Innovation of gonadotropin releasing hormone (GnRH)-
modified nanocarrier delivered to induce testicular and mammary cell apoptosis

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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นวัตกรรมการนำส่งยีนเหนี่ยวนำการตายแบบอะพอพโทซิสด้วยพาหะนำส่งระดับนาโนเชื่อมติดกับ
ฮอร์โมนโกนาโดโทรปินรีลิสซิ่งในเซลล์อัณฑะและเต้านม

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วัตถุประสงค์ของการวิจัยนี้คือ การตรวจสอบการนำไปใช้ของอนุภาคไคโตซานระดับนาโนที่ถูกดัดแปลงให้มีคุณสมบัติเป็นตัวนำส่งยีนเหนี่ยวนำการตายแบบอะพอพโทซิสเข้าสู่เซลล์ที่มีตัวรับฮอร์โมนโกนาโดโทรปินรีลิสซิ่ง (เซลล์อัณฑะ และเซลล์มะเร็งเต้านม) การศึกษานี้ได้มีการรายงานผลของอนุภาคไคโตซานระดับนาโนเชื่อมติดกับฮอร์โมนโกนาโดโทรปินรีลิสซิ่ง (Gonadotropin Releasing Hormone-modified Chitosan; GnRH-CS) เพื่อนำส่งยีนอย่างมีเป้าหมาย และการใช้ GnRH peptideในการระบุเป้าหมายการนำส่งยีนไปสู่เซลล์ที่มีตัวรับฮอร์โมน (GnRH receptor; GnRHR) สำหรับการวิเคราะห์คุณสมบัติทางกายภาพของอนุภาคด้วยเทคนิคทางเคมีต่างๆ ผลที่ได้คือ GnRH-CS สามารถจับตัวกับพลาสมิดดีเอ็นเอด้วยแรงทางประจุไฟฟ้า และก่อให้เกิดอนุภาคที่เป็นประจุบวกระดับนาโน จากการศึกษาเพาะเลี้ยงเซลล์แบบ 2 มิติ และ 3 มิติพบว่าอนุภาค GnRH-CS สามารถนำส่งยีนรายงานผลไปสู่เซลล์เพาะเลี้ยงจากเซลล์ไตของตัวอ่อนมนุษย์ (HEK293T cell) ที่มีการดัดแปลงให้มี GnRHR เพื่อใช้เป็นเซลล์จำลองที่มีการแสดง GnRHR, เซลล์สืบพันธุ์เพศผู้จากหนูเมาส์ (GC-1 cell) และเซลล์มะเร็งเต้านมจากมนุษย์ (MDA-MB-436 cell) ได้อย่างจำเพาะ และยังพบว่า GnRH-CS สามารถนำส่งยีนได้ดีกว่า และเป็นพิษต่อเซลล์น้อยกว่าอนุภาคไคโตซานระดับนาโนที่ไม่ได้ดัดแปลง สำหรับการศึกษาภายในร่างกายสัตว์ ได้มีการใช้ยีน Tumor Necrosis Factor alpha (TNF-alpha) เพื่อเหนี่ยวนำการตายในเซลล์อัณฑะของหนูแรทโดยการฉีดสารเข้าอัณฑะโดยตรง (Intra-testicular injection) ผลการศึกษาพบว่ามีการลดลงอย่างมีนัยสำคัญทางสถิติ ($P<0.001$) ของขนาดอัณฑะในวันที่ 7 หลังฉีดในทุกกลุ่มทดลอง และลดลงอย่างต่อเนื่องอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ในวันที่ 14 และ 28 หลังฉีด ในกลุ่มอนุภาคที่มียีน TNF-alpha (GnRH-CS/TNF-alpha) และกลุ่มอนุภาคเปล่า (GnRH-CS) ตามลำดับ สำหรับวันที่ 35 ซึ่งเป็นวันสิ้นสุดการทดลองพบว่าขนาด และน้ำหนักอัณฑะในกลุ่ม GnRH-CS/TNF-alpha และกลุ่ม GnRH-CS ลดลงจากกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.001$) พบว่าไม่มีการเปลี่ยนแปลงอย่างมีนัยสำคัญทางสถิติของระดับฮอร์โมนเทสโทสเตอโรนหลังฉีด จากการตรวจทางจุลพยาธิวิทยา และการตรวจอัลตราซาวน์พบว่ามีการเสื่อม และการตายของเซลล์อัณฑะในกลุ่ม GnRH-CS/TNF-alpha และกลุ่ม GnRH-CS ขณะที่กลุ่มควบคุม และกลุ่ม TNF-alpha มีลักษณะเซลล์อัณฑะปกติ โดยระดับความรุนแรงของการเสื่อม และการตายของเซลล์อัณฑะในกลุ่ม GnRH-CS/TNF-alpha มีมากกว่ากลุ่ม GnRH-CS นอกจากนั้นยังมีการศึกษาวิจัยแบบนำร่องเพื่อค้นหาข้อมูลเบื้องต้น โดยใช้หนูเมาส์ไร้ขนที่ได้รับการปลูกถ่ายเซลล์มะเร็งเต้านมเข้าใต้ผิวหนัง พบว่ายีน TNF-alpha ที่ถูกนำส่งด้วยอนุภาค GnRH-CS ทำให้เกิดการแบ่งตัวของเซลล์มะเร็งที่ช้าลง สรุปได้ว่างานวิจัยนี้อาจนำไปประยุกต์ใช้ในการทำหมันสัตว์เพศผู้แบบไม่ผ่าตัด และการรักษามะเร็งเต้านมด้วยยีนบำบัดได้ในอนาคต

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Chatwalee Boonthum : Innovation of gonadotropin releasing hormone (GnRH)-modified nanocarrier delivered to induce testicular and mammary cell apoptosis. Advisor: Asst. Prof. Dr. SUPPAWIWAT PONGLOWHAPAN Co-advisor: Prof. Dr. KAYWALEE CHATDARONG,Dr. Teerapong Yata

The main overall goal of this study is to investigate the application of modified chitosan as a potential vector for apoptotic gene delivery to gonadotropin-releasing hormone receptor (GnRHR)-expressing cells (i.e. testicular cells and mammary cancer cells). This study reported Gonadotropin Releasing Hormone-modified Chitosan (GnRH-CS) nanoparticle as a promising vector for targeted gene delivery, and a GnRH peptide was used for active targeting of a transgene in GnRHR expressing cells. The physiological characterizations of the prepared nanoparticle were investigated by proton nuclear magnetic resonance spectroscopy (^{1}H NMR), Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and Nanosizer. The prepared GnRH-CS was able to condense DNA to form positively charged nanoparticles. In both two-dimensional (2D) and three-dimensional (3D) cell cultures systems, this alternative gene transfer strategy could specifically deliver the reporter genes to a transiently transfected Human Embryonic Kidney cell line model system expressing GnRHR, mouse-derived spermatogonia cells (GC-1 cell line) and mammary cancer cells (MDA-MB-436). Importantly, GnRH-CS exhibited higher transfection activity and lower cell toxicity compared to unmodified CS at the same ratio. From *in vitro* to *in vivo* study, Tumor Necrosis Factor alpha (TNF-α) was exploited as a therapeutic gene delivered by GnRH-CS in order to induce testicular cell death in male rat via intra-testicular injection. Significant differences in a reduction in testicular volume on day 7 ($P<0.001$) were found in all treated groups, compared to the pre-treatment volume. The testicular volume continued to reduce on day 14 ($P<0.05$) and 28 ($P<0.05$) in GnRH-CS/TNF-alpha and GnRH-CS groups, respectively. On day 35, after castration, testicular weight and volume of dissected testis were significantly lower in GnRH-CS/TNF-alpha and GnRH-CS groups ($P<0.001$). Serum testosterone levels did not differ ($P>0.05$) throughout the observation period. Ultrasonographically and histopathologically, GnRH-CS/TNF-alpha and GnRH-CS induced testicular degeneration and death while TNF-alpha and control groups showed normal findings. GnRH-CS/TNF-alpha treated animals showed higher severity degree of testicular degeneration compared to GnRH-CS treated animals. Moreover in pilot study using mammary gland tumor-bearing nude mice showed promising results in tumor suppression when TNF-α was delivered by the GnRH-CS. It was possible that our study might be apply for non-surgical sterilization and mammary cancer treatment in the future.

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Field of Study:	Theriogenology	Student's Signature ....
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ABBREVIATIONS

^{1}H-NMR:	^{1}H nuclear magnetic resonance spectroscopy
2D:	Two-dimensional
3D:	Three-dimensional
ATR-FTIR:	Attenuated total reflectance Fourier transform infrared spectroscopy
BTB:	Blood-testis barrier
$CaCl_2$:	Calcium chloride
$CdCl_2$:	Cadmium chloride
CMIA:	Chemiluminescent microparticle immunoassay
CS:	Chitosan
CULAC:	Chulalongkorn University Laboratory Animal Center
DADs:	Degrees of amidation
DC:	Dystrophic calcification
DDA:	Degree of deacetylation
DI:	Deionized
DMA:	N-dimethylacetamide
DMSO:	Dimethylsulfoxide
DNS:	Degree of N-succinylation
DS:	Degree of substitution
E.coli:	*Escherichia coli*
EAO:	Experimental autoimmune orchitis
EDC:	N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride
EP:	Enhanced permeation and retention
FBS:	Fetal bovine serum
FDA:	Food and drug administration
FSH:	Follicle-stimulating hormone
GCV:	Ganciclovir
GFP:	Green fluorescent protein
GLD:	Generalized lymph proliferative disease

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GnRH:	Gonadotropin releasing hormone
GnRHR:	GnRH receptor
GPC:	Gel permeation chromatography
H&E:	Hematoxylin and Eosin
HEK:	Human embryonic kidney
HOBt:	1-Hydroxybenzotriazole hydrate
HPLC:	High performance liquid chromatography
HRs:	Hormone receptors
HSV-tk:	Herpes simplex virus thymidine kinase
IRE:	Internal reflection element
LH:	Luteinizing hormone
LUC:	Luciferase
Mn:	Number-averaged molecular weight
Mw:	Weight average molecular weight
NANOTEC:	National nanotechnology centre
NHS:	N-Hydroxysuccinimide
NIMT:	National Institute of Metrology
NLC:	Nanostructured lipid nanocarrier
NPs:	Nanoparticle
NSCS:	N-succinyl chitosan
NSTDA:	National Science and Technology Development Agency
PCR:	Polymerase chain reaction
PDI:	Polydispersity index
pDNA:	Plasmid DNA
PEG:	Polyethylene glycol
pGFP:	Plasmid green fluorescence protein
pLuc:	Plasmid luciferase
SCO:	Sertoli cell only syndrome
SEM:	Standard error of the mean
STF:	Seminiferous tubular fluid
TCCF:	Total corrected cellular fluorescence

TMGT:	Testis mediated gene transfer
TNBC:	Triple-negative breast cancer
TNF-α:	Tumor necrosis factor alpha
TRAIL:	TNF-related apoptosis-inducing ligand
TRF:	Thailand Research Fund
ULA:	Ultralow attachment
WHO:	World health organization

CHAPTER I

1.1 Importance and rationale

The direct use of nucleic acid as a therapeutic agent to treat gene-associated diseases (so-called gene therapy) by transferring exogenous nucleic acids into the appropriate cells has attracted great interest over the past few decades [1]. To achieve successful gene delivery, development of appropriate gene carriers is an important factor. A large number of gene delivery strategies have been investigated to improve both the stability and uptake of these therapeutic genes. Viral-based methods are typically the most efficient and intensively used delivery systems due to their highly evolved natural infection. However, their use as vectors for gene delivery immunogenicity [2, 3], and the potential for insertional mutagenesis, which can be oncogenic [4, 5]. These limitations have led to the development of non-viral delivery systems which are less immunogenic and can incorporate larger genetic units. Non-viral methods rely on the use of lipids, polymers and organic nanoparticles. However, the effectiveness of this system is hindered by its low transfection efficacy and low cell specificity [6]. To overcome this limitation, ligands with high affinity and specificity have been integrated into gene delivery vector for receptor-mediated endocytosis [7].

In this study we used Gonadotropin Releasing Hormone (GnRH) as a targeting ligand to increase cell specificity of chitosan polymer. GnRH binds to GnRH receptor (GnRHR) that was found to be expressed in normal reproductive tissues (e.g. mammary, endometrium, ovary, and prostate) [8]. Interestingly, GnRHR are overexpressed in reproductive related cancer tissues (i.e. prostate, mammary, endometrial, and ovarian cancers) [9]. Therefore, it is possible that targeted gene delivery to GnRHR-expressing cells could be a valuable tool for 1) the treatment of certain gene disorders affecting sexual development and reproductive function , 2) contraception and fertility control [10], and 3) gene therapy of cancer associated with reproductive system [9].

One aim of this study, we propose the induction of apoptosis in testicular tissue (as a male nonsurgical sterilization) instead of using commercial chemical agents to induce necrotic cell death. To generate the efficient method in order to address the pet overpopulation that is the big obstacle in the world [9, 11] and to reduce the

severe adverse reactions of commercial non-surgical sterilants for male animals such as massive necrosis and severe irritation of testicular tissue following intratesticular injection [12] The other goal of this study is to investigate the use of the prepared nanoparticle to mediate apoptotic gene (Tumor Necrosis Factor -alpha; TNF-alpha) delivery for mammary cancer treatment using GnRH-receptor expressing mammary gland tumor as a model. It is possible that our study can improve the quality of life in animals suffering from mammary cancer from the side effects of chemotherapy, surgical stress and postoperative complications [13].

1.2 Literature review

1.2.1 Gene therapy

Gene therapy also known as the insertion of exogenous nucleic acids into the target cells or tissue is also problematic [1]. With regard to testis, Testis mediated gene transfer (TMGT) has studied for making transgenic animal and reported that TMGT was a potential method for producing transgenic animal [14]. There are some routes to transfer exogenous gene to sperm cell such as DNA injection into seminiferous tubules, epididymis and testis [15]. However the research study reported that transgene transmission in mice by non-surgical TMGT using different transfect ants such as dimethylsulfoxide (DMSO), N-dimethylacetamide (DMA) or liposome (Lipofectin) caused reduction of germ epithelium of testes after DNA complex injections. So it must be elucidated to further applications of TMGT for in vivo gene transfer technology [14]. As for cancer treatment, a number of apoptosis-inducing genes have been identified and proposed as therapeutic genes in order to eliminate unwanted cells [16]. The Fas ligand, tumor necrosis factor -alpha, growth factor withdrawal, oncogenes, irradiation, ceramide and chemotherapeutic drugs can induce apoptosis [17]. In this study, we focus on a suicide gene (TNF-alpha) that can induce apoptosis, can be a valuable tool for testicular germ cell apoptosis as a sterilization [10] and reproductive related cancer treatment [9]. In order to make great progress in gene delivery, it is of paramount importance to develop effective carriers capable of delivering a gene of interest to target cells to achieve sufficient and sustained transgene expression. Viral vectors have

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received considerable attention and become powerful tools of gene transfer. Unfortunately, the clinical application of viral vectors is limited because of unfavorable immunological features and mutagenic integration for some viruses. These concerns have driven extensive attention to the development of non-viral vectors [18].

1.2.2 Non-viral carrier

Non-viral gene transfection carriers can be divided into polymer-based and lipid-based systems. As for polymer-based nanoparticles, Ideal points are the following properties 1) capable of DNA-condensing that generates particle complexes that are enough small to entry into cellular tissue by endocytosis, 2) endosomolytic properties, 3) biocompatibility, 4) easy to formulate and purification in large scale manufacture, and 5) easy to add high affinity targeting ligand. It has been approved as a food additive and incorporated into a wound-healing product [19]. In this study concentrates on Chitosan (CS) that is a linear polysaccharide derived from the deacetylation of chitin (found in the exoskeletons of crustaceans) with a typical degree of acetylation of less than 0.35 [20]. Many analytical tools are used to examine the degree of acetylation, such as ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR), Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), pyrolysis gas chromatography, gel permeation chromatography, UV spectrophotometry, first derivative of UV spectrophotometry, solid state NMR, thermal analysis, various titration schemes, acid hydrolysis and High Performance Liquid Chromatography (HPLC), separation spectrometry and near-infrared spectroscopy [20]. Chitosan are digested by lysosomal enzyme that resides in cellular tissue, therefore this non-viral carrier can be used for drug delivery system or controlled release applications. There are many applications of chitosan such as photography, cosmetic, artificial skin, wound healing product, food and nutrition, ophthalmology, water engineering, paper finishing, solid-state batteries, drug delivery system, biotechnology and fat trapper [20]. Among non-viral vectors, chitosan has been exploited as gene-delivery vectors due to its excellent biocompatibility and biodegradability [21]. However, the effectiveness of this system is hindered by its low transfection efficacy and low cell specificity. To overcome this

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limitation, ligands with high affinity and specificity, such as transferrin-, folate-, mannose-, and galactose-conjugated chitosan, have been integrated into gene delivery vector for receptor-mediated endocytosis [7]. In this study concentrates on how to develop nanocarrier mediated apoptotic gene for transferring gene of interested specifically to testicular tissue and mammary cancer cells. With regard to reproductive system, Gonadotropin-releasing hormone (GnRH) is the most important sex hormone that provides the primary driving force upon the reproductive axis, its receptor (GnRHR) is localized on testis [22] and interestingly, GnRHR are overexpressed in reproductive related cancer tissues (i.e. prostate, mammary, endometrial, and ovarian cancers) [9]. As a result, it may be good that GnRH peptide-modified non-viral carriers can bind specifically to GnRHR-expressing tissues.

1.2.3 Gonadotropin- releasing hormone (GnRH)

GnRH is a 10 amino acid peptide produced and secreted by the hypothalamus. GnRH binds to GnRHR on gonadotrope cells in the anterior pituitary, stimulating the biosynthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), affecting sex development and reproductive functions [23]. In addition to gonadotrope cells, GnRHR was found to be expressed in normal reproductive tissues (e.g. mammary, endometrium, gonads, and prostate) [8]. Interestingly, GnRHR are overexpressed in cancer tissues, either related (i.e. prostate, mammary, endometrial, and ovarian cancers) or unrelated (i.e. melanoma, glioblastoma, lung, and pancreatic cancers) to the reproductive system. Therefore, it is possible that targeted gene delivery to GnRHR-expressing cells could be a valuable tool for 1) the treatment of certain gene disorders affecting sexual development and reproductive function [24], 2) contraception and fertility control [10], and 3) gene therapy of cancer associated with reproductive system [9].

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1.2.4 Sterilization and population control

Pet overpopulation is the problem in many countries [11] There are over 500 million free roaming dogs in the world [25]. For example in US, 30 million puppies and kittens are born each year [26]. Moreover intact males can reproduce an immeasurable number of offspring, especially cats [26]. Stray dogs suffer from poor quality of life, chronic disease, parasites and malnutrition. Because of these, euthanasia techniques are method to control animal population in some countries such as the US, the UK and the EU. With regard to zoonosis, the World Health Organization (WHO) showed that canine rabies threatens over 3 billion people in Asia and Africa, tens of thousands of people dying from the disease every year [26]. As a result, pet overpopulation is a global crisis now. The mainstay to control the population of dogs and cats is surgical sterilization via ovariohysterectomy (spaying) and orchiectomy (castration). The most common approach to estrus prevention differs among countries. The spectrum of invasive and non-invasive methods allow for different treatment modalities depending on the cultural background of the pet owner, the clinician's preference or various factors related to the animal such as age, breed, temperament, intended use, household environment or the social, ethical, economic and regulatory features of each country. For example, Boonthum et al., 2015 [27] studied current attitudes of the pet owners in Bangkok, Thailand, towards species bias and gender differences in dog and cat surgical sterilization over the 5- year observation (2010-2014), dogs were more than cats to be sterilized and females were more than males to be sterilized in cat and dog. Females are more important from a population control point-of-view, interestingly a single intact male may produce more offspring in one year than a single female is capable of producing in a lifetime. However there are many adverse effects of surgical sterilization such as surgical risk, a few behavioral abnormalities, obesity and urinary incontinence [28]. Nonsurgical sterilization offer an alternative method to control population.

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1.2.5 Nonsurgical sterilization

There are many nonsurgical sterilization methods such as Immunocontraception (contraception vaccine),Hormonal down-regulation, Intratesticular/Intraepididymal/Intravas-deferens injections, Chemical targeting, Cytotoxin conjugates and other methods [29]. The nonsurgical techniques to control breeding in owned animals are to be suitable for the temporary sterilization (contraception). On the other hand, nonsurgical sterilization methods to control breeding in stray animal populations require for the mass sterilization (the permanent infertility). The ideal points of nonsurgical sterilization method are safe, effective, affordable, permanent and delivered in a single injection with predictable effects on behavior and health [30].

Nowadays, there are many commercial product for nonsurgical sterilization, although safety information of these are available, do not have detailed histopathological descriptions. Especially, Intratesticular, intraepididymal and intravasdeferens injections know as chemical castration, is a nonsurgical approach to male contraception because of inducing massive necrosis of testicular tissue, it was found the incidence of severe adverse reactions such as necrosuppurative orchitis and ulcerative dermatitis after administration. The chemical castration, require injection directly into the testis and cause sterilization. However the manufacturer claims that administration of these injections is not painful, in order to facilitate handling, there are recommendation by manufacturer to use sedative and anesthetic drug for dog and cat respectively. Neutersol® or EsterilSol™, a commercial solution of zinc gluconate neutralized by arginine, although it was approved by Food and Drug Administration (FDA), reported on histomorphology of the testis after administration [12] as well as zinc gluconate with dimethyl sulfoxide, no detailed histopathological descriptions of cases that required surgical sterilization with scrotal ablation could be found [31, 32]. The research study was showed that EsterilSol™ caused necrosuppurative orchitis and scrotal necrotizing dermatitis after injection [12]. Necrosis is one type of cell death, is

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caused by infection, toxins or trauma [33]. Another type of cell death is apoptosis, is effected by a program cell death, does not cause cell lysis and inflammatory reaction [33]. An important point to overcome the adverse effect of chemical castration is avoidance of necrosis and replaces with apoptosis.

1.2.6 Apoptosis in testis

Apoptosis is affected by a program cell death, does not cause cell lysis and inflammatory reaction. Pathway of cell death in testis, 75% of all male germ cells are usually eliminated by apoptosis in order to control testicular homeostasis, a ratio between sertoli cells and maturing germ cells, to eliminate cells with genetic defects [33]. There are three stages of apoptosis in the testis: 1) apoptosis in the fetal period, 2) the first wave of apoptosis during prepuberty and 3) sporadic apoptosis throughout adult life [34]. Apoptosis during male germ cell development is divided into intrinsic and extrinsic pathway. As for intrinsic pathway or mitochondrial pathway and molecules of one pathway can influence the other, during the developing gonad there is apoptosis of excess cells and this period apoptosis is largely dependent on Bcl-xL and Bax [35]. The lower levels of Bcl-xL demonstrate increased germ cell death similar to Bax overexpression therefore the normal development of functional spermatogenesis should balance between apoptosis-protecting and apoptosis-inducing proteins [36]. When the gonocytes differentiate into spermatogonia, the first wave of spermatogenesis occurs. In rats and mice this event takes place between days 10 and 30 after birth. The first wave of spermatogenesis in mice and rats involves capsize 2 [37], capsize 3, 8 and 9 indicating involvement of both extrinsic and intrinsic pathway of apoptosis [38]. As for extrinsic pathway or death receptor pathway, TNFR1 is found in most tissues and can interact with either a cytoplasmic TNFR1-associated death domain protein (a 34-kDa adaptor protein that is also a TNFR1-associated signal transducer) or the Fas-associated death domain protein (a 23-kDa adaptor protein), which can lead to TNF-alpha induced death signaling through a capsize-dependent apoptotic pathway [39] such as to regulate Sertoli/germ cell survival or apoptosis in the testis. Thus, TNF-alpha plays a significant role in determining the size of the germ

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cell population in the seminiferous epithelium via its effects on germ cell apoptosis [33]. Fas receptor and Fas ligand are expressed in the testis and considerable interest has been generated in understanding the role of these proteins in the control of apoptosis in the testis [40]. The Fas mutant GLD (Generalized Lymph proliferative Disease) mice that lack a functional Fas-signaling pathway have a small but significant increase in testis weight and numbers of spermatid heads per testis compared with wild type mice. In addition, GLD mice show spontaneous incidence of germ cell apoptosis [41]. In addition, knockout and transgenic mouse models in the study of male germ cell apoptosis has helped to elucidate the molecular mechanisms of action of the cellular death machinery during germ cell apoptosis thereby providing the basis for the identification of novel targets for male contraception and effective management of male infertility [33].

