EFFECTS OF PRO-INFLAMMATORY CYTOKINES TUMOUR NECROSIS FACTOR ALPHA AND INTERLEUKIN-6 ON GHRELIN EXPRESSION IN PANCREATIC AR42J CELL LINE

By

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ABSTRACT

EFFECTS OF PRO-INFLAMMATORY CYTOKINES TUMOUR NECROSIS FACTOR ALPHA AND INTERLEUKIN-6 ON GHRELIN EXPRESSION IN RAT PANCREATIC AR42J CELL LINE

LAO KAH MENG

Ghrelin is a 28 amino acid peptide endogenous ligand for growth hormone secretatgogue receptor (GHS-R) that functions to stimulate growth hormone, regulate inflammation, appetite and energy balance. Tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) are pro-inflammatory cytokines which are involved in inflammation. The main objectives of this study are to determine the effects of different concentration of TNF-α and IL-6 on ghrelin gene expression in stimulated AR42J rat pancreatic cell line. Cells were treated with different concentrations of cytokine for 24 hours. Real-Time RT-PCR, Western blot and densitometry analysis were carried out to quantify ghrelin mRNA and protein expression, respectively. The mRNA and protein expression for the control sample in TNF-α and IL-6 treated cell was attributed as 100%. As compared to the control sample, the mRNA expression was up-regulated by 10% at 5 ng/mL of TNF-α treatment and down-regulated by 10%, 14% and 45% at 10 ng/mL, 25 ng/mL and 50 ng/mL of TNF-α treatment, respectively, while the

protein expression was up-regulated by 1.4% at 5 ng/mL of TNF-α treatment and down-regulated by 9.0%, 20.4% and 22.1% at 10 ng/mL, 25 ng/mL and 50 ng/mL of TNF-α treatment, respectively. For IL-6 treated cell, the mRNA expression for 5 ng/mL of IL-6 was up-regulated by 41% and down-regulated by 43%, 75% and 80% at 10 ng/mL, 25 ng/mL and 50 ng/mL of IL-6, respectively, while the protein expression for 5 ng/mL of IL-6 was up-regulated by 57.8% and down-regulated by 5.5%, 32.4% and 51.9% at 10 ng/mL, 25 ng/mL and 50 ng/mL of IL-6, respectively, as compared to the control sample. It is hypothesised that TNF-α and IL-6 indirectly down-regulate ghrelin gene expression by altering the level of insulin resistance, glucose and cAMP. Overall, there is a dose-response relationship between ghrelin and the pro-inflammatory cytokines, TNF-α and IL-6; ghrelin mRNA and protein expression decrease as the cytokine concentration was increased.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LAO KAH MENG

APPROVAL SHEET

This project report entitled **"EFFECTS OF PRO-INFLAMMATORY CYTOKINES TUMOUR NECORSIS FACTOR ALPHA AND INTERLEUKIN-6 ON GHRELIN EXPRESSION IN PANCREATIC AR42J CELL LINE"** was prepared by LAO KAH MENG and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that **LAO KAH MENG** (ID No: **08ADB07075**) has completed this final year project entitled **"EFFECTS OF PRO-INFLAMMATORY CYTOKINES TUMOUR NECORSIS FACTOR ALPHA AND INTERLEUKIN-6 ON GHRELIN EXPRESSION IN PANCREATIC AR42J CELL LINE"** supervised by Dr. Chew Choy Hoong from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that any name is included as one of the authors for this article. This arrangement of the name depends on my supervisor.

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LIST OF ABBREVATIONS

α	Alpha
β	Beta
g	Gravity
µL	Microliter
%	Percent
˚C	Degree celcius
AMPK	Adenosine 5’ monophosphate activated protein kinase
ATCC	American Type Culture Collection
BCP	1-Bromo-3-Chloropropane
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cm	Centimeter
CO_2	Carbon dioxide
dH_2O	Distilled water
ddH_2O	Deionized distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
g	Gram

GADPH	Glyceraldehydes-3-phosphate dehydrogenase
GH	Growth hormone
GHS	Growth hormone secretatgogue
GHS-R	Growth hormone secretatgogue receptor
Gln	Glutamine
GOAT	Ghrelin *O*-acyltransferase
gp130	Glycoprotein 130
HI-FBS	Heat inactivated fetal bovine serum
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IL-1α	Interleukin 1 alpha
IL-4	Interleukin 4
IL-6	Interleukin 6
IRS-1	Insulin receptor substrate 1
kDa	kiloDalton
kPa	kiloPascal
L	Liter
LPS	Lipopolysaccharide
M	Molar
mg	Milligram
mL	Milliliter
Mm	Milimolar

mRNA	Messenger ribonucleic acid
MOPS	3-(N-morpholino)propanesulfonic acid
NaCl	Sodium chloride
NF-κb	Nuclear factor kappa B
NF-IL6	Nuclear factor-IL-6
ng	Nanogram
nm	Nanometer
PCR	Polymerase chain reaction
PBS	Phospahate buffer saline
pg	picogram
PVDF	Polyvinylidene fluoride
Q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrilamide gel electrophoresis
Ser	Serine
TACE	Tumour necrosis factor alpha converting enzyme
TBS	Tris-buffer saline
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha

TNF-R	Tumour necrosis factor alpha receptor
UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/v	Weight per volume

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Chapter 1

INTRODUCTION

Ghrelin, a 28 amino acids orexigenic hormone is produced by X/A-like cells and is well known as the endogenous ligand for growth hormone secretagogue receptor (GHS-R) (Kojima & Kangawa, 2005). It is produced in stomach, hypothalamus, pancreas, pituitary, intestine, adrenal gland, testis, ovary, placenta and brain (Silvia & Kumar, 2010; Yin, Li, Xu, An, & Zhang, 2009). Ghrelin has two isoforms, ghrelin and des-Gln14-ghrelin and can be further categorised into two groups, des-acyl ghrelin and acylated ghrelin. Acylated ghrelin is the active form of ghrelin where it is acylated at the third serine residue of the peptide. This modification is essential for the binding of ghrelin to GHS-R in order to exert its effect (Soares & Moreira, 2008). Des-acyl ghrelin is the form of ghrelin that does not undergo acylation. Ghrelin's main function is to stimulate growth hormone release. Besides, it also regulates food intake, energy homeostasis, adipogenesis and immune system (Kojima & Kangawa, 2005; Soares & Moreira, 2008).

Ghrelin has been studied in various diseases for the investigation of its therapeutic role. Moreira and Soares (2007) studied the role of ghrelin in bone, immune system, gastrointestinal tract and cardiovascular system. Using their studies on cardiovascular system as an example, ghrelin inhibits the basal and

TNF-α-induced chemokines production and mononuclear cell adhesion. This inhibition prevents endothelial dysfunction and inflammation of the cardiovascular system. Therefore, they concluded that ghrelin could be used to treat atherosclerosis. In the same study, they mentioned that ghrelin improves pancreaticobiliary inflammation by inhibiting neutrophil action.

Tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) are pleiotropic cytokine produced by different immune cells. TNF-α is produced by macrophages, while IL-6 is produced by T-cells, monocytes and dendritic cells (Coico & Sunshine, 2009). These two cytokines are known as the pro-inflammatory cytokines where they are involve in acute phase response. Besides, research by Dixit and his friends (2004) also mentioned that these two cytokines plays important role in the development of aneroxia-cachexia syndrome which causes negative energy balance. Immune cells like T-cells, B-cells and neutrophils express ghrelin and GHS-R (Dixit et al., 2004). Therefore, there is a relationship between ghrelin and the immune system. A study done by Mafra and his friends (2010) showed that ghrelin levels were positively correlated with TNF-α and IL-6. Therefore, they suggest that TNF-α and IL-6 may be an important regulator in ghrelin synthesis.

Many studies have been done to investigate the role ghrelin in the immune system. Most data suggested that ghrelin is involved in regulating the transcription and mRNA expression of pro-inflammatory cytokines, concluding that ghrelin has

anti-inflammatory effects. According to Dixit and Taub (2005), ghrelin inhibits the production of TNF-α and IL-6 through GHS-R pathway due to the present of this receptor on the immune cells.

Previous studies were carried out looking at the effects of ghrelin on cytokines expression, but limited studies were done for vice versa. No study has been carried out yet to investigate the effects of TNF-α and IL-6 on ghrelin expression. Therefore, this study is aimed to investigate the effects of pro-inflammatory cytokines, TNF-α and IL-6, on ghrelin expression. It is hypothesised that TNF-α and IL-6 would decrease the expression of ghrelin for them to exert their pro-inflammatory activities.

Therefore, the objectives of this study were:

i. To investigate the effects of pro-inflammatory cytokines TNF-α and IL-6 on ghrelin mRNA expression in AR42J cells
ii. To investigate the effects of pro-inflammatory cytokines TNF-α and IL-6 on ghrelin protein expression in AR42J cells
iii. To compare the mRNA and protein expression of ghrelin in TNF-α and IL-6- treated AR42J cells to determine the presence of post-translational modification

Chapter 2

LITERATURE REVIEW

2.1 Growth Hormone Secretagogue and Its Receptor

Growth hormone (GH) is an important hormone in the body which stimulates cell growth, regulates body metabolism and homeostasis. It is secreted from somatotrophs found in the anterior pituitary. The production of this hormone is tightly regulated in which its release is stimulated by GH-releasing hormone from the hypothalamus and is inhibited by somatostatin (Kojima & Kangawa, 2005). Other than that, GH secretagogues (GHS) also play a role in regulating GH release. GHS is a synthetic compound that has the ability to stimulate GH release through a specific G-protein coupled receptor known as the GHS receptor (GHS-R). Since GHS is a synthetic compound, an endogenous ligand is needed to bind to GHS-R to produce the effect of GHS. Ghrelin has been identified as one of the specific endogenous ligand to bind specifically to GHS-R (Kojima et al., 1999).

GHS-R is present in two different forms; GHS-R1a and GHS-R1b, which is produced as a result of alternative splicing. According to Dixit and Taub (2005), GHS-R1a is a 336 amino acids peptide with seven transmembrane proteins G-protein coupled receptor that has a ligand binding site in the third transmembrane loop while GHS-R1b is a 289 amino acids peptide with five

transmembrane proteins. Current studies mostly focus on GHS-R1a as it is the functional receptor between these two forms that carry out all the functions of a GHS. Meanwhile, GHS-R1b is non-functional as no specific function has been assigned to it yet. Since ghrelin binds to GHS-R1a, this receptor is also known as the ghrelin receptor.

