รายงานฉบับสมบูรณ์

โครงการวิจัยเรื่อง

การพัฒนาพาหะนำส่งยีนเหนี่ยวนำการตายของเซลล์แบบอะพอพโทซิสระดับนาโน เพื่อการคุมกำเนิดสัตว์เพศผู้แบบไม่ผ่าตัดทำหมัน: ต้นแบบเพื่อการคุมจำนวนประชากรสุนัขและแมวจรจัด

Development of nanocarrier-mediated delivery of apoptosis-inducing gene for non-surgical sterilization in male animals: a model for stray dog and cat population control

ผู้วิจัย

1. ผู้ช่วยศาสตราจารย์ น.สพ.ดร. ศุภวิวัธน์ พงษ์เลาหพันธุ์

2. อาจารย์ ดร. ธีรพงศ์ ยะทา

ภาควิชาสูติศาสตร์-เธนุเวชวิทยา และวิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์

จุฬาลงกรณ์มหาวิทยาลัย

สิงหาคม 2562

กิตติกรรมประกาศ (Acknowledgement)

โครงการนี้ได้รับทุนอุดหนุนการวิจัยจาก "ทุนอุดหนุนโครงการวิจัย เงินอุดหนุนทั่วไปจากรัฐบาล ประจำปี พ.ศ. 2562 (งบประมาณแผ่นดิน)" สัญญาเลขที่ GB-A_61_039_31_04 ระยะเวลาดำเนินโครงการ ตั้งแต่วันที่ 1 ตุลาคม พ.ศ. 2560 ถึงวันที่ 30 กันยายน 2561 เป็นจำนวนเงิน 787,500 บาท

บทคัดย่อ

การศึกษาวิจัยนี้มีวัตถุประสงค์ เพื่อตรวจสอบการนำไปใช้ของอนุภาคไคโตซานระดับนาโนที่ถูกดัดแปลงให้มีคุณสมบัติเป็นตัวนำส่งยีนเหนี่ยวนำการตายแบบอะพอพโทซิสเข้าสู่เซลล์อัณฑะที่มีตัวรับฮอร์โมนโกนาโดโทรปินรีลิสซิ่ง โดยการออกแบบตัวนำส่งยีนนี้สามารถนำไปใช้ประโยชน์ในการทำหมันสัตว์เพศผู้แบบไม่ผ่าตัด การศึกษานี้ได้มีการรายงานผลของอนุภาคไคโตซานระดับนาโนเชื่อมติดกับฮอร์โมนโกนาโดโทรปินรีลิสซิ่ง (Gonadotropin Releasing Hormone-modified Chitosan; GnRH-CS) เพื่อนำส่งยีนอย่างมีเป้าหมาย และการใช้เปปไทด์โกนาโดโทรปินรีลิสซิ่งในการระบุเป้าหมายการนำส่งยีนไปสู่เซลล์ที่มีตัวรับฮอร์โมนโกนาโดโทรปินรีลิสซิ่ง (GnRH receptor; GnRHR) จากการศึกษาในห้องปฏิบัติการ (*In vitro* study) พบว่าอนุภาค GnRH-CS สามารถนำส่งยีนรายงานผล (Green Fluorescent Protein; GFP และ Luciferase; LUC) ไปสู่เซลล์เพาะเลี้ยงจากเซลล์ไตของตัวอ่อนมนุษย์ (Human Embryonic Kidney cell line) ที่มีการดัดแปลงให้มี GnRHR เพื่อใช้เป็นเซลล์จำลองที่มีการแสดง GnRHR และเซลล์สืบพันธุ์เพศผู้จากหนูเมาส์ (Spermatogonia cells; GC-1 cell) ได้อย่างจำเพาะ สำหรับการศึกษาภายในร่างกายสัตว์ (*In vivo* study) ได้มีการใช้ยีน Tumor Necrosis Factor alpha (TNF-alpha) เพื่อเหนี่ยวนำการตายในเซลล์อัณฑะของหนูแรทโดยการฉีดสารเข้าอัณฑะโดยตรง (Intra-testicular injection) ผลการศึกษาพบว่ามีการตายของเซลล์อัณฑะ (จากการตรวจทางจุลพยาธิวิทยา และการตรวจคลื่นเสียงความถี่สูงหรืออัลตราซาวน์) มีการลดลงอย่างมีนัยสำคัญทางสถิติของขนาดอัณฑะ (จากการตรวจทางกายภาพ การวัดด้วยคาลิปเปอร์แบบดิจิตอล และเครื่องชั่งน้ำหนักแบบดิจิตอล) ไม่พบผลข้างเคียงหลังการฉีด และพบว่ายังมีระดับฮอร์โมนเทสโทสเตอโรนในเลือดคงที่ (จากการตรวจด้วยหลักการ chemiluminescent microparticle immunoassay; CMIA) สรุปได้ว่างานวิจัยนี้สามารถนำไปประยุกต์ใช้ในการทำหมันสัตว์เพศผู้แบบไม่ผ่าตัด พบว่าวิธีนี้ช่วยลดการเกิดผลข้างเคียงจากการฉีดด้วยสารทำหมันอื่นที่มีรายงานก่อนหน้า