1.2.7 Methods to induce apoptosis

The precise factor for inducing apoptosis in the germ cells is not yet known. Although apoptosis can be triggered by diverse stimuli, it is usually initiated through two major cell intrinsic pathways, 1) the mitochondrial pathway and 2) the membrane pathway. Apoptosis is characterized by characteristic morphological changes that include cell shrinkage, chromatin condensation, membrane blebbing, internucleosomal DNA degradation, and fragmentation of cells into apoptotic bodies [42]. Hormone-induced male germ cell apoptosis, follicle stimulating hormone (FSH), luteinizing hormone, human chorionic gonadotropin and testosterone have all been shown to regulate germ cell survival. Exposure to excess hormones or deprivation of hormones can lead to cellular apoptosis in the testis [33]. Toxicant-induced male germ cell apoptosis, the effects of toxins can induce apoptosis [33]. Temperature-induced male germ cell apoptosis, cryptorchidism, a condition in which the testes are exposed to body temperature rather than scrotal temperature, the study was suggested that Fas is involved in heat-induced testicular germ cell apoptosis and that Fas-dependent apoptosis is responsible for the p53-independent phase of germ cell loss in the cryptorchid testis [33]. Orchitis-induced male germ cell apoptosis, the research study

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was concluded that extrinsic, mitochondrial and possibly ER pathways are inducers of germ cell apoptosis in Experimental Autoimmune Orchitis (EAO) and that Bax and Bcl-2 proteins modulate this process [43]. In term of gene-induced apoptosis, is biological induction, as gene therapy, transferring exogenous nucleic acids (apoptotic gene) into the appropriate cells and inducing apoptotic pathway. There are three types such as 1) Bcl-2 Family Genes that involve in mitochondrial pathway of apoptosis, 2) Tumor Necrosis Factor Family that promote cell death by activating a death receptor pathway, and 3) Caspases that work in both the mitochondrial and death-receptor apoptosis pathways. Moreover there are many interested suicide genes such as Herpes Simplex Virus Thymidine Kinase (HSV-tk), in combination with Ganciclovir (Antiviral drug; GCV) has been used for inducing cancer cell apoptosis. GCV is a nucleoside analog that is converted to a monophosphate form in the cell by HSV-tk and then phosphorelated by host cellular kinase to the triphosphate form that will block DNA elongation and result in cell death [44].

1.2.8 TNF-alpha induces apoptosis

TNF-alpha is a cytokine that kill tumor cell and enhance tumor endothelium permeability to increase accumulation of chemotherapeutic drugs into tumor. However there are many side effects of the systemic administration of TNF-alpha protein at therapeutically active dose, an earlier study has shown that a single intravenous injection of TNF-alpha gene vector is one option to treat cancer cell by increasing the accumulation of liposomal doxorubicine (Doxil) into tumor [45]. As for cancer gene therapy, contains the down-regulation of multidrug resistance proteins and cancer resistance proteins, the up-regulation of apoptotic induced proteins, TNF-related apoptosis-inducing ligand (TRAIL), p53, TNF-alpha and cytotoxic immune cytokines [46]. TNF-alpha in testis, plays a significant role to regulate sertoli:germ cell ratio by inducing apoptosis [47]. Moreover germ cell and sertoli cell can secrete TNF-alpha to help germ cell migration by disrupting the Blood-testis barrier (BTB), acute intratesticular administration of recombinant TNF-alpha lead to transiently disrupt the BTB [48]. Furthermore chronic systemic administration of this cytokine can induce germ

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cell loss from the seminiferous epithelium [49]. In several different cell types including fibroblast can be induced apoptosis by TNF-alpha [50].

1.2.9 Tumor targeted gene delivery

Side effects of chemotherapy are seriously problems, the use of high doses of cytotoxic drugs can damage normal tissues. To overcome this problem, different types of nanocarrier-based delivery systems have been developed with the aim to carry and deliver anticancer drugs/ anticancer genes directly at the level of tumor cells. Moreover the blood vessels surrounding tumor cell are leaky and exhibit hyperpermeability compared to normal tissues. The leakiness in leads to penetration and retention of NPs in the tumor bed which is known as enhanced permeation and retention (EPR) effect. The pore size in the leaky tumor vasculatures has been reported to range from 380 to 780 nm (Figure 1). Therefore, nanoparticles (NPs) that rely on EPR to target tumors should possess size below this cut-off [51]. There are many studies about tumor targeted gene delivery. The nanotechnology platforms have recently been used to deliver suicide gene to tumor cell.

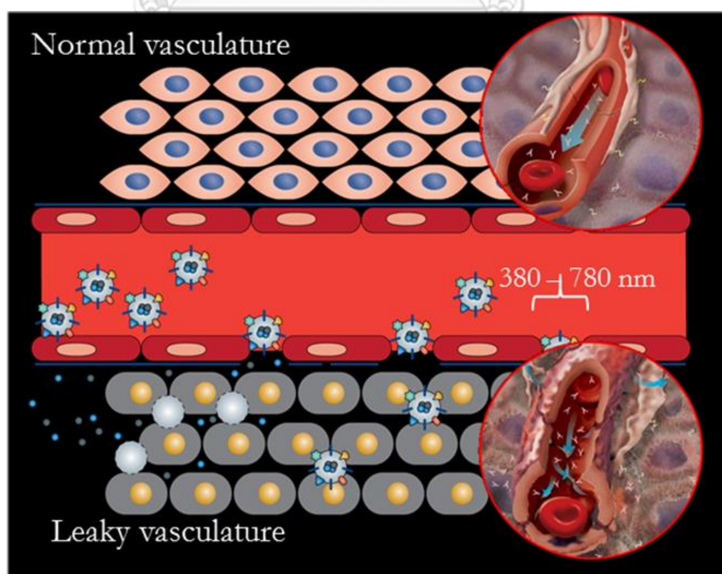

Figure 1. *Tumor vasculature*

1.2.10 GnRH Receptor (GnRHR) overexpressing cancers

Many studies demonstrated that GnRH receptors (GnRHR) are overexpressed in reproductive related cancer cells such as mammary, endometrial, ovarian and prostatic cancer. Interestingly, there are non-reproductive related cancer cells that also overexpressed GnRHR such as melanoma, glioblastoma, lung and pancreatic cancer [9]. In Side effects of chemotherapy are seriously problems, the use of high doses of cytotoxic drugs can damage normal tissues. To overcome this problem, different types of nanocarrier-based delivery systems have been developed with the aim to carry and deliver anticancer drugs/ anticancer genes directly at the level of tumor cells. There are many studies about tumor targeted gene delivery. The nanotechnology platforms have recently been used to delivery suicide gene to tumor cell.

normal tissue, GnRHR are expressed in ovary, testes, endometrium, myometrium, prostate, mammary and placenta [52]. The level of GnRHR expression in ovary varies in the follicular stages [53].While the level of GnRHR expression in endometrium and placenta changes at different weeks of gestation [9]. In normal mammary and prostate, the data were reported that there are only mRNA of these receptors in prostate biopsies, with lower levels in normal prostate than in prostate cancer specimens [9]. Because of GnRHR overexpressing cancers and the limitation of GnRH expression in normal tissues, the novel targeted therapeutic strategies were studied. Furthermore GnRHR can be activated by GnRH agonist since it can increase these receptors expression. On the other hand GnRH antagonist can inactivate these receptors by competitive blocking [54]. Therefore in our study we should evaluate the specific GnRHR binding by GnRH agonist and antagonist competition assay.

1.3 Objectives and hypothesis

Objectives of Study:

The main overall goal of this study is to investigate the application of modified chitosan as a potential vector for apoptotic gene delivery to GnRHR-expressing cells (i.e. testicular cells and mammary cancer cells). Such design of gene carrier could be useful in particular for male animal nonsurgical sterilization and gene therapy for mammary cancers.

Specific objectives, to achieve this goal, three specific objectives are defined:

1) To design, synthesize, characterize nanoparticles (Chitosan) and evaluates these for targeted gene delivery *in vitro*

2) This aim evaluates the potential use of the prepared nanoparticle as a promising carrier to mediate apoptotic gene delivery in order to induce germ cell apoptosis.

3) This purpose investigates the utilization of the prepared nanoparticle as a promising carrier to mediate apoptotic gene delivery for mammary cancer treatment.

Hypothesis:

1) N-succinyl-chitosan could be synthesized by reaction of chitosan with succinic anhydride, which was subsequently reacted with GnRH peptide by using EDC/NHS as a coupling agent, yielding GnRH-conjugated chitosan.

2) GnRH-CS polymers are capable of condensing and directing plasmid DNA (pDNA) into GnRHR-expressing cells and exhibit higher transfection activity compared to unmodified CS.

3) Nonsurgical sterilization for male animals could be achieved through activation of the apoptosis pathway within a gonadal stem cell population, eliminating the entire testicular germ cell or activating apoptosis within all of Sertoli nurse cells by using gene inducing apoptosis delivered by GnRH-CS polymers.

4) Deconstruction of GnRH-associated mammary gland tumor could be achieved by using gene inducing apoptosis delivered by GnRH-CS polymers.

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1.4 Conceptual framework

The thesis is divided into 3 parts (As shown in Figure 2.)

Part I: Synthesis and characterization of the GnRH-conjugated chitosan/pDNA complexes

Part II: Examination of the *in vitro* gene delivery (transfection) efficacy and targeting property mediated by the GnRH-CS/pDNA complexes

Part III: Investigation of the *in vivo* effect after administration with the GnRH-CS carrying a gene of interest

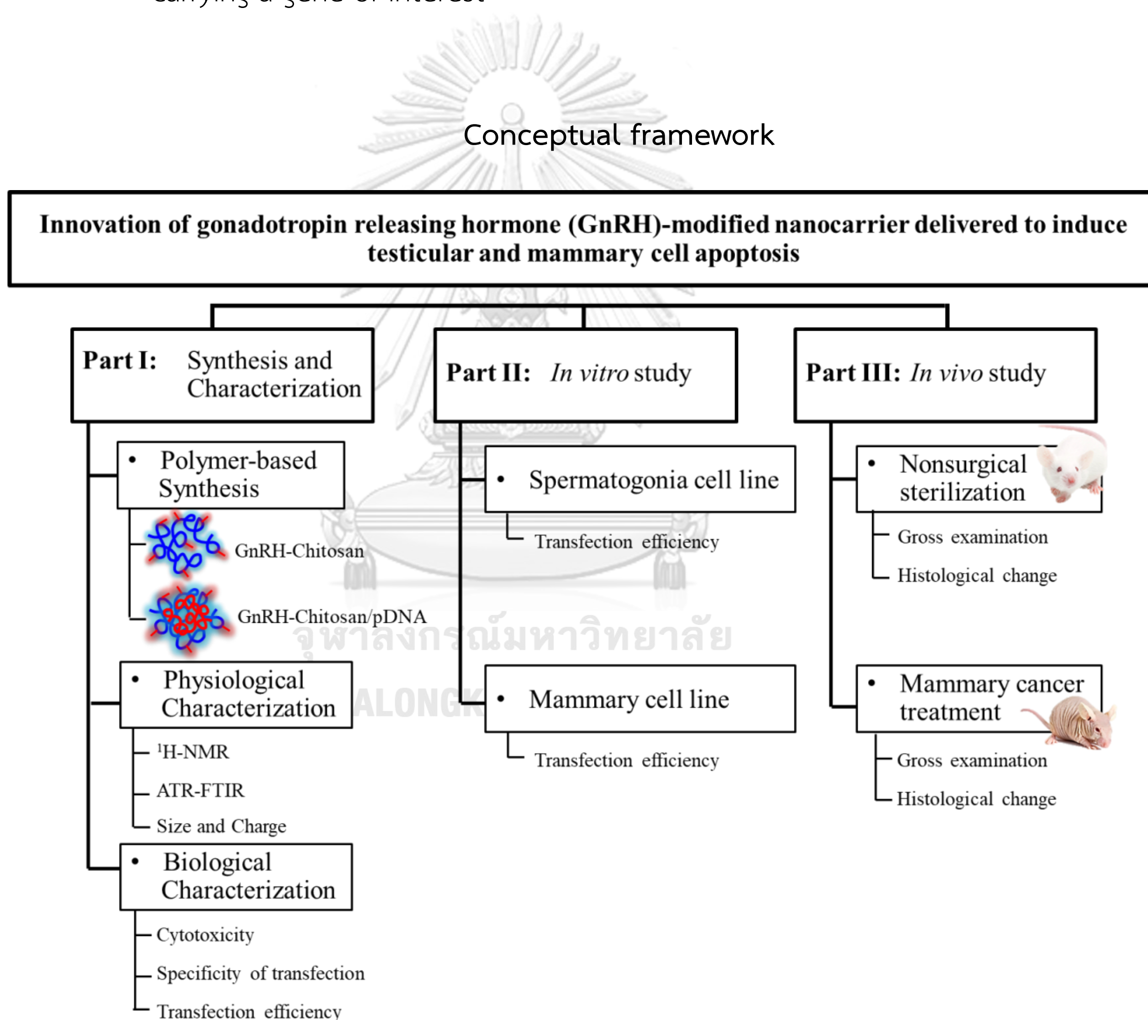

Figure 2. A diagram representing the conceptual framework of the thesis

1.5 Advantages of the study

1) Formulate GnRHR-targeted CS delivering gene of interest for the potential use in contraception, fertility control and reproductive related cancer treatment.
2) Develop nonsurgical sterilization method based on nanocarrier/pDNA complexes in animals.
3) Develop the alternative method to treat in reproductive related cancer that does not respond to chemotherapy and surgery
4) Increase knowledge of DNA delivery system and gene therapy in veterinary medicine.
5) Provide sufficient scientific data for international publication

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CHAPTER II

2.1 Chitosan-based DNA delivery vector targeted to gonadotropin-releasing hormone (GnRH) receptor

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Abstract

The main purpose of this study was to investigate the application of modified chitosan as a potential vector for gene delivery to gonadotropin-releasing hormone receptor (GnRHR)-expressing cells. Such design of gene carrier could be useful in particular for gene therapy for cancers related to the reproductive system, gene disorders of sexual development, and contraception and fertility control. In this study, a decapeptide GnRH was successfully conjugated to chitosan (CS) as confirmed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). The synthesized GnRH-conjugated chitosan (GnRH-CS) was able to condense DNA to form positively charged nanoparticles and specifically deliver plasmid DNA to targeted cells in both two-dimensional (2D) and three-dimensional (3D) cell cultures systems. Importantly, GnRH-CS exhibited higher transfection activity compared to unmodified CS. In conclusion, GnRH-conjugated chitosan can be a promising carrier for targeted DNA delivery to GnRHR-expressing cells.

Keywords: Gonadotropin releasing hormone (GnRH), Chitosan, Gene delivery

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Introduction

The direct use of nucleic acid as a therapeutic agent to treat gene-associated diseases (so-called gene therapy) by transferring exogenous nucleic acids into the appropriate cells has attracted great interest over the past few decades [1]. In order to make great progress in gene therapy, it is of paramount importance to develop effective carriers capable of delivering a gene of interest to target cells to achieve sufficient and sustained transgene expression. Viral vectors have received considerable attention and become powerful tools of gene transfer. Unfortunately, the clinical application of viral vectors is limited because of unfavorable immunological features and mutagenic integration for some viruses. These concerns have driven extensive attention to the development of non-viral vectors [18]. Chitosan (CS) is a linear polysaccharide derived from the deacetylation of chitin found in the exoskeletons of crustaceans. Among non-viral vectors, chitosan has been exploited as gene-delivery vectors due to its excellent biocompatibility and biodegradability [55]. However, the effectiveness of this system is hindered by its low transfection efficacy and low cell specificity [56]. To overcome this limitation, ligands with high affinity and specificity, such as transferrin-,folate-, mannose-, and galactose-conjugated chitosan, have been integrated into gene delivery vector for receptor-mediated endocytosis [57].Gonadotropin-releasing hormone (GnRH) is a 10 amino acid peptide produced and secreted by the hypothalamus. GnRH binds to its receptor (GnRHR) on gonadotrope cells in the anterior pituitary, stimulating the biosynthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), affecting sex development and reproductive functions [58]. In addition to gonadotrope cells, GnRHR was found to be expressed in normal reproductive tissues (e.g. breast, endometrium, ovary, and prostate) [8]. Interestingly, GnRHR are overexpressed in cancer tissues, either related (i.e. prostate, breast, endometrial, and ovarian cancers) or unrelated (i.e. melanoma, glioblastoma, lung, and pancreatic cancers) to the reproductive system. Therefore, it is possible that targeted gene delivery to GnRHR-expressing cells could be a valuable tool for 1) the treatment of certain gene disorders affecting sexual development and reproductive function [24], 2) gene therapy of cancer

associated with reproductive system (i.e. prostate, breast, endometrial, and ovarian cancers) [9], and 3) contraception and fertility control [10].

The main overall aim of this study is to investigate the application of modified chitosan as potential vectors for gene delivery to GnRHR-expressing mammalian cells. Throughout this study, the abbreviation GnRH-CS will be used to refer to GnRH-conjugated chitosan. In this study, we prepared GnRH-CS. The physiochemical properties of the synthesized GnRH-CS as well as the complex of GnRH-CS and plasmid DNA (pDNA) carrying a gene of interest (GnRH-CS/pDNA complexes) were analyzed, and their cytotoxicity and cell specificity were also characterized. Transfection efficiency was investigated in established mammalian cell line model system expressing GnRHR. We hypothesized that GnRH-CS polymers are capable of directing pDNA into GnRHR-expressing cells via receptor-mediated pathways and exhibit higher transfection activity compared to unmodified CS. A schematic diagram of the GnRH-CS/pDNA complex and its proposed mechanism of trafficking were shown in Figure 3.

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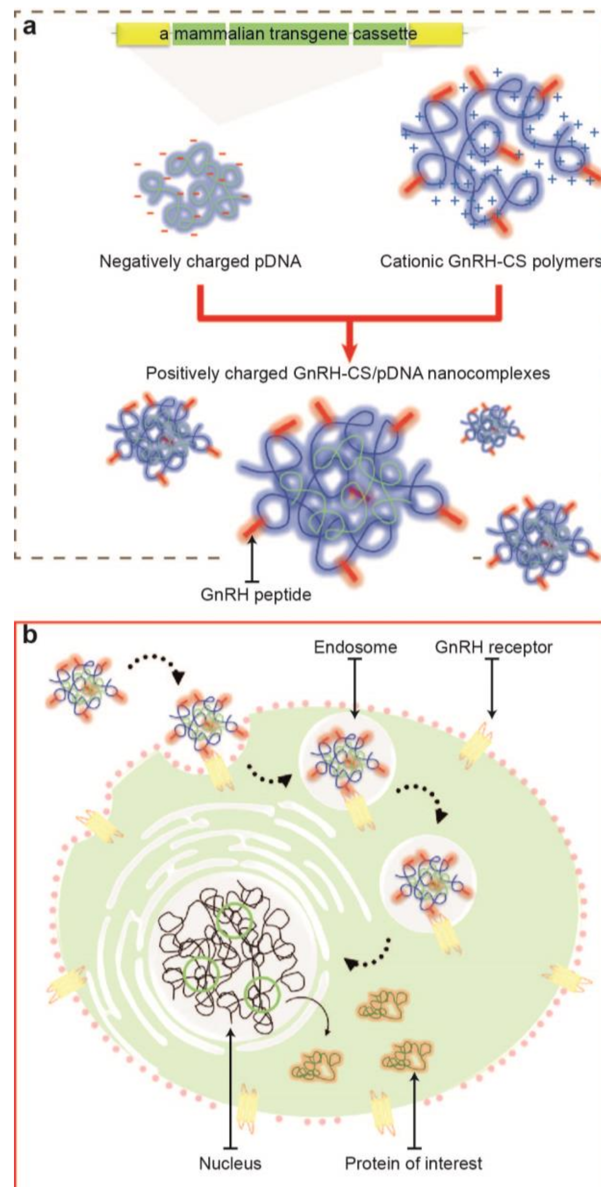

Figure 3. *Schematic illustration showing the formation of GnRH-CS/pDNA complex and its delivery procedure. a) pDNA condensation in the presence of cationic GnRH-CS polymer by electrostatic interactions. b) The proposed mechanism of gene delivery process by GnRH-CS/pDNA complex. GnRH peptides displayed GnRH/pDNA complexes facilitate active targeting of GnRHR-expressing cells via specific interaction between the GnRH motif and the GnRH receptor. Following internalization, the pDNA must be released and transported to the nucleus where gene expression occurs.*

Materials and methods

Gonadotropin-releasing hormone (GnRH) was purchased from Sigma. Chitosan (CS) was purchased from Oil Zac Technologies Co., Ltd. The degree of deacetylation (DDA = 98%) was determined by ^{1}H NMR method [59]. The number-averaged molecular weight (Mn), weight average molecular weight (Mw), and polydispersity index (PDI; Mw/Mn) of CS were determined to be 5483 g/mol, 11158 g/mol and 2.03, respectively using gel permeation chromatography (GPC). Succinicanhydride, acetic acid, 1-Hydroxybenzotriazole hydrate (HOBt), N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), and N-Hydroxysuccinimide (NHS). All chemicals and reagents were used without further purification. Dialysis tubing (MWCO = 3500 Da) was purchased from Cellu Sep T1 (Membrane Filtration Products, Inc.). Syringe fillers were purchased from Sartorius Stedim Biotech. MilliQ Plus (18.2 MΩ, Millipore) purified water was used to make all aqueous solutions. The Dulbecco's modified eagle medium (DMEM), antibiotics, and fetal bovine serum (FBS) were purchased from Gibco. MGC Mouse GnRH receptor cDNA (Clone ID: 30249439) was purchased from Dharmacon. LIVE/DEAD® Viability/Cytotoxicity kit and Lipofectamine™ 3000 were obtained from Invitrogen. Cell Titer-Glo® Luminescent Cell Viability assay system and Steady-Glo® Luciferase assay system were provided by Promega.

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Polymer characterization

All samples were characterized by using ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR) and Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). The ^{1}H NMR spectra were measured on a Bruker AVANCE 500 MHz spectrometer (Bruker, Switzerland), using D2O/CD3COOD as solvent at a 10 mg/ml concentration of polymers. The GnRH-CS with different the degrees of amidation (DADs) value, GnRH-CS1 and GnRH-CS2, for DAD 0.014 and 0.018, respectively were used in this study. All measurements were performed at 300 K, using the pulse accumulation of 64 scans and LB parameter of 0.30 Hz. The degree of N-succinylation (DNS) value of NSCS was calculated using the following equation 1, while the degree of amidation (DAD) value of GnRH-CS was calculated using the following equation 2. All DNS and DAD were determined using ^{1}H nuclear magnetic resonance spectroscopy

(^{1}H NMR). ATR-FTIR spectra were collected on a Nicolet 6700 spectrometer (Thermo Company, USA) using a single-bounce ATR-FTIR Smart Orbit accessory with a diamond internal reflection element (IRE), at ambient temperature (25°C).

Synthesis of N-succinyl chitosan

N-succinyl chitosan (NSCS) was synthesized by N-succinylation CS with succinic anhydride. Briefly, 1.00 g of CS (6.17 × 10–3mol)was dissolved in 50 mL of 1% (v/v) aqueous acetic acid, and then 0.03 g of succinic anhydride (0.05 meq/GlcN) was added. The reaction mixture was stirred at room temperature for 24 h and dialyzed against deionized (DI) water for three days to remove free succinic anhydride. The NSCS was then obtained by lyophilization. The degree of N-substitution (DS) of NSCS units in the polymers was determined using ^{1}H-NMR spectroscopy.

Synthesis of NSCS conjugated with GnRH.

The conjugation of N-succinyl chitosan (NSCS) with gonadotropin hormone (GnRH) was carried out by using EDC/NHS as a coupling agent in water as shown in Figure 4. Briefly, 0.30 g of NSCS (1.79×10^{-3} mol) was suspended in 30 mL of DI water that containing 0.24 g of 1-hydroxybenzotriazole hydrate (HOBt) (1.0 meq/GlcN). The reaction mixture was stirred at room temper-ature for 24 h, and the clear solution was obtained. Afterwards, 0.34 g of EDC (1.0 meq/GlcN) and 0.21 g of NHS (1.0 meq/GlcN) were added and stirred at room temperature for 1 h. Then, 1.50 mg (0.72×10^{-3} meq/GlcN) or 3.50 mg (1.65×10^{-3} meq/GlcN) of gonadotropin hormone (G) was added into the solution. The reaction mixture was stirred at room temperature for 24 h. The clear solution was dialyzed with DI water for 3 days to remove impurity. The N-succinyl CS conjugated with gonadotropin hormone (NSCS-G) was then obtained by lyophilization. The degree of N-substitution (DS) of NSCS-G units in the polymers was determined using ^{1}H-NMR spectroscopy.

