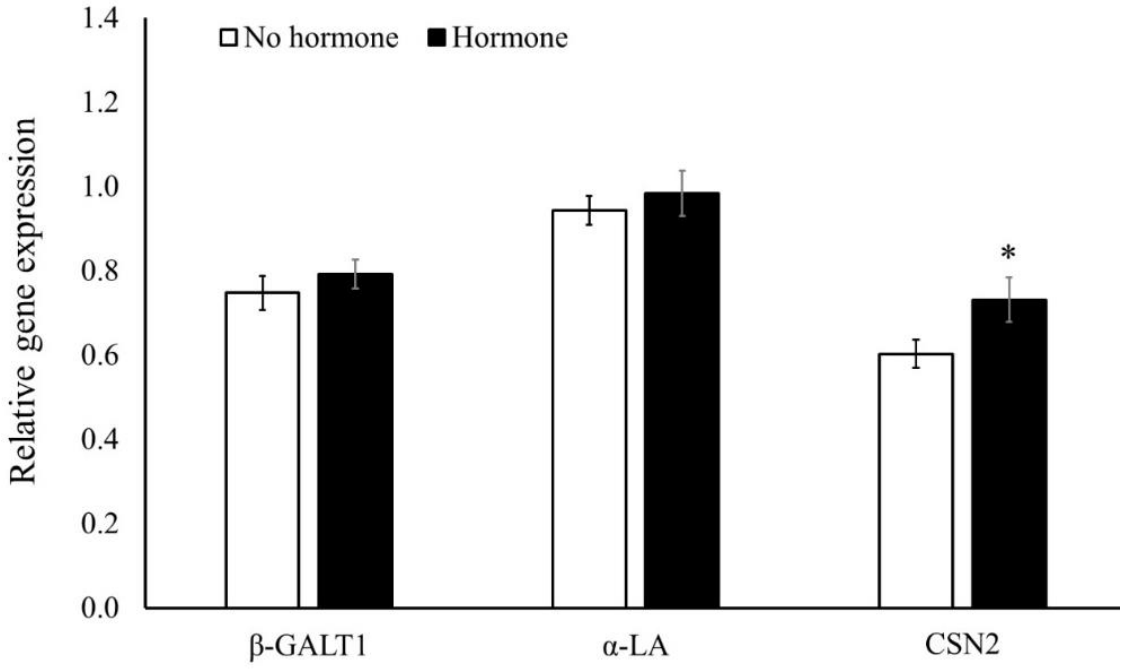


Figure 17 Effect of lactogenic hormone on relative gene expression of MECs culture.
MECs were allowed to differentiate for 12 days in the differentiation medium with or without lactogenic hormone supplementation. Total RNA of MECs were collected and subjected for gene expression analyses. The PCR products were visualized by electrophoresis images, measured band intensities, and normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The relative expression of CSN2 but not β-GALT1 and α-LA genes of MECs cultured in the differentiation medium supplemented with lactogenic hormone, is significantly higher than MECs cultured in the differentiation medium without supplementation of lactogenic hormone. Each procedure is carried out in duplicate. Data are shown as mean±SE. (*P<0.05, β-GALT1; β-1,4-galactosyltransferase, α-LA; α-lactalbumin, CSN2; β-casein)