Abstract

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). Such design of gene carrier could be useful in particular for male animal nonsurgical sterilization. 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. In in vitro study, this alternative gene transfer strategy could specifically deliver the reporter genes (Green Fluorescent Protein; GFP and Luciferase; LUC) to a transiently transfected Human Embryonic Kidney cell line model system expressing GnRHR and mouse-derived spermatogonia cells (GC-1 cell line). From in vitro to in vivo study, Tumor Necrosis Factor alpha (TNF-alpha) was exploited as a therapeutic gene delivered by GnRH-CS in order to induce testicular cell death in male rat via intra-testicular injection. Testicular cell death (by histopathological examination and ultrasonography), significant reductions of testis size and weight, no adverse reactions and unchanged testosterone levels (by chemiluminescent microparticle immunoassay; CMIA) were observed. It was possible that our study could be applied for non-surgical sterilization. As for non-surgical sterilization in male animals, this strategy can minimize the chemical sterilant side effect of intratesticular injection.

Table of Contents

List of Tables

List of Illustration

List of Abbreviation

1H-NMR:	1H 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
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
DMSO:	Dimethylsulfoxide

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 (Badiga, Chetty et al. 2011). 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 (Muruve, Barnes et al. 1999, Wolins, Lozier et al. 2003), and the potential for insertional mutagenesis can be oncogenic (Buckley 2002, Marshall 2002). 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 (Prabaharan and Mano 2005). To overcome this limitation, ligands with high affinity and specificity have been integrated into gene delivery vector for receptor-mediated endocytosis (Jiang, Kwon et al. 2007).

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) (Cheung and Wong 2008). 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 (Ono and Harley 2013), 2) contraception and fertility control (Dissen, Lomniczi et al. 2012), and 3) gene therapy of cancer associated with reproductive system (Limonta, Montagnani Marelli et al. 2012).

In this study, we proposed 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 (Coleman, Veleanu et al. 2010) 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 (Forzan, Garde et al. 2014).

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 which were testicular cells. Such design of gene carrier could be useful in particular for male animal nonsurgical sterilization. Three specific objectives of the present study were as follows.