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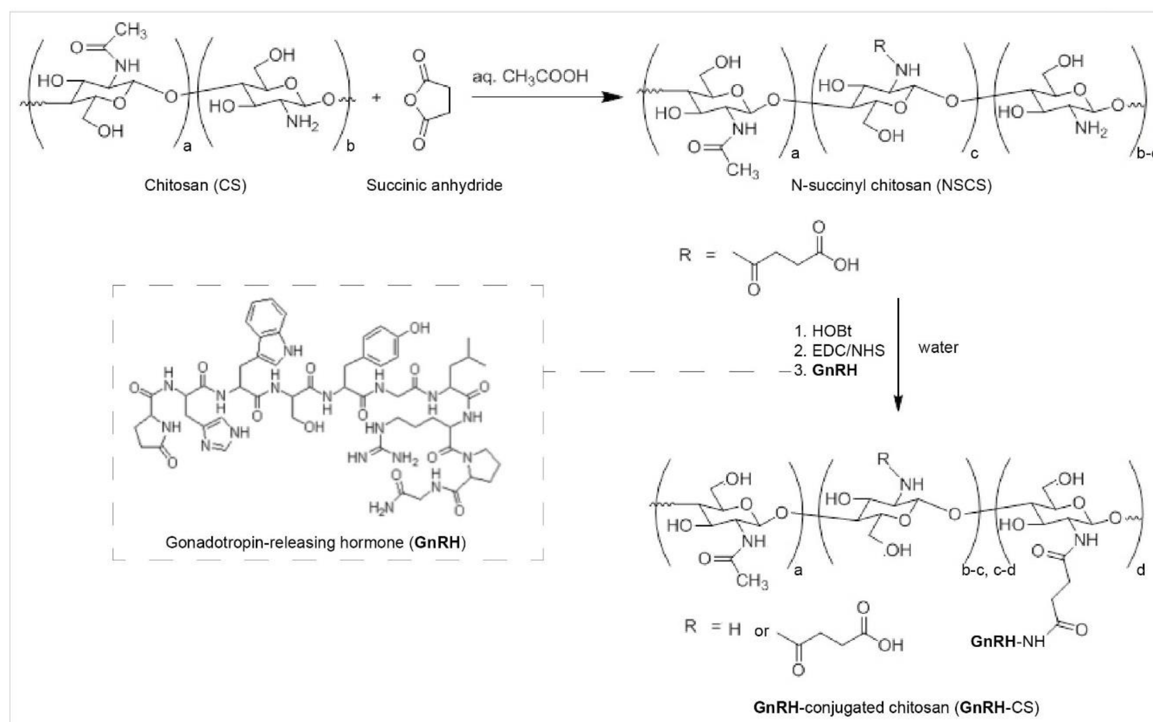

Figure 4. *Proposed reaction scheme for synthesis of GnRH-grafted chitosan (GnRH-CS). N-Succinyl chitosan (NSCS) was synthesized by N-succinylation of CS with succinicanhydride followed by conjugation with GnRH peptide by using EDC/NHS as a coupling agent.*

Construction of pDNA carrying a cDNA encoding mouse GnRH receptor.

The cDNA encoding a GnRH receptor was introduced into pSF plasmid. Briefly, the 50 L polymerase chain reaction (PCR) reaction was performed with 10 pg DNA templates, 0.5 µM primer pairs, 200 µM dNTP and 0.02 U Phusion Hot Start II DNA polymerase. The reaction was initiated by pre-heating the reaction mixture to 98°Cfor 30 s; 25 cycles of 98°C for 15 s, 61°C for 30 s and 72°C for 1 mins (30 s/500 bp). The PCR products were evaluated by agarose gel electrophoresis. The digested PCR product was ligated to the pSF plasmid using the T4 Quick ligase. An aliquot of ligation reaction was transformed into E. coli competent cells strain DH5 alpha and inoculated on LB agar plate containing 50 µg/ml tetracycline. Single colonies were picked and their plasmids were isolated by using a QIAprep Spin Miniprep kit (QIAGEN). The positive clones were selected by restriction digestions.

Cell culture.

The human skin fibroblast (BJ) and the human embryonic kidney(HEK293T) cell lines were maintained in complete D-MEM medium supplemented with 10% Fetal Bovine Serum (FBS), Penicillin (100units/ml), Streptomycin (100 µg/ml) and L-glutamine (2 mM). Cells were grown at 37°C in a humid atmosphere of 5% CO_2.

Cytotoxicity

In vitro cytotoxicity of GnRH-CS was evaluated by Cell Titer-Glo® Luminescent Cell Viability assay system. BJ cells were seeded and cultured in medium containing cationic polymers with different concentrations. The cytotoxicity of GnRH-CS was examined 24 h post incubation. The cell viability was performed according to the manufacturer's instructions, and the results are expressed as thepercentage of cell viability. Cells were also stained with the reagents in the LIVE/DEAD® Cell Viability/Cytotoxicity Assay Kit according to the manufacturer's instructions, and visualized under the fluorescence microscope.

Amplification of plasmid DNA

After transformation, plasmids were isolated and purified from the overnight Escherichia coli (*E.coli*) strain DH5alpha culture by using HiSpeed Plasmid Midi Kit. The concentration of plasmid DNA was determined by measuring the absorbance at 260 nm. The plasmid was stored at -20°C until used.

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Preparation and characterization of polyplexes

GnRH-CS and unmodified CS were each dissolved in 0.2% acetic acid to obtain 1 mg/ml polymer solutions. Plasmid DNA solutions were added drop wise into each of the polymer solutions under high-speed vortex for 1 min. The pDNA concentration was fixed. Various ratios of the polymer/pDNA complexes (weight/weight) were prepared depending on the experiment. The complete formation of complexes was determined by gel retardation assay. The same amount of naked DNA was used as the control. Gel Electrophoresis was carried out at 100 V for approximately 30 min. DNA was visualized

by illumination on a long wave UV light box and photographed. Particle size and zeta-potential charge measurements were conducted using Nanosizer.

Generation of cells transiently expressing GnRHR

Sub-confluent monolayer cultures of HEK293T were transfected with pDNA vectors containing a gene encoding GnRHR under the control of CMV promoters for constitutive expression in mammalian cells. Transiently transfected HEK293T cells were prepared following Lipofectamine transfection (Invitrogen) of mouse GnRHR cDNA in pSF, CMV promoter plasmid, which was constructed and produced in our laboratory. For 3D spheroid generation, a suspension of transiently transfected HEK293T cells after trypsinization was seeded into ultralow attachment (ULA) surface plates. After 24 h incubation, a multicellular spheroid spontaneously formed in each well. Parental non-transfected cells were used as controls.

Cell transfection

GnRH-CS/pDNA or CS-pDNA preparations in 0.2% acetic acid was directly added to the cell supernatant and incubated at 37°C. Four hours later, the medium was replaced with fresh medium. Depending on the experiment, specificity or efficiency of transfection as determined by the expression of reporter transgenes was assessed at indicated time points.

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Examination of reporter gene expression

Plasmid green fluorescence protein (pGFP) and plasmid luciferase (pLuc) were used in this study. GFP expression was analyzed using a Nikon Eclipse TE2000-U fluorescence microscope. Photographic images were obtained by using 4× magnification and fluorescent setting. Bright field photographs were also obtained. The Steady-Glo® luciferase assay kit was used to evaluate expression of the luciferase reporter transgene in transfected cells

Statistical analysis

GraphPad Prism software (version 5.0) was used to perform statistical analyses. Data were presented as mean ± standard error of the mean (s.e.m). P values were generated by one-way or two-way ANOVA, considered significant when <0.05 and denoted as follows:*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Results and discussion

Synthesis and characterization of GnRH-CS

The scheme for the overall synthesis is shown in Figure 5. By taking advantage of the previously developed N-succinyl chitosan(NSCS) derivative [60-62], this strategy was exploited in order to incorporate GnRH into CS polymer. NSCS was therefore synthesized in the first step by reacting primary amino groups of low molecular weight CS with succinicanhydride under mild acidic condition. However, high degree of N-succinylation could interfere the electrostatic interaction between positively charged primary amino groups of CS and negative charge plasmid DNA. A vast majority of amino groups must be available to maintain the overall positive charge of CS [63]. We therefore modified CS polymer with low degree of N-substitution. The use of GnRH as a targeting ligand for different classes of nanocarrier (namely, linear polymers, branched star-like dendrimers, liposomes, and lipid nanoparticles) has previously reported for delivery of anticancer compound [64-67]. These nanocarriers include linear Tax-polyethylene glycol (PEG)-GnRH polymers, Tax-PAMAM (polyamidoamine)-GnRH dendrimers, Tax-DSPE-PEG (1,2-distearoyl-sn-glycero- 3-phosphoethanol amine-N-amino polyethylene glycol)- GnRH liposomes, and nanostructured lipid nanocarrier (NLC) respectively, which have been developed for the targeted delivery of chemotherapeutic drugs and the last one for co-delivery of siRNA and chemotherapeutic drug. However, the specific purpose of this study was to develop GnRH-CS derivertives as nanocarrier for targeted gene delivery. Following synthesis of NSCS with low degree of N-substitution, conjugation between NSCS and GnRH was carried out in water by using EDC/NHS as covalent coupling agent to yield GnRH-CS. It is also interesting to note that conjugation of succinyl chitosan with other agents was

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mostly carried out in aqueous organic acid or some organic solvents. In contrast, in our work, the reaction was performed in water (see material and methods section) under mild condition without requiring any organic solvents or acid and heat that might affect the biological activity of GnRH peptide.

The chemical structures of NSCS and GnRH-CS were investigated using ATR-FITR (Figure 5a.) and ^{1}H NMR spectroscopy (Figure 5b.) com-pared to CS and GnRH. The ATR-FTIR spectrum of GnRH showed characteristic absorption bands at 3273, 1630, 1523, 1514, 1465, and 850–600 cm^{-1} resulting from N─H and O─H stretching, C=O stretching of amide bonds (Amide I band), N─H bending (Amide II band), C=C stretching of aromatic groups, and C─H out-of-plane bending aromatic groups in the GnRH molecule, respectively. The ATR-FTIR spectra of NSCS is similar to that of CS except for the presence of additional absorption bands at 1554 and 1407 cm^{-1}corresponding to N─H bending (Amide II band) and O─H bending of succinic acid while the ATR-FTIR spectra of GnRH-CS showed characteristic absorption band at 1638 and 750 cm−1is assigned to C=O stretching of amide bonds (Amide I band), and C─H out-of-plane bending aromatic groups of GnRH moiety.

The ^{1}H NMR spectrum of GnRH in D_2O showed a multiplet protons in the range of δ 6.5–8.0 ppm and 4.5–1.0 ppm, indicating the presence of aromatic protons and protons of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly. In comparison to1H NMR spectra of CS in D_2O/CD_3COOD, the ^{1}H NMR spectra of NSCS in D_2O/CD_3COODshowed an additional triplet protons at δ 2.50 ppm, corresponding to the succinyl protons, while other multiplet proton signals at δ 5.23–2.90 ppm were assigned to the H1–H6 protons of CS and singlet at δ 1.90 ppm corresponds to the CH3protons from GlcNAc. The GnRH CS spectra exhibited multiplets protons at δ 8.0–7.0 ppm due to aromatic protons of GnRH, while other multiplet protons in the range of δ 4.5–1.0 ppm, indicating the presence of protons of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly, were not observed. This could be due to very low mole ratio of GnRH used in the reaction. These results indicate that the NSCS and GnRH-CS were successfully incorporated into the CS backbone. Since the succinyl and aromatic region did not overlap the proton resonances assigned to the GlcN groups of CS, the proton signals attributed to the

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succinyl and aromatic moieties were used to determine the degree of substitution (DS) values. Based on ^{1}H NMR spectra, the degree of N-succinylation (DNS) value of NSCS was calculated using the following equation 1, while the degree of amidation (DAD) value of GnRH-CS was calculated using the following equation 2.

$$DNS = (I_{Su}/4)/(I_{H2-H6}/6) \quad (1)$$

$$DAD = (I_{Ar}/10)/(I_{Su}/4) + (I_{H2}) \quad (2)$$

I_{Su} represents the total area (integration) of N-succinyl protons, I_{Ar} represents the total area (integration) of aromatic protons, $I_{H2\text{-}H6}$ representing the peak area (integration) of protons C2–C6, and I_{H2} representing the peak area (integration) of protons C2 in the CS backbone. In this study, the DNS was found to be 0.05 based on primary amino groups of CS. The homogeneous reaction was occurred easily by ring opening of succinic anhydride under mile acidic condition. This result revealed that succinyl groups are effectively N-substituted onto the primary amino groups of CS backbone. As shown in Table 1, The DADs were found to be in the ranges of0.014–0.018 based on succinyl moieties of NSCS. The homogeneous reaction was carried out in water by simply mixing CS and HOBt in water, and arginine groups of GnRH were conjugated to carbonyl groups by covalent coupling agent in the NSCS backbone. These results in conjunction with the IR and1H NMR spectra discussed above indicate successful introduction of both these functionalities onto the CS backbone. Our results also confirmed that the low degree of modification not only preserve the DNA condensation ability of chitosan, but is also sufficient for receptor-mediated gene delivery (see Sections 3.3 and 3.4).

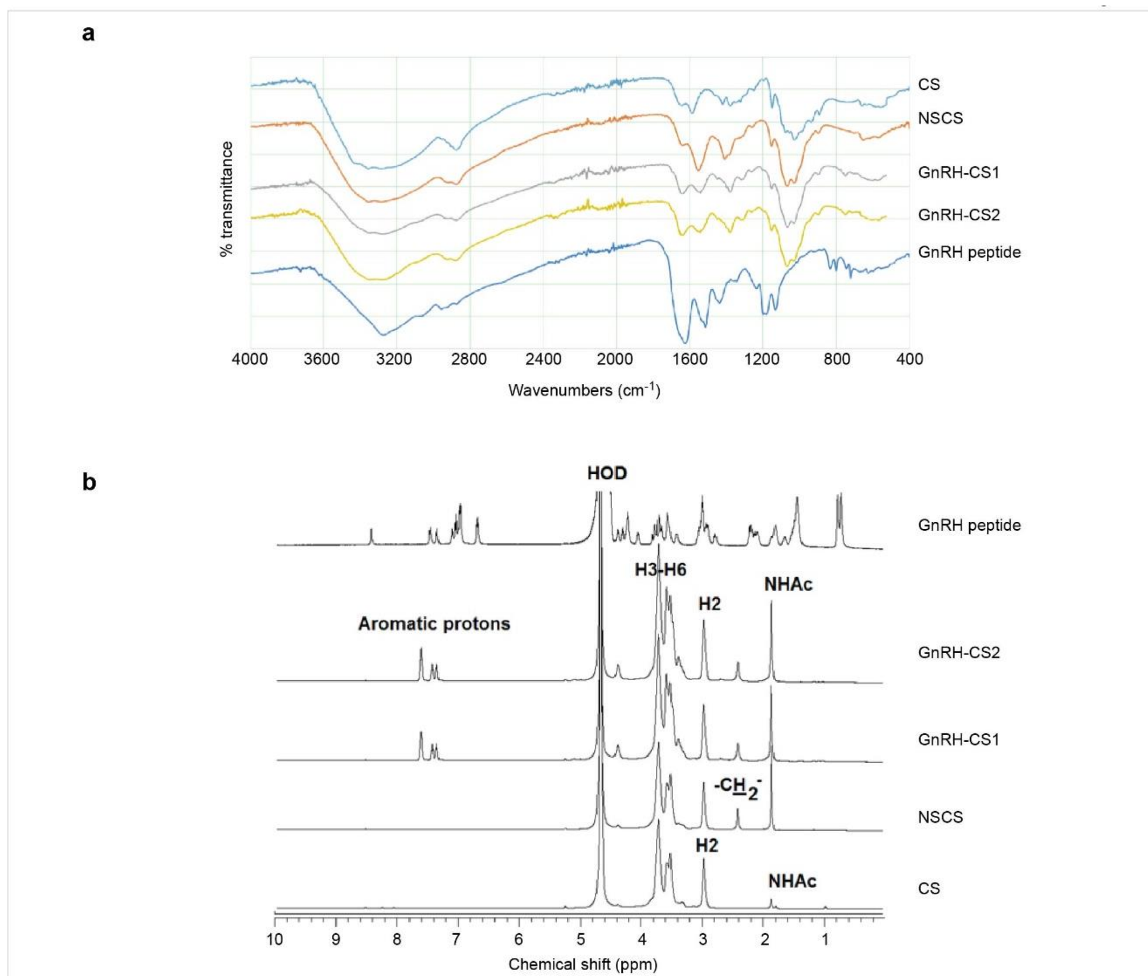

Figure 5. *Characterization of GnRH-grafted chitosan (GnRH-CS). a) Structure characterization by FTIR spectra. b) Representative ^{1}H NMR spectrum of GnRH-CS compared to NSCS, CS and GnRH peptide.*

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Table 1. The degree of substitution (DS) and percentage of recovery of chitosan derivatives.

Entry	Sample	DS	Recovery (%)[c]
1	CS	–	–
2	NSCS	0.05[a]	87.42
3	GnRH-CS1	0.014[b]	38.43
4	GnRH-CS2	0.018[b]	41.90

[a] DNS is the degree of *N*-succinylation as determined by ^{1}H NMR.
[b] DAD is the degree of amidation as determined by ^{1}H NMR.
[c] Recovery (%) = [Weight of product (g)/Weight of starting material (g)] × 100

Cytotoxicity of GnRH-CS

We sought to investigate whether the prepared GnRH-CS was toxic for BJ cells. This cell line is a normal fibroblast that represents normal tissues and has been widely used for in vitro cytotoxicity of material of interest in order to evaluate the biocompatibility of newly synthesized materials or natural products [68, 69]. In this experiment, polyethylenimine (PEI) which has been described as one of the most widely used cationic polymer for in vitro gene delivery [70] was also tested in parallel. The cell viability of PEI-treated cells was drastically decreased with increasing concentration as shown in Figure 6a. This observation can be attributed to a number of previous investigations which have highlighted its high cytotoxicity [71]. In contrast, no cytotoxicity was observed in the concentration ranges of unmodified CS or GnRH-conjugated CS tested. Similar results were observed in both GnRH-CS1 and GnRH-CS2. Figure 6b shows a large number of dead cells (Red fluorescence) in cells treated with 200 µg/ml of PEI polymer compared to normal morphology of viable cells (green fluorescence) treated with the same concentration of unmodified CS or GnRH-CS. This result is consistent with the previous cell viability study [72] suggesting that unmodified CS and the prepared GnRH-CS were more biocompatible than PEI.

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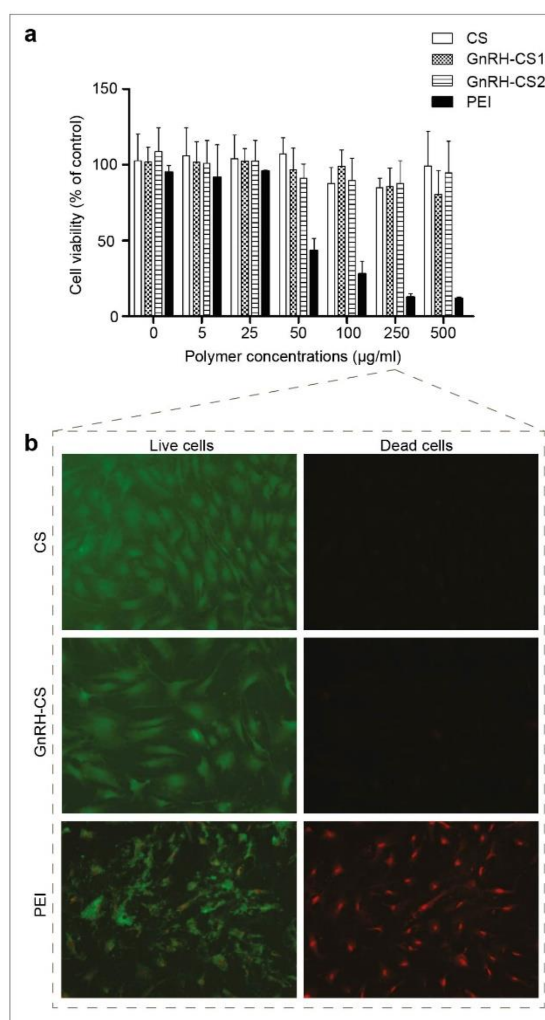

Figure 6. *Cytotoxicity of polymers at various concentration in BJ cells (human skin fibroblast). a) Following 24 h incubation with polymers, cell viability was determined by the Cell Titer-Glo® cell viability assay. Mean cell viability was normalised to non-treated controls, with the mean of n = 3 + SEM, from one representative experiments of three independent experiments. Statistical analysis was performed using two way ANOVA with tukey's post hoc test, n.s.-not significant, * p < 0.05, ** p < 0.01, ***p < 0.001. b) Morphological characteristics of human fibroblast cells were visualized under the fluorescence microscope. Cells were also stained with the reagents in the LIVE/DEAD® Cell Viability/Cytotoxicity Assay Kit and visualized under the fluorescence microscope. Dead and live cells fluoresce red-orange and green, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)*

Characterization of GnRH-CS/DNA complexes

Gel retardation assay

The ability of cationic polymers to condense DNA molecule is a critical criteria for an effective gene delivery system. The complete formation of GnRH-CS/pDNA complex was therefore evaluated by agarose gel electrophoresis. Figure 7a. demonstrates migration of free plasmid DNA through the gel resolved into distinct bands corresponding to supercoiled or circular forms of plasmids. DNA migration was completely retarded when GnRH-CS: pDNA weight ratios were above 40:1. The critical complex ratio of unmodified CS:pDNA was around 5:1. Larger amount of GnRH-CS required for the complete DNA condensation can be attributed to the relatively weaker DNA-binding ability of GnRH-CS compared with that of unmodified CS. There was no difference between GnRH-CS1 and GnRH-CS2 for their ability to condense DNA. GnRH-CS1 was chosen for further studies.

Particle size and zeta potential

Cationic polymers are widely use to condense negatively charged DNA by electrostatic interactions into small particles (polyplexes), for protecting DNA from degradation and facilitating cell internalization. It has been suggested that a size of polyplex below 300 nm is required for maximum endocytosis by cells [72]. Having defined the critical ratio that produced the complete formation of polyplexes as determined by the earlier gel retardation assay (Figure 7a.), we analyzed the size of GnRH-CS/pDNA and Sizer, the average particle sizes of GnRH-CS/pDNA and unmodified CS/pDNA were 210 and 370 nm, respectively (Figure 7b.). We also analyzed the zeta potential of the prepared complexes. At critical ratios, the zeta potential of GnRH-CS/pDNA and CS/pDNA complexes were 46.8 and 48.6 mV, respectively (Figure 7b.). The presence of excessive amounts of nitrogen residues compared to phosphate groups on DNA results in positive charges on polyplexes, facilitating their binding to negatively charged cell surfaces [73]. Taken together, the prepared GnRH-CS/pDNA and CS/pDNA polymers were able to condense plasmid DNA to form positively charged and nanosized particles, which are the characteristics of a desirable vector for gene delivery.