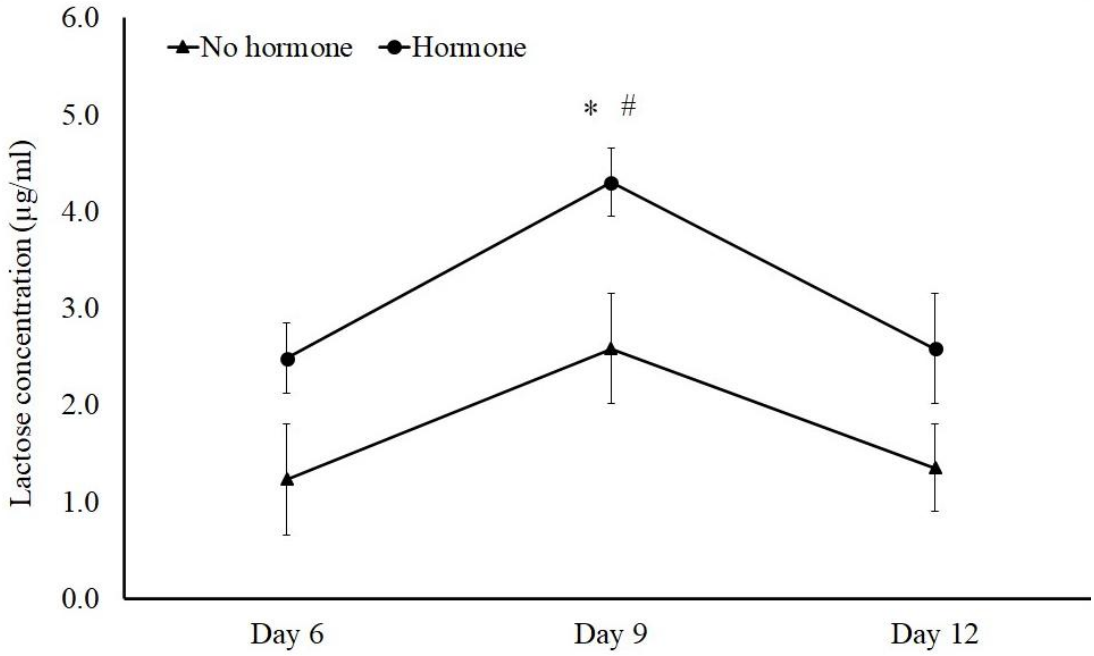


Figure 18 Lactose synthesis of MECs culture after differentiation with lactogenic hormone. MECs were allowed to differentiate for 12 days in the differentiation medium with or without lactogenic hormone supplementation. Lactose concentration of MECs culture in the differentiation medium with lactogenic hormone, is significantly higher than MECs culture medium without lactogenic hormone (*P<0.05). At day 9, lactose concentration from MECs culture supplemented with lactogenic hormone was significantly higher than that day 6 and day 12 ($^{\#}$P<0.05). Each group is carried out in duplicate. Data are shown as mean±SE. *Significant lactogenic hormone effect, $^{\#}$Significant time effect.

II: Investigation of the effect of HTa on lactose synthesis and Akt expression of MECs culture

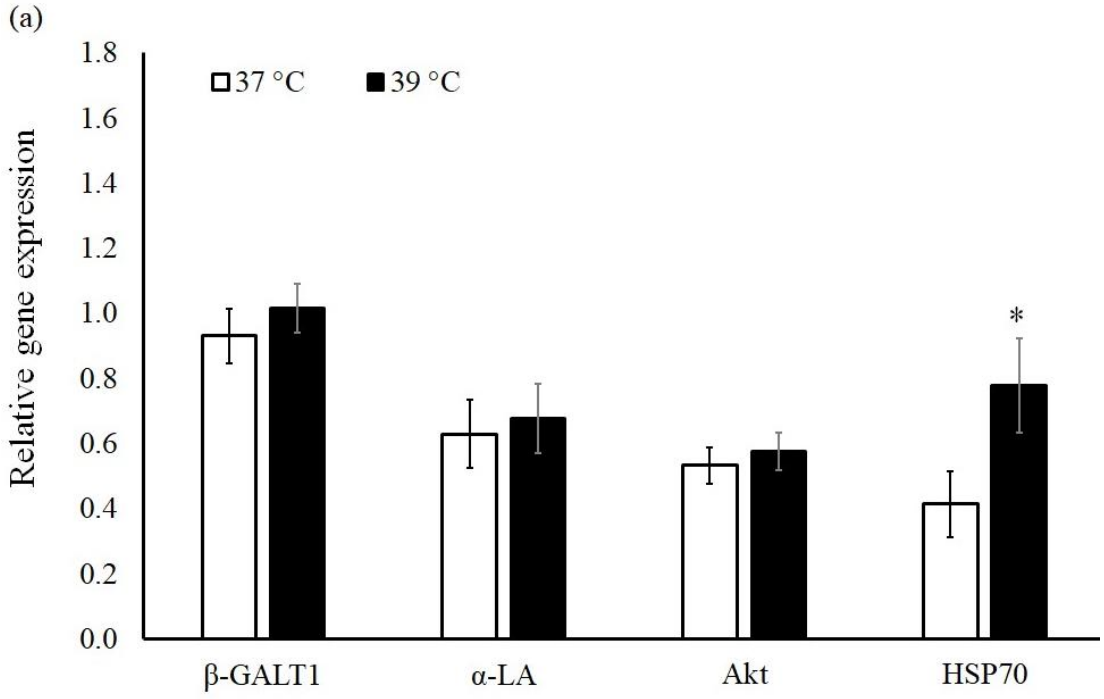
The effect of HTa on relative gene expression and lactose concentration of MECs culture