2.2 Ghrelin

2.2.1 Ghrelin and Des-acyl Ghrelin

Ghrelin is a 28 amino acid peptide that was first purified from rat stomach by a Japanese scientist in 1999 (Kojima et al., 1999). Two types of ghrelin isoforms has been identified; ghrelin and des-Gln14-ghrelin. Ghrelin is a full length peptide with 28 amino acids, while des-Gln14-ghrelin is a 27 amino acids peptide. For des-Gln14-ghrelin, it undergoes alternative slicing which uses the CAG codon that encodes glutamine at position 14 as the splicing signal thus producing a peptide with one amino acid lesser (Kojima & Kangawa, 2005; Soares & Moreira, 2008). Both ghrelin and des-Gln14-ghrelin are identical in function and could undergo the same post-translational modification such as the acylation of the third serine residue. In fact, ghrelin is the only peptide known to be octanoylated (van der Lely, Tschop, Heiman, & Ghigi, 2004; Yin et al., 2009). This modification is important as it is essential for the binding to GHS-R to exert most of its biological effects (Mager et al., 2008; Soares & Moreira, 2008). Among these two forms, des-Gln14-ghrelin is the minor

form as it is present in a very low amount while ghrelin is the major form found in the circulation.

According to Kojima and Kangawa (2005), ghrelin can also be classified into few groups; octanoylated, non-acylated, decanoylated and decenoylated. The form that is mainly studied would be the octanoylated and non-acylated form. Octanoylated ghrelin is the form in which an N-octanoyl group is covalently bound to the hydroxyl group located on the third serine residue of the ghrelin peptide while non-acylated ghrelin is the form where no octanoyl group is attached to the peptide (Yin et al., 2009). Figure 2.1 shows the structure of non-acylated and acylated ghrelin.

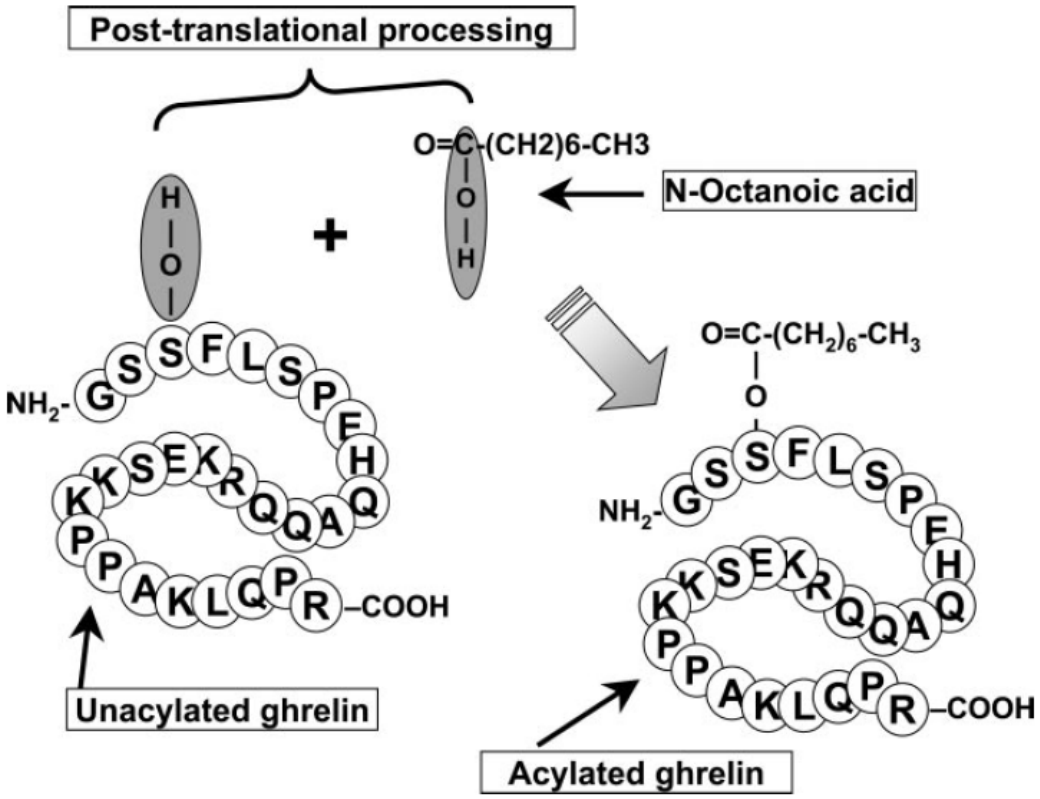


Figure 2.1: Post-Translational Modifications of Ghrelin Peptide.
The post-translational modification of ghrelin occurs at the third serine residue. When a hydroxyl group binds the third Ser residue, it produces unacylated ghrelin which is the inactive form of ghrelin while addition of N-octanoic acid to the same amino acid produces acylated ghrelin, the active form of ghrelin. The acylation process is important as it is essential for the binding of ghrelin to its receptor, GHS-R1a. Adapted from van der Lely et al., (2004).

2.2.2 Ghrelin Structure and Gene Expression

Ghrelin gene is located on chromosome 3p25-26 and is made up of four exons and three introns (Soares & Moreira, 2008). Exon 1 is the non-coding exon while exon 2 and exon 3 form the mature ghrelin (Kojima & Kangawa, 2005). Transcription of ghrelin gene produces a 117 amino acid pre-pro-ghrelin. Pre-pro-ghrelin would be cleaved to form 94 amino acids pro-ghrelin. Pro-ghrelin is subsequently processed into the 28 amino acids mature ghrelin by pro-hormone convertase 1/3 (PC1/3) (Dixit & Taub, 2005; Yin et al., 2009). This peptide shares 82% homology similarity between human and rat (Dixit & Taub, 2005). The difference in the amino acid sequence of ghrelin gene between human and other species is shown in Table 2.1. From the table, human ghrelin and rat ghrelin only differs in two amino acids at the position 11 and 12. Arginine and valine is replaced with lysine and alanine at these positions, respectively. Meanwhile, Figure 2.2 shows the structure of human ghrelin gene and its processing.

Table 2.1: Structure of Ghrelin from Domesticated Species Aligned to Human Ghrelin.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Species
G	S	S	F	L	S	P	E	H	Q	R	V	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Human
G	S	S	F	L	S	P	E	H	Q	***K***	***T***	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Gerbil
G	S	S	F	L	S	P	E	H	Q	***K***	***A***	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Mouse
G	S	S	F	L	S	P	E	H	Q	***K***	***A***	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Rat
G	S	S	F	L	S	P	E	H	Q	***K***	***L***	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Dog
G	S	S	F	L	S	P	E	H	Q	***K***	V	Q	Q	R	K	E	S	K	K	P	***A***	A	K	L	***K***	P	R	Pig
G	S	S	F	L	S	P	E	H	Q	***K***	***L***	□	Q	R	K	E	***A***	K	K	P	***S***	***G***	***R***	L	***K***	P	R	Cattle
G	S	S	F	L	S	P	E	H	Q	***K***	***L***	□	Q	R	K	E	***P***	K	K	P	***S***	***G***	***R***	L	***K***	P	R	Sheep
G	S	S	F	L	S	P	***T***	***Y***	***K***	***N***	***I***	Q	Q	***Q***	K	***D***	***T***	***R***	K	P	***T***	A	***R***	L	***H***	***R***	R	Chicken
G	S	S	F	L	S	P	***S***	□	Q	R	□	***P***	Q	***G***	K	***D***	□	K	K	P	P	□	***R***	***V***	***G***	***R***	R	Eel
G	S	S	F	L	S	P	***S***	□	□	□	□	□	Q	***K***	***P***	***Q***	***N***	K	***V***	***K***	***S***	***S***	***R***	***I***	***G***	***R***	***Q***	Tilapia

Bold and *italic* indicates that residue is different from that in human; □ indicates space added for alignment to the human ghrelin primary structure. Adapted from van der Lely et al. (2004).

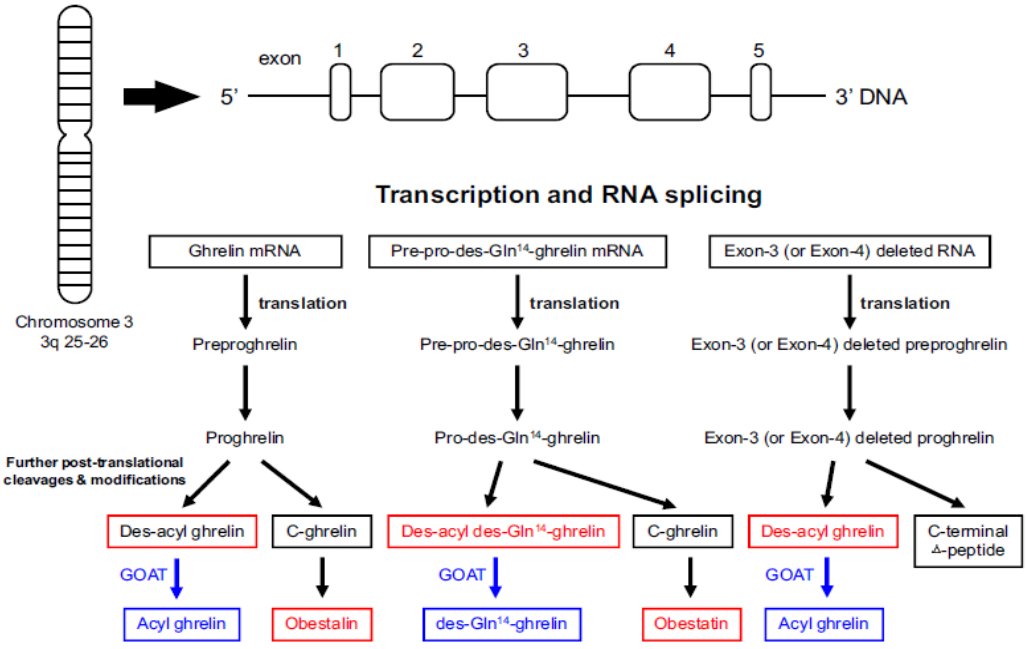


Figure 2.2: Sequential Steps in Ghrelin Gene Production.
The alternative splicing of the ghrelin gene produces three different ghrelin mRNAs. These mRNA undergoes translation and post-translational modifications to form different products. The products are acyl ghrelin, obestation, des-Gln14-ghrelin. Adapted from Chen, Asakawa, Fujimiya, Lee, and Inui (2009).

There are several factors regulating the transcription of ghrelin. The identified activators of the ghrelin gene promoter are glucagon and cAMP (Silvia & Kumar, 2010). The decrease in this two components would decrease the expression of ghrelin mRNA. Besides there are other transcriptional factors influencing ghrelin expression too. The binding site for the transcriptional factors is located on the 5'-upstream site of the gene (Nakai et al., 2004). The examples of these elements are cAMP response element binding protein (Yin et al., 2009), nuclear factor-IL-6 (NF-IL6), nuclear factor kappa B (NF-κB) and hepatocyte nuclear factor-5 (Kojima & Kangawa, 2005).

After the transcription process, the ghrelin mRNA would be translated into few protein forms. This means that ghrelin gene can produce more than one product. The products are ghrelin, obestatin and des-Gln14-ghrelin. There is only one post-translational modification for ghrelin in which is the octanoylation at the third serine residue of the peptide. The enzyme that catalyzes this process is known as ghrelin *O*-acyltransferase (GOAT) (Mafra et al., 2010). On the other hand, acylated-ghrelin can be des-acylated by plasma esterase for the regulation circulating acylated-ghrelin level (De Vriese et al., 2004). Meanwhile, plasma protease is responsible for the degradation of ghrelin in the circulation. Through these two enzymes, the circulating ghrelin level could be maintained (Yin et al., 2009).