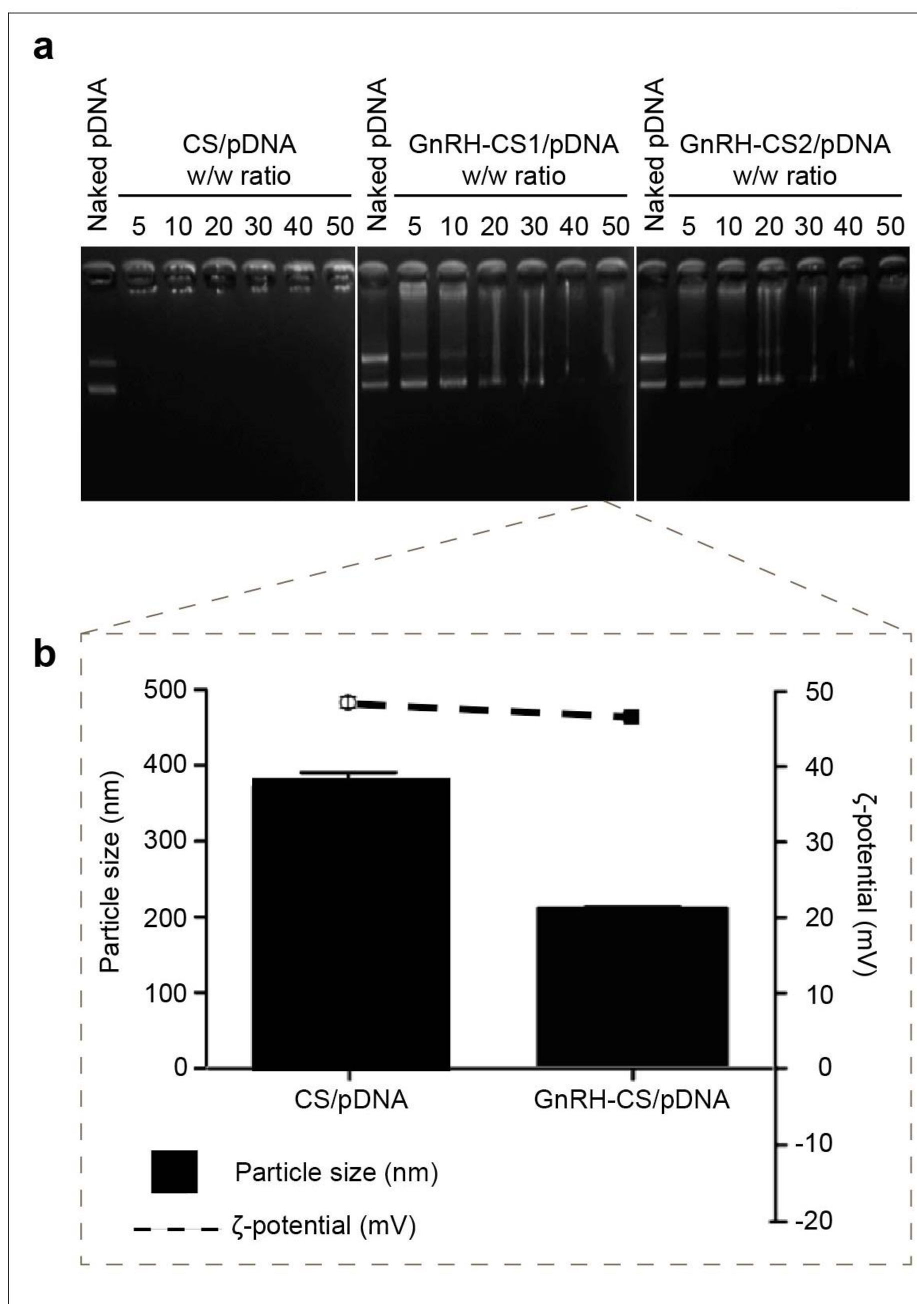

Figure 7. *Characterization of GnRH-CS/DNA complexes. a) Agarose gel retardation assay of the GnRH-CS/pDNA and unmodified GnRH-CS/pDNA polyplexes. Weight ratios of polymer:DNA are 5:1, 10:1, 20:1, 30:1, 40:1, and 50:1, respectively. b) Particle size and zeta potential of pDNA complexed with unmodified CS or GnRH-CS at optimal ratio of polymer/pDNA (5 or 50, respectively). All values are reported as mean ± standard error.*

Analysis of gene expression

Specificity of transfection

HEK293T cell line is one of the most intensively used mammalian cells in cell biology and biotechnology, ranging from the study of gene expression to biopharmaceutical production [74-76]. A previous study has generated the transfected HEK293T expressing GnRH receptor and used this established cell line as a model [77]. Similarly, we used the same strategy and generated a transiently transfected HEK293 expressing GnRH receptors as a representative for the aforementioned cells that express GnRH receptor and used them as target cells compared to original HEK293 as non-targeted cells in our study. We therefore sought to explore the targeting property of the GnRH-CS/pDNA complex to confirm that transfection is specific and mediated by interaction between the GnRH ligand and GnRH receptor. Targeted gene delivery to transiently transfected HEK293Texpressing GnRHR (targeted cells) and nontransfected HEK293T (non-targeted cells lacking GnRHR) by the GnRH-CS was compared to unmodified CS prepared with increasing weight ratios of polymers and pDNA carrying a GFP reporter gene. As shown in Figure 8a, there were no significant differences in GFP expression between targeted and non-targeted cells when treated with unmodified CS/pDNA complex at any weight ratios tested, suggesting inability of unmodified CS to discriminate between targeted and non-targeted cells. In contrast, GnRH-CS was able to specifically deliver a GFP gene to targeted cells. When GnRH-CS/pDNA complexes were used to transfect cells, GFP expression could be observed exclusively in targeted cells. No GFP expression was observed in non-targeted cells at any weight ratios tested as shown in Figure 8a. Two critical factors that strongly affect the physiochemical properties and biological activities of compounds and involved in drug target interactions are electrostatic and steric effects [78]. Our observation that the GnRH-CS/pDNA complex was able to discriminate between targeted and non-targeted cells despite of positive charge similar to unmodified CS/pDNA complex suggested that its biological activity is more affected by steric effect (resulted from the conjugation of GnRH ligands). It is possible that the superiority of steric effect could prevents non-specific binding to cells via electrostatic interactions, resulting in specific gene delivery via receptor-mediated endocytosis. It has been reported that multicellular spheroids

better mimic the microenvironment of tissues because they exhibit different features compared to traditional monolayer cultures. These 3D cultures serve as more relevant model systems for a better investigation of gene delivery vectors [79]. We therefore evaluated the specificity of GnRH-CS/pDNA complexes in a 3D multicellular spheroid in addition to 2D monolayer cultures. Similar results were obtained from both 2D and 3D cell culture model systems as shown in Figure 8b. Taken together, these findings confirm that gene delivery by the GnRH-CS is targeted, specific and dependent on GnRH receptor.

Transfection efficiency

Next, to further explore the superiority of the GnRH-CS, we treated targeted cells expressing GnRHR with GnRH-CS/pDNA complex carrying a Luc reporter gene, which were prepared with various polymer:pDNA ratios. This was compared to unmodified CS and transfection efficiency was subsequently assessed. The GnRH-CS/pDNA complexes resulted in much higher transfection efficiency than the unmodified CS/pDNA complexes. A dose response of Luc expression at increasing concentrations of polymer is shown in Figure 9a. Levels of Luciferase expression increased with increasing GnRH-CS:pDNA ratios and maximized at critical ratios when the complexes were completely formed. At similar ratios, the transfection efficacy of the GnRH-CS was clearly higher than those of the unmodified CS. The expected patterns of Luciferase gene expression were reproduced in 3D cell culture system (Figure 9b.).

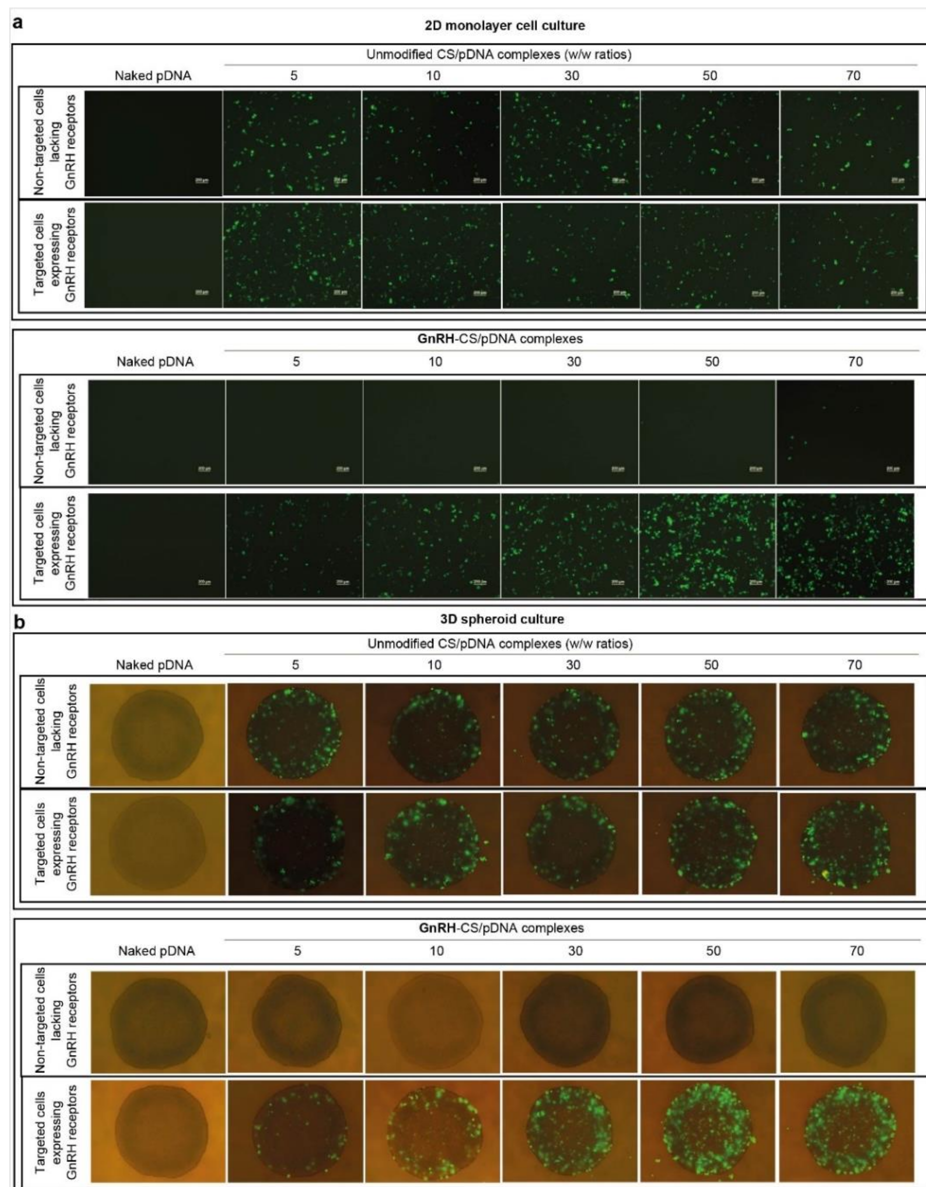

__Figure 8.__ Evaluation of the specificity of targeted cell transfection by GnRH-CS/pDNA complexes. a) Assessment of targeted gene transfer by GnRH-CS/pDNA compared to the unmodified CS/pDNA complexes carrying the GFP reporter gene using a range of polymer concentrations in the targeted cells expressing GnRH receptor and the non-targeted cells lacking GnRH receptor. b) Transfection of 3D spheroids by polyplexes. The spheroids were transfected with polyplexes (i.e. GnRH-CS/pDNA and unmodified CS/pDNA).Representative images showing GFP expression in the monolayer cultures and spheroids were taken at day 3 post transfection.

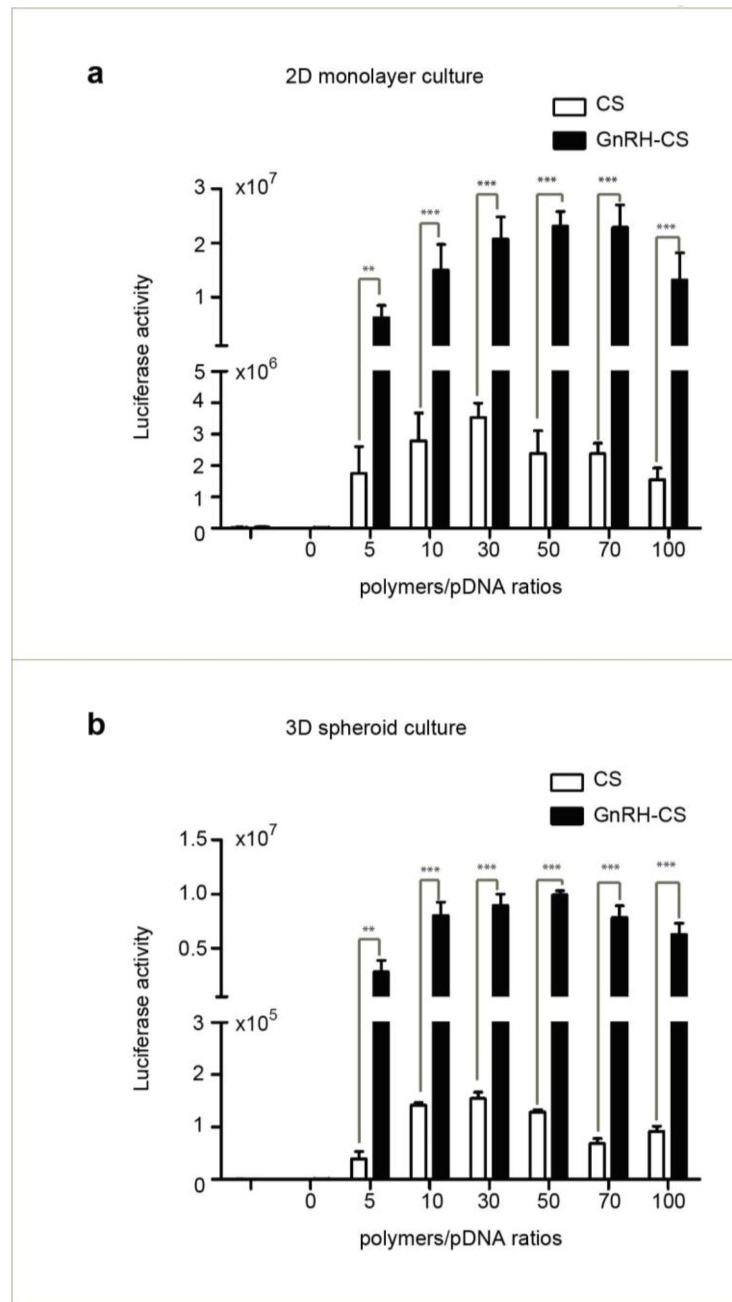

Figure 9. *Comparison of transfection efficacies of GnRH-CS/pDNA and unmodified CS/pDNA complexes in GnRHR-expressing cells. a) 2D monolayer cultures b) 3Dmulticellular spheroids. Data represent the mean + SEM of triplicate samples from one representative experiments of three independent experiments, significant difference; n.s.-not significant, * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ (one way ANOVA with tukey's post hoc test).*

Conclusion

In this study, we have developed and successfully tested an improved version of chitosan-based vector concept by using a GnRH peptide as a targeting moiety for the delivery of a gene of interest to mammalian cells expressing GnRH receptor. GnRH-CS was successfully synthesized and investigated for their physiochemical properties, biocompatibility, and transfection activity. The formation of GnRH-CS/pDNA complexes yielded the positively charged nanoparticle. GnRH-CS proved superior to unmodified CS for specificity and transfection efficiency. The non-toxicity, specificity and high transfection efficiency of GnRH-CS make it ideal for targeted gene delivery to GnRHR-expressing cells, which is necessary for the potential treatment of associated diseases (i.e. some gene disorders of sexual development, reproductive system-related cancers,) as well as for contraception and fertility control. (Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.09.015)

Acknowledgments

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2.2 Gonadotropin-releasing hormone (GnRH)-modified Chitosan as a safe and efficient gene delivery vector for spermatogonia cells

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Abstract

The use of male gonadal tissue as a site for the local delivery of DNA is an interesting concept. Previously, we reported synthesis, physiochemical, and biological properties of gonadotropin releasing hormone (GnRH)-conjugated chitosan as a carrier for DNA delivery to GnRH receptor-overexpressing cells. In this study, the application of modified chitosan as a potential vector for gene delivery to testicular cells was carried out. Transfection efficiency was investigated in mouse-derived spermatogonia cells (GC-1 cells) using green fluorescent protein (GFP) as a reporter gene. GnRH-conjugated chitosan exhibited higher transfection activity and specificity compared to the unmodified chitosan. Furthermore, the GnRH-modified chitosan showed less cytotoxicity. In conclusion, we have developed and successfully tested the GnRH-modified chitosan for delivery of a transgene of interest to spermatogonia cells in vitro. Such vector could be useful in particular for testis-mediated gene transfer.

Keywords: chitosan, gonadotropin releasing hormone, spermatogonia, testis-mediated gene transfer

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Introduction

DNA transfer to male germ cells such as spermatogonia can be carried out by direct introduction of foreign DNA into interstitial cells (Leydig cells) that localized between seminiferous tubules (called testis-mediated gene transfer; TMGT) or into seminiferous tubules [80]. In order to make great progress in TMGT, it is of paramount importance to develop safe vectors capable of delivering a gene of interest to testicular cells at levels of effective and sufficient transgene expression.

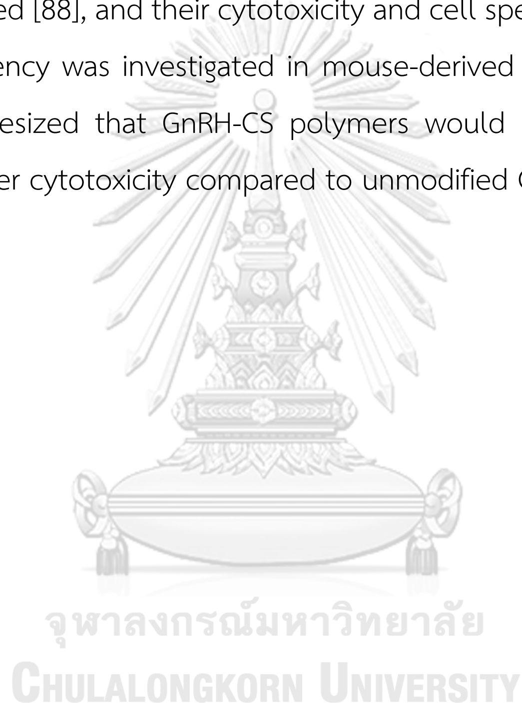
The design and construction of gene delivery vectors have followed two parallel paths. One has taken an advantage of animal viruses which highly evolve their gene delivery capacity. Retroviral and adenoviral vectors have been exploited for the delivery of transgenes into spermatogonia cells [81, 82]. The main limitations for this approach have been unfavorable immunological features and mutagenic integration for some viruses [18]. The other path has been to develop non-viral vectors, such as polymeric nanoparticles and liposomes capable of packaging and delivering DNA to target cells for transgene expression [83, 84].

Chitosan (CS) is a biodegradable polysaccharide composed of D-glucosamine repeating units derived from the deacetylation of chitin found in the exoskeletons of crustaceans. Among non-viral vectors, chitosan has been explored as a gene delivery vector by several research groups [85-87]. Chitosan can efficiently condense DNA, protect DNA from enzymatic degradation, and deliver plasmid DNA to various cell types. Moreover, chitosan is considered to be a safe biomedical material for clinical applications due to its excellent biocompatibility and biodegradability [21]. However, the usefulness of this system is limited by its poor transfection efficiency [6]. To increase affinity and specificity, chitosan can be readily modified and conjugated with targeting ligands (such as transferrin, folate, mannose, galactose) in order to improve its transfection efficiency [7].

Recently, synthesis and physiochemical properties of GnRH-conjugated chitosan (GnRH-CS) as a DNA delivery vector targeted to gonadotropin-releasing hormone receptor (GnRHR) have been studied [88]. Specifically, N-succinyl-chitosan was synthesized by reaction of chitosan with succinic anhydride, which was subsequently reacted with a *decapeptide* GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-

Gly-NH2) by using ethyl (dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) as a coupling agent, yielding GnRH-conjugated chitosan. The results showed that synthesized GnRH-CS was able to condense DNA to form positively charged nanoparticles and specifically deliver plasmid DNA to GnRHR-expressing cells, and GnRH-CS exhibited higher transfection efficiency compared to unmodified CS [88].

The overall aim of this study was to investigate the application of modified chitosan as a potential vector for gene delivery to spermatogonia cells. Complexes of GnRH-CS and plasmid DNA (pDNA) (GnRH-CS/pDNA complexes) were prepared as previously described [88], and their cytotoxicity and cell specificity were characterized. Transfection efficiency was investigated in mouse-derived spermatogonia cells (GC-1 cells). We hypothesized that GnRH-CS polymers would exhibit higher transfection efficiency and lower cytotoxicity compared to unmodified CS.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Materials and methods

Preparation and characterization of polyplexes

The procedure for preparing and characterizing polyplexes was previously described [88]. Briefly, GnRH-CS or unmodified CS solution (1 mg/mL) were each prepared by dissolving chitosan in 0.2% acetic acid. The polymer and plasmid DNA solutions were mixed together under high-speed vortex for 1 min. The ratios of polymer/pDNA complexes (weight/weight) tested were 1:20, 1:40, 1:60 and 1:80. Untreated and naked pDNA-treated cells were used as controls. Particle characterizations (size and zeta-potential) were also performed as previously described [88].

Cell culture and transfection

The mouse-derived spermatogonia cell line (GC-1; ATCC) was maintained in complete D-MEM medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM). Cells were grown at 37°C in a humid atmosphere of 5% CO_2. One day after incubation, the polyplexes solution was directly added to the medium and incubated at 37°C. The optimal ratio of chitosan polymer to pDNA was determined by keeping the pDNA fixed at 1 µg per well and adding increasing concentrations of polymers using pDNA carrying green fluorescence protein gene. Four hours later, the medium was replaced with fresh medium. Untreated and naked pDNA-treated cells were used as controls. Transfection efficiency was assessed by the expression of reporter transgenes at 72 h post-treatment. The experiment was performed in triplicate.

Cytotoxicity

The effect of pDNA/polymer complex on GC-1 spermatogonia cells was examined to determine the cytotoxicity of the delivery system. Cells were seeded at 5×10^4 cells/mL in 48 well microtiter plates in DMEM with FBS. The cells were incubated overnight for attachment. Four different ratios of pDNA/polymer complexes were added in triplicates and incubated for 24 h at 5% CO_2 at 37°C. Following 24 h incubation, cell viability was determined by the CellTiter-Glo® cell viability assay

(Promega). Cells were also stained with the reagents in the LIVE/DEAD® Cell Viability/Cytotoxicity Assay Kit (Invitrogen). Mean cell viability derived from 3 independent experiments was normalized to non-treated controls.

Fluorescence imaging and quantification

Green fluorescent protein (GFP) expression in GC-1 cells was visualized on a fluorescence microscope (Olympus IX71) and captured using a digital camera. Fluorescent quantification was performed with ImageJ software program (version 1.51j8, National Institutes of Health, Bethesda, MD, USA) [89, 90]. Each fluorescent image contained 4,080×3,072 pixels, and each pixel was scored to a value ranging from 0 to 255 on an 8-bit digital scale. On this scale, the maximum background intensity was 80 (indicated as GFP fluorescence) and the minimum background intensity was 0 (assigned as black color). Fluorescence was measured using ImageJ software program on ten cells that were randomly selected. The shape of the cells was outlined and the area, mean fluorescence value and integrated density measured. Several background readings were also measured. The total corrected cellular fluorescence (TCCF) was calculated as the integrated density minus the sum of the area of the selected cell, multiplied by the mean background fluorescence of background readings [91, 92].

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Statistical analysis

Statistical differences were calculated using a one-way ANOVA with Tukey's post hoc test (GraphPad Prism software, version 5.0) and denoted as follows: *$p<0.1$, **$p<0.01$ and ***$p<0.001$.

Results

Transfection efficacy of GnRH-CS

Differences in the transfection efficacy of GnRH-CS/pDNA and unmodified CS/pDNA complexes were observed. Complexes of pDNA with GnRH-CS augmented gene transfer to *GC-1* cells. Treatment with an optimal ratio of GnRH-CS/pDNA complexes (1:40) resulted in enhanced GFP expression (TCCF) (Figure 10a). Representative images of GC-1 cells expressing GFP are shown in Figure 1b. At 72 h post transfection, maximum GFP gene transfer levels were achieved with the GnRH-CS polymer at polymer concentrations of 40 µg. No GFP gene expression was detected in control untreated or naked pDNA-treated cells. Unmodified CS failed to enhance expression over a range of polymer/pDNA ratios (Figure 10b).

Evaluation of cytotoxicity by the pDNA/polymer complex

At 24 h post incubation, there was a dramatic increase in the number of live cells by the GnRH-CS/pDNA compared to the unmodified CS/pDNA complex (Figure 11a). Cell death was more evident with unmodified CS/pDNA, while GnRH-CS/pDNA showed less cytotoxicity (Figure 11b). Cell death also was less evident in both controls (untreated or naked pDNA-treated cells) Figure 11b).