During the experimental period, the relative HSP70 gene expression from MECs was significantly higher than that in comparison with the 37 ℃ group within 1 hour (0.41±0.20) after being incubated at 39 ℃ for 1 hour (0.78±0.33) (P<0.05, Figure

19a). However, there was no differences in the relative expression of HSP70 gene after continuously incubated at 39 ℃ for 48 hours (0.53±0.14) when compared with MECs incubated at 37 ℃ (0.69±0.12). In addition, the relative expression of β-GALT1, α-LA, and Akt genes of MECs culture incubated at 37 or 39 ℃ for 1 or 48 hours showed no significant difference in this study (P>0.05, Figure 19a-b).

Lactose concentration from MECs culture incubated at 37 or 39 ℃ for 1 or 48 hours are shown in Figure 20. Lactose concentration of MECs culture incubated at 37 ℃ for 1 and 48 hours was 2.29±0.66 and 3.03±0.88 µg/ml, whereas at 39 ℃ for 1 and 48 hours, lactose concentration was 2.67±0.77 and 2.02±0.58 µg/ml, respectively.

However, there was no difference in lactose concentration of MECs cultures incubated at 37 and 39 ℃ groups in the present study (P>0.05).



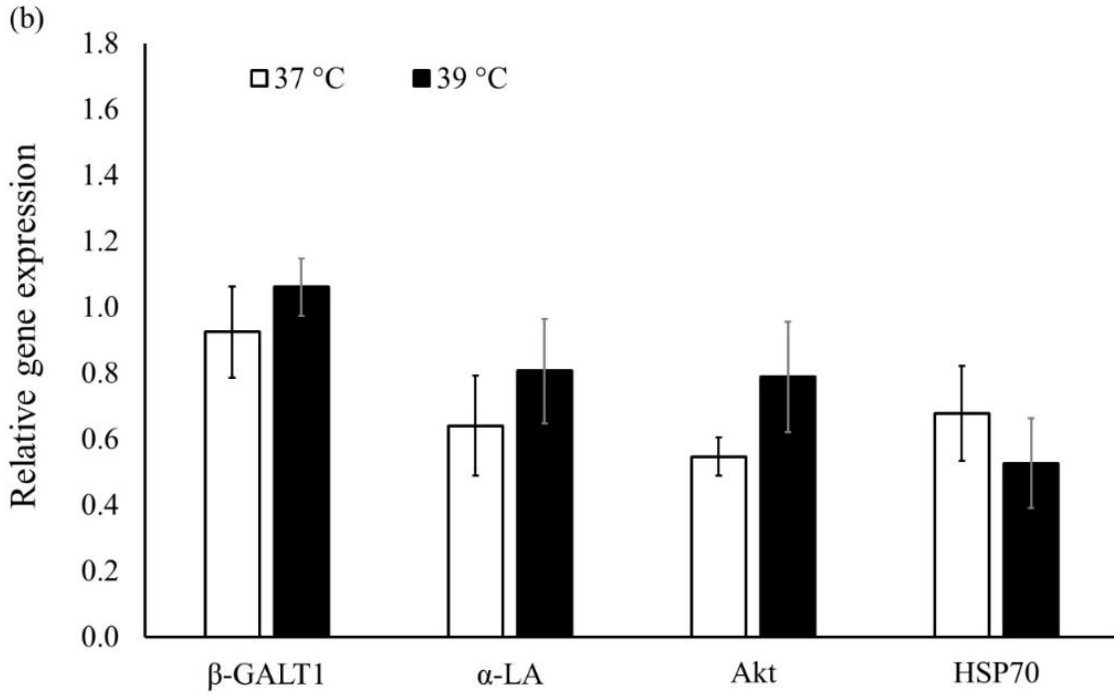


Figure 19 Effect of HTa incubation on relative gene expression of MECs isolated from goat milk. The incubation temperature is raised from 37 ℃ up to 39 ℃. Cells are allowed to expose to an increasing of temperature (39 ℃) for 1 hour or 48 hours. The expression of HSP70 gene is significantly upregulated (*P<0.05), however, there are no significantly different of expression of β-GALT1, α-LA and Akt genes between MECs cultured in 37 ℃ and 39 ℃ for 1 hour (a). After 48 hours incubation, the relative expression of all β-GALT1, α-LA, Akt, and HSP70 genes of MECs are incubated at both 37 ℃ and 39 ℃ show no significantly different (b) (P>0.05). Each group is carried out in duplicate. Data are shown as mean±SE.