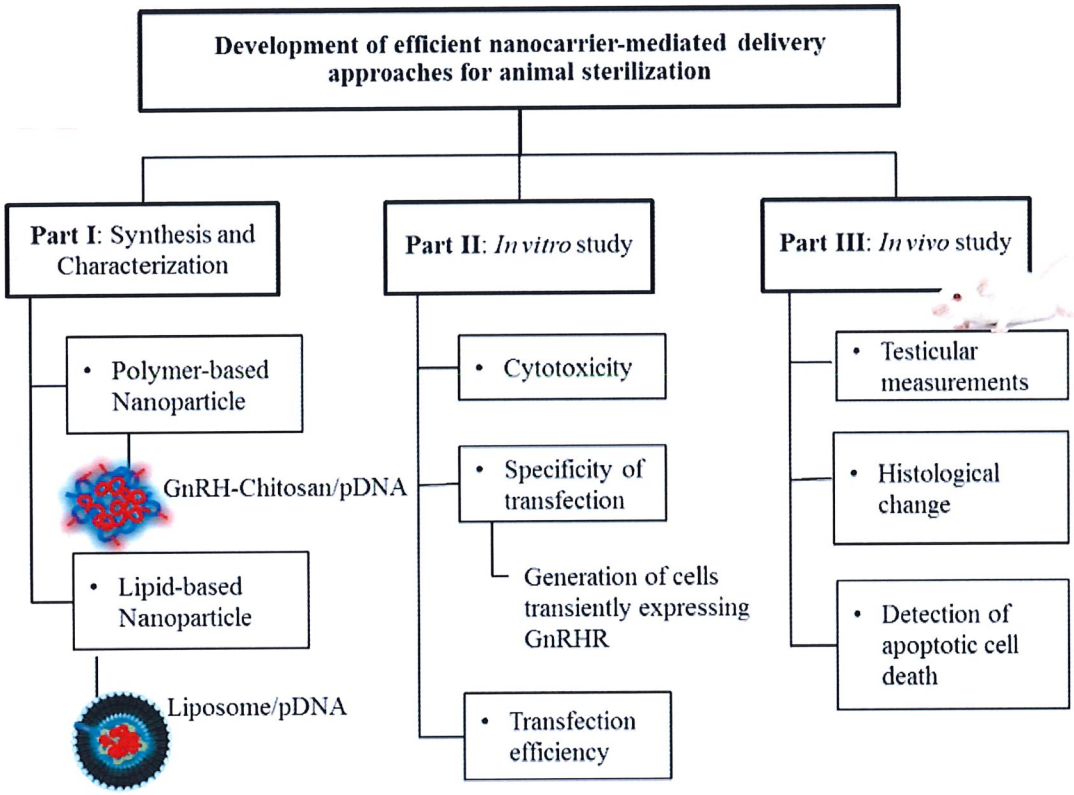
(i). To design, synthesize, characterize nanoparticles (polymer- and lipid-based nanocarriers)

(ii). In vitro study: to evaluate their biological activities for targeted gene delivery in cell lines, i.e. cytotoxicity, specificity of transfection and transfection efficiency

(iii). In vivo study: the designed nanocarriers carrying apoptosis-inducing gene will be injected intratesticularly in mice and the in vivo effect after injection of nanoparticles/pDNA will be observed, i.e. testicular measurement, histological change of testicular tissue, detection of apoptotic cell death.

The advantages of the study were (i) to formulate GnRHR-targeted CS delivering gene of interest for the potential use in contraception, fertility control and reproductive related cancer treatment, (ii) to develop nonsurgical sterilization method based on nanocarrier/pDNA complexes in animals, (iii) to increase knowledge of DNA delivery system and gene therapy in veterinary medicine and (iv) to provide sufficient scientific data for international publications.

Conceptual framework



Chapter I

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

1.1 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 (Badiga, Chetty et al. 2011). 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 (Morille, Passirani et al. 2008). 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 (Saranya, Moorthi et al. 2011). However, the effectiveness of this system is hindered by its low transfection efficacy and low cell specificity (Prabaharan and Mano 2005). 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 (Jiang, Kwon et al. 2007).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 (Millar 2005). In addition to gonadotrope cells, GnRHR was found to be expressed in normal reproductive tissues (e.g. breast, endometrium, ovary, and prostate) (Cheung and Wong 2008). 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 (Ono and Harley 2012), 2) gene therapy of cancer

associated with reproductive system (i.e. prostate, breast, endometrial, and ovarian cancers) (Limonta, Marelli et al. 2012), and 3) contraception and fertility control (Dissen, Lomniczi et al. 2012).

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 1.