2.2.3 Distribution of Ghrelin

Ghrelin is produced by the X/A-like cell which is mainly found in the stomach (Seim, Herington, & Chopin, 2009; St-Pierre, Wang, & Tache, 2003). This cell is also found in small intestine, pancreas, hypothalamus, kidney, pituitary gland, testis, lymphocytes and brain (Silvia & Kumar, 2010; Yin et al., 2009). The various distribution of ghrelin suggests that it may have other functions besides stimulating the release of GH. After the production of ghrelin, it will be directly secreted into the blood stream where it would circulate to other parts of the body. This peptide has the ability to cross the blood-brain barrier too.

As mentioned in section 2.2.1, ghrelin can be found in the form of octanoylated and non-acylated. Silvia and Kumar (2010) found that actanoylated ghrelin comprises 5% of total ghrelin whereas non-acylated ghrelin made up 95% of total ghrelin in the circulation. They also mentioned that majority of the circulating ghrelin comes from stomach and total ghrelin in the plasma is approximately 200-600 pg/mL. Between octanoylated ghrelin and non-acylayed ghrelin, non-acylated ghrelin has longer life span and thus it is present in a higher concentration compared to octanoylated ghrelin (Dixit & Taub, 2005).

2.2.4 Regulation of Ghrelin Production

Ghrelin is produced in a regulated manner to maintain the body homeostasis. Yin and colleagues (2009) wrote a review on the substances that can cause ghrelin fluctuation. In their review, glucose, insulin, growth hormone, insulin-like growth

factor, leptin and somatostatin inhibits ghrelin secretion while fatty acids, glucagon and estrogen increases ghrelin secretion. Table 2.2 is adapted from their review showing the summary of condition that alters ghrelin level.

Table 2.2: Alteration of Serum Ghrelin Level under Different Conditions.

Group	Elevated ghrelin level	Depressed ghrelin level
Nutrients	Fatty acids[a], amino acids[a]	Glucose, fatty acids[a]
Hormones	Glucagon[a], IGF-1, estrogen[a]	Insulin, growth hormone, somatostatin, leptin[a], estrogen[a]
Autonomic nervous system	Vagus nerve activation	Sympathetic nerve activation
Physiological status	Fasting, lean, youth	Feeding[a], obesity, aging[a]
Pathological status	Prader–Willi syndrome, anorexia nervosa, cachexia	Metabolic syndrome, diabetes mellitus

[a]Information incomplete or controversial.

Adapted from Yin et al. (2009).

Since pancreatic cell line is used for this study, the hormone glucagon and insulin is given more attention. For glucagon, it may increase or decrease ghrelin secretion. Glucagon has been shown to increase ghrelin by activating the promoter of the ghrelin gene (Silvia & Kumar, 2010). Meanwhile according to Arafat et al. (2005), their studies proved that glucagon decreases ghrelin level. One of glucagon function is to catalyze the conversion of glycogen back into glucose. In their study, glucose was shown to markedly inhibit ghrelin secretion. Therefore, high levels of glucagon would increase glucose levels and hence decreasing ghrelin levels.

Insulin also decreases ghrelin levels. A number of researches mentioned in the review by Yin and friends (2009) proved that the administration of insulin decrease ghrelin levels in human and rats. The levels of insulin may explain the low ghrelin levels in type 2 diabetes mellitus patient. A study done by Kempa, Krzyzanowska-Swiniarska, Miazgowski, and Pilarska (2007) shows that insulin sensitivity plays a more important role in regulating ghrelin levels compare to insulin itself. To date, the exact mechanism on how insulin affects ghrelin levels, either directly by influencing the gene expression or indirectly due to the physiological changes cause by it, is still unknown (Date et al., 2001; Mager et al., 2008).

2.3 Physiological Functions of Ghrelin

2.3.1 Growth Hormone

Ghrelin is a well known endogenous ligand for GHS-R and its main function is to stimulate GH release from anterior pituitary. Ghrelin binds to its specific G-protein coupled receptor, GHS-R1a and activates phospholipase C signaling pathway. This increases the intracellular calcium ions that stimulate GH release (Kojima & Kangawa, 2005; Silvia & Kumar, 2010). With such ability, ghrelin is used to diagnose and treat GH deficiency. The efficacy of ghrelin in treating GH deficiency is not well established yet, but ghrelin is proven as a great tool in diagnosing GH deficiency (Kojima & Kangawa, 2005; Moreira & Soares, 2007). According to the review by Moreira and Soares (2007), intravenous injection of ghrelin can be used as an alternative to insulin-tolerance test in investigating the

pituitary capacity to release GH for the diagnosis of GH deficiency as ghrelin can provide a more reproducible result.

2.3.2 Appetite Regulation

Ghrelin is also a well known orexigenic hormone that regulates appetite and body weight. Studies have shown that ghrelin levels increases before meal and decreases after meal (Moreira & Soares, 2007). Since ghrelin has the ability to cross the blood-brain barrier, it acts on the hypothalamus directly to induce hunger (Dixit & Taub, 2005). Adenosine 5' monophosphate activated protein kinase (AMPK) plays an important role in the orexigenic effect of ghrelin. Ghrelin increases appetite through AMPK phosphorylation which increases the expression of neuropeptide Y or agouti-related protein by cells in the arcuate nucleus (Moreira & Soares, 2007; Silvia & Kumar, 2010). Besides, ghrelin also stimulate appetite through the inhibition of leptin expression (Dixit & Taub, 2005). This property of ghrelin and also its involvement in regulating energy expenditure is used for the treatment of cachetic patients like those with anorexia nervosa, bulimia nervosa and cancer cachexia (DeBoer et al., 2007).

2.3.3 Gastrointestinal Function

Ghrelin has been shown to provide protection to the epithelial, stimulate motility and gastric secretion (Moreira & Soares, 2007). Ghrelin exhibit protective effects to the epithelium of the gastrointestinal tract by affecting the cell proliferation and differentiation. The structural similarity between ghrelin and motilin give rise to

the study of the motility effects of ghrelin. It has been demonstrated that ghrelin administration exhibits a strong prokinetic effect and accelerates gastric emptying in both human and rodents. The prokinetic effect of ghrelin is mediated through the acetylcholine system. Besides, intravenous administration of ghrelin increases gastric acid secretion through the activation of vagus nerve (Masuda et al., 2000).

2.3.4 Immunoregulation

Immune cells like T-cells, B-cells and neutrophils express ghrelin and GHS-R (Dixit et al., 2004; Lensiak & Roth, 1976). Thus, many research have been carried out to study the effects of ghrelin in the immune system and to date, the functional role of ghrelin in the immune system is not well defined. From the studies performed, ghrelin functions to increase the growth and differentiation of thymus which in turn, increases the levels of T-cells production and modulate the production of pro-inflammatory cytokines (Hattori, 2009). Ghrelin inhibits the release of TNF-α, IL-6 and IL-1α through GHS-R specific pathway, thus this relieves the inflammatory responses (Sung et al., 2008). Besides, ghrelin also inhibits leptin-induced cytokine synthesis and LPS-induced cytokine production (Dixit & Taub, 2005; Smith, Jiang, & Sun, 2005). In a study performed on rat, ghrelin was shown to attenuate pancreatic damage by decreasing the production of IL-1β. Ghrelin also improves experimental-induced arthritis by decreasing the synthesis of IL-6 (Sung et al., 2008). This anti-inflammatory effect of ghrelin may be applied as the treatment for wasting diseases, arthritis and aging.

2.3.5 Cardiovascular Function

Gnanapavan et al. (2002) discovered the presence of ghrelin and GHS-R1a mRNA in heart and aorta. This discovery provides evidence that ghrelin may play a role in the cardiovascular function. In congestive heart failure, atherosclerosis and septic shock, the levels of pro-inflammatory cytokines are high due to vascular inflammation. Ghrelin exerts its anti-inflammatory effects by decreasing the levels of the pro-inflammatory cytokines as mentioned in section 2.3.4. Specifically, ghrelin inhibits mononuclear cell adhesion and the basal and TNF-α-induced cytokine production. Besides relieving the inflammation levels, the action of ghrelin in a heart failure model improves the left ventricular function and attenuates the development of cardiac cachexia (Smith et al., 2005). Thus, ghrelin may be useful for treating atherosclerosis. Besides all these functions, ghrelin also carry many other functions. Table 2.3 summarises the effects of ghrelin and the therapeutic potential of ghrelin agonist and antagonists.

Table 2.3: Effects of Ghrelin and the Therapeutic Potential of Ghrelin Agonists and Antagonists.

Effects of ghrelin and the therapeutic potential of ghrelin agonists and antagonists		
Function	**Physiological or pathological role**	**Therapeutic potential**
GH-release	Stimulates GH-release	Ghrelin agonists in GH-deficiency states such as aging and short stature
Appetite and body weight	Stimulates appetite and food intake and increases body weight	Ghrelin agonists in cachexia and anorexia Ghrelin antagonists in obesity and Prader–Willi syndrome
Metabolism	Inhibits insulin secretion and action; induces hyperglycaemia; stimulates lipogenesis and proliferation of adipocytes; and inhibits lipolysis	Ghrelin agonists in metabolic syndrome
Reproduction	Inhibits LH secretion, embryo development, spermatogenesis, Leydig-cell development and testosterone secretion	Unknown
Gastrointestinal	Stimulates gastric secretion; offers epithelial protection; and stimulates motility	Ghrelin agonists in gastroparesis and in gastric ulcer and colitis
Cardiovascular	Decreases blood pressure; improves endothelial function; increases stroke volume; decreases inotropism; decreases cardiomyocytes apoptosis; and protects against ischaemia/reperfusion injury	Ghrelin agonists in atherosclerosis, hypertension, chronic heart failure, dilated cardiomyopathy, sepsis and cardiopulmonary bypass surgery
Pulmonary	Unknown	Ghrelin agonists in pulmonary hypoplasia and hypertension
Cell proliferation	Stimulates the proliferation of several normal cell lines	Ghrelin agonists or antagonists, depending on the tumour. Ghrelin agonists in diseases associated with abnormal angiogenesis
Immunology	Enhances immune cell proliferation and inhibits secretion of proinflammatory cytokines	Ghrelin agonists in either immunodeficiency or inflammatory states, such as wasting diseases and sepsis
Bone	Stimulates proliferation and function of osteoblasts	Ghrelin agonists in osteoporosis and metabolic bone disease

Adapted from Moreira and Soares (2007).

2.4 Pro-inflammatory cytokines

Pro-inflammatory cytokines is produced by various immune cells to work as the signaling molecules in the immune system. The examples of pro-inflammatory cytokines are interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α). Cytokines also act on the central nervous system to regulate energy balance and food intake

(Dixit et al., 2004). Patients with inflammatory diseases or injury usually exhibits the symptoms of low appetite or decrease food intake and this has been associated with the function of the cytokines that are produced in a high levels in these patients. Cytokines are useful in the immune system but its production needs to be tightly regulated to prevent negative effect on one's health. High levels of cytokines may cause excessive inflammatory reaction and eventually will lead to inflammatory diseases.