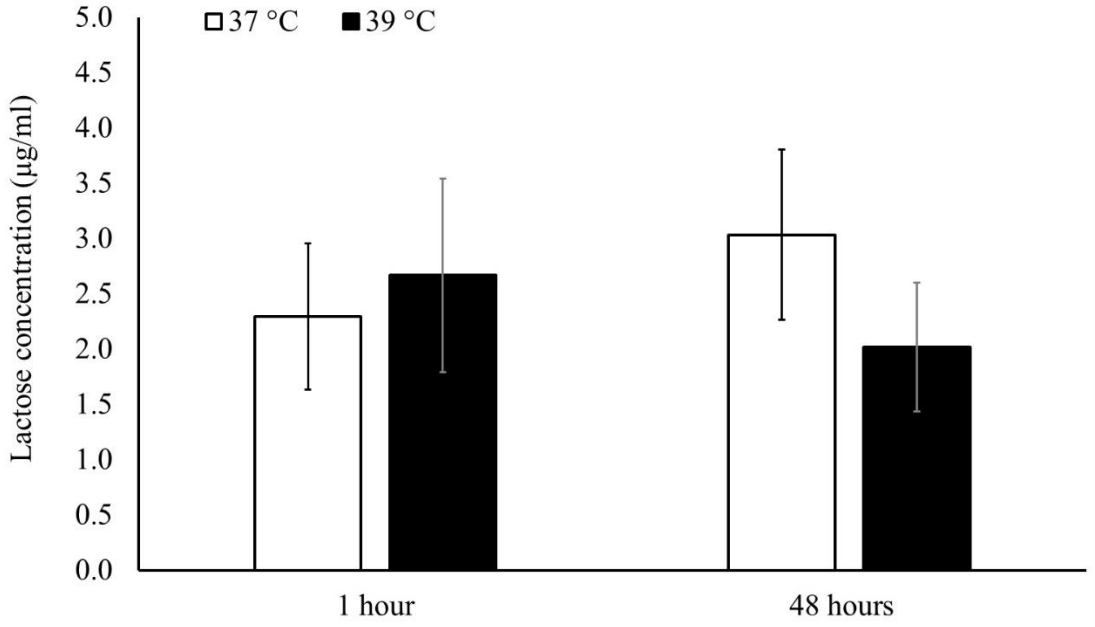


Figure 20 Lactose synthesis of MECs after 37 or 39 ℃ incubation for 1 or 48 hours.
Lactose concentration of MECs culture incubated at 39 ℃ for 1 or 48 hours, is not significantly differences between incubated at 37 ℃ group (P>0.05). Each group is carried out in duplicate. Data are shown as mean±SE.

The effect of HTa on MDA concentration and GPx activity of MECs culture

MDA concentration and GPx activity of MECs culture incubated at 37 and 39 ℃ are shown in Figure 21 and Figure 22. The average MDA concentration of MECs culture incubated at 37 ℃ for 1 and 48 hours were 12.45±5.08 and 11.44±4.67 nmol/ml, whereas at 39 ℃ for 1 and 48 hours, MDA concentration were 12.61±5.15 and 13.01±5.31 nmol/ml, respectively. During the experiment, there was no difference in MDA concentration of MECs cultures incubated at 37 or 39 ℃ groups (P>0.05, Figure 21). Similarly, the average GPx activity of MECs culture incubated at 37 for 1 and 48 hours was 196.70±98.35 and 281.33±114.85 nmol/ml and GPx activity at 39 ℃ was 318.05±129.84 and 341.80±139.54 nmol/ml, respectively. There was no difference between 37 or 39 ℃ incubation for 1 or 48 hours on GPx activity (P>0.05, Figure 22).