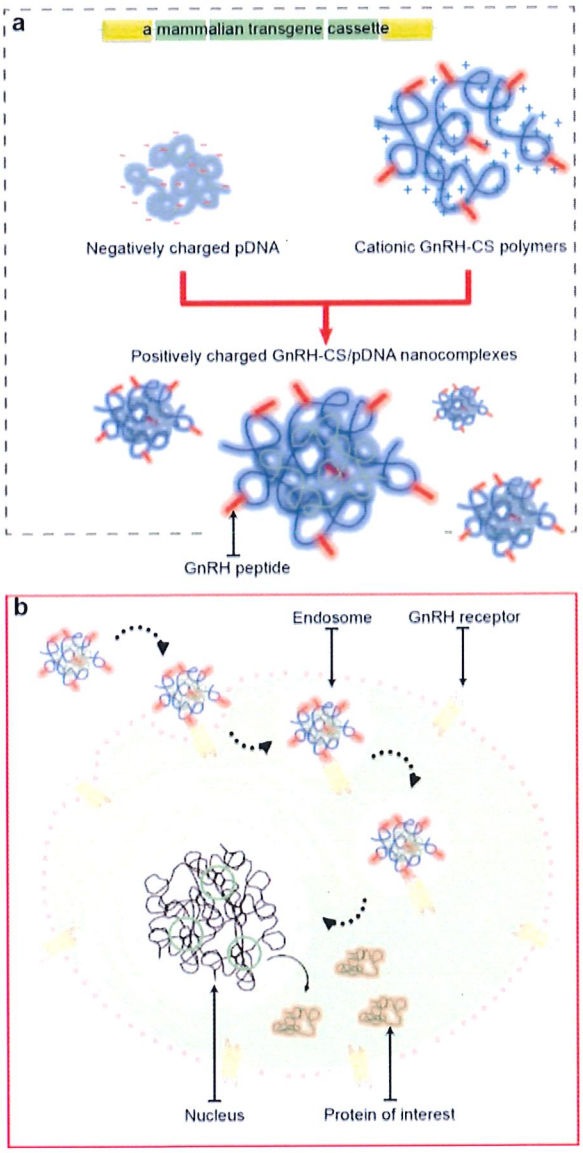


Figure1. Schematic illustration showing the formation of GnRH-CS/pDNA complex and its delivery procedure. a) pDNA condensation in the presence of cationic GnRH-CS polymer by electrostatic interactions. b) The proposed mechanism of gene delivery process by GnRH-CS/pDNA complex. GnRH peptides displayed 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.

1.2 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 (Lavertu, Xia et al. 2003). 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 M, 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.

Polymer characterization

All samples were characterized by using1H nuclear magnetic resonance spectroscopy (1H NMR) and Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). The1H NMR spectra were measured on a Bruker AVANCE 500 MHz spectrometer (Bruker, Switzerland), using D2O/CD3COOD as solvent at a10 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 using1H 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 then0.03 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—3mol) 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—3meq/GlcN) or 3.50 mg (1.65 × 10—3meq/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 (Figure 2.).

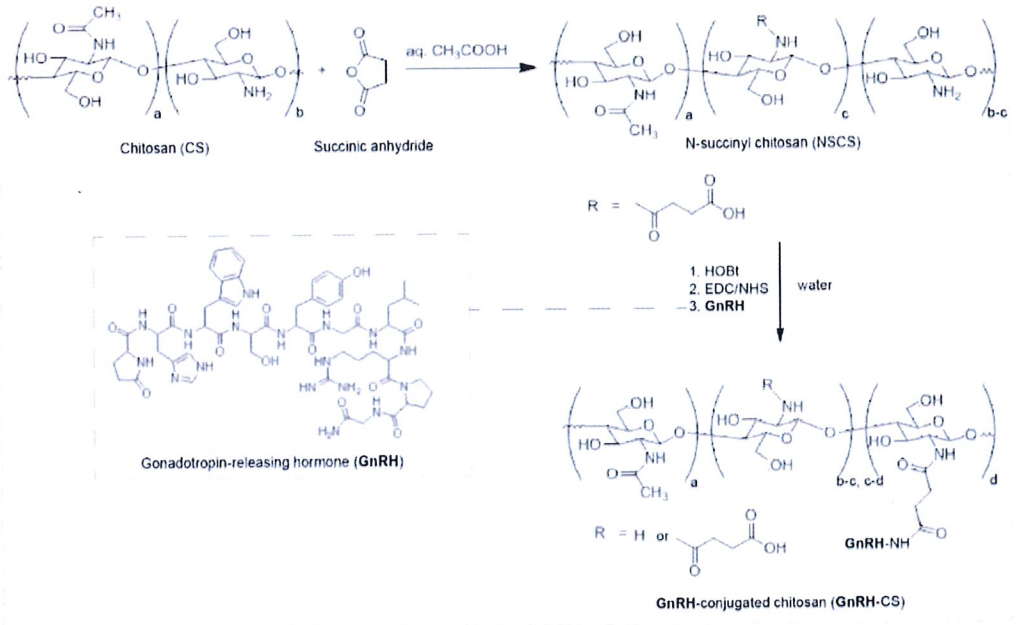


Figure 2. Proposed reaction scheme for synthesis of GnRH-grafted chitosan (GnRH-CS). N-Succinyl chitosan (NSCS) was synthesized by N-succinylation of CS with succinicanhydride followed by conjugation with GnRH peptide by using EDC/NHS as a coupling agent.