2.4.1 Cytokine: Tumour Necrosis Factor Alpha (TNF-α)

TNF-α is a pleiotropic cytokine that was first isolated by Carswell et al. (1975). It is a homotrimeric 17 kDa protein produced by several types of cells like mast cell, macrophages, endothelial cells, adipocyte, astrocytes, smooth muscle cells and fibroblasts (Wacknik, Eikmeier, Simone, Wilcox, & Beitz, 2005). Among all the cells involve in the production of TNF-α, macrophages are the main producer of TNF-α. It is initially synthesised as a 26 kDa transmembrane monomer and then, it undergoes proteolytic cleavage by TNF-α converting enzyme (TACE) to form a 17 kDa protein (Cawthorn & Sethi, 2007). There are two receptors identified as the TNF-α receptor (TNF-R), 55 kDa TNF-R1 and 75 kDa TNF-R2. TNF-R1 is mostly expressed in all somatic cells while TNF-R2 is commonly found in hemapoietic cells (Apostolaki, Armaka, Victoratos, & Kollias, 2010). Most TNF-α effect is produce through the activation of TNF-R1. The activation of TNF-R2 is associated with the proliferation of thymocytes, while the cytotoxicity effect of TNF-α is mediated through both receptors (Soliven & Szuchet, 1995).

It is only produced following bacterial lipopolysaccharide stimulation and other bacterial products (Mukai et al., 2009). TNF-α has many roles. In the immune system, it functions to stimulate acute phase response, act as a chemoattractant for neutrophils, increase the adhesion molecule on the endothelial cells for neutrophil migration and stimulate phagocytosis (Morjaria, 2008). In the central nervous system, TNF-α stimulates the production of corticotrophin releasing hormone from the hypothalamus, suppresses appetite and induces fever (Endo, Masaki, Seike, & Yoshimatsu, 2007). TNF-α have catabolic roles too where it may cause cachexia. Besides, another role of TNF-α is that it induces insulin resistance (Ruan & Lodish, 2003). Overproduction of this cytokine would cause arthritis, inflammatory diseases and multiple sclerosis (Apostolaki et al., 2010).

2.4.2 Cytokine: Interleukin-6 (IL-6)

IL-6 is a pleiotropic cytokine with the structure of four anti-parallel helices connected with two long and one short loop. Its molecular weight ranges from 21 kDa to 28 kDa. This is caused by the different glycosylation and phosphorylation modifications at the amino acid position 73 and 172 (Ibelgaufts, 2001). It is produced by lymphoid and non-lymphoid cells like T-cells, macrophages and endothelial cells. IL-6 receptor consist of two molecules; the α chain and β chain. The α chain is a 80 kDa IL-6 binding protein while the β chain is 130 kDa signal transducer, gp130 (Kishimoto, 2003). This receptor can be found in T-cells, myeloid cell lines, hepatoma cell lines, activated

B-cells and more. The synthesis of IL-6 is inhibited by glucocorticoid, IL-4 and TGF-β.

It is released during infection, burns, trauma and neoplasia in response to bacterial endotoxin, IL-1 and TNF-β. Thus, IL-6 is involved in acute phase response, oncogenesis and haematopoiesis. In the immune system, IL-6 functions to stimulate acute phase reaction, proliferation and differentiation of B-cells and T-cells and also stimulate the release of interferon from B-cells and T-cells. As in haematopoiesis, IL-6 stimulates the proliferation of murine pluripotential hematopoietic progenitors and together with IL-3, they stimulate the maturation of megakaryocytes (Kishimoto, 2003). This role of IL-6 relates it to Fanconi anaemia as mentioned by Rosselli, Sanceau, Wietzerbin and Moustacchi (1992) where this disease is caused by deficiency in the production of IL-6. Other roles of IL-6 includes stimulate the release of anterior pituitary hormones such as prolactin, and growth hormone, enhancing osteoclast development, inducing insulin resistance and regeneration of hepatocyte (Kishimoto, 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials used in this project were purchased from the suppliers as shown in Table 3.1.

Table 3.1: Materials Used and Their Respective Suppliers.

Materials	Supplier (s)
AR42J Cell Line	American Type Culture Collection, USA
Fetal Bovine Serum	i-DNA, Singapore
Tri-Reagent® LS	Molecular Research Center, USA
Cell Scrappers	Techno Plastic Products (TPP), Switzerland
1 mL Cryo-vials	Nunc, USA
Tissue Culture Flask (25 cm^2)	
Agarose	Cambrex, USA
Glycerol	QRëc™, New Zealand
Prestained Protein Size Markers, Broad Range	New England Biolabs, USA
TEMED	Bio Basic, Canada
Guanidine Hydrochloride	Fluka Chemica, USA
Bovine Serum Albumin	Fisher Scientific, USA
Hydrochloric acid	
Sodium dodecyl sulphate (SDS)	
Glycine	
Coomassie blue G-250	

Table 3.1, continued.

Materials	Supplier(s)
Isopropanol	Merck, Germany
2-mercaptoethanol	
Sodium acetate	Systerm® , Malaysia
Sodium chloride	
Acetic acid	
Bromophenol blue	
95% Ethanol	HmbG® Chemicals, Germany
Absolute Ethanol	
Butanol	
Ethidium Bromide	Amresco, USA
MOPS (sodium salt)	
Formaldehyde	
Phosphate Buffer Saline (PBS)	
Acrylamide:bisacrylamide (37.5:1)	
Methanol	LabScan, Ireland
Acetone	Bendosen, Norway
Tween® 20	ACRŌS, USA
Nutrient Mixture F-12 Ham	Sigma Aldrich, USA
1-Bromo-3-Chloropropane (BCP)	
Tissue Culture Grade DMSO	
Trypsin EDTA	
Tris	
Glycine	
Anti-rabbit IgG (HRP-linked antibody)	Cell Signaling Technology, USA
Anti β-actin antibody	AbFrontier, Korea
Anti- ghrelin antibody	Santa Cruz Biotechnology, USA
Immobilon™ Western Chemiluminescent HRP Substrate	Milipore, USA
Polyvinylidene fluoride membrane	
Cytokine: Tumour Necrosis Factor Alpha	
: Interleukin-6	

3.2 Cell Culture Media

Nutrient Mixture F-12 Ham from Sigma Aldrich was used as the growing medium for the cells. This medium was prepared from the Nutrient Mixture F-12 Ham powder which was dissolved in 900 mL of sterile deionised water. Then, the medium was supplemented with 15.7 mL of 7.5% (w/v) sodium bicarbonate solution. After that, the pH of the medium was adjusted to 0.1- 0.3 pH units. The solution was subsequently top up to the final volume of 1 L and it was sterilised by filtration through a membrane with the porosity of 0.22 micron.

3.3 Stock Solutions

All stock solutions used are shown and categorised as shown in Table 3.2, 3.3, 3.4, 3.5 and 3.6.

Table 3.2: Solutions Used for Electrophoresis of RNA.

Solution	Composition
RNA Loading Dye Stock	50% (v/v) glycerol
	1 mM EDTA, pH 8.0
	0.25% (w/v) bromophenol blue
10X MOPS, pH 7.0	400 mM MOPS (sodium salt)
	100 mM sodium acetate
	10 mM EDTA, pH 8.0

Table 3.3: Solutions Used for Protein Extraction.

Solution	Composition
Guanidine Hydrochloride: Ethanol: Glycerol	0.3 M Guanidine Hydrochloride 2.5% Glycerol Top up with 95% Ethanol
Ethanol: Glycerol	2.5% Glycerol Top Up with 95% Ethanol

Table 3.4: Solutions Used for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Solution	Composition
SDS-PAGE Upper Gel Buffer, pH 6.8	0.5 M Tris-Cl 10% (w/v) SDS
SDS-PAGE Lower Gel Buffer, pH 8.8	1.5 M Tris-Cl 10% (w/v) SDS
SDS-PAGE Running Buffer (10X)	250 mM Tris 1.92 M Glycine 1% (w/v) SDS
SDS-PAGE Gel Loading Buffer (Reducing), pH 6.8	0.12 M Tris-Cl 25% (v/v) glycerol 2% (w/v) SDS 0.004% (w/v) bromophenol blue 5% (v/v) 2-mercaptoethanol

Table 3.5: Solutions Used for Coomassie Blue Staining.

Solution	Composition
Coomassie Blue Staining Solution	50% Methanol
	10% Acetic Acid
	0.05 g Coomassie blue
Coomassie Blue De-staining Solution	50% Methanol
	10% Acetic Acid

Table 3.6: Solutions Used for Western Blot Analysis.

Solution	Composition
Towbin Transfer Buffer	25 mM Tris
	192 mM Glycine
	20% (v/v) methanol
Tris-buffer saline (TBS), pH 7.4	10 mM Tris-Cl
	200 mM NaCl
	0.1% Tween® 20
Blocking Solution	0.1% Tween® 20
	1% BSA
Stripping Solution, pH 2.2	0.4 M Glycine
	0.2% (w/v) SDS
	2% Tween® 20
Detection Reagent	1 mL HRP Substrate Peroxidase
	1 mL HRP Substrate Luminol

3.4 Preparation of Glassware and Plasticware

All glassware and plasticware like pipette tips, microcentrifuge tubes, PCR tubes and centrifuge tubes were autoclaved at 121˚C for 15 minutes with the pressure of

975 kPa before use. Certain solutions used in the project especially the solutions in RNA and protein works were autoclaved prior to use too.

3.5 Cell Culture Techniques

3.5.1 Maintenance of Cells in Culture

The AR42J cells were grown in 4 mL of F12K medium supplemented with 20% (v/v) of fetal bovine serum (FBS). The cells were then maintained and grown in a humid incubator of 5% (v/v) CO_2 at 37˚C. The cell culture medium in the tissue culture flask was replaced every three days. Phosphate buffer saline (PBS) was used as the wash solution during the change of growth medium. This saline was prepared by the addition of distilled water into the tablet form PBS which was purchased. The solution was then autoclaved to sterilise it.

3.5.2 Subculturing of Cells

Subculturing of cells was performed after the cells had achieved approximately 80% confluence. Firstly, the cell culture medium in the tissue culture flask was discarded. The cells were washed twice with phosphate buffer saline (PBS) to remove all traces of serum which contained trypsin inhibitor. Then, 1 mL of 0.25% (w/v) trypsin which functions to disperse the cells from the growth surface was added and incubated at 37˚C, 5% (v/v) CO_2 for 10 to 15 minutes. After incubation, the cells were inspected under the inverted phase contrast microscope to check if all the cells had detached from the growth surface. Next, 2 mL of complete growth medium was added into the tissue culture flask. The medium

was then gently transferred into a 15 mL centrifuge tube and was centrifuged at 500 *g* for 5 minutes.

After centrifugation, the supernatant was removed and the pellet was resuspended gently in 2 mL of complete growth medium. Subsequently, the cells were divided equally and plated into seven new tissue culture flasks. In each flask, complete growth medium supplemented with 20% (v/v) of fetal bovine serum (FBS) was added to achieve a final volume of 4 mL. Finally, the cells were incubated in a humid incubator of 5% (v/v) CO_2 at 37˚C.