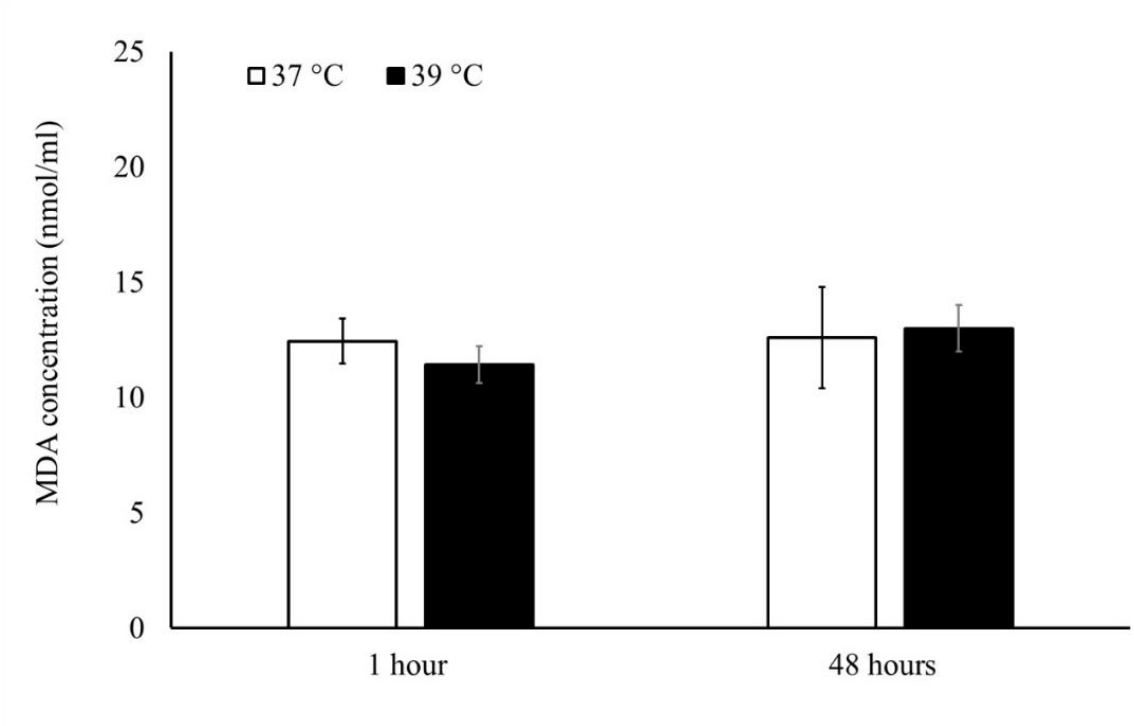


Figure 21 Effect of HTa incubation on MDA concentration of MECs culture.

MDA concentration is measured of MECs culture after an exposure to 39 ℃ for 1 hour or 48 hours. No significant difference of the average MDA concentration of MECs cultured at 37 ℃ versus at 39 ℃ is observed, ($P>0.05$). Each of procedures is conducted in duplicate. Data are shown as mean±SE.

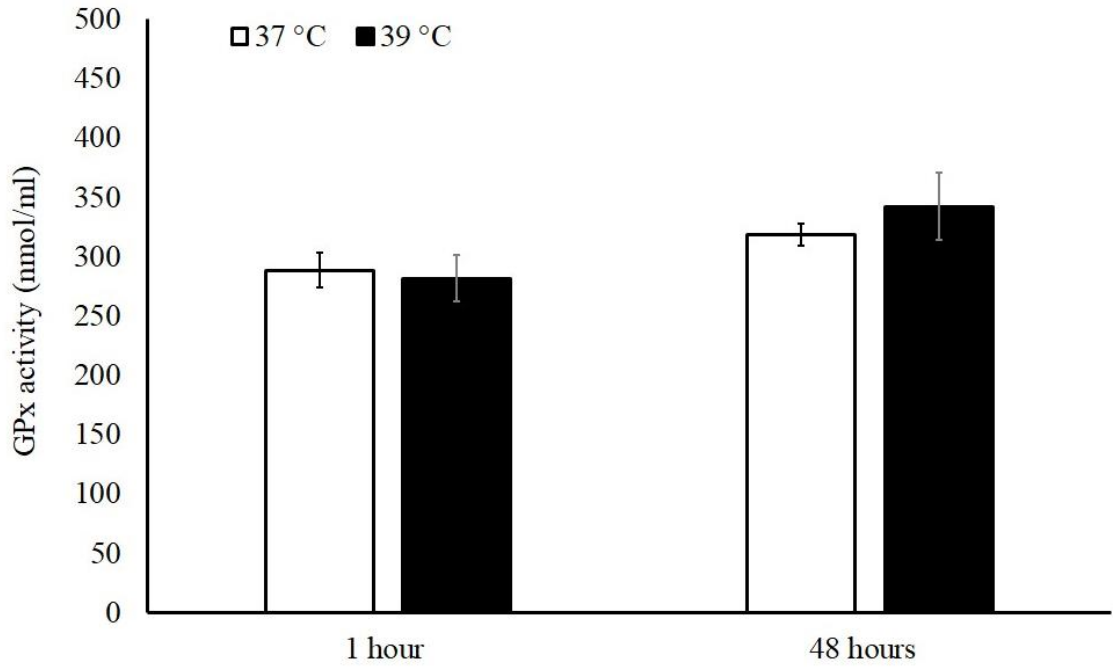


Figure 22 Effect of HTa incubation on GPx activity of MECs culture.