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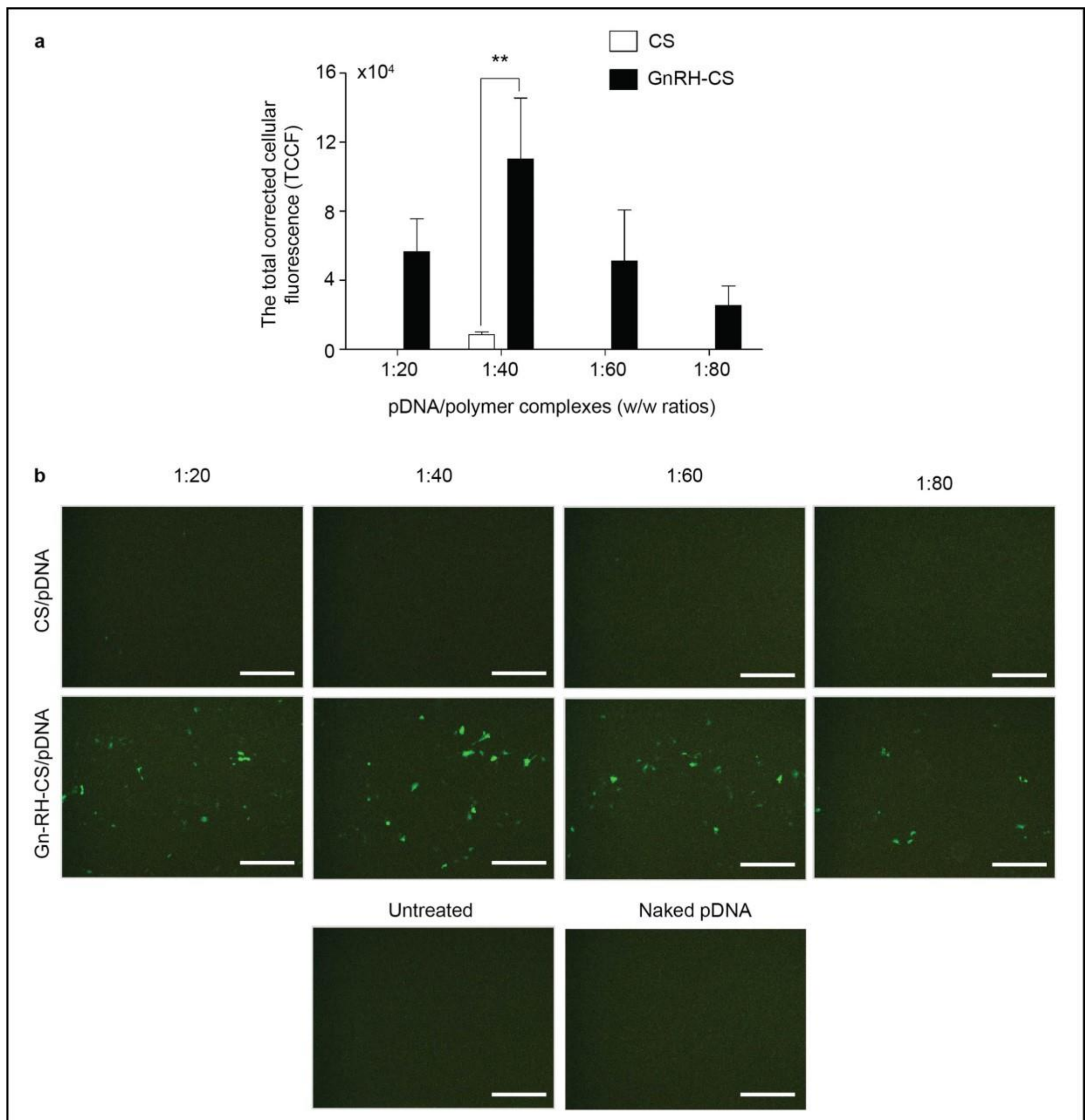

Figure 10. Evaluation of spermatogonia cell transfection efficacy by GnRH-CS/pDNA complexes. (a) Assessment of targeted gene transfer by GnRH-CS compared with the unmodified CS carrying the GFP reporter gene using a range of polymer concentrations in spermatogonia GC-1 cells. Significant differences in TCCF was denoted; n.s.-not significant, * $p<0.05$, ** $p<0.01$, ***$p<0.001$. (b) GFP expression observed after complex treatment of spermatogonia GC-1 cells with the GnRH-CS/pDNA complexes. Spermatogonia GC-1 cells were transfected with different polymer/pDNA ratios (0-80 μg of unmodified CS or GnRH-CS per 1 μg of pDNA).

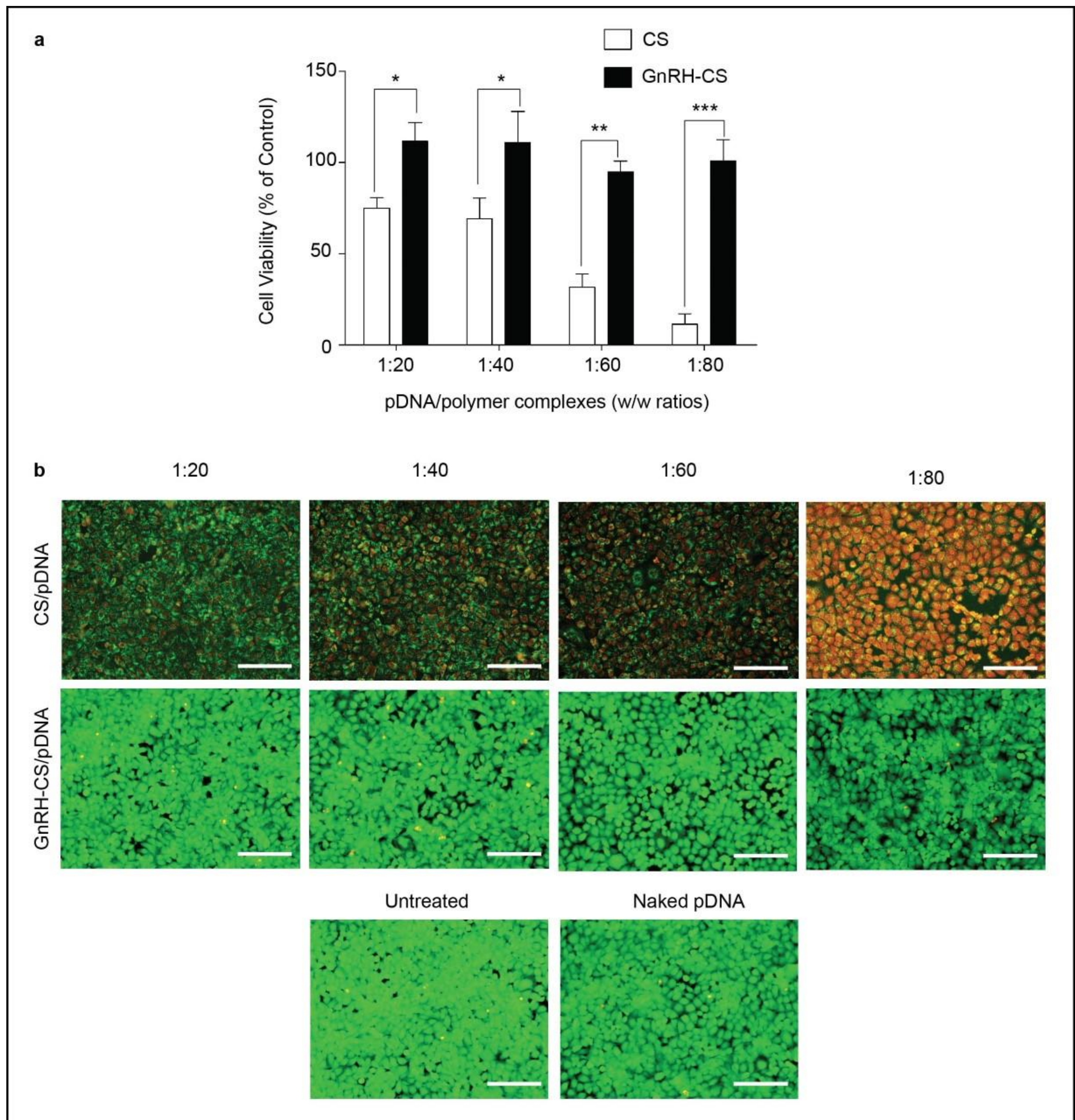

Figure 11. *Spermatogonia cell viability after exposure to four different ratios of pDNA/polymer complexes. (a) Mean cell viability (% of control) between unmodified CS and GnRH-CS/pDNA complexes (*p<0.1, **p<0.01, ***p<0.001). (b) Representative figures of GC-1 cells were treated with pDNA/polymer complexes. Dead and live cells fluoresce red-orange and green, respectively.*

Discussion

In vitro transfection of testicular cells is an alternative method to animal experimentation for simple and rapid evaluation of transfection efficiency of newly developed vectors designed for TMGT [93]. The transfection ability of an improved version of a chitosan-based vector was previously demonstrated using a GnRH peptide as a targeting moiety for the delivery of a gene of interest to established mammalian cells expressing GnRHR *in vitro* [88]. Similar results were observed in the current study.

T*estes* contain *two types* of cells: germ cells (spermatogonia, spermatocytes and spermatids) and somatic cells (Sertoli cells and Leydig cells). Specifically, the current study aimed at investigating *in vitro* gene delivery to male germ cells. However, the *in vitro* germ cells production is limited due to the lack of a culture system for long-term maintenance [94]. To overcome this limitation, the immortalized GC-1 cell line was used. This mouse-derived spermatogonia cell line was immortalized by transfection with a plasmid containing coding sequences for the SV40 large T antigen. More importantly, this cell line shows characteristics of a stage between type B spermatogonia and primary spermatocytes [95].

An important finding in the current study was that unmodified chitosan-based vectors suffer from high cytotoxicity in spermatogonia cells. We believe that this phenomenon is associated with cytotoxicity observed at high concentrations of cationic polymers. It has been suggested that uncontrolled cationic density may compromise the plasma membrane integrity, leading to the pore formation and/or erosion and, subsequently, cytotoxicity [96]. One interesting aspect of GnRH-CS is their ability to reduce cytotoxicity for spermatogonia cells in comparison with unmodified CS. Our findings represent an important step forward in the development of GnRH-CS as a gene delivery vehicle for testis-mediated gene delivery.

Direct introduction of exogenous nucleic acid into testis (referred as TMGT) has attracted great interest over the past few decades [15]. In addition to the production of transgenic animals, TMGT could be a valuable tool for the treatment of certain gene disorders affecting sexual development and reproductive function [24], as well as for contraception and fertility control [10]. Intratesticular injection is an alternative of the local delivery of DNA of interest into the testis. However, injection of a radiolabeled

substance into the rat testes to trace absorption from the testis showed that although the majority of injected substance remained in the testis, some (1%) localized in the liver, indicating the transportation via vascular system (Russell et al., 1987). Because, the expression of GnRHR has been found in other normal reproductive tissues (e.g. breast, endometrium, ovary, and prostate) [8], not limited to spermatogonia cells (Ciaramella et al. 2015), further *in vivo* study of GnRH-CS as a carrier for DNA delivery to GnRHR expressing cells is warranted to see if there is any adverse effect on other tissues.

Conclusion

Unmodified CS-based vectors suffer from low transfection efficiencies and high cytotoxicity in spermatogonia cells. The results of the present study demonstrated that modification of CS with GnRH is a promising strategy to overcome these challenges associated with chitosan mediated gene delivery. This improved version of chitosan can be employed to reduce cytotoxicity or promote transfection efficacy, both of which benefit testis-mediated gene transfer.

Acknowledgments

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2.3 Intra-testicular injection of Gonadotropin Releasing Hormone-modified Chitosan mediated Tumor Necrosis Factor alpha gene as a nonsurgical sterilization in rat

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Manuscript is in process

Abstract

Recently, the intra-testicular injection of chemical sterilants play an alternative method in addressing the issue of animal overpopulation control. The big obstacle of this method is a severe adverse inflammatory reaction. The challenges are to minimize the side effect using the natural compound for reducing the severity of testicular toxicity, remaining the hormone for social ranking while it is still effective in infertility. The present study used Gonadotropin Releasing Hormone-modified Chitosan (GnRH-CS) as a gene vector mediated Tumor Necrosis Factor alpha gene (TNF-alpha) to induce testicular cell death via intra-testicular injection. Sixteen mature male rats were divided into four groups: GnRH-CS/TNF-alpha (Nanoparticle/DNA), GnRH-CS (Naked nanoparticle), TNF-alpha (Naked DNA) and control (without injection). The animals were anesthetized before single bilateral intra-testicular injection, 700 µL of solution per testis. Body weight, testicular volume, testicular weight, testicular ultrasonography and serum testosterone were evaluated. The animals were euthanized on day 35. The testis were removed, weighted and examined histologically. Moreover, liver, kidney and urinary bladder were subjected to histological procedure because they also contain GnRH receptors. The results showed no significant difference in body weight throughout the study period. Significant differences in a reduction in testicular volume on day 7 ($P<0.001$) were found in all treated groups, compared to the pre-treatment volume. The testicular volume continued to reduce on day 14 ($P<0.05$) and 28 ($P<0.05$) in GnRH-CS/TNF-alpha and GnRH-CS groups, respectively. On day 35, after castration, testicular weight and volume of dissected testis were significantly lower in GnRH-CS/TNF-alpha and GnRH-CS groups ($P<0.001$). Serum testosterone levels did not differ ($P>0.05$) throughout the observation period. Ultrasonographically and histopathologically, GnRH-CS/TNF-alpha and GnRH-CS induced testicular degeneration while TNF-alpha and control groups showed normal findings. GnRH-CS/TNF-alpha treated animals showed higher severity degree of testicular degeneration compared to GnRH-CS treated animals. Taken together, intra-testicular injection of GnRH-CS/TNF-alpha provided an alternative non-surgical castration method to induce testicular cell death without any side effects. Long-term effects of intra-testicular injection of GnRH-CS/TNF-alpha on male fertility remain an interesting issue for further research.

Keywords: gonadotropin releasing hormone, chitosan, tumor necrosis factor alpha gene, nonsurgical sterilization, intra-testicular injection, testicular cell death

Introduction

In the present time, nonsurgical sterilization has become an interesting issue for population control in animals. This alternative method has been studied by many researchers using intra-testicular injection of chemical sterilants to induce tissue necrosis [12, 97, 98]. However, serious adverse effects have been indicated in treated animals such as necrosuppurative orchitis and/or ulcerative dermatitis after administration [99, 100]. These weak points remain a major problem to allow the user to convince this alternative method. The main challenge is to compromise the severity of testicular inflammation and necrosis, while the infertility effect remains permanently after a single bilateral injection into the testes. One of the most important issues in the conventional surgical sterilization was the loss of testosterone, the hormone produced by the testes. Testosterone has been suggested to have an impact on dominance rank in humans, monkeys and other species [101-105]. Development of a new version of nonsurgical castration that shows no adverse health effects and less severity of the testicular tissue is required as an alternative method of population control, particularly in the dog and cat.

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Recently, we successfully established our nanostrategy (Gonadotropin Releasing Hormone-modified Chitosan: GnRH-CS) that delivers gene of interest to GnRH receptor (GnRHR)-expressing cell model and spermatogonia cell line [88]. In the present study, we tested our nanoparticle mediated apoptotic gene (Tumor Necrosis Factor alpha: TNF-alpha) to induce testicular cell death using a single bilateral intra-testicular injection in rats. The objectives of this study were to determine the effects of gene inducing testicular cell death delivered by GnRH-CS polymers on testicular changes after a single bilateral intra-testicular injection in rats.

Materials and methods

Experimental design

Animal experimentation was performed according to procedures approved by the Chulalongkorn University Laboratory Animal Center (CULAC), Bangkok, Thailand (Protocol number 1773017). Sixteen adult male Wistar rats (*Rattus norvegicus*) were randomly divided in 4 groups. Group A, B and C were received GnRH-CS/TNF-alpha (Nanoparticle/DNA), GnRH-CS (Naked nanoparticle) and TNF-alpha (Naked DNA), repectively, via intra-testicular injection of each testicle. Group D served as a control group without intra-testicular injection. On Day 0, 7, 14, 28 and 35 days after injection [106], rats were examined (body weight, testicular volume, serum testosterone, testicular ultrasound). At the end point (Day 35), all animals were euthanized; the testes, liver, kidney and urinary bladder were removed. The testes were weighted and then subjected to standard histological procedure. Other tissues were also examined for any histological changes.

Formulation of DNA nanoparticles

Detailed methodology for amplification of DNA and preparation of nanoparticles has been shown in our previous publication [88]. The nanoparticle and DNA solutions were mixed together under high-speed vortex for 1 minute. The optimal ratio of nanoparticle/DNA complexes (1/40) that showed the highest transfection efficiency in the previous study were prepared [107].

Intra-testicular injections

Before testicular injection, animals were anesthetized using Isoflurane inhalation (Attane™, Boise, USA). The testes were exposed in scrotal sac and were fixed by a digital pressure, 70% alcohol pad applied on skin for aseptic technique [106, 108], and 700 μL [98] of solutions (GnRH-CS/TNF-alpha, GnRH-CS or TNF-alpha) was slowly injected into cranio-dorsal aspect of each testicle with 30G × ½" needle attached to 1-ml plastic disposable syringe at the 3-4 mm injection depth [106]. The needle was removed slowly after injection to avoid leakage of the solution to the scrotal sac. Both testes were injected.

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Body weight, testicular weight and testicular volume

Body weight and testicular dimensions were monitored on day 0, 7, 14, 28 and 35. The testicular dimensions (height, width and length) measured with a digital caliper. This study used the formulation of a solid ellipsoid triaxial body to calculate the testicular volume [109, 110]. After euthanasia on day 35, the testicle of each animal was taken, weighed and measured (length, width and depth) in order to compare the testicular weight and volume between groups.

Serum testosterone

Blood samples were obtained from saphenous vein on day 0, 7, 14, 28 and 35 in all rats. The sample was centrifuged and serum was collected for hormonal analysis. Testosterone levels were assayed via a chemiluminescent microparticle immunoassay (CMIA) [111]. The detection limit is 0.05 nmol/L (1.44 ng/dL).

Testicular ultrasonographic examination

The testes were sonographically scanned using a portable Mindray M9Vet® ultrasound machine (Mindray Medical Limited, Thailand) with 4–16 MHz linear transducer (Mindray L16-4Hs probe). Two planes (transverse and sagittal) were examined to compare the echotexture of the testis before and after injection and also between groups [112, 113].

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Pathological analysis of the testes, liver, kidney and urinary bladder

On Day 35, after humane euthanasia with carbon dioxide, the testes were taken and examined for gross examination. Testicular samples together with liver, kidney and urinary bladder were dissected and fixed in 4% paraformaldehyde solution for 24 h, and embedded in paraffin. The sections were stained with Hematoxylin and Eosin (H&E) and observed using a light microscope [98]. The degree of testicular degeneration was classified as normal, mild, moderate and severe. The classification of pathological changes in the testis was modified from previous studies [114-117] (Table 2).

Table 2. The severity score of testicular degeneration

Groups	Normal	Mild degeneration	Moderate degeneration	Severe degeneration
Gross testicular size	normal size	slightly small	small	marked small
Percentage of testicular volume reduction	<20%	≥20-40%	≥40-60%	≥60-80%
Percentage of cellular abnormality, degeneration and death	0%	>50%	>75%	100%

This table was modified from previous studies [114-117]. The histopathology of the testis includes

- Seminiferous tubule hyalinization, tubules show fibrosis with an absence of germ cells and Sertoli cells.
- Sertoli cell only syndrome (SCO), tubules contain only Sertoli cells with an absence of germ cells.
- Maturation arrest, complete interruption of spermatogenesis was found in tubules.
- Hypospermatogenesis, tubules indicate spermatogenesis disorder.
- Vacuolization, there was cytoplasmic vacuoles in the seminiferous tubules due to degeneration.
- Coagulative necrosis, cell death appears pale eosinophilic region due to infarction.
- Mineralization, Dystrophic calcification (DC) occurs in degenerated or necrotic tissue.

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Statistical analysis

GraphPad Prism software (version 5.0) was used to perform statistical analyses. Data were presented as mean ± standard error of the mean (SEM). P values were generated by two-way ANOVA followed with Bonferroni post-test, considered significant when <0.05.

Results

Body weight

Body weight is present in Figure 12. At 0, 7, 14, 28 and 35 days after testicular injection, no significant differences were found between groups (*P*>0.05). Within GnRH-CS/TNF-alpha, GnRH-CS and TNF-alpha groups, body weight on Day 35 after injection showed significantly increases in comparison with pre-treatment.

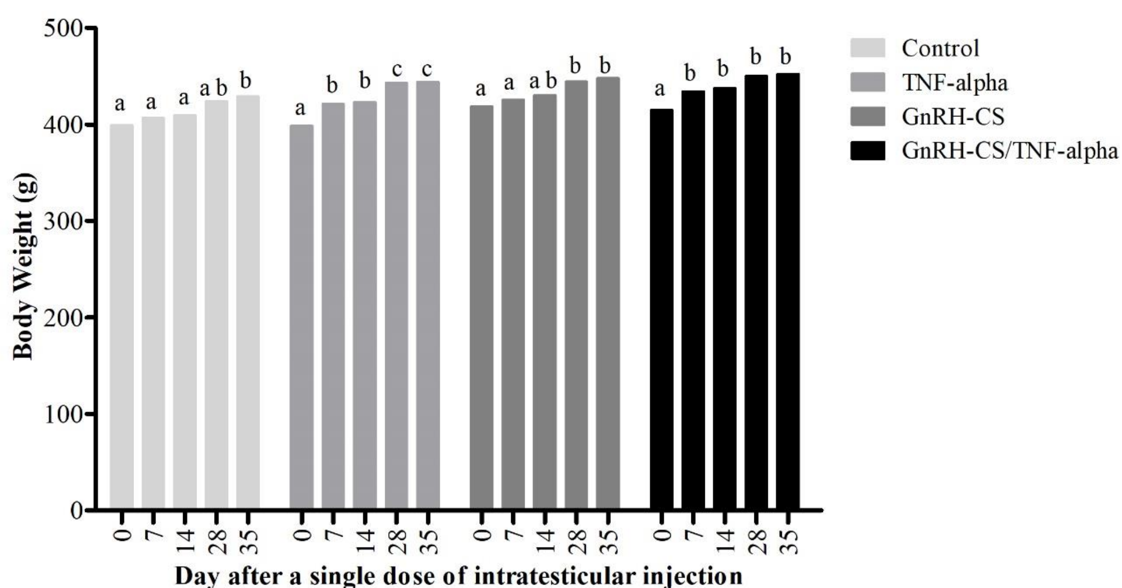

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Figure 12. *Body weight (mean ± SEM) of rats in different groups at each time point after intra-testicular injection. The letter a, b and c indicate differences in body weight among Day 0, 7, 14 and 28 within each group. Different letters indicate a significant difference (P<0.05).*

Testicular volume

The control group showed no significant differences in testicular volume throughout the study period (Figure 13). On the other hand, three treated animal groups showed significantly decreases in testicular volume on Day 7 post injection (*P*<0.001) (Fig. 13). There were 39.7% ± 15.45%, 33.4% ± 10.50% and 28.51% ± 14.18% reduction of testicular volume in GnRH-CS/TNF-alpha, GnRH-CS and TNF-alpha groups, respectively. The testicular volume continued to significantly reduce on Day 14 (*P*<0.05) in GnRH-CS/TNF-alpha group, but not in the GnRH-CS and TNF-alpha groups. The results on Day 28 after injection showed 67.47% ± 11.40%, 59.00% ± 8.38% and 33.67% ± 22.49% reduction compared to the pre-treatment volume in GnRH-CS/TNF-alpha, GnRH-CS and TNF-alpha groups, respectively.

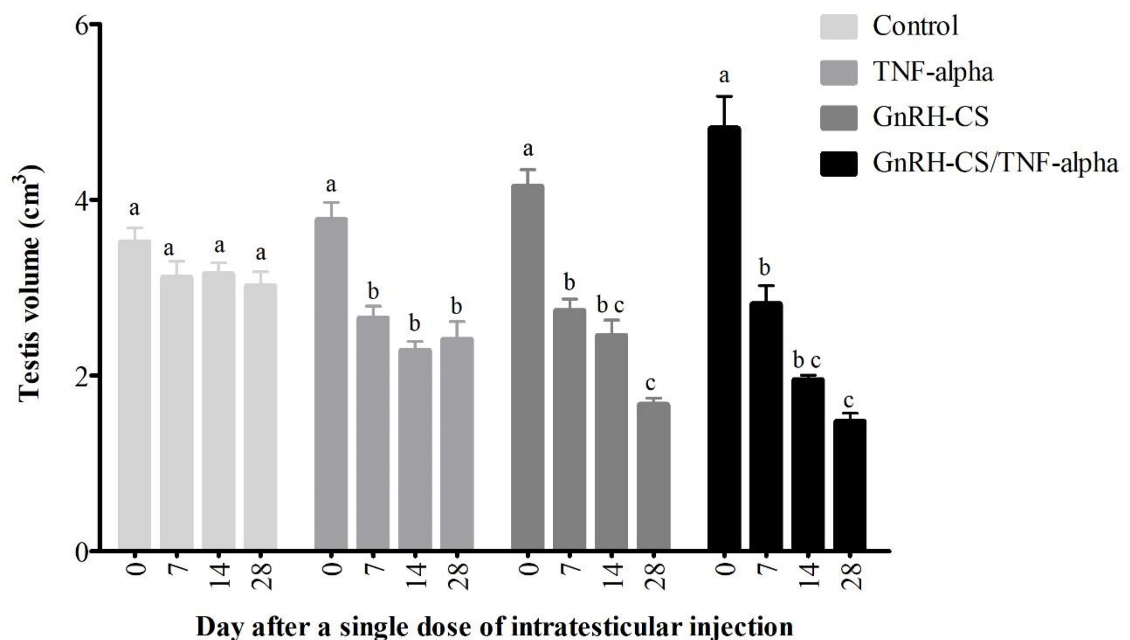

Figure 13. *Testicular volume (mean ± SEM) of the 4 animal groups (control, GnRH-CS/TNF-alpha, GnRH-CS and TNF-alpha) before (Day 0) and after treatment (Day 7, 4 and 28). (n=8 testes per group) The letter a, b and c indicate differences in testicular volume among Day 0, 7, 14 and 28 within each group. Different letters indicate a significant difference (P<0.05).*

Testicular weight and volume of dissected testis

On day 35, testicular weight and volume of dissected testes of treated animals were compared with control animals, and the results were shown in Figure 14. In comparison to the control, testicular weight and volume significantly reduced (*P*<0.001) in GnRH-CS/TNF-alpha and GnRH-CS groups.