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 1mins (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 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 g/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 DH5α 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

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.

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.

1.3 Results and discussion

Synthesis and characterization of GnRH-CS

The scheme for the overall synthesis is shown in Figure 3. By taking advantage of the previously developed N-succinyl chitosan(NSCS) derivative (Xiangyang, Ling et al. 2007, Xu, Bajaj et al. 2010, Toh, Chen et al. 2011), 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 (Bravo-Anaya, Soltero et al. 2016). 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 (Dharap, Wang et al. 2005, Khandare, Chandna et al. 2006, Saad, Garbuzenko et al. 2008, Taratula, Kuzmov et al. 2013). 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 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 3a.) and ^{1}H NMR spectroscopy (Figure 3b.) 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_3COOD showed 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 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-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 not only confirmed that the low degree of modification not only preserve the DNA condensation ability of chitosan, but also sufficient for receptor-mediated gene delivery.

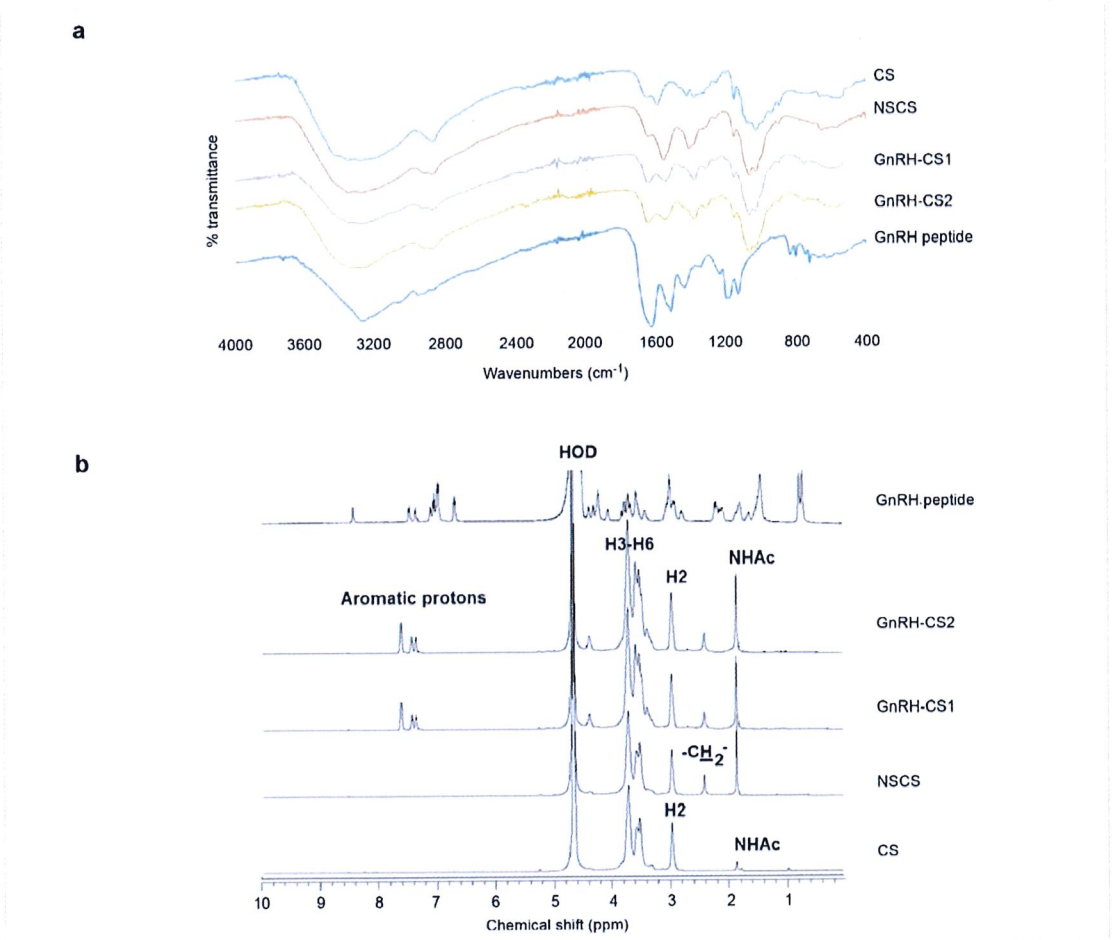


Figure 3. Characterization of GnRH-grafted chitosan (GnRH-CS). a) Representative1H NMR spectrum of GnRH-CS compared to NSCS, CS and GnRH peptide. b) Structure characterization by FTIR spectra.