GPx activity is measured from MECs after an exposure to 39 ℃ for 1 hour or 48 hours. No significant difference of the average GPx activity in MECs cultured at 37 ℃ versus at 39 ℃ is observed, (P>0.05). Each of procedures is conducted in duplicate. Data are shown as mean±SE.

CHAPTER V
DISCUSSION

Experiment 1: The effect of seasons on lactation performance and oxidative stress status in Saanen goat

The present study focused on the environmental conditions and the effect of HTa on the physiological responses of the goats such as rectal temperature and respiration rate. Normally, in tropical countries, HTa condition is a major factor leading to an abnormal physiological response. The high value of THI is an important factor causes stress in lactating animals. According to the previous studies (Chaiyabutr et al., 2008; Habeeb et al., 2018; Nguyen et al., 2018), THI was used as an indicator of thermal conditions. Goat and sheep can be fed without heat stress at THI less than 82. The value of THI between 82 and 84 could induce moderate heat stress. The value of THI between 84 and 86 is the range of severe heat stress. The value of THI greater than 86 is extremes severe heat stress (Silanikove, 2000). THI levels used for goats are higher than that of dairy cows, this supposes due to the small animal high tolerance to heat stress more than cows. The present results show that THI and Ta increased during summer when compared with the winter season throughout the experimental period. This study shows that the goats in the present study were exposed to the natural HTa condition especially during summer months. Tropical countries are in a tropical zone which prolongs exposure to the sun. This condition is different from the temperate zone that the ambient temperature is lower and shorter exposure to the sun than that tropical zone. This is an important reason for the differences in conditions that affect the natural conditions and cause a detrimental effect on the management of farm animals.

The goats in the study showed an increase in heat load during exposure to HTa condition in the summer months which increased in Tr during the daytime was observed. The effect of HTa can be evaluated from physiological responses of the goats such as increasing Tr and RR (Chaiyabutr et al., 2008; Nguyen et al., 2018). The present results indicated that the goats adapted to these uncomforted conditions by changes the physiological responses such as increased RR and panting, which are heat loss process by the evaporative mechanism for the maintenance of body temperature (Chaiyabutr et al., 1990; Alam et al., 2013). Furthermore, the negative effect of HTa exposure during summer months in the present study were decreasing DMI and MY. These results confirm that the indirect effect of HTa decreased in DMI and thereby decreased MY. It is well known that HTa decreases feed intake of the dairy animals. Decreasing feed intake during HTa is one of the thermoregulation mechanisms of the animal by preventing an increased heat load. In ruminants, it is known that the amounts of metabolizable energy from a diet based on roughage raise heat production greater than from based on concentrates diet (Orskov and MacLeod, 1990; Sudarman and Ito, 2000). Interestingly, lower DMI from summer was mainly due to the lower roughage intake in the present study. This suggested that lower roughage intake of the goats supposed to be another physiological response for reducing heat increment. Whereas, the concentrate DMI unchanged is an effort to increase nutrient intake to meet their requirement.

In the present study, decreasing blood glucose levels of the goat after morning milking in the winter group was similar to result in a previous study of the dairy cows (Allcroft, 1933). It was suggested that the level of blood glucose was high before milking and fell during milking. The supposed reason was that, after morning milking, blood glucose was consumed by the mammary glands and used for milk synthesis.

Plasma cortisol of the goats during the afternoon was higher than in the morning in both seasons in the present study. This result was similar to the previous study in the dairy cows which showed the plasma cortisol concentration of non-cooled cow tended to be higher than that of the cows housed in a close-sided barn under an evaporative cooling system (Chaiyabutr et al., 2008). During stress, the hypothalamic-pituitary adrenal axis (HPA) is stimulated and synthesis of adrenocorticotrophic hormone (ACTH) by the anterior pituitary (Kannan et al., 2000; Saidu et al., 2016; Isaac et al., 2017). Therefore, increasing plasma cortisol concentration during the afternoon from both seasons in the present study supposedly related to the stress response of HTa effect of the goats.