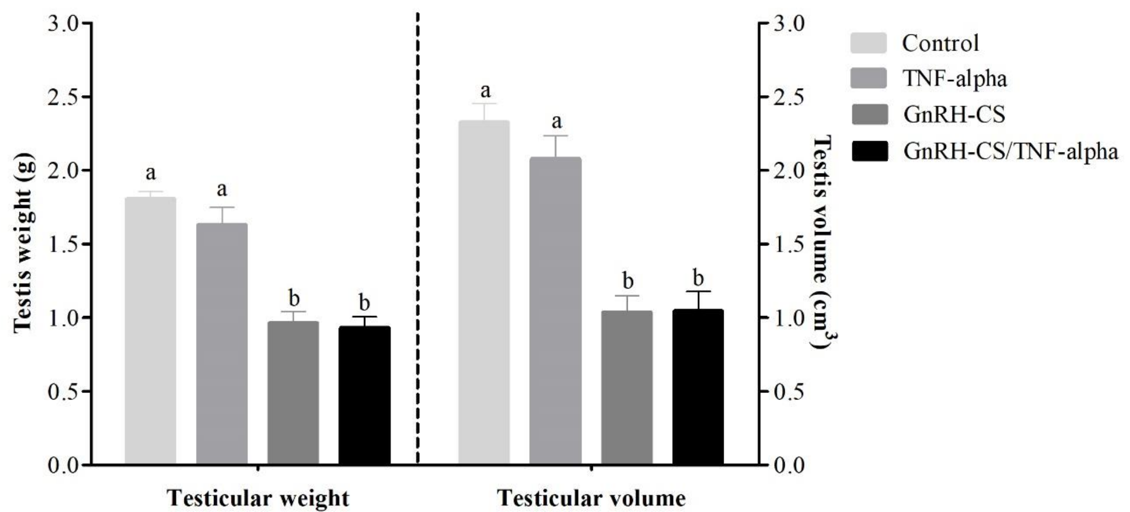

Figure 14. *Testicular weight and volume (mean ± SEM) of the 4 animal groups after castration (Day 35) were significantly lower in GnRH-CS/TNF-alpha and GnRH-CS groups (P<0.001) than those of control group. The letter a, b and c indicate differences in testicular weight or testicular volume among the 4 groups. Different letters indicate a significant difference (P<0.05).*

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Serum testosterone level

Serum testosterone levels of 16 rats categorized by treatment groups are shown in Figure 15. No significant differences in testosterone levels between Day 0 and 35 were observed in each group (*P*>0.05).

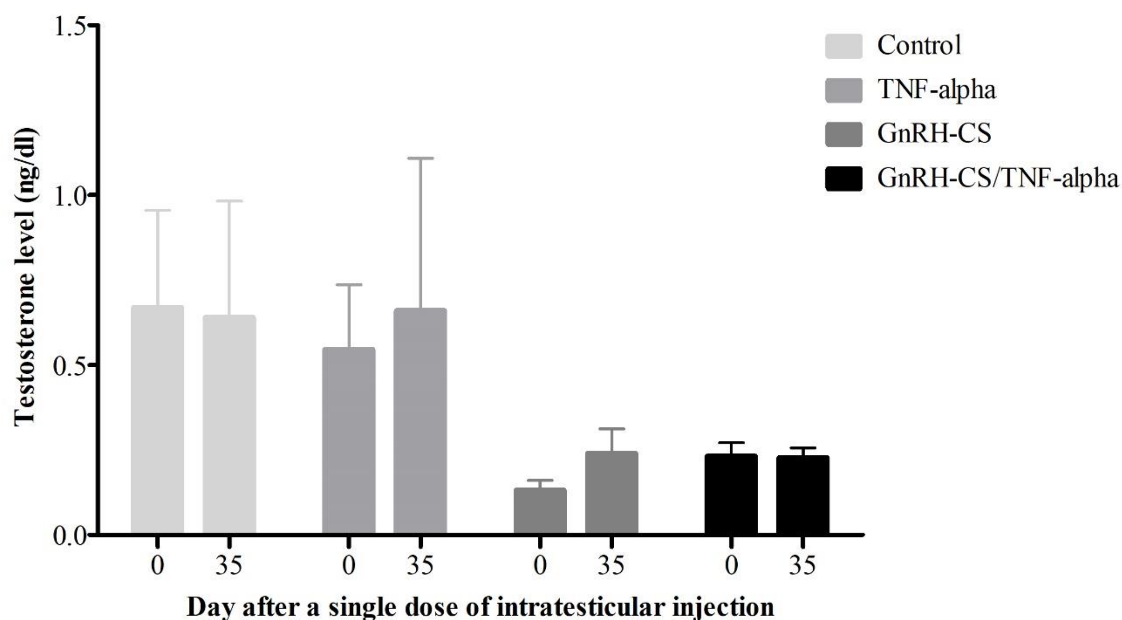

Figure 15. *Testosterone levels (ng/dl) (mean ± SEM) on Day 0 and 35 days after intra-testicular injection in the 4 animal groups. No significant differences (P>0.05) were observed between Day 0 and 35 in all groups.*

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Testicular ultrasonographic examination

As shown in Figure 16, the ultrasonographic results a control rat demonstrated a uniform echotexture of the testes. On Day 7 after injection, the dilatation of testicular vessels with mild heterogeneity of the surrounding parenchyma was showed in three treatment group. On Day 35 after injection, GnRH-CS/TNF-alpha and GnRH-CS groups showed irregular shaped hypoechoic nodule with or without hyperechoic edge, calcification within the testicular parenchyma, a global reduction in testicular size and reflectivity.

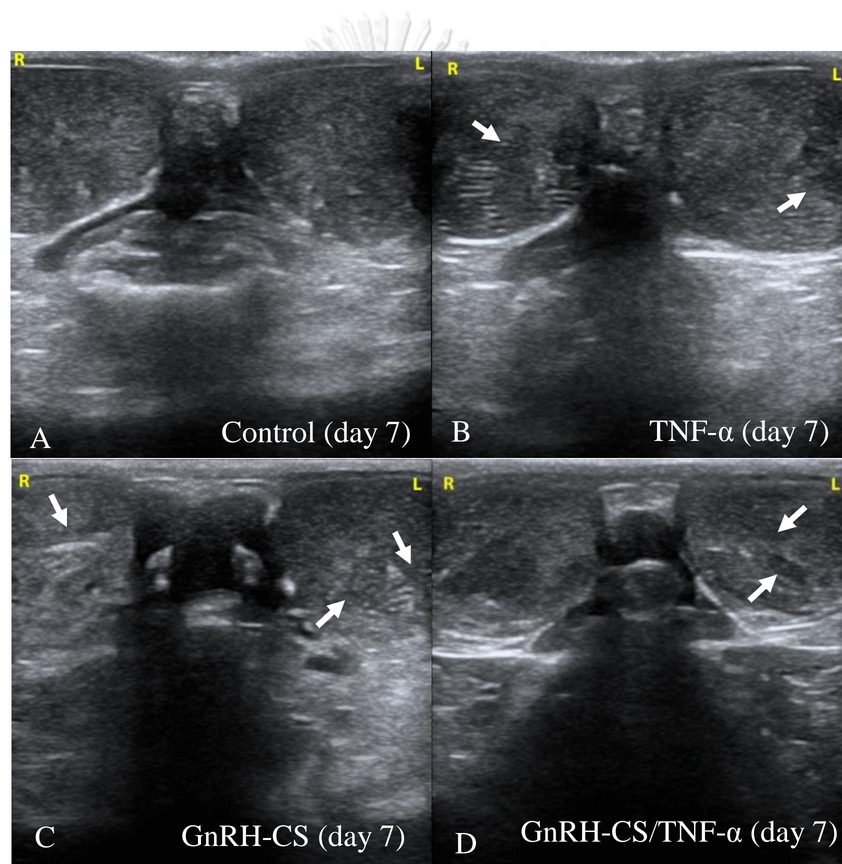

Figure 16. *Testicular ultrasonographic examination on Day 7 after intra-testicular injection (transverse plane of right and left testicles). Control (A), TNF-alpha (B), GnRH-CS (C) and GnRH-CS/ TNF-alpha (D). The dilatation of testicular vessel (thin arrows) with mild heterogeneity of the surrounding parenchyma are found in the 3 treatment groups on Day 7 after intra-testicular injection (B, C and D).*

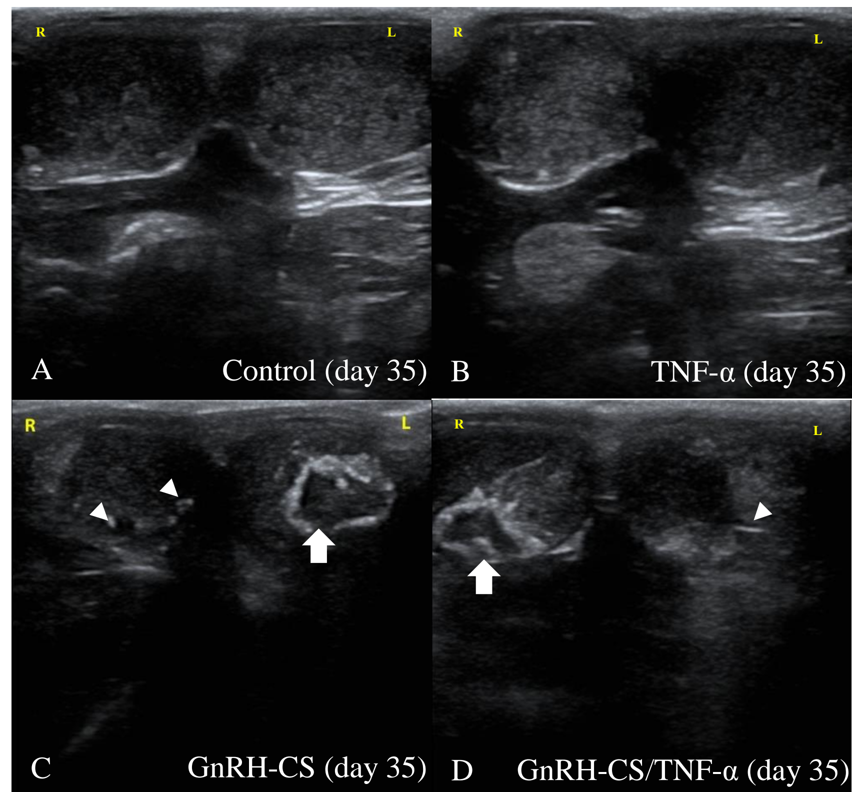

Figure 17. Testicular ultrasonographic examination on Day 35 after intra-testicular injection (transverse plane of right and left testicles). Control (A), TNF-alpha (B), GnRH-CS (C) and GnRH-CS/ TNF-alpha (D). On Day 35, GnRH-CS/TNF-alpha and GnRH-CS groups showed irregular shaped hypoechoic nodules with or without hyperechoic edge (broad arrows), calcification (arrowheads) (C and D).

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Pathological analysis of the testes, liver, kidney and urinary bladder

Macroscopic findings of the testes on Day 35 can be seen in Figure 18. The testes obtained from TNF-alpha group showed similar appearance to the control. GnRH-CS and GnRH-CS/TNF-alpha groups showed a slightly small testicular size compared to the control. Histologically, control and TNF-alpha groups showed well organized seminiferous tubules and interstitial tissue (Figure 19 & 20, A and B). Histopathological analysis of the testicle showed >50% and >75% of germ cell abnormalities in GnRH-CS and GnRH-CS/TNF-alpha groups, respectively (Figure 19, C and D). Leydig cells are present in all groups. Higher magnifications (200×) of histological findings (Figure 20) showed normal seminiferous tubules in the control and TNF-alpha groups. Moderate degeneration in the germinal epithelium, extensive intratubular hyalinization due to fibrosis, coagulative necrosis, lymphocytic infiltration, collapsed seminiferous tubules, mineralization and spermatid retention were consistently observed in animals treated with GnRH-CS/TNF-alpha. Testicular parenchyma in animal treated with GnRH-CS showed mild testicular degeneration lined by Sertoli cells remain, coagulative necrosis, lymphocytic infiltration and tubular vacuolization. There was no pathology of liver, kidney and urinary bladder in all groups (Figure 21-23).

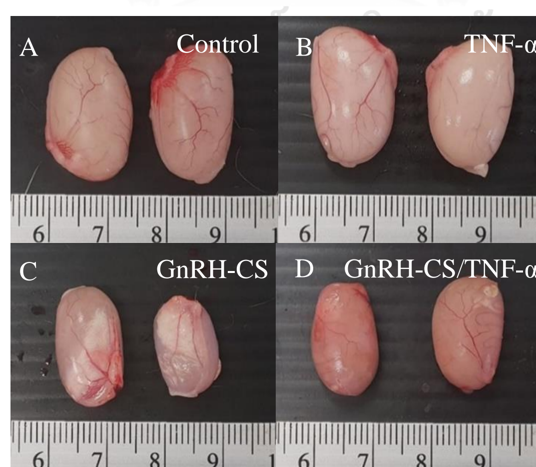

Figure 18. *Macroscopic appearance of testes from control (A), rat treated with TNF-alpha (B), rat treated with GnRH-CS (C) and rat treated with GnRH-CS/TNF-alpha (D). GnRH-CS and GnRH-CS/TNF-alpha groups showed a slightly small testicular size.*

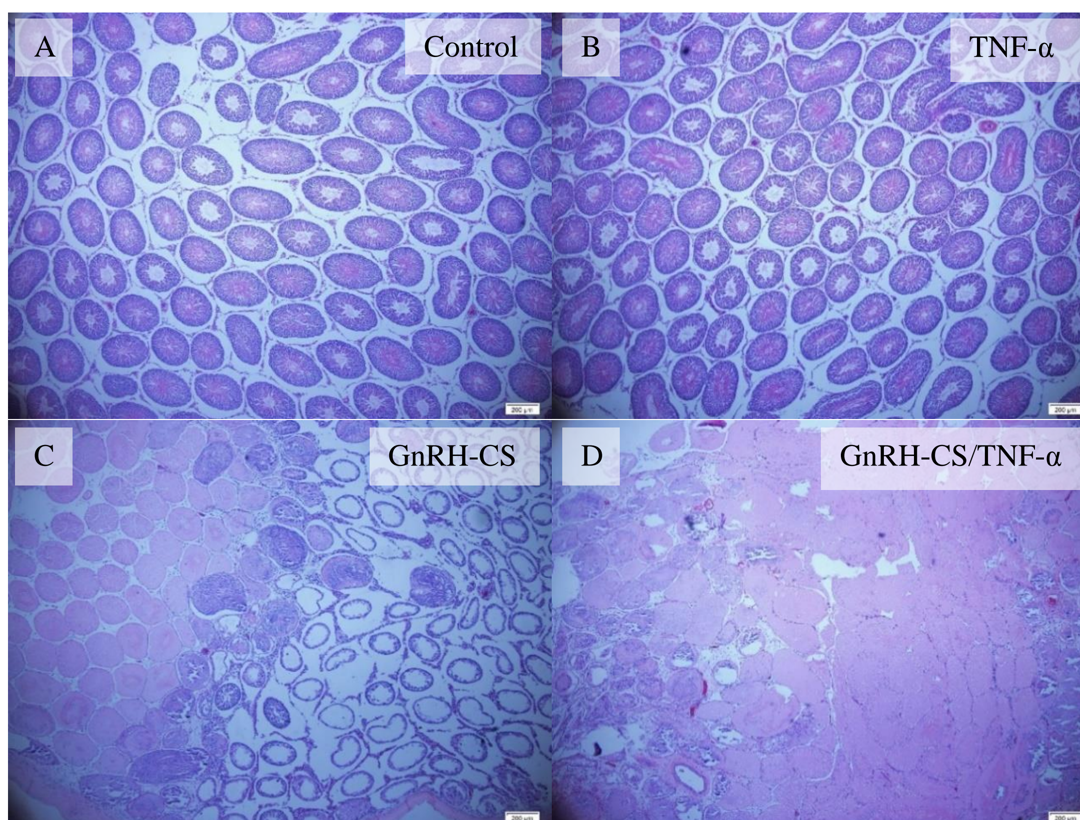

Figure 19. *Microscopic appearance of the testes (×40 magnification, H&E) from the control (A), a rat treated with TNF-alpha (B), a rat treated with GnRH-CS (C) and a rat treated with GnRH-CS/TNF-alpha (D).*

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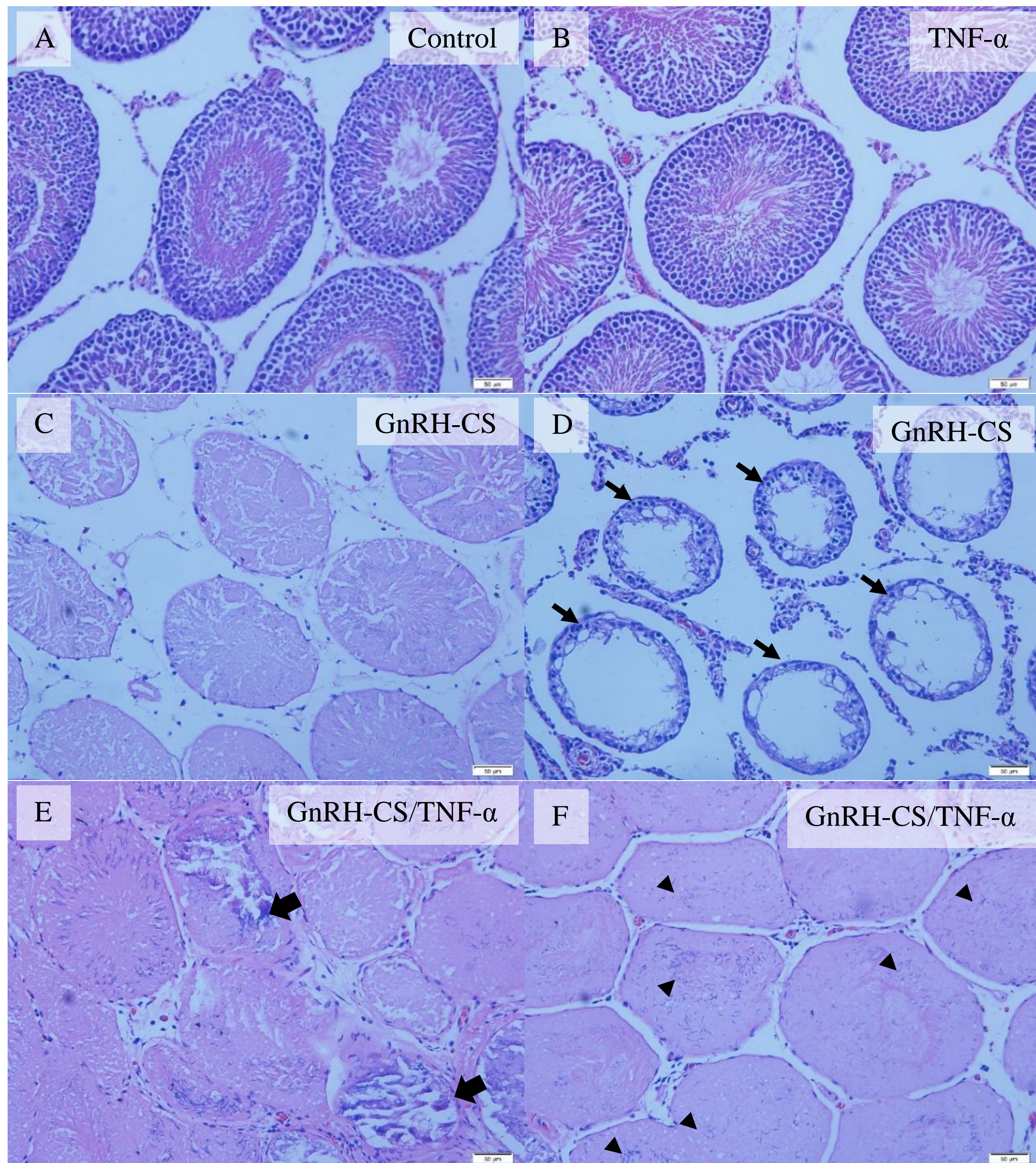

Figure 20. *Microscopic appearance of the testes (×200 magnification, H&E). Testicular histological section of a control rat (A) demonstrated normal seminiferous tubules and interstitial tissue. Seminiferous tubules from GnRH-CS group (C and D) appeared normal interstitial tissue, intratubular coagulative necrosis (C), tubules lined by Sertoli cells remain (thin arrows) and tubular vacuolization (D). GnRH-CS/TNF-alpha (E and F) induced extensive intratubular hyalinization, coagulative necrosis, lymphocytic infiltration, collapsed seminiferous tubules, mineralization (broad arrows) and spermatid retention (arrowheads).*

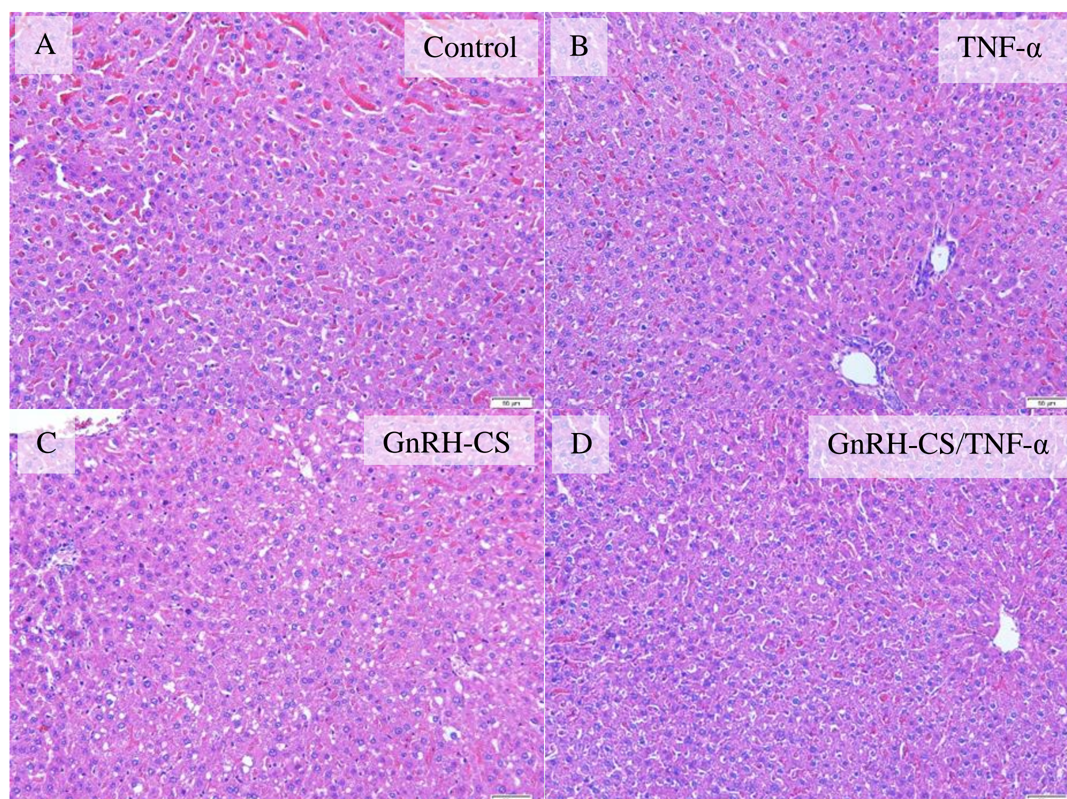

Figure 21. *Microscopic appearance of the liver (×200 magnification, H&E). Histological section of liver in a control rat (A), a rat treated with TNF-alpha (B), a rat treated with GnRH-CS (C) and a rat treated with GnRH-CS/TNF-alpha (D). No pathology of liver was found in all groups.*

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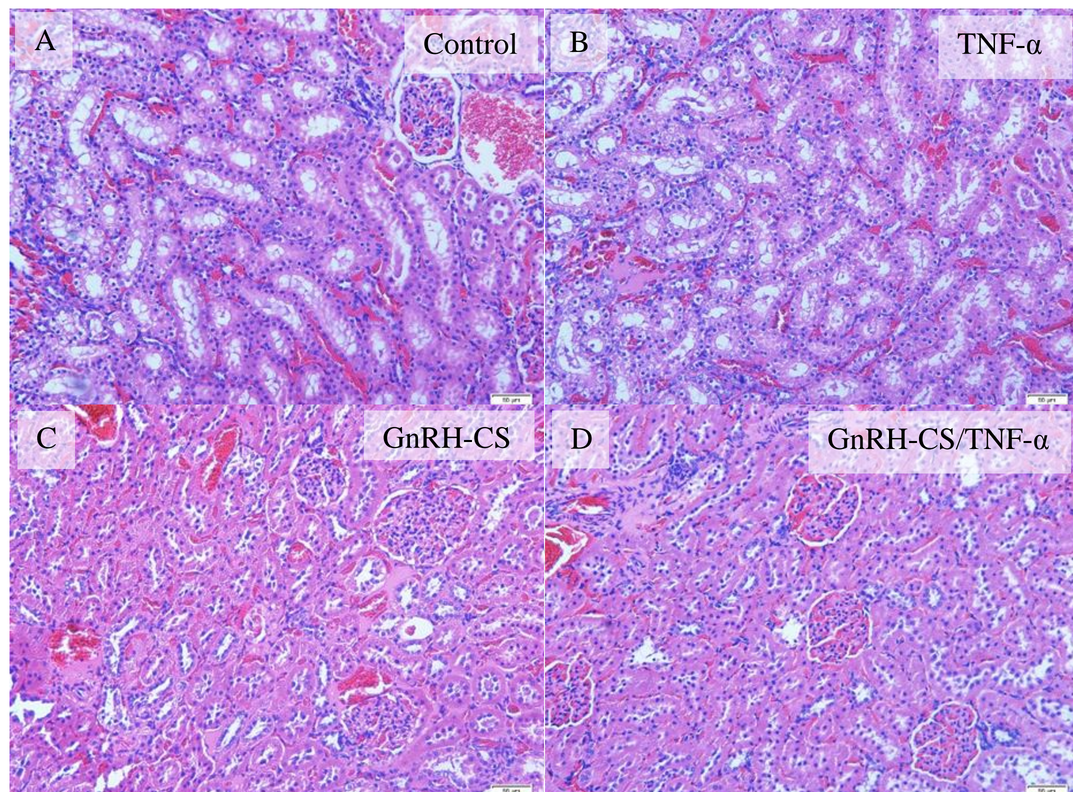