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 (Uram, Szuster et al. 2013, Saleh and Pecina 2014). In this experiment, polyethylenimine (PEI) which has been described as one of the most widely used cationic polymer for in vitro gene delivery (Demeneix and Behr 2005) was also tested in parallel. The cell viability of PEI-treated cells was drastically decreased with increasing concentration as shown in Figure 4a. This observation can be attributed to a number of previous investigations which have highlighted its high cytotoxicity (Kafil and Omidi 2011). In contrast, no cytotoxicity was observed in the con-centration ranges of unmodified CS or GnRH-conjugated CS tested. Similar results were observed in both GnRH-CS1 and GnRH-CS2. Figure 4b 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 (Peng, Lai et al. 2014) suggesting that unmodified CS and the prepared GnRH-CS were more biocompatible than PEI.

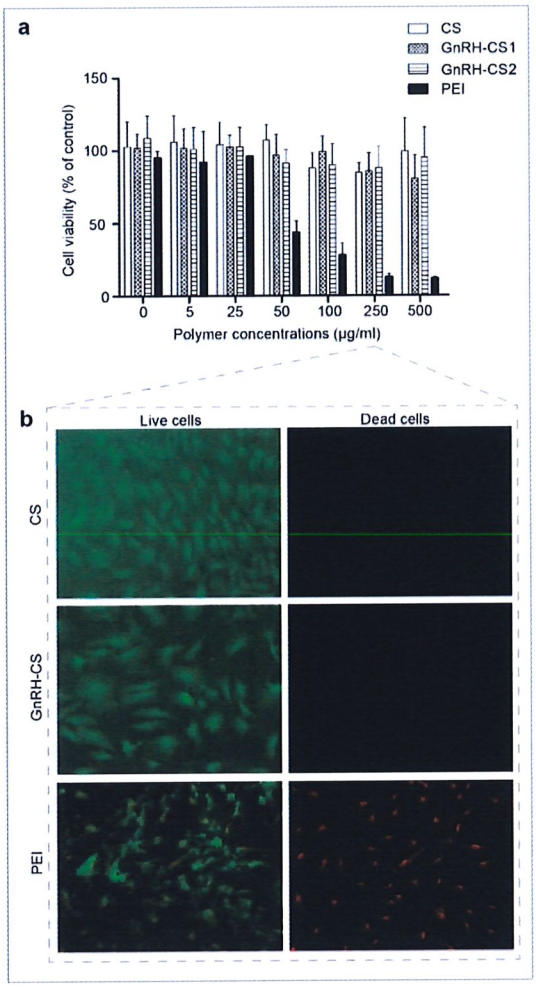


Figure 4. Cytotoxicity of polymers at various concentration in BJ cells (human skin fibroblast). a) Following 24 h incubation with polymers, cell viability was deter-mined by the Cell Titer-Glo® cell viability assay. Mean cell viability was normalised to non-treated controls, with the mean of n = 3 + 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 5a. 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 andGnRH-CS2 for their ability to condense DNA. GnRH-CS1 was chosen for further studies.3.3.2. 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 below300 nm is required for maximum endocytosis by cells (Peng, Lai et al. 2014). Having defined the critical ratio that produced the complete formation of polyplexes as determined by the earlier gel retardation assay (Figure 5a.), 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 5b.). 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 5b.). 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 (Pack, Hoffman et al. 2005). 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.