3.5.3 Preserving and Storing of Cells

One flask of cell from every passage was stored. Firstly, the cells were trypsinised as described in section 3.5.2. They were centrifuged at 500 *g* for 5 minutes. The supernatant was discarded and the cells were resuspended in 10% DMSO, 30% FBS and 60% growth medium. The cell suspension was dispensed in 1 mL aliquots into sterile cryo-vials which were subsequently covered with many layers of parafilm and placed in a polysterene box. The box was then stored in -80˚C overnight. On the next day, the cryo-vials were stored in a liquid nitrogen container.

3.5.4 Thawing of Frozen Cells

A cryo-vial was removed from the liquid nitrogen container and placed in a 37˚C waterbath until the cells thawed. Then, the cryo-vial was cleaned thoroughly using 70% (v/v) ethanol. Next, the content was transferred to a 15 mL centrifuge tube containing 9 mL growth medium and centrifuged at 500 *g* for 5 minutes. The supernatant was discarded and the cells were resuspended in 2 mL of growth medium and were plated out into the tissue culture flask. On the next day, the cells were washed twice with PBS and were cultured in the appropriate medium.

3.5.5 Treatment of AR42J Cells with Cytokines

The cells were allowed to grow until it reached approximately 70% confluence. Before treatment of cells with cytokine, the growth medium was first discarded and the cells were washed twice with PBS. Then the cells were pre-incubated one hour with 4 mL of complete growth medium but with reduced amount of heat inactivated- fetal bovine serum (HI-FBS) which was from 20% to 1% (v/v) HI-FBS. Following the incubation, the growth medium was discarded and the cells were washed twice with PBS again. Subsequently, different concentration of cytokines were added into the fresh medium supplemented with 1% FBS. For the control medium, no cytokines were added. Next, the cells were incubated in a humid incubator of 5% (v/v) CO_2 at 37˚C for 24 hours. After 24 hours, the total cellular RNA and protein were extracted as described in section 3.6.1 and 3.7.1 respectively.

3.6 RNA-Associated Techniques

3.6.1 Isolation of Total Cellular RNA Using TRI-Reagent LS

Total cellular RNA was extracted from cells cultured using Tri-Reagent® LS according to the manufacturer's instruction. The cells medium was discarded and were washed twice with PBS. Then, 0.75 mL of TRI-Reagent LS was added into the cells and was homogenised by pipetting the cells up and down. The cells were scrapped aside using a scrapper and was transferred into a 1.5 mL microcentrifuge tube which was incubated at room temperature for 5 minutes. Next, 100 µL of BCP was added into the homogenate and was vortex for 15 seconds. The mixture was left at room temperature for 15 minutes and was centrifuged at 12 000 *g* for 15 minutes at 4˚C. Following centrifugation, the mixture would be separated into three phases, the aqueous phase containing RNA, interphase containing DNA and organic phase containing proteins. The aqueous phase was transferred into a new microcentrifuge tube and 500 µL of isopropanol was added to precipitate the RNA. Meanwhile, the remaining interphase and organic phase was used for protein extraction in section 3.7.1. The mixture were stored at room temperature for 10 minutes and was centrifuged at 12 000 *g* for 8 minutes at 4˚C. The supernatant was removed, the pellet were washed using 75% ethanol and was centrifuge at 7500 *g* for 5 minutes at 4˚C. After the centrifugation, the ethanol wash was discarded and the pellet was air-dried for 10 to 15 minutes. Finally, the RNA was resuspended in 30 µL of nuclease-free water and incubated at 55˚C for 15 minutes. The RNA was stored in -80˚C when not in use.

3.6.2 Spectrophotometric Measurement of RNA

Spectrophotometry was performed to determine the purity and concentration of the RNA extracted. From each sample of RNA, 1 µL of sample was added onto the measuring point of the spectrophotometer and the optical density was measured at 230 nm, 260 nm and 280 nm. Nuclease-free water was used as blank.

3.6.3 Electrophoresis of RNA on Denaturing Agarose-Formaldehyde Gel

Electrophoresis of RNA was carried out in on 1% (w/v) denaturing agarose-formaldehyde gel with the final concentration of 1X MOPS and 2.2 M of formaldehyde. Firstly, 0.2 g of agarose was dissolved in 14.4 mL of sterile H_2O by heating at 100˚C. When all the agarose had dissolved, it was allowed to cool until 55˚C and 2 mL of 10X MOPS and 3.6 mL of 37% (v/v) formaldehyde were added. This mixture was poured into a gel-casting tray with comb and allowed to solidify. Samples and RNA loading dye were prepared in PCR tube, and were then incubated at 65˚C for 15 minutes, chilled on ice before they were loaded onto the gel. Electrophoresis was carried out using 1X MOPS as the electrophoresis buffer at 90 V for 45 minutes.

3.6.4 DNase Treatment of RNA

DNase treatment of RNA was performed by adding 2 µg of RNA to 1 µL of RQ1 buffer and 2 µL of RNase-free DNase in a final volume of 10 µL. Then, the sample was incubated for 30 minutes at 37˚C. After incubation, 1 µL of RQ1 DNase stop solution was added into the sample to stop the reaction of the DNase.

Following that, the sample was incubated at 65˚C for 10 minutes for the enzyme inactivation.

3.6.5 Real Time Reverse Transcriptase- Polymerase Chain Reaction (Real-Time RT-PCR)

Real-Time RT-PCR was carried out using QIAGEN Quantifast SYBR Green RT-PCR Kit (Bio-Rad) according to the manufacturer's instruction. All reactions were performed in a final volume of 25 µL. The mixture was store on ice until use. The components into the Real-Time mixture were shown in Table 3.7.

Table 3.7: Components for Real Time RT-PCR.

Components	Volume, µL
2x Quantifast SYBR Green RT-PCR Master Mix	12.5
RNase- Free Water	5.3
10 µM Forward Primer	2.5
10 µM Reverse Primer	2.5
Quantifase RT-Mix	0.2
50 µg/µL RNA	2.0
Total volume	25.0

The samples were placed in iCycler IQ5 Real-Time PCR Detection System (Bio-Rad) for quantitative PCR (Q-PCR). The parameter used in this assay was as shown in the Table 3.8.

The quantity of the target mRNA was normalised against a house-keeping gene, β-actin, which served as an internal control for the determination of the relative mRNA expression of the target gene.

3.6.6 Primers Used

The nucleotide sequences of primers used for Real-Time RT-PCR are shown in Table 3.9.

3.7 Western Blot Analysis

3.7.1 Preparation of Protein Samples

Total cellular protein was extracted using Tri-Reagent® LS. The interphase and organic phase from section 3.6.1 was used to extract the protein. First, 300 µL of 100% ethanol was added into the mixture, mixed and stored at room temperature for 2 to 3 minutes. Then, the mixture was centrifuged at 2000 *g* for 5 minutes at 4˚C to precipitate the DNA in the mixture. After centrifugation, 300 µL of the supernatant was transferred to a new tube and 900 µL acetone was added. The mixture was mixed and stored in room temperature for 10 minutes before subjecting it to centrifugation at 12, 000 *g* for 10 minutes at 4˚C. Following centrifugation, the supernatant was removed and 500 µL of guanidine hydrochloride with 95% ethanol and 25% of glycerol mixture (GEG) was added to disperse the pellet. Another 500 µL of GEG was added to make the solution final volume as 1 mL. The mixture was stored at room temperature for 10 minutes and centrifuged at 8000 *g* for 5 minutes at 4˚C. The pellet was then washed and

dispersed using vortex with 1 mL of GEG twice. Next, 1 mL of 95% ethanol with 25% glycerol was added to the pellet and was stored at room temperature for 10 minutes. The mixture was centrifuge at 8000 *g* for 5 minutes at 4˚C. The supernatant was discarded and the pellet was air dried for 7 - 10 minutes and was dissolved in 1% (v/v) SDS by incubating at 50˚C. The protein samples were stored at -80˚C until use.

3.7.2 Bio-Rad Protein Assay

The concentration of the protein samples extracted was determined using the Bio-Rad Dc Protein Assay reagent kit (Bio-Rad, USA) according to the manufacturer's instruction. A standard curve was produced in each assay through suitable dilutions of a 1.7 mg/mL bovine serum albumin (BSA) stock solution in order to achieve the concentration of 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, 1.0 mg/mL, 1.2 mg/mL and 1.4 mg/mL. For the protein samples, 5 µL of samples was diluted with 20 µL of 10% SDS. From the diluted solution, 5 µL was loaded into a 96-wells micro-titer plate followed by the addition of the protein assay reagents. The samples and the reagents were mixed properly and were incubated at room temperature for 15 minutes. The absorbance was read at 750 nm using a microplate reader (TECAN). Lastly, the protein sample concentrations were calculated from the standard curve using Microsoft Excel.

3.7.3 Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE stacking gels and separating gels were prepared using 10% (w/v) and 5% (w/v) of acrylamide respectively. The gels were prepared from the stock solutions in Table 3.4 with the composition as shown in Table 3.10.

Table 3.10: Composition of SDS-PAGE Separating and Stacking Gel.

Gel Component	10% (w/v) Separating Gel	5% (w/v) Stacking Gel
SDS-PAGE upper gel buffer	-	0.625 mL
SDS-PAGE lower gel buffer	1.25 mL	-
40% acrylamide: bisacrylamide (37.5:1)	1.250 mL	0.313 mL
ddH_2O	2.445 mL	1.535 mL
10% (w/v) ammonium persulfate	50 µL	25 µL
TEMED	5.0 µL	2.5 µL
Total volume	5.0 mL	2.5 mL

Electrophoresis was carried out using the Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad, USA) and the gel apparatus being assembled as described by the manufacturer. First, the resolving gel was poured until 2.5 cm from the upper edge of the inner glass plate. Then, butan-1-ol was added forming a layer over the

gel to exclude air bubbles. The gel was allowed to polymerise for 30- 40 minutes. When the gel was set, butan-1-ol was removed by washing off using distilled water and upper surface was dried using filter paper. Next, stacking gel was poured in and comb was added. After the polymerisation of the stacking gel, the comb was removed and the wells were washed with distilled water. Following that, the gel was placed into the electrophoresis tank. The upper and lower chambers were filled with 1X SDS-PAGE running buffer which was diluted from the stock solution in Table 3.4.

Protein from different samples was prepared in an equal volume, 50 µg and was mixed with 5 µL of SDS-PAGE gel loading buffer (Table 3.4). The mixture was heated at 100˚C for 2 minutes. Then, the samples were cooled and loaded into the gel. A protein marker (8 µL) (Appendix A) was loaded into the first lane of the gel. Electrophoresis was carried out at a voltage of 100 V for 1 hour or until the bromophenol blue marker dye was within 0.5 cm of the lower end of the gel. The gel was then subjected to Western blotting.