Figure 22. *Microscopic appearance of the kidney (×200 magnification, H&E). Histological section of kidney in a control rat (A), a rat treated with TNF-alpha (B), a rat treated with GnRH-CS (C) and a rat treated with GnRH-CS/TNF-alpha (D). No pathology of kidney was found in all groups.*

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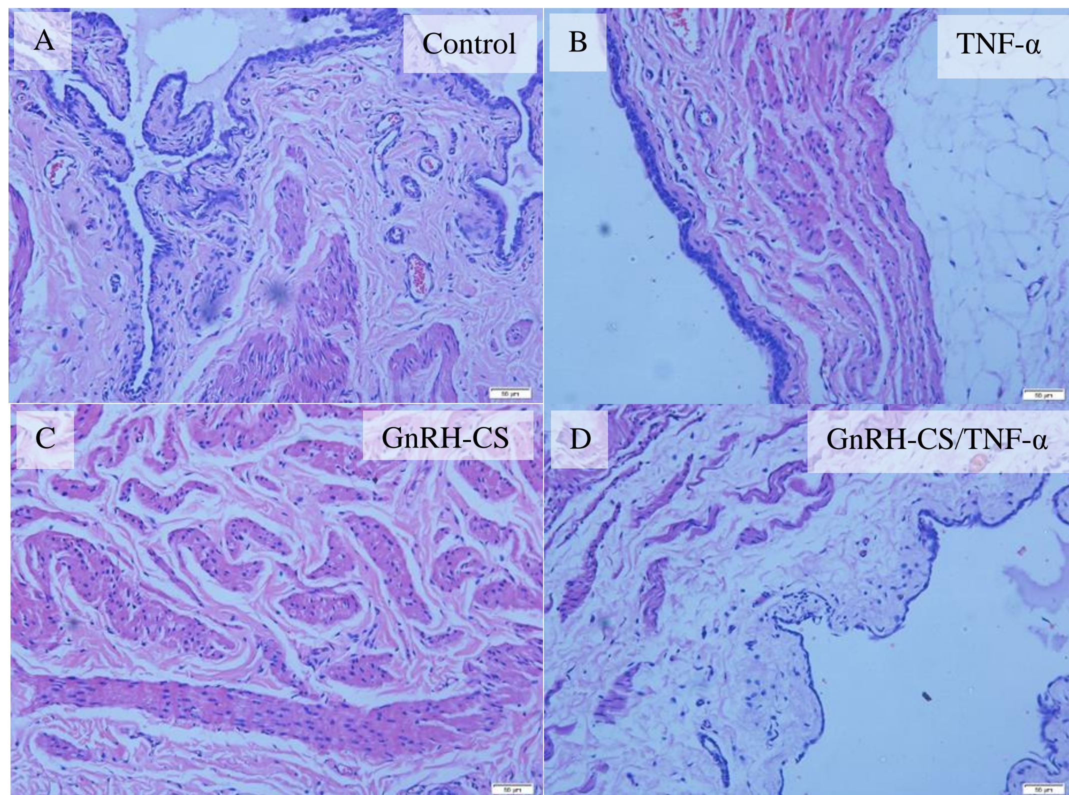

Figure 23. *Microscopic appearance of the urinary bladder (×200 magnification, H&E). Histological section of urinary bladder in a control rat (A), a rat treated with TNF-alpha (B), a rat treated with GnRH-CS (C) and a rat treated with GnRH-CS/TNF-alpha (D). No pathology of urinary bladder was found in all groups.*

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Discussion

Intra-testicular injections of chemosterilants have been suggested as an alternative tool to control large-scale animal population, especially in dogs and cats, because the technique is less time-consuming and cheap compared to surgical castration. Many chemical sterilants have been developed and tested in animals such as zinc-based solution in dogs [118, 119], cats [120] and bears [121]; 20% hypertonic saline in rats [97, 122] and cattle [123]; chlorhexidine in bovines [124]; calcium chloride ($CaCl_2$) in rats [125, 126], cats [127], dogs [30], goats [128] and cattle [129]; $CaCl_2$ with DMSO in cats [130]; cadmium chloride ($CdCl_2$) in lamps [131] and clove oil in dogs [132]. The main point of chemical castration via intra-testicular injection was either safe but not effective or effective but not safe [118]. The challenges are to provide permanent infertility after a single injection and also to minimize side effects thus reducing the severity of testicular toxicity.

The present study used chitosan that is a natural polysaccharide derived from chitin. Chitosan has anticancer and anti-inflammatory properties [133, 134]. Chitosan was modified its chemical structure by conjugated with GnRH as a targeting moiety for delivering a gene of interest to cells expressing GnRHR such as mammary, endometrium, ovary, testis, prostate and urinary bladder [8, 22, 135]. A gene of interested in this study is TNF-alpha as a gene inducing apoptosis. Moreover, under normal physiological conditions, TNF-alpha are produced in the testis and play a role in maintaining testicular function [136]. Our previous studies showed that chitosan was able to condense DNA and specifically deliver DNA to GnRHR-expressing cells [88] and spermatogonia cells [107]. In this study, GnRH-CS was used to deliver TNF-alpha gene to induce testicular cell death in male rats as an animal model.

The most interesting finding was a progressive decrease in testicular volume post intra-testicular injection with GnRH-CS/TNF-alpha and GnRH-CS. The control animals showed no significant decrease in testicular volume during study period. Moreover, the testicular volume and weight recorded after castration were significantly lower in GnRH-CS/TNF-alpha and GnRH-CS groups than those of control and TNF-alpha groups. These findings accorded with earlier observations showing that the testicular weight should be considered equal to volume because of its density (1.03–1.04). In

addition to the reduced total testicular volume and testicular weight, it might be affected by testicular germ cell degeneration, a loss in germ cell number and seminiferous tubular fluid (STF) [137]. However, many studies of chemical sterilants revealed individual variation in testicular size after injection, no changes were observed in some animals while others showed small testcles [32, 121]. The presence of testis in animal treated with some chemical sterilants that might be blind observers to indicate castrated or non-castrated animal. Therefore, identification of non-surgically castrated animals in large scale population must be concerned.

Body weight was measured to monitor general health effects on weight loss due to stress/pain after treatment. During the study period, animals could eat normally and no weight loss was detected in all treated groups. It is possible that treated animals did not suffer from pain or stress. With regard to pain, the literature reviewed that peripheral nerve pain located only on the scrotal skin and in the capsule of the testis. Therefore, it is possible that pain due to capsular distension may occur within 24 hours and remain for 7-15 days [138]. This study showed no change that indicated pain after recovering from anesthesia.

Surgical sterilization eliminates gonadal sources of testosterone production. Testosterone is needed for secondary sexual characteristics, including male dominance rank [101-105]. Unlike the conventional method, non-surgical sterilization does not eliminate Leydig cell that is the sources of testosterone production [127, 138]. The present study revealed no significant changes in serum testosterone levels in all treated groups. Our strategy might not prevent the development of benign prostatic hyperplasia (BPH) that is common in adult intact male dogs under androgenic control. It might be useful for controlling the animal population in some species that need male sex hormone for dominance hierarchy.

Ultrasonography is a common used imaging for detection of pathologic conditions of the testes [139]. Normal ultrasound images of the testis revealed a uniform texture and reflectivity as observed in control animals. On Day 7 after injection, three treatment groups showed the dilatation of testicular vessel due to testicular atrophy [139], this finding supported the results of reduced testicular volume. However, the color doppler ultrasound should be used to confirm dilated testicular

vessel [140]. Testicular ultrasound examinations of GnRH-CS/TNF-alpha and GnRH-CS groups, on Day 14 showed irregular-shaped hypoechoic nodule with or without hyperechoic edge, and on Day 28 and 35 calcification within the testicular parenchyma and hypoechoic nodule with/without irregular hyperechoic margins were observed. A global reduction in testicular volume and reflectivity were found especially on Day 35 after injection, these findings refered to testicular atrophy [141]. The hypoechoic nodules found in some animals might relate to the site of injection that showed more heterogeneity of echotexture than other surrounding tissues. Interestingly, the abnormalities expressed only part of the injection site or not the entire testicle. The challenge is to develop the solution property able to diffuse through the whole testicular tissue or adjust the technique of injection.

An ideal chemosterilant is safe, effective, inexpensive, permanent and required single injection [30, 126]. Permanent sterilization needs irreversible damage of testicular cells. In this study, GnRH-CS/TNF-alpha group showed higher degree of severity of testicular cell death than others. The severity of testicular degeneration in this study divided in to normal, mild, moderate and severe. The current study found that GnRH-CS/TNF-alpha and GnRH-CS induced moderate and mild testicular cell death, respectively. The plain GnRH-CS also induced testicular cell death by its anticancer and anti-inflammatory properties [134]. While TNF-alpha groups did not show significant changes in testicular weight, volume or histologic appearance at the end of the study compared to the control animals. It is possible that the naked TNF-alpha is degraded by the body enzyme after injection. Germ cell abnormalities include seminiferous tubule hyalinization (absence of tubule), Sertoli cell only syndrome (presence of Sertoli cells only in tubule), maturation arrest (incomplete spermatogenesis) and hypospermatogenesis (presence of spermatozoa and absence of spermatogonia) [117]. Testicular degeneration can be defined as the mixture of vacuolization in germinal epithelium of the seminiferous tubules, partial loss of germ cells, disorganization of germ cell layer and leaving tubules lined only by Sertoli cells [137]. Coagulative necrosis can be seen as a big area of pale eosinophilic region due to infarction. Histologically, our findings were in agreement with previous studies using other chemical sterilants [142]. Testicular necrosis is not reversible and the affected

area is replaced by fibrous tissue. According to testicular necrosis, spermatids fail to be moved and still remained at the lumen. In addition, because liver and kidney that are important organs of metabolism, detoxification, storage and excretion [143], our study showed no histological changes in the liver and kidney of treated animals. In particular, as the urinary bladder represents cells expressing GnRHR [135], we also found no histologic changes of the urinary bladder. It is suggested that intra-testicular injection of the modified vectors used in the present study are generally safe.

Conclusion

The main goal of the current study was to determine the effect of single bilateral intra-testicular injection of gene inducing testicular cell death delivered by GnRH-CS polymers. In this study, intra-testicular injection of GnRH-CS/TNF-alpha resulted in seminiferous tubules necrosis (>75% of the entire biopsy samples) without any adverse health effects on injection site and serum testosterone levels. In the future, an in-depth study of long-term effects of intra-testicular injection of GnRH-CS/TNF-alpha on male infertility should be explored.

Acknowledgments

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2.4 Gonadotropin-releasing hormone (GnRH)-conjugated Chitosan for targeted DNA delivery to GnRH receptor overexpressing mammary gland tumor

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Manuscript is in process.

Abstract

In a previous study, we generated the Gonadotropin-releasing hormone (GnRH)-conjugated Chitosan (CS) and tested it in the GnRH receptor (GnRHR)-expressing cell model. This version of chitosan could be a promising carrier for targeted DNA delivery to GnRHR-expressing cells because it showed higher transfection activity compared to unmodified chitosan, no cytotoxicity and the capacity of DNA condensation. The main purpose of this study was to investigate the efficacy of GnRH-modified chitosan (GnRH-CS) for GnRHR overexpressing mammary gland tumor targeted delivery of apoptotic gene as a gene therapy. We used the nanoparticle mediated reporter gene (Green Fluorescent Protein; GFP) and therapeutic gene (Herpes Simplex Virus Thymidine Kinase/Ganciclovir; HSV-TK/GCV and Tumor Necrosis Factor alpha; TNF-α) delivery to two types of mammary cancer cell line (MDA-MB-436 and MCF-7) that were generally difficult to transfect. Moreover we studied this nanoparticle mediated apoptotic gene (TNF-α) delivery for mammary cancer treatment using mammary cancer bearing nude mice as a pilot *in vivo* study. The result showed that GnRH-CS had higher efficiency reporter gene transfection compared to unmodified CS. As for the HSV-TK/GCV induced toxicity, MDA-MB-436 revealed a less decrease in cell viability than MCF-7 because of its properties (Triple-negative breast cancer, poor prognosis, high risk of recurrence, etc.) that appeared resistance to suicide gene/pro-drug combinations induced cytotoxicity. TNF-α offered an alternative suicide gene to treat the mammary cancer cell used in the present study. Our *in vivo* study using mammary gland tumor-bearing nude mice demonstrated promising results in tumor suppression/ regression when TNF-α was delivered by the GnRH-CS to targeted mammary tumor cells. It is possible that our study could improve the quality of life in animals suffering from mammary cancer from the side effects of chemotherapy, surgical stress and postoperative complications.

Keywords: gonadotropin releasing hormone (GnRH), chitosan, gene delivery

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Introduction

The goal of cancer gene therapy is to modify the genetic material of cancer cells by introducing a therapeutic gene to compromise or inhibit the cell growth. Many types of gene have been used for cancer treatment such as suicide genes, anti-angiogenesis genes and cellular stasis genes [144]. However, when the naked nucleic acid therapeutics are administered intravenously to systemic circulation they encounter many obstacles, such as the enzymatic degradation and the rapid clearance, leading to low efficiency of their therapeutic capacity to targeted cells. Moreover, cellular entry limitations of nucleic acids such as the electrostatic repulsion from cell membranes and poor endosomal escape are challenging to develop nanocarriers for gene therapy [145]. Therefore, a number of different gene carriers have been used to deliver a therapeutic gene to targeted cell. In our previous study, we developed and successfully tested an improved version of chitosan (CS)-based vector concept by using a Gonadotropin-releasing hormone (GnRH) peptide as a targeting moiety for the delivery of a gene of interest to mammalian cells expressing GnRH receptor (GnRHR).

The aim of this study is to investigate the efficacy of chitosan for targeted DNA delivery to GnRHR overexpressing mammary gland tumor. We used the chitosan as a gene carrier because it is a natural linear polysaccharide from biological origin (marine polymer chitin) which has biodegradability, biocompatibility, low immunogenicity, low cytotoxicity and the capacity of nucleic acids condensation. Although chitosan is one of the most candidate natural polymers in gene delivery system (especially in cancer therapy), its limitation is low transfection efficiency and low cell specificity. To address these hurdles, it should be conjugated with targeting ligands [146]. Our targeting ligand is GnRH peptide because GnRH can bind to its receptor that are overexpressed in cancer tissues related to the reproductive system especially breast cancer.[9, 147] We hypothesized that our GnRHR targeted Chitosan can deliver a gene of interest to GnRHR overexpressing mammary gland tumor.

Materials and methods

Polymer synthesis and characterization

The polymers were prepared as described in our previous study [88]. Briefly, N-Succinyl chitosan (NSCS) was synthesized by reacting CS with succinic anhydride. The conjugation of NSCS with GnRH was carried out by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) as a coupling agent. ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) and attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) were used for measurement of the degree of N-substitution (DS) of NSCS units in the polymers and the successful conjugation of polymers with GnRH.

Cell culture

Two types of mammary cancer cell line (MDA-MB-436; ATCC® HTB-130™ and MCF-7; ATCC® HTB-22™) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), Penicillin (100 units/ml), Streptomycin (100 μg/ml) and L-glutamine (2 mM) at 37˚C and 5% CO_2.

Amplification of plasmid DNA (pDNA)

After transformation, plasmids were isolated and purified from the overnight *Escherichia coli* (*E.coli*) strain DH5 α culture by using HiSpeed Plasmid Midi Kit. The concentration of plasmid DNA was determined by measuring the absorbance at 260 nm. The plasmid was stored at -20°C until used.

Preparation and characterization of polyplexes

To prepare 1 mg/ml polymer solutions, GnRH-CS and unmodified CS were each liquefied in 0.2% acetic acid. The polymer and plasmid DNA solutions were mixed together under high-speed vortex for 1 minute. The ratio of polymer/pDNA complexes (weight/weight) were prepared depending on the experiment. The naked DNA was used as the control. Particle size and ζ-potential charge were similar in the previous study [88].

Cell transfection

The polyplexes solution was directly added to the cell supernatant and incubated at 37°C. Four hours later, the medium was replaced with fresh medium. Depending on the experiment, specificity or efficiency of transfection as determined by the expression of reporter transgenes was assessed at indicated time points.

Examination of reporter gene expression

Plasmid Green Fluorescence Protein (pGFP) and plasmid Luciferase (pLuc) were used as a reporter gene. GFP expression was analyzed using a Nikon Eclipse TE2000-U fluorescence microscope. The Steady-Glo® luciferase assay kit was used to evaluate expression of the luciferase reporter transgene in transfected cells.

Examination of therapeutic gene expression

As for the Herpes Simplex Virus Thymidine Kinase/Ganciclovir (HSV-TK/GCV) experiment, cells were incubated with the HSV-TK for 2 days and then the medium was replaced with fresh medium containing varying 20 uM of GCV. The cell viability was examined 5 days post incubation by CellTiter-Glo® Luminescent Cell Viability assay system (Promega Inc, Madison, WI, USA).

Tumor model

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The study and animal use were performed according to procedures approved by the Chulalongkorn University Laboratory Animal Center (CULAC), Bangkok, Thailand (Protocol number 1773017). The mammary cancer cell line (MDA-MB-436) were maintained at 37˚C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), Penicillin (100 units/ml), Streptomycin (100 µg/ml) and L-glutamine (2 mM). MDA-MB-436 cells (1×10^6 cells suspended in Corning Matrigel matrix; Sigma-Aldrich Pte. Ltd., Singapore) were inoculated subcutaneously on the dorsal side between the shoulder blades of nude mice [148].

In vivo pilot studies

Thirty days after tumor induction, tumors were palpable; the mice were randomly divided into 3 groups, with each group consisting of 2 mice. In a control (Group A), PBS was injected into tail vein. Unmodified CS/TNF complex (Group B) or GnRH CS/TNF complex (Group C) was injected intravenously. Seven days after injection, mice were sacrificed and tumors were excised. The tumor volume as measured by digital caliper was compared between groups.

Statistical analysis

GraphPad Prism software (version 5.0) was used to perform statistical analyses. Data were presented as mean±standard error of the mean (s.e.m). P values were generated by one-way or two-way ANOVA, considered significant when <0.05 and denoted as follows: *p < 0.05, **p <0.01 and ***p < 0.001.

Results and discussion

Analysis of reporter gene expression

Specificity of transfection

We transfected the GnRH-CS/pDNA complex to MDA-MB-436 cell line as a representative of mammary gland tumor. The comparison of MDA-MB-436 cell specificity between GnRH-CS and unmodified CS prepared with increasing weight ratios of polymers and pDNA carrying a GFP reporter gene as shown in Figure 24. MDA-MB-436 basal like human mammary cancer is the hormone receptors (HRs)-negative mammary cancer that is associated with unresponsiveness to the usual endocrine therapies, aggressive histology, poor prognosis, and shorter survival [149]; however, this cell line could be specifically transfected with GnRH-CS/pDNA by receptor mediated endocytosis. Cell specificity of GnRH-CS was more than unmodified CS that deliver gene into the cell via the electrical charge. This result confirm that GnRH ligands could improve the targeting property of chitosan to send gene to mammary cancer cell.

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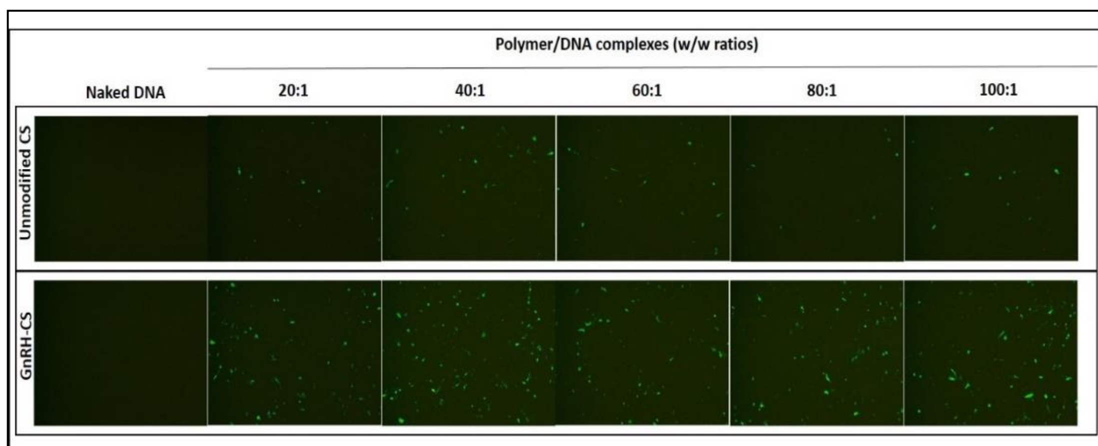

Figure 24. *Comparison of the specificity of transfection and transfection efficiency of GnRH-CS/pDNA and unmodified CS/pDNA complexes carrying a GFP reporter gene in MDA-MB-436 cell. Assessment of targeted gene transfer by GnRH-CS/pDNA compared to the unmodified CS/pDNA complexes carrying the GFP reporter gene using a range of polymer concentrations in MDA-MB-436 cell line (Day 4 after transfection). Weight ratios of polymer:DNA are 0:1, 20:1, 40:1, 60:1, 80:1, and 100:1, respectively. GnRH-CS can enhance the gene delivery efficiency more than unmodified chitosan in the same polymer:pDNA ratio.*

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Transfection efficiency

GnRH-CS can enhance the gene delivery efficiency more than unmodified chitosan in the same polymer:pDNA ratio. We tested the optimal ratio (polymer:pDNA, 1:40) of GnRH-CS/pDNA complexes carrying a Luc gene to the MDA-MB-436 cell. The experiment showed that the GnRH-CS/pLuc complexes had higher transfection efficiency than unmodified CS/pLuc complexes as showed in Figure 25.

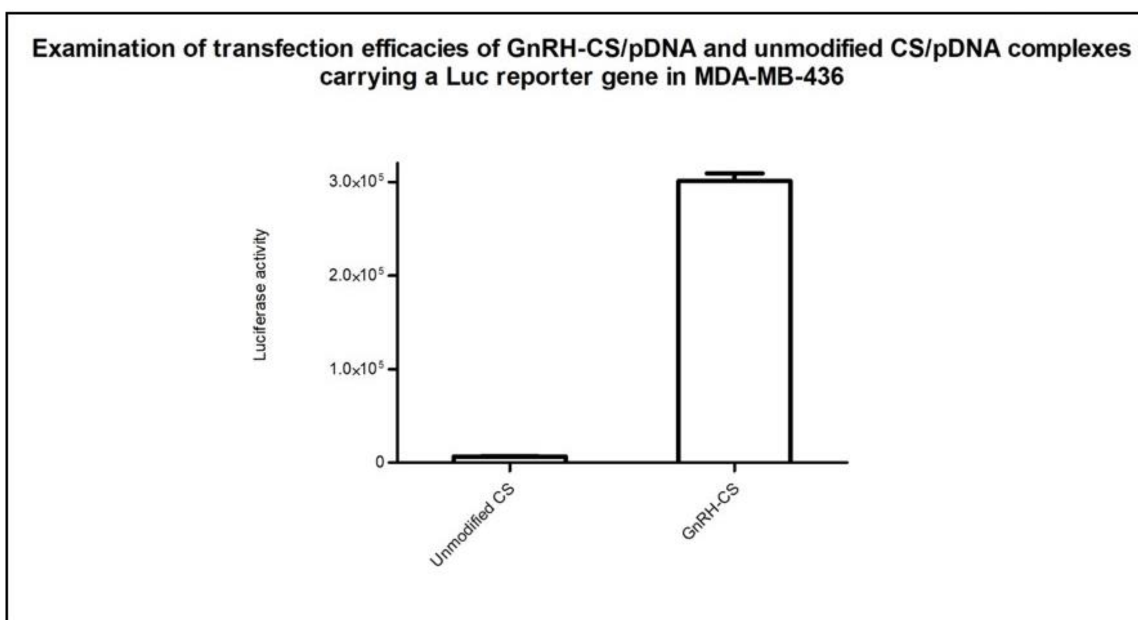

Figure 25. *Examination of transfection efficacies of GnRH-CS/pDNA and unmodified CS/pDNA complexes carrying a Luc reporter gene in MDA-MB-436 cell. At similar GFP reporter gene expression ratios, the transfection efficacy of the GnRH-CS was clearly higher than the unmodified CS.*

Analysis of therapeutic gene expression

Evaluation of the effect of HSV-TK expression on GCV-induced cytotoxicity in MDA-MB-436 and MCF-7 cells

In both cell lines, average cell survival rate of GnRH-CS/pDNA complexes carrying a HSV-TK gene transfected cells prior to GCV administration (20 uM) was lower than that of cells transfected with unmodified CS/pDNA complexes carrying a HSV-TK gene with GCV treatment (at same concentration) as shown in Figure 26a and 26b . MDA-MB-436 revealed a less decrease in cell viability than MCF-7 because MDA-MB-436 is the triple-negative breast cancer (TNBC), which is an aggressive subtype with no specific treatment, poor prognosis and high risk of recurrence so it appeared resistance to suicide gene/pro-drug combinations induced cytotoxicity. Because MDA-MB-436 has low sensitivity to the HSV-TK/GCV system, we used TNF-α gene that is the alternative choice to induce mammary cancer cell apoptosis.