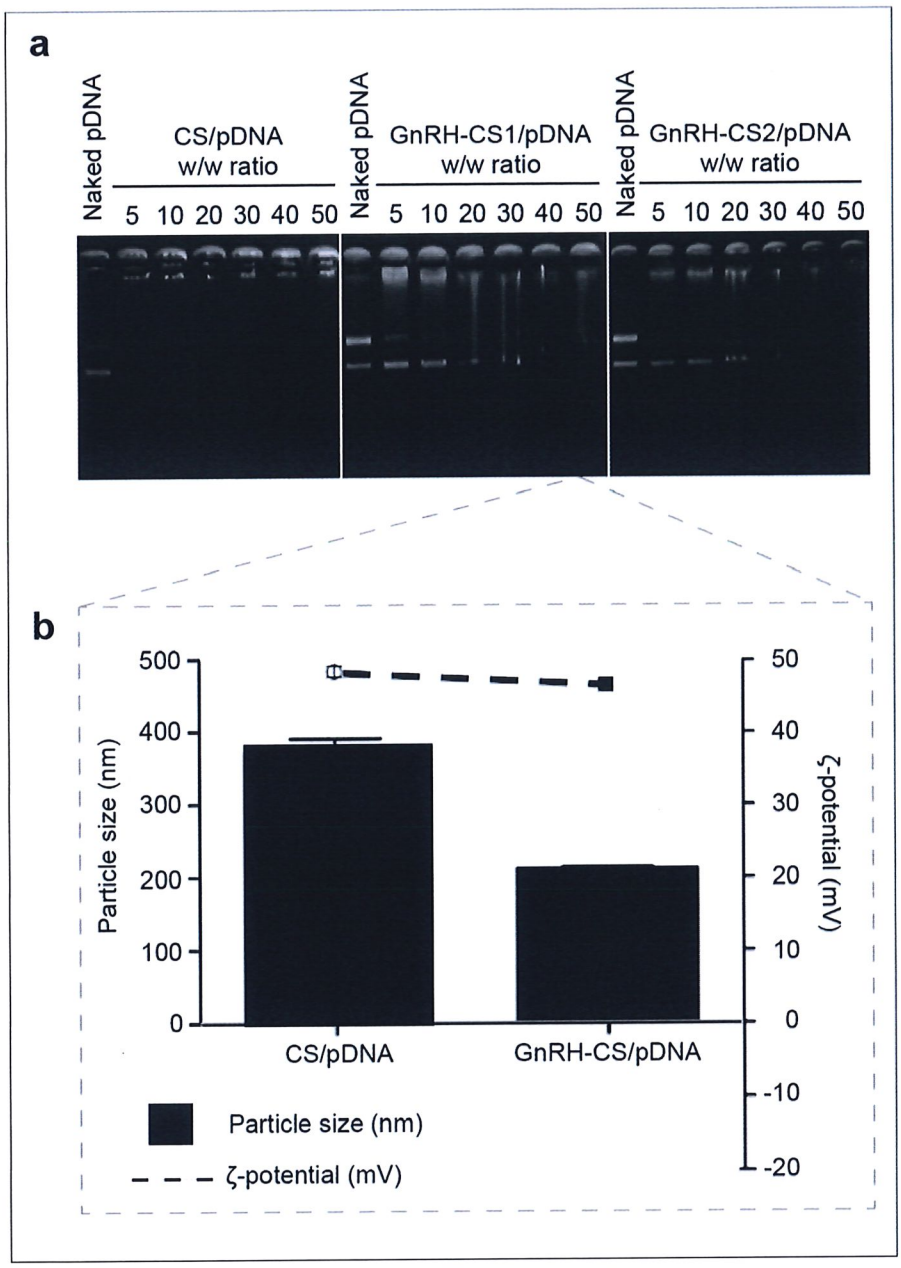


Figure 5. Characterization of GnRH-CS/DNA complexes. a) Agarose gel retardation assay of the GnRH-CS/pDNA and unmodified GnRH-CS/pDNA polyplexes. Weight ratios of polymer:DNA are 5:1, 10:1, 20:1, 30:1, 40:1, and 50:1, respectively. b) Particle size and zeta potential of pDNA complexed with unmodified 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 (Thomas and Smart 2005, Atwood, Lopez et al. 2011, Lin, Boone et al. 2014). A previous study has generated the transfected HEK293T expressing GnRH receptor and used this established cell line as a model (Morgan, Stewart et al. 2008). 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 com-pared to unmodified CS prepared with increasing weight ratios of polymers and pDNA carrying a GFP reporter gene. As shown in Figure 6a, 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 6a.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 (Cheng and Yuan 2006). 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 (He, Tabata et al. 2010). 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 6b. Taken together, these findings confirm that gene delivery by the GnRH-CS is targeted, specific and dependent on GnRH receptor.