3.7.4 Coomassie Blue Staining of the SDS-PAGE Gel

The gel was removed from the glass plate and the stacking gel was cut away. Next, the gel was soaked into the Coomassie Blue staining solution (Table 3.5) for one hour. After that, the gel was soaked into the de-staining solution (Table 3.5) for four hours to remove the excess stain and allow the protein bands to be visible. Lastly, the gel picture was captured.

3.7.5 Western Blotting

Electrophoretic transfer of proteins was done using Bio-Rad Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA). The proteins were transferred from the SDS-PAGE gel to polyvinylidene fluoride (PVDF) transfer membrane (Milipore, USA) as described by the manufacturer. First, the gel was removed from the glass plates and the stacking gel was cut away carefully. The gel was then soaked in Towbin transfer buffer (Table 3.6) at room temperature for 15 minutes. During this period, four filter papers and PVDF membrane were cut into the size of the gel. Then, the membrane was soaked in methanol for two minutes then in distilled water for two minutes and finally being stored in transfer buffer until ready for transfer. Meanwhile, the filter paper and the sponge pads were soaked in the transfer buffer. When all the materials were ready, the membrane, gel, filter paper and filter pads were assembled onto the blotting cassette as described by the manufacturer. The cassette was subjected to electro-blotting in the blotting tank which was filled with transfer buffer. It was electrophoresed at 100 V with the temperature of 4˚C for 75 minutes. After the transfer, the membrane was stored in -80˚C until use or used immediately for immunodetection.

3.7.6 Probing of Blotted PVDF Transfer Membranes

The PVDF membranes were probed immunochemically using SNAP i.d.™ (Milipore, USA) according to the manufacturer's instruction. The membrane was assembled properly onto the block holder which was previously wet with distilled

water. The non-specific protein binding sites were blocked by incubating the membrane with blocking buffer solution (Table 3.6) for 5 minutes. Then, the blocking solution was removed followed by the addition of primary antibody that had been diluted in the ratio of 1:1000. The membrane was incubated for 10 minutes. Next, the membrane was washed three times with 10 mL of Tris-buffer saline (TBS). After finished washing, secondary antibody, peroxidase-conjugated the anti-rabbit IgG, diluted in the ratio of 1:2500 was added onto the membrane and incubated for another 10 minutes. Another set of washing with TBS was performed. For the detection of ghrelin, ghrelin antibody was used as the primary antibody while for β-actin, the house keeping gene, β-actin primary antibody was used.

3.7.7 Chemiluminescent Detection of Membrane Bound Antigen-Antibody Complexes

After blotting the membrane with secondary antibody, the membrane was placed on the flat board in the FluoroChem Chemiluminescent (Alpha Innotech, USA) cabinet. Immobilon^{TM} Western detection reagent (Milipore, USA) which was prepared as shown in Table 3.6 was added onto the membrane surface. The detection reagent was prepared and incubated for 15 minutes before use. Prior to the detection of the antigen-antibody complexes, the camera focus and the parameters for the capture of the membrane image was adjusted according to the appearance and clarity of the bands viewed on the monitor. The protein marker was aligned with the image captured to verify the protein size of the band.

3.7.8 Stripping of the Antibody Blotted Membrane

After the detection of the antigen-antibody complex, the membrane-bound antibody was stripped off using stripping reagent (Table 3.6). The membrane was soaked in the stripping reagent for 15 minutes and shake on a rocker. Then, the membrane was washed twice with Tris-buffer saline through shaking also for 10 minutes each. Subsequently, the wash solution was removed and the membrane was briefly soaked in methanol, air dried and stored at -80˚C. The membrane could be used again for blotting.

3.7.9 Densitometry analysis of Western Blotting Results

Immunodetected protein bands were quantified using the FluoroChem System Software (Alpha Innotech, USA). The percentage of ghrelin protein expression was calculated by normalising its level against β-actin protein expression. The values obtained were converted into percentage that was relative to the control sample which was assigned as 100%.

CHAPTER 4

RESULTS

4.1 AR42J Cell Culture

The AR42J rat pancreatic cell line was used in this experiment as ghrelin is expressed in pancreas (Wierup, Svensson, Mulder, & Sundler, 2002) and therefore the expression of ghrelin mRNA and protein could be studied. This cell was an epithelial cell obtained from the Winstar strain rat. The cell was stimulated with different concentration of cytokines (5, 10, 25, 50 ng/mL) for 24 hours after it reached 60-70% confluence.

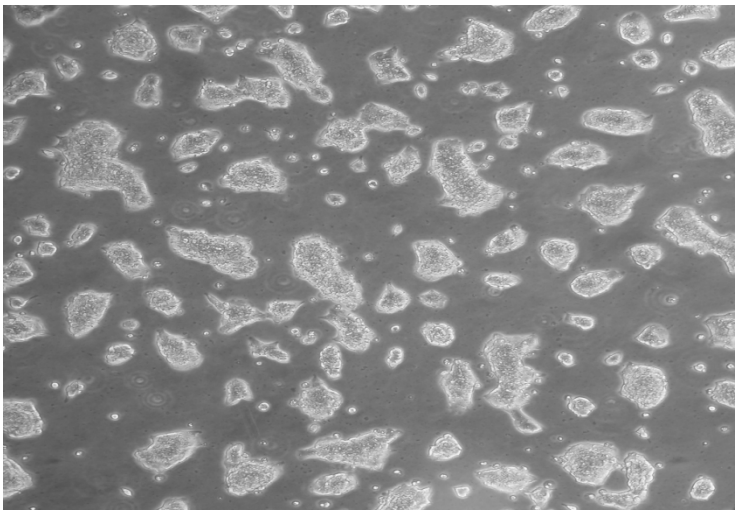


Figure 4.1: The AR42J Cell Line.

The AR42J cells are a type of epithelial cells. The confluence of the cell shown in the figure is approximately 60%.

4.2 Total Cellular RNA Extraction

4.2.1 Concentration and Purity of Total Cellular RNA Extracted

The concentration and purity of the RNA extracted was measured using spectrophotometer as described in section 3.6.2. The concentration of the RNA in the sample was determined through the absorbance value obtained at the wavelength of 260 nm and 280 nm. On the other hand, the purity of the RNA sample was determined using the ratio between absorbance at 260 nm and absorbance at 280 nm (A_{260}/ A_{280}). The results for the RNA concentration and purity are as shown in Table 4.1 and 4.2 for TNF-α and IL-6 stimulated cells, respectively. From the results obtained, all the samples A_{260}/ A_{280} ratio were in the range of 1.7 to 2.0. This indicated that the RNA extracted in this study was pure and free from contaminants (Molecular Research Center, 2011).

Table 4.1: Total Cellular RNA Concentration and A_{260}/ A_{280} Ratio for TNF-α Stimulated AR42J Cells.

Amount of TNF-α Treatment	260 nm	280 nm	A_{260}/ A_{280}	Concentration, ng/µL
0 ng/mL	1.12	0.58	1.93	2224.00
5 ng/mL	0.91	0.48	1.90	1806.00
10 ng/mL	0.70	0.37	1.89	1400.00
25 ng/mL	0.83	0.43	1.92	1644.00
50 ng/mL	0.53	0.29	1.87	1058.00

Table 4.2: Total Cellular RNA Concentration and A_{260}/ A_{280} Ratio for IL-6 Stimulated AR42J Cells.

Amount of IL-6 Treatment	260 nm	280 nm	A_{260}/ A_{280}	Concentration, ng/µL
0 ng/mL	0.78	0.43	1.82	1552.00
5 ng/mL	0.53	0.28	1.85	1052.00
10 ng/mL	0.87	0.50	1.75	1738.00
25 ng/mL	0.96	0.54	1.79	1906.00
50 ng/mL	0.44	0.25	1.73	884.00

4.2.2 Integrity of Total Cellular RNA Extracted

The RNA of the AR42J cells were extracted after 24 hours of stimulation with different concentrations of cytokines and was subjected to 1% (w/v) denaturing agarose-formaldehyde gel electrophoresis (section 3.6.3) to check its integrity. Figure 4.2 shows the gel image which was captured under UV transilluminator for TNF-α and IL-6 stimulated cells, respectively.

There were two distinct bands observed on the gel and was labeled as 28S rRNA and 18S rRNA. The integrity of the total cellular RNA extracted was evaluated through the presence of 2:1 ratio between the 28S rRNA and 18S rRNA band on the gel image. The presence of this ratio indicates that the RNA extracted was intact (Applied Biosystems, 2011). Thus, the sample can be used to proceed with further investigation. There was some smearing between these two bands. This is most probably due to the presence of other RNAs like mRNA.

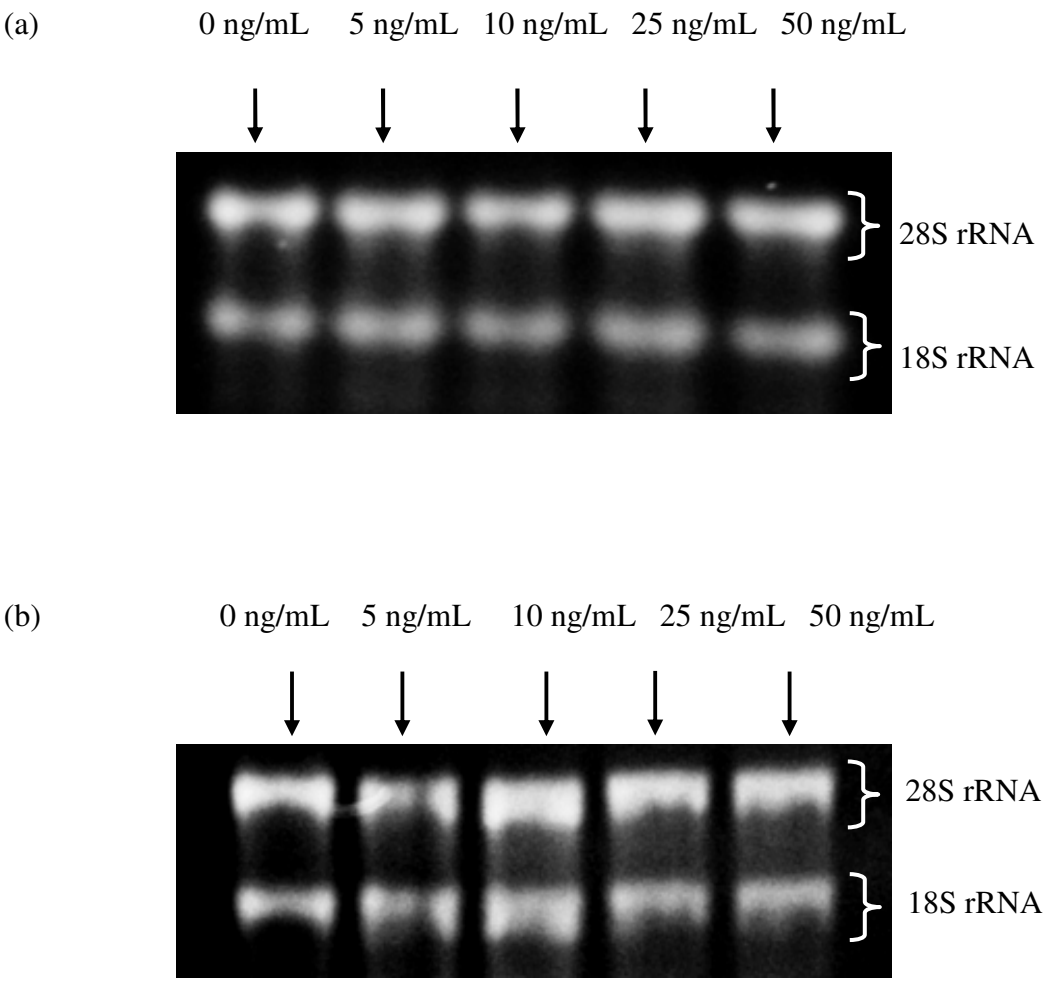


Figure 4.2: Denaturing Agarose-Formaldehyde Gel Electrophoresis of Total Cellular RNA Extracted From Cytokine Stimulated AR42J Cells.