In the present study, the effect of HTa on plasma GPx activity in the afternoon during summer showed higher than that in the winter group, whereas, plasma MDA concentration was unchanged. This indicates that HTa during summer the goats were maintained their adaptive responses by increased in defense mechanisms against ROS damage capacity (Bernabucci et al., 2002). Heat stress might be defined by HTa condition together with the physiological responses of the animals at least 3 levels. Firstly, the success of maintaining body temperature of the animal and compromised with HTa condition is absent heat stress (Dikmen and Hansen, 2009). The secondly and thirdly levels are the mild degree or reversible heat stress and severe degree or irreversible heat stroke, respectively. These two levels of heat stress risen together with increasing plasma cortisol (Isaac et al., 2017). Since RR, plasma GPx activity, and plasma cortisol from both winter and summer were increased significantly during the afternoon, this suggested that the goats from both winter and summer groups were mild degree of heat stress.

The study of HTa effect on the gene related to the biochemical pathway of synthesis and secretion of milk components was performed in the current study. The

relative gene expression of α-LA and HSP70 gene in the goat milk cells increased significantly in summer when compared with the winter. However, there were no significant differences between the relative gene expression of β-GALT1 and Akt genes in the present study. Even though the previous study has been shown the closed relationship between β-GALT1, and Akt on lactose synthesis (Lin et al., 2016). The result suggested that HTa effect during summer might not influence the expression of genes related to lactose synthesis.

The present results agree with a study of α-LA protein production in milk during the summer (Gellrich et al., 2014). The influence of summer on the concentration of α-LA protein trend to higher than it was in winter and autumn. Although it seems hard to explain the effect of HTa on an up-regulation of α-LA gene expression in the current study. However, there might be a possible explanation that α-LA is a small whey milk protein that has been studied extensively its functions. It has been found that α-LA functions, is a protein with Ca^{2+} strong binding site (Permyakov and Berliner, 2000), it probably increased protein stability against heat stress effect or various denaturing agents. The relative gene expression of HSP70 gene in the goat milk cells increased significantly in summer when compared with the winter. These results in accordance with the previous study (Dangi et al., 2012). It is reported that HSP70 gene expression in caprine peripheral blood mononuclear cells (PBMCs) was significantly up-regulated in summer season when compared with winter season. The study has confirmed that HSP70 plays an important role in response to the effect of HTa. It has been known that HSP has increased synthesis following exposure to elevated temperatures (Morimoto, 1999; Santoro, 2000). The function of HSP is involved in folding/refolding of the denatured protein after heat exposure back to native conformations. An increase in HSP is a homeostatic mechanism that protects the cell from damage during HTa (Hu et al., 2016).

Moreover, another function of HSP is play a role in associate with the proteasome. HSPs function is not only refolding disaggregate proteins but also degraded dysfunction protein in the proteasome and lysosomes (Ran et al., 2007). This suggested that an increase in HSP70 genes expression probably responded to the negative effects of mammary gland function during HTa exposure in summer months of this study.

In conclusion, these studies reveal the discomfort of ambient temperature and humidity index especially during the afternoon in both winter and summer months. These conditions have contributed to the discomfort of the dairy goats fed under HTa. The goats in the study showed an increase in heat load during exposure to HTa condition in the summer months which increased in Tr. The alleviating of the heat increment of the goat was responded by increased RR, Moreover, HTa effect during summer influenced decrease DMI, especially roughage DMI and followed by decrease MY. The effect of HTa on milk synthesis might be related to an increase in antioxidative capacity of the goat and expression of HSP70 gene but this effect is not related with Akt expression.

Experiment 2: The effect of in vitro HTa on lactation synthesis and oxidative stress status in MECs

In the present study, the investigation of HTa incubation on lactose concentrations and the expression of gene-related lactose synthesis as well as Akt and HSP70 genes from MECs culture was performed. The characterization of MECs showed positive for CK 18 protein similar to what was detected in the mammary epithelial cell line (Fu et al., 2014) and goat mammary gland tissue (Zhang et al., 2013). This result reveals that the primary MECs culture isolated from goat milk expresses epithelial markers. The established goat MECs in this study show positive for the epithelial cell marker. Moreover, the cells express of the mammary function