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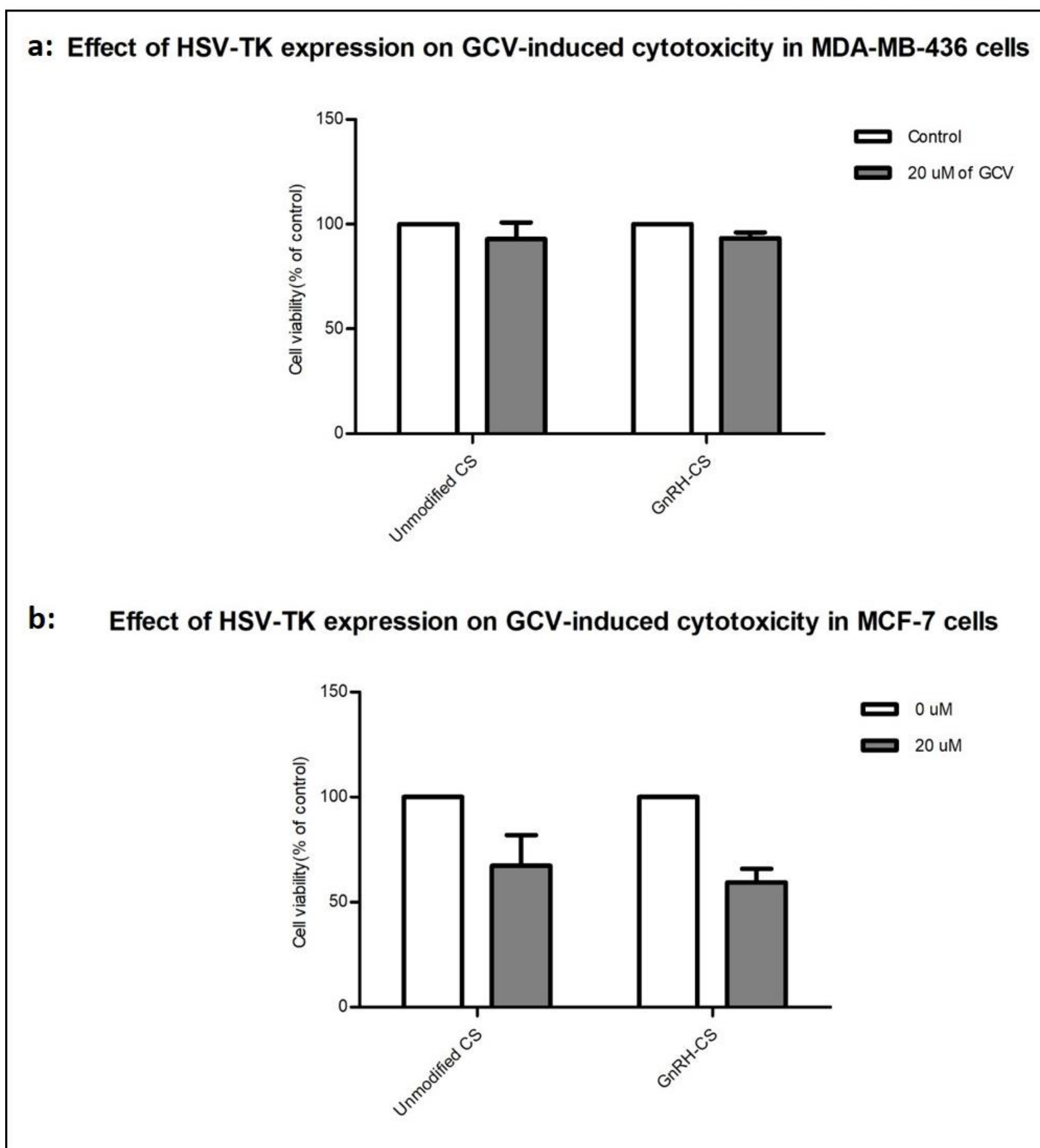

Figure 26. *Evaluation of the effect of HSV-TK expression on GCV-induced cytotoxicity in MDA-MB-436 and MCF-7 cells. In both of cell lines, average cell survival rate of GnRH-CS/pDNA complexes carrying a HSV-TK gene transfected cells prior to GCV administration (20 uM) was lower than that of cells transfected with unmodified CS/pDNA complexes carrying a HSV-TK gene with GCV treatment (at the same concentration).*

Specificity of transfection and transfection efficiency of GnRH-CS/pDNA complexes carrying a TNF-α gene in MDA-MB-436 cell

To confirm the utilization of GnRH-CS for cancer treatment, we tested this version of modified chitosan in animal model of cancer. At the reporter gene expression ratio, GnRH-CS/pDNA complexes carrying a TNF-α gene had a single dose-response effect on tumor regression of *in vivo* experimental mammary cancer as shown in Figure 27.

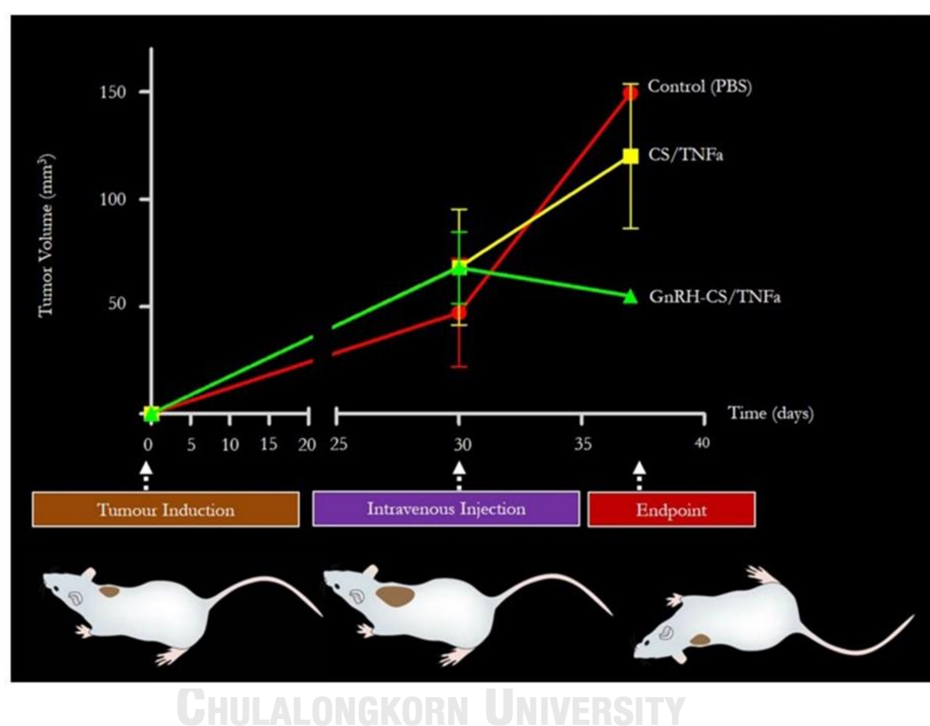

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Figure 27. *A single dose-response effect of GnRH-CS/TNF-α on tumor regression of in vivo experimental mammary cancer (Pilot study). In vivo pilot study using mammary tumor-bearing nude mice showed promising results in tumor suppression when a suicide gene (Tumor Necrosis Factor alpha; TNF-α) was delivered by the GnRH-CS.*

Conclusion

In this study, we confirmed GnRH peptide as a targeting ligand could improve cell specificity of chitosan to send suicide gene to mammary cancer cell line and animal model of cancer. We found the favorable results in tumor suppression when a suicide gene (TNF-α) was delivered by the GnRH-CS. The GnRH-CS was a good gene carrier for delivering gene to targeted mammary cancer. Surgical excision or mastectomy is the treatment of choice but it is an invasive treatment. In addition, post-operative pain and recurrence of the tumor may occur. So our strategy may be offer an alternative choice to improve the quality of animal’s life.

Acknowledgments

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CHAPTER III

2.1 General discussion and conclusion

The general concept of gene therapy is to introduce a therapeutic gene material into target cell in order to treat or slow down the progression of a disease. It is well documented that naked gene was degraded by the *in vivo* clearance. Transgene should be loaded in a vehicle, so called gene vector, to protect it from enzymatic degradation in the body. The great vector can ferry the gene of interest to the patient target cells. The most important step in achieving gene therapy is assorting the vectors. The optimal delivery system depends on type of target cells. Moreover, there are four barriers to provide a successful gene therapy, i.e. 1) vector uptake, transport and uncoating 2) vector genome persistence 3) sustained transcriptional expression and 4) the host immune response [150]. The viral and non-viral vectors are two broad categories of gene vector. Viral vectors are derived from viruses by replacing the viral gene with the therapeutic gene. The principle of viral vector design is the removal of genes encoding viral components essential for viral propagation reduces the risk of generating infectious virus [151]. The viral vectors are divided into two general categories such as integrating and non-integrating. Retrovius, lentivirus and adeno-associated virus can be integrated in recipient cells. On the other hand, adenovirus is a non-integrating viral vector. The disadvantages of using virus vectors are its immunogenicity and cytotoxicity. Non-viral vectors have safety advantage over viral vectors such as pathogenicity, less immunotoxicity, low cost, ability to transfer large size genes and ease of production [152]. These vectors can be divided into the plasmid DNA molecule and the delivery constituent [150]. At present, non-viral DNA delivery is powerful in many culture systems especially in *ex vivo* application but there has been little success in *in vivo* application. The critical steps in attaining a successful non-viral gene transfer are low efficiency of transportation into target cells, transfection and specificity [150, 152, 153]. This approach requires a modification of gene vector with high affinity and specificity ligands to use for a wide variety of cells. To create delivery platforms, DNA should be engaged to macromolecules including those based on polymer and/or lipid-based systems [154, 155].

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Chitosan is the second most important natural polymer worldwide [156] because of its high positive charges and low cytotoxicity. It is obtained by deacetylation of chitin shells of shrimp and other crustaceans. Chitosan is a polysaccharide composed of d-glucosamine and N-acetyl-d-glucosamine. The obstacle of chitosan is its solubility that is dissolved only in acid solution however low molecular weight chitosan is highly soluble in physiological buffer solutions [157]. According to the solubility of chitosan, the present study used low molecular weight chitosan that was synthesized by reacting primary amino groups with succinic anhydride [60-62] to generate N-Succinyl chitosan. The synthesis of NSCS with low degree of N-substitution was carried out in water. Moreover we modified CS polymer with low degree of N-substitution in order to remain the overall positive charge of CS that magnetizes with negative charge plasmid DNA. Because the positively charged primary amino groups of CS can be minimize in high degree of N-substitution [63].

The current study used GnRH peptide as a targeting ligand to develop nanocarrier for patient target cells expressed GnRHR and the reaction was performed in water under mild condition without requiring any organic solvents or acid and heat that might affect the biological activity of GnRH peptide. Previous studies used GnRH as a targeting ligand for nanocarriers in anticancer gene therapy [64-67]. As for the cytotoxicity, more positive charges are more toxic due to interference with mitochondrial function [158]. The unmodified CS and the prepared GnRH-CS were non-toxic to fibroblast cell line while PEI showed high cytotoxicity [88]. Moreover, this carrier had no cytotoxicity in spermatogonia cell in comparison with unmodified CS [107]. These findings support the earlier study on cell viability of chitosan and PEI in human embryonic kidney cell line [159]. Our study used two modalities to confirm viability and toxicity of cells, i.e. Cell Titer-Glo® Luminescent Cell Viability assay system (quantitative cytotoxicity tests) and LIVE/DEAD® Cell Viability/Cytotoxicity Assay Kit (qualitative cytotoxicity tests). Cell Titer-Glo® Luminescent Cell Viability assay system is the commercial kit containing reagents to measure ATP in living cells. These are the steps to detect viable cells. Firstly cells are lysed by detergent, the lysed cells release ATP, ATPase inhibitors stabilize the ATP and then luciferase catalyze the reaction of

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luciferin and ATP that generate light. The advantages of this assay are fast to use, high sensitivity and low artifacts. The onset of luminescent signal is 10 minutes and half-life is greater than 5 hours. This also eliminates an incubation step to generate signal. However, the ATP assay sensitivity is restricted by reproducibility of pipetting replicate samples [160]. LIVE/DEAD® Cell Viability/Cytotoxicity Assay Kit is the commercial kit containing two probes (calcein AM and ethidium homodimer; EthD-1) that measure cell viability. The intracellular esterase activity and plasma membrane integrity are the parameters of cell viability in this assay. Non fluorescent cell-permeant calcien AM was converted to the intensely fluorescent calcein (green fluorescence) by intracellular esterase enzyme in viable cells. While dead cells have a loss of membrane integrity, EthD-1 can enter into cell and produces a bright red fluorescence. This assay is fast, high sensitivity and low cytotoxicity.

In 2D and 3D *in vitro* study using two reporter genes (GFP for qualitative result and LUC for quantitative result), we used the transfected HEK293T cells expressing GnRH receptor [77]. This strategy represents GnRHR-expressing cells as target cells compared to original HEK293T cells as non-targeted cells in our study [88]. This part concerned the electrostatic effect of chitosan that affected on vector-target interactions. Unmodified chitosan showed non-specifically delivered reporter gene to cell via electrostatic interaction. While GnRH-CS could discriminate between targeted and non-targeted cells, it carried transgene to target cell via receptor-mediated endocytosis. From these findings, the GnRH-CS is targeted, specific and dependent on GnRHR. However the competitive binding assay should be used to confirm the GnRHR-mediated endocytosis.

We also investigated the analysis of gene expression such as specificity of transfection and transfection efficiency in spermatogonia cell line or GC-1 cell [107]. The spermatogonia cell was used as a representative of male germ cells (spermatogonia, spermatocytes and spermatids) [81, 82]. However the limitation of the *in vitro* study of male germ cell is not capable of long-term maintainance [94]. To address this model, the immortalized GC-1 cell line was studied. The findings exhibited that the modification of chitosan with GnRH peptide could reduce cytotoxicity and

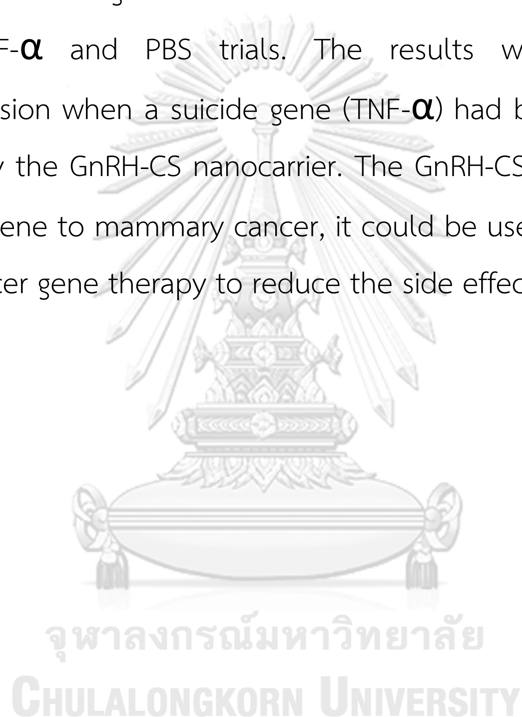
enhance the specificity and transfection efficiency of unmodified chitosan in spermatogonia cell line [107]. Therefore, this version of chitosan can be used as a gene delivery vehicle for testis-mediated gene transfer or TMGT.

As for the *in vitro* study of mammary cancer gene therapy, the mammary cancer cell line, MDA-MB-436 was used to test the efficacy of GnRH-CS/pDNA complex before testing in animal model. MDA-MB-436 basal like human mammary cancer is the hormone receptors-negative mammary cancer that is associated with unresponsiveness to the usual endocrine therapies, aggressive histology, poor prognosis, and shorter survival [149]. However this cell line could be specifically transfected with GnRH-CS/pDNA (Green fluorescent protein and Luciferase) by receptor mediated endocytosis. Cell specificity of GnRH-CS was more than unmodified CS that deliver gene into the cell via the electrical charge. The result confirmed that GnRH ligands could improve the targeting property of chitosan to send gene to mammary cancer cell.

A single bilateral intra-testicular injection of Gonadotropin Releasing Hormone-modified Chitosan mediated Tumor Necrosis Factor alpha gene was investigated in rats as a model for intra-testicular injection, non-surgical sterilization. A gradual decrease in testicular volume post-injection with GnRH-CS/TNF-α was found in comparison with GnRH-CS (Naked nanoparticle), TNF-α (Naked DNA) and control group. In addition to the reduced total testicular volume (*in vivo* over the scrotal skin and *in vitro* after castration) and testicular weight (after castration), it was most likely to be affected by testicular germ cell degeneration, a loss in germ cell number and seminiferous tubular fluid (STF) [137]. Some studies revealed individual variation in testicular volume after testicular injection [32, 121]. With regard to the pain during and after intra-testicular injection, because capsular distension occurs within 24 hours and may remain for 7-15 days, pain may take place due to peripheral nerve pain that located only on the scrotal skin and in the capsule of the testis [138]. No changes, e.g. weight and behavior, that indicated pain after recovering from anesthesia were found in this study. It might infer that all treated rats had normal food intake after injection. The strength of this research study is that our strategy showed no clinical signs of

scrotal/testicular inflammation and low severity of testicular cell death, and serum testosterone levels remained unchanged. Moreover, the current study used ultrasonographic and histopathological assessment to confirm testicular degeneration [139].

In this study, a single dose-response effect of GnRH-CS/TNF-α on tumor regression of *in vivo* experimental mammary cancer (a pilot study) was performed. Using the MDA-MB-436 cell line, we induced the cancerous cell growth to nude mice subcutaneously. We investigated tumor volume after treatment with GnRH-CS/TNF-α, unmodified-CS/TNF-α and PBS trials. The results were favorable in tumor suppression/regression when a suicide gene (TNF-α) had been delivered to targeted cancerous cells by the GnRH-CS nanocarrier. The GnRH-CS is a good gene carrier for delivering TNF-α gene to mammary cancer, it could be used as an alternative choice for mammary cancer gene therapy to reduce the side effects of chemotherapy.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2.2 Limitation of the study

The overall objective of this study is to examine the application of modified chitosan as a potential vector for transgene delivery to gonadotropin-releasing hormone receptor-expressing cells. In the first *in vitro* study, we generated the cells transiently expressing GnRHR by transfection the human embryonic kidney cell lines cells with pDNA vectors containing a gene encoding GnRHR under the control of CMV promoters for constitutive expression in mammalian cells. In the second *in vitro* study was investigated in type B spermatogonia and primary spermatocytes. The hormone receptors-negative mammary cancer cell line was used in the third *in vitro* study of mammary cancer gene treatment. These *in vitro* studies were carried out in 3D spheroid. However the current study was limited by GnRHR-expressing cell model, one type of reproductive cell line and reproductive related cancer cell line.

In the study of single bilateral intra-testicular injection in rats, gross examination of cut surface of testicles was ignored. The cut surface of testis is the important macroscopic findings to assess after testicular injection with chemosterilants. Being limited to assess the pain of treated animal, this study evaluated decreased body weight as a parameter to indicate stress-associated pain. It is unfortunate that apoptosis (early stage) could not be assessed due to the design of our study to castrate at the end of the study (Day 35 after injection). Therefore, only testicular degeneration could be evaluated histologically. At the late stage of apoptosis, apoptosis and necrosis were not able to be differentiated. Although the current study is based on the short-term effects, the findings suggest this version of chitosan can furry apoptotic gene to induce testicular cell death without adverse reaction. The issue of long-term effects and mating fertility should be explored in further research.

In vivo pilot study using mammary tumor-bearing nude mice, notwithstanding the relatively limited sample, this work exhibited the promising results in tumor suppression. Additionally, the findings recommend that a larger sample size, the daily measurement of tumor size, macroscopic finding of tumor, histopathological examination of tumor and immunohistochemical detection of tumor suppression should be evaluated in the future study.

2.3 Suggestion from the study

Many reproductive tissues express GnRH receptor such as mammary, endometrium, ovary and prostate [8]; however, reproductive-related cancer tissues overexpressed GnRH receptor, e.g. prostate, endometrial and ovarian cancers [9]. More broadly, research is also needed to determine the use of GnRH-modified chitosan for gene delivery to GnRH receptor-expressing cells in these cell lines. To make sure that GnRH-CS can carry the gene of interest to target cell via receptor-mediated endocytosis, competitive assay of GnRH binding can be examined.

Further investigation of intra-testicular injection of GnRH-CS/TNF-α in other species should be examined. Data of the gross examination of cut surface of testicles, serum or fecal testosterone levels, testicular testosterone levels, immunohistochemical detection of apoptosis in early stage and cell death should be examined. To identify if the animals have pain after testicular injection, serum cortisol level, fasting blood sugar level, stress leukogram and behavioral pain score should be monitored. Considerably more work will need to be done to add more endpoints to detect the early apoptosis of the entire testicular germ cells that leads to permanent male infertility. Long-term effects of GnRH-CS/TNF-α adminstration via intra-testicular injection on male fertility are of interest to be elucidated, particularly in the dog and cat. Additionally, further investigations using mammary tumor-bearing nude mice are needed to estimate the daily measurement of tumor size, macroscopic finding of tumor, histopathological examination of tumor and immunohistochemical detection of tumor suppression.

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PUBLICATION	1. Boonthum, C., K. Namdee, S. Boonrungsiman, K. Chatdarong, N. Saengkrit, W. Sajomsang, S. Ponglowhapan and T. Yata (2017). “Chitosan-based DNA delivery vector targeted to gonadotropin-releasing hormone (GnRH) receptor.” Carbohydrate polymers 157: 311-320. 2. Yostawonkul, J., S. Surassmo, K. Namdee, M.Khongkow, C. Boonthum, S. Pagseesing, N. Saengkrit, U. Rungsardthong Ruktanonchai, K. Chatdarong, S. Ponglowhapan and T. Yata (2017). “Nanocarrier-mediated delivery of alpha-mangostin for non-surgical castration of male animals.” Scientific Reports 7:16234. 3. Pugseesing, S., J. Yostawonkul, S. Surassmo, S. Boonrungsiman, K. Namdee, M. Khongkow, C. Boonthum, T. Lempridee, U. Rungsardthong Ruktanonchai, N. Saengkrit, K. Chatdarong, S. Ponglowhapan and T. Yata (2017). “Formulation, physical, in vitro and ex vivo evaluation of nanomedicine-based chemosterilant for non-surgical castration of male animals.” Theriogenology 108:167-175 4. Boonthum, C., K. Namdee, K. Khongkrow, S. Temisak, K. Chatdarong, W. Sajomsang, S. Ponglowhapan and T. Yata (2018). “Gonadotropin-releasing hormone (GnRH)-modified

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AWARD RECEIVED

1. Best student oral presentation, In vitro study of gonadotropin-releasing hormone (GnRH)-modified Chitosan to deliver a transgene to spermatogonia cells: for male animal contraception, The 20th European Veterinary Society For Small Animal Reproduction Congress, Vienna, Austria, June 29th - July 1st, 2017

2. Best oral presentation award, Development of a Smart Biopolymer for Breast Cancer Gene Therapy by Grafting Chitosan with Gonadotropin Releasing Hormone (GnRH), The First Materials Research Society of Thailand International Conference
(1st MRS Thailand International Conference), Convention Center, the Empress Hotel, Chiang Mai, Thailand, October 31st - November 3rd, 2017

3. Elanco Best Research award (นักวิจัยดีเด่นด้านสัตวแพทย์: นิสิตบัณฑิตศึกษา), คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และบริษัท Elanco

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