(a) Gel image for TNF-α treated cells. (b) Gel image for IL-6 treated cells. The numbers labeled above the picture shows the concentration of the cytokine used for the stimulation. From both picture, the 28S rRNA bands are larger and wider than the 18S rRNA. Therefore, the 28S rRNA:18S rRNA ratio shown is approximately 2:1. The RNA extracted was intact.

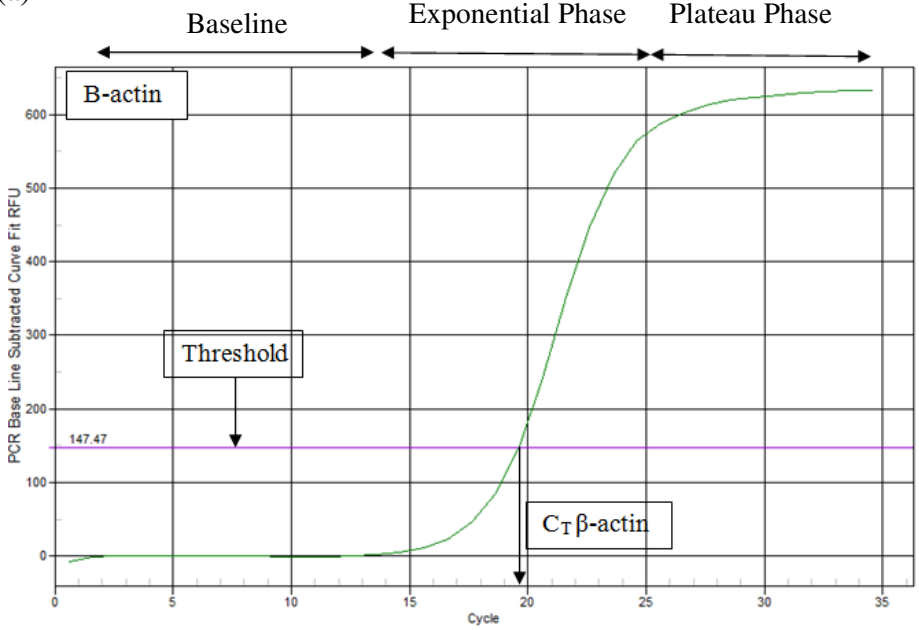
4.3 Real-Time RT-PCR

4.3.1 PCR Amplification of TNF-α and IL-6 Treated AR42J Cells

The Real-Time RT-PCR amplification curve was obtained from iCycler IQ5 Real-Time RT-PCR amplification system software and Quantifast SYBR Green RT-PCR Kit. The amplification chart in Figure 4.3 was a sigmoid curve showing that the target mRNAs were amplified. The Y-axis represents the amount of target mRNA against the background fluorescence and is known as the arbitrary fluorescent units while the X-axis represents the amplification cycle.

From the chart, the threshold for β-actin is 147.47 while ghrelin is 140.90. The C_T value which is the number of cycles needed for the arbitrary unit to cross the threshold level was calculated from the software itself. The C_T value for β-actin is approximately 20 cycles while ghrelin is approximately 28 cycles.

(a)



(b)

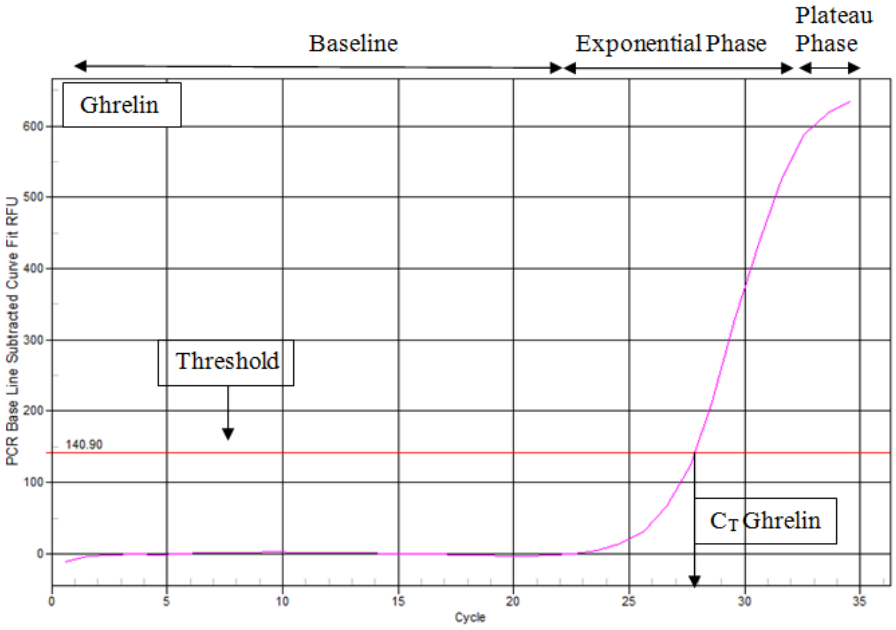


Figure 4.3: Real-Time RT-PCR Ghrelin and β-actin mRNA Amplification Chart.

Amplification chart for (a) β-actin, (b) ghrelin. The sigmoid curve of the PCR amplification chart shows that there was an increase in the fluorescence unit of the sample. The C_T was calculated by the amplification software and it indicates the minimum number f cycles needed for the arbitrary unit to cross the threshold level.

4.3.2 Melting Curve Analysis for β-actin and Ghrelin

Melting curve analysis was performed to verify the specificity of the primers to the target gene. A well designed and specific primer would produce only one peak in the melting curve chart. From Figure 4.4, the melting temperature for β-actin and ghrelin are 85.5˚C and 84.5˚C, respectively. Since there is only one sharp peak observed in the melting curve chart, the primer used was specific to the target gene which in turn dictates that the result for mRNA expression was specific to the target gene.

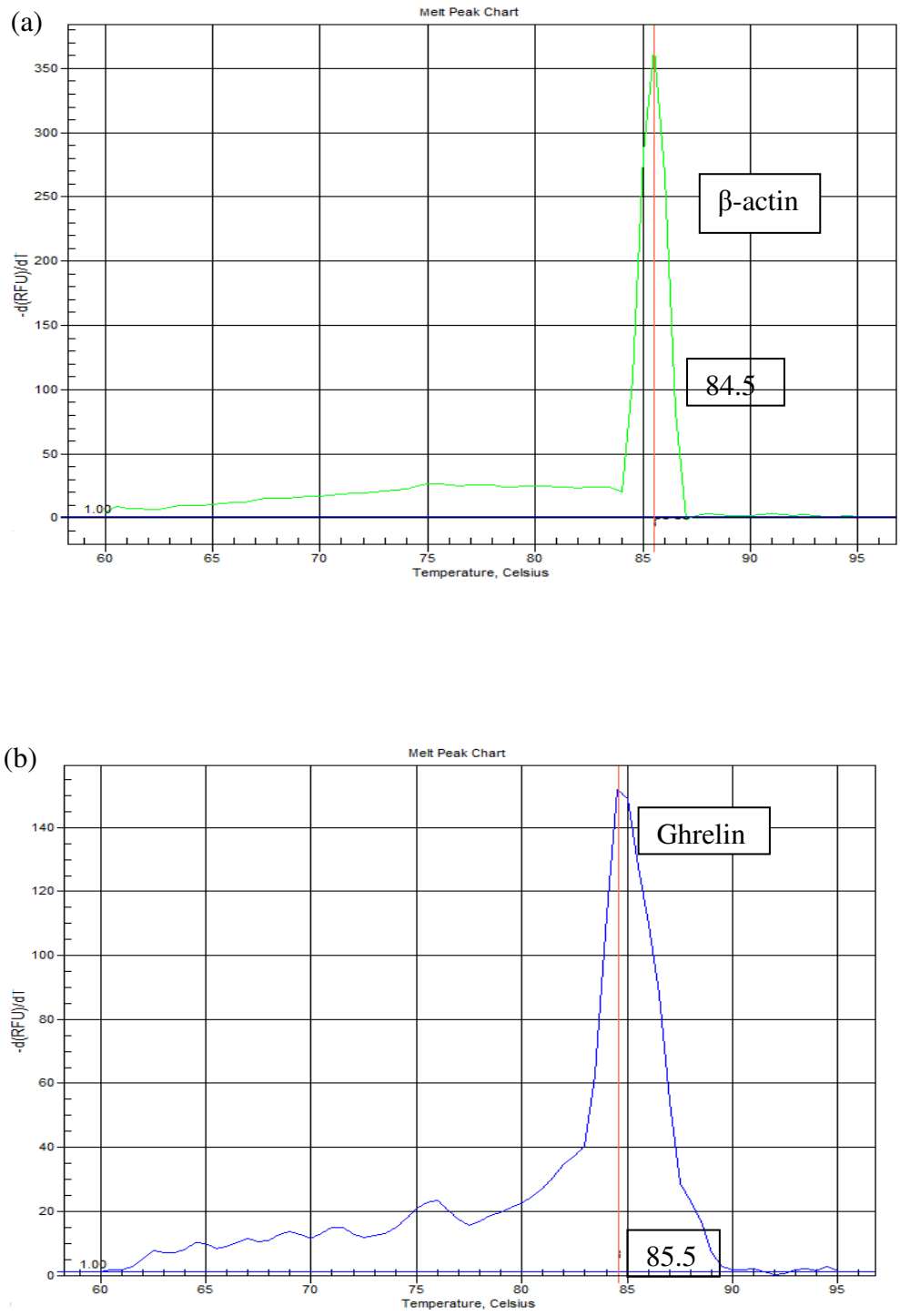


Figure 4.4: Melt Curve Analysis Chart.

(a) Melt curve analysis for β-actin. The melting temperature is 84.5˚C. (b) Melt curve analysis for ghrelin. The melting temperature is 85.5˚C. For both chart, only one peak was observed. This shows that the primer used only amplified one gene.

4.3.3 Ghrelin mRNA Expression in TNF-α and IL-6 Treated AR42J Cells

Ghrelin gene mRNA expression was detected using Real-Time RT-PCR. Delta delta C_t formula was used for the calculation of the relative mRNA expression (Appendix B). The gene expression was normalised against β-actin, a housekeeping gene. The controls used were the cells that were not treated with any cytokines and their respective gene expression was assigned as 1.00. Table 4.3 and 4.4 shows the ghrelin mRNA expression in TNF-α and IL-6 treated cells, respectively while Figure 4.5 is the representation of the mRNA gene expression of ghrelin in TNF-α and IL-6 treated cells in a chart form.