gene, and lactose production secreted in culture medium was detected. CSN2, β-GALT1, and α-LA gene were used to demonstrate the mammary function of the cells (Sigl et al., 2014). The induction differentiation of MECs function was performed by lactogenic hormone supplementation. After differentiation was induced for 12 days, the results found that MECs express the mammary function genes such as CSN2, β-GALT1, and α-LA. Moreover, the expression of CSN2 gene was upregulated in MECs cultured in differentiation medium supplemented with lactogenic hormone. This result was in agreement with former studies which the addition of glucocorticoid and PRL to the *in vitro* culture medium of MECs increased the rate of CSN2 mRNA expression (Guyette et al., 1979; Travers et al., 1996). The results from the present study reveal that the lactogenic hormone not only increased the relative expression of CSN2 gene in MECs culture but also increased lactose concentration. Lactose concentration was detected in the culture medium of MECs culture and increased in 6 days after it was differentiated by being supplemented with lactogenic hormone. This is consistent with the study of a concentration of 12 mM glucose enhanced lactose content, which was detected in the culture medium of the mammary epithelial cells of dairy cow (Lin et al., 2016). This study confirms that MECs culture isolated from goat milk can secrete lactose into the culture medium.

The study of the effect of temperature on MECs function was performed. The incubation temperature of 37 °C has been used for the culture of bovine (Lin et al., 2016) and goat MECs culture (Ogorevc and Dovč, 2015). However, the variation in vivo of rectal temperature between 39 and 40 °C has been reported in dairy cattle (Chaiyabutr et al., 2008) and goats (Nguyen et al., 2018) fed under HTa condition. Therefore, this study focuses on the in vitro MECs culture incubation temperature that similar to the body temperature of the goat in cases of HTa exposure. The effect of temperature on the mammary function of MECs in the current study was

conducted by incubating MECs culture at 37 or 39 ℃ for 1 or 48 hours. The investigation of the expression of β-GALT1, α-LA, Akt, and HSP70 genes and lactose concentration of MECs culture was performed.

The results show that HSP70 peaked rapidly within 1 hour after HTa exposure similar to the study of bovine MECs (Hu et al., 2016). The results confirmed that HSP70 is sensitive to HTa and responded as the dominant subtype to protect MECs from acute heat exposure in goat (Luengrattana et al., 2000) and cow (Gaughan et al., 2013; Min et al., 2015). However, after 48 hours exposure to 39 ℃, the average relative HSP70 gene expression of MECs showed no significantly different between MECs culture incubated at 37 or 39 ℃. This result is consistent with the previous study (Hu et al., 2016) which upregulation of HSP70 genes under the acute increasing temperature. Increasing HSP70 gene expression after 1 hour of HTa exposure and decrease thereafter in this study might play a role in protecting MECs and supporting the cells in order to maintain its functions under acute temperature elevation. In contrast, there were no differences between the average relative β-GALT1, α-LA, Akt gene expression of MECs culture incubated at 37 or 39 ℃ for 1 or 48 hours. The results demonstrated that goat MECs continue differentiated under 39 ℃ which similar to their normal body temperature consistent with the study of lactation capacity in mouse MECs (Kobayashi et al., 2018). Furthermore, there were no differences in lactose concentration of MECs culture between incubated at 37 or 39 ℃ for 1 or 48 hours. The present result suggests that the temperature used in this study may not influence both the lactose synthase enzyme and Akt gene expression and also lactose concentration of MECs culture.

It is known that HTa is the cause of changes in the oxidative stress status in MECs culture (Kapila et al., 2016; Li et al., 2019). In the present study, there was no difference in MDA concentration and GPx activity of MECs culture incubated at 37 or

39 ℃ for 1 or 48 hours. MECs culture in this experiment was challenged to maintain the homeostasis capacity after HTa exposure. Furthermore, HSP70 rapidly increased in MECs after exposure to 39 C. This response may lead to the cytoprotective effect from thermal or oxidative stress by HSP70.

In conclusion, HTa did not affect the lactose synthase enzyme both β-GALT1 and α-LA genes as well as did not relate to Akt expression of MECs culture. Additionally, there was no HTa effect on lactose concentration in the culture medium of MECs. Therefore, the temperature used in this study was similar to the body temperature which demonstrated that MECs might continue to maintain its functions under 39 ℃ incubation.

Advantages of the study

The study reveals that does from both seasons were suffered from the HTa of the tropical conditions. HTa during summer months on mammary gland function was directly affected an increase in GPx activity and HSP70 gene expression but not β-GALT1 and Akt genes. Therefore, decreasing MY is the indirect effect rather than the direct effect of mammary gland function. In addition, the direct effect of HTa might be involve the metabolic pathway of lactose synthesis. Thus, further study needs to determine the effects of HTa on the metabolic pathway of lactose synthesis in dairy goats fed under HTa and the MECs culture.

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