4.4 Protein Analysis

4.4.1 Concentration of Total Cellular Protein Extracted

The amount of cellular protein extracted was measured using spectrophotometry as mentioned in section 3.7.2. A standard curve was plotted (refer to Appendix C) and the concentration of the protein extracted was calculated from the standard curve and recorded in Table 4.5.

Table 4.5: Concentration of Total Cellular Protein Extracted in TNF-α and IL-6 Treated AR42J Cells.

Amount of Cytokine Treatment	Total Cellular Protein Extracted	
	TNF-α	IL-6
0 ng/mL	1.4 µg/µL	3.0 µg/µL
5 ng/mL	2.3 µg/µL	2.8 µg/µL
10 ng/mL	2.0 µg/µL	3.5 µg/µL
25 ng/mL	2.1 µg/µL	2.9 µg/µL
50 ng/mL	2.2 µg/µL	2.3 µg/µL

4.4.2 Coomassie Blue Staining of SDS-PAGE Gel

Coomassie blue staining was performed to detect the presence of proteins on the SDS-PAGE gel. The gel image was as shown in Figure 4.6. There were many protein bands present on the gel and it was hard to identify and confirmed the presence of the protein band for ghrelin and β-actin. Thus, Western blotting was performed to specifically detect the protein band for ghrelin and β-actin. Another set of membrane were used for Western blotting.

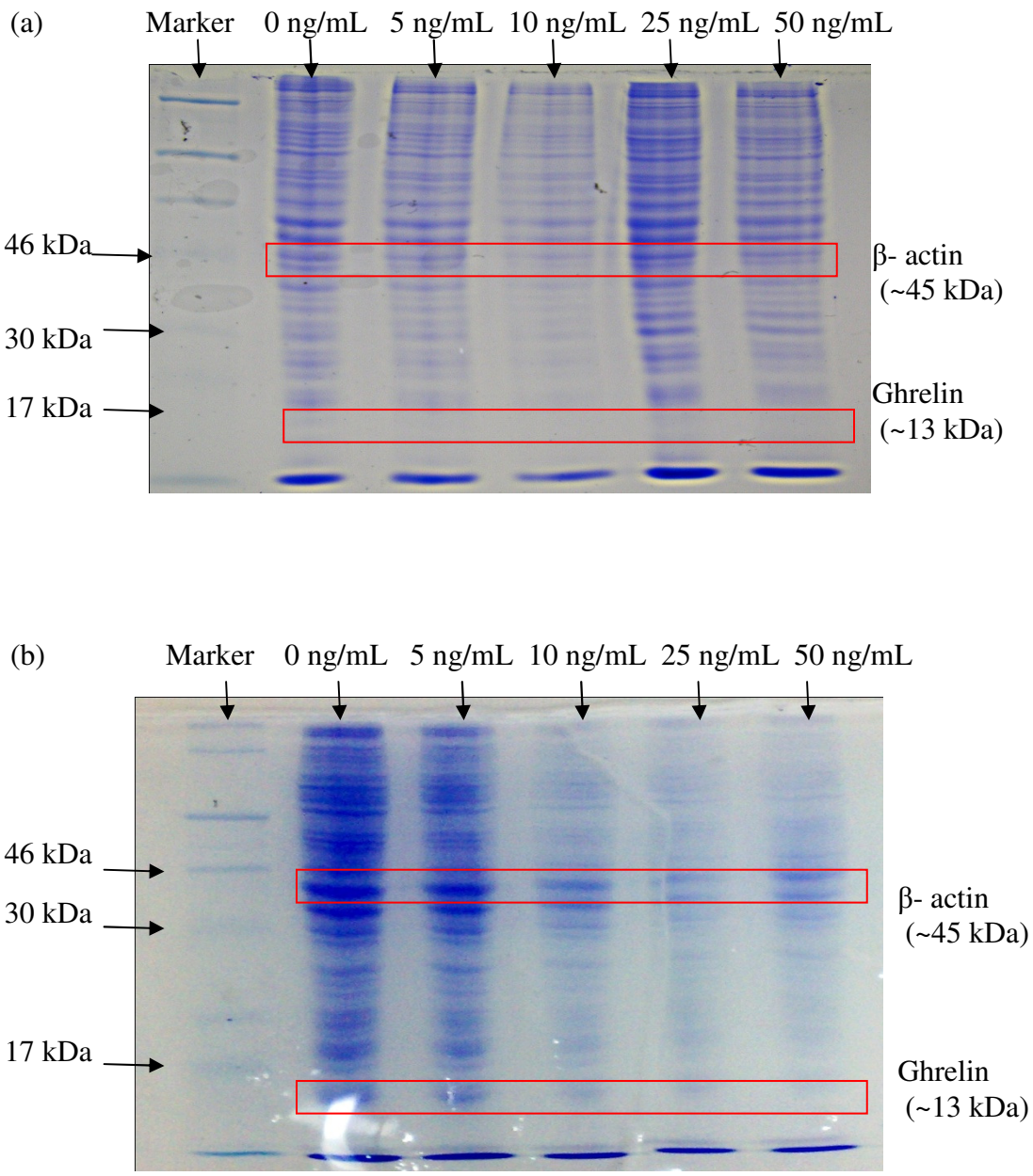


Figure 4.6: Coomassie Blue Staining of Protein Extracted from Cytokine Stimulated AR42J Cells.

The image shows protein extracted from (a) TNF-α stimulated cells and (b) IL-6 stimulated cells. The protein size for β-actin is 45 kDa while ghrelin is 13 kDa. Some of the protein bands were very fade and could not be observed due to the low concentrations of sample loaded into each well. Thus, Western blot was performed to confirm the presence of ghrelin and β-actin.

4.4.3 Ghrelin Protein Expression in TNF-α and IL-6 Treated AR42J Cells

After blotting the membrane with protein, immunodetection of ghrelin protein was performed using ghrelin antibody (Santa Cruz Biotechnology, USA) as described in section 3.7.6 and 3.7.7. Figure 4.7 shows the immunodetection of ghrelin protein extracted from cells treated with different cytokines. After Western blotting, densitometry anaylsis was performed to quantitatively measure the protein expression. This is because observing the protein bands on the membrane itself is not accurate as the concentration of protein load into each lane may not be precise (Figure 4.7). β-actin is a housekeeping gene where its protein is constantly expressed and is not affected by the cytokine treatment (Chew, Chew, Najimudin, & Tengku-Muhammad, 2007).

CHAPTER 5

DISCUSSION

5.1 AR42J Cells as the Model of Study

AR42J cells are epithelial cells extracted from a Winstar Rat pancreas (ATCC, 2011). Lai, Cheng, Ko and Leung (2005) successfully detected the presence of ghrelin mRNA and its receptor in this cell line. This shows that this cell line could be used as a model system for the study of ghrelin gene expression. Indeed, this cell line has been used for the study of the pancreas exocrine and endocrine function (Rosewicz, Riecken, & Wiedenmann, 1992), signal transduction (Lai et al., 2005) and gene expression studies. TNF-α's and IL-6's receptor are present in all somatic cells (Apostolaki et al., 2010). The AR42J cell is an example of somatic cell and thus, it expresses TNF-α and IL-6 receptors. Studies on these cytokines have been performed on this particular cell line and these cytokines were shown to be involved in the progression of acute pancreatitis (Boole, 2006; Chan & Leung, 2009; Langley, 2008). Hence this cell line could be used as the model system for the study of the roles played by these cytokines in this disease's progression (Sandoval et al., 2010; Yu, Lim, Kim, & Kim, 2005).

Ghrelin's role in the immune system has been widely studied. A few studies have shown that ghrelin has anti-inflammatory roles, in which it decreases the production of pro-inflammatory cytokines (Dixit et al., 2004; Mager et al., 2008;

Moreira & Soares, 2007). These studies provide evidence of the relationship between ghrelin and the pro-inflammatory cytokines. Since TNF-α and IL-6 are involved in the progression of acute pancreatitis and ghrelin was shown to exert anti-inflammatory effects on these cytokines, the cell line was used as the model of study to investigate ghrelin gene expression in a condition similar to acute pancreatitis; where by the levels of TNF-α and IL-6 are high in this disease.

5.2 Future Studies

In this study, many factors are suggested to have caused the decrease in ghrelin gene expression by the TNF-α and IL-6. It is also suggested that both cytokines act indirectly in down-regulating ghrelin gene expression. However, these are only suggestions. Further investigation should be performed to confirm them.

For both TNF-α and IL-6, the development of insulin resistance is suggested to be the cause of decreased in ghrelin expression. In this case, the levels of insulin receptor and insulin itself should be investigated. Real-Time RT-PCR and Western blot can be performed for the quantitative analysis of insulin and its receptor in cells stimulated with these cytokines. Besides, immunoassay could also be performed to detect the levels of insulin. Since there are some ambiguities on either insulin itself or the level of insulin resistance plays a role in affecting ghrelin gene expression, further studies should be carried out.

Besides, increase in glucagon and decrease in cAMP was also suspected as the cause of decrease ghrelin synthesis. Glucose levels were suggested to be high that it inhibits ghrelin production. Thus, the levels of these three substances should be investigated along with ghrelin gene expression in cells stimulated with TNF-α and IL-6.

Since the relationship between TNF-α and IL-6 with ghrelin gene expression was established, more detailed studies could be performed. A time-course ghrelin mRNA and protein expression could be investigated. Besides, there were two molecular forms of ghrelin; acylated ghrelin and des-acyl ghrelin. The exact form of ghrelin that is affected by the cytokines could be studied as each molecular forms of ghrelin carry different functions. There are many ELISA kits available in the market nowadays for the specific detection of either ghrelin or des-acyl ghrelin and this could be used in the future study of this project.

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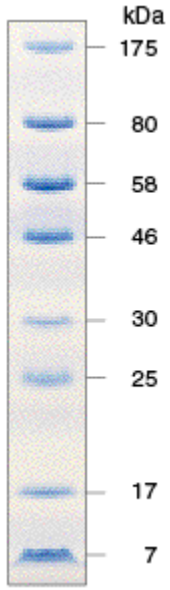
APPENDICES

Appendix A

The protein marker used for SDS-PAGE was as shown below:



The picture was adapted from New England Biolabs (2010).

Appendix B

For the quantification of relative mRNA expression of ghrelin using Real-Time RT-PCR, the delta delta C_t formula was used (Dharmaraj, 2011).

$$[delta][delta]C_t = [delta]C_{t,sample} - [delta]C_{t,reference}$$

[delta] C_t = C_t value for any sample normalized to the housekeeping gene

$[delta]C_{t,reference}$ = C_t value for the calibrator which is also normalized to the housekeeping gene

Appendix C

The picture below shows the standard curve of different concentration of BSA. The concentration of the protein extracted was calculated from the standard curve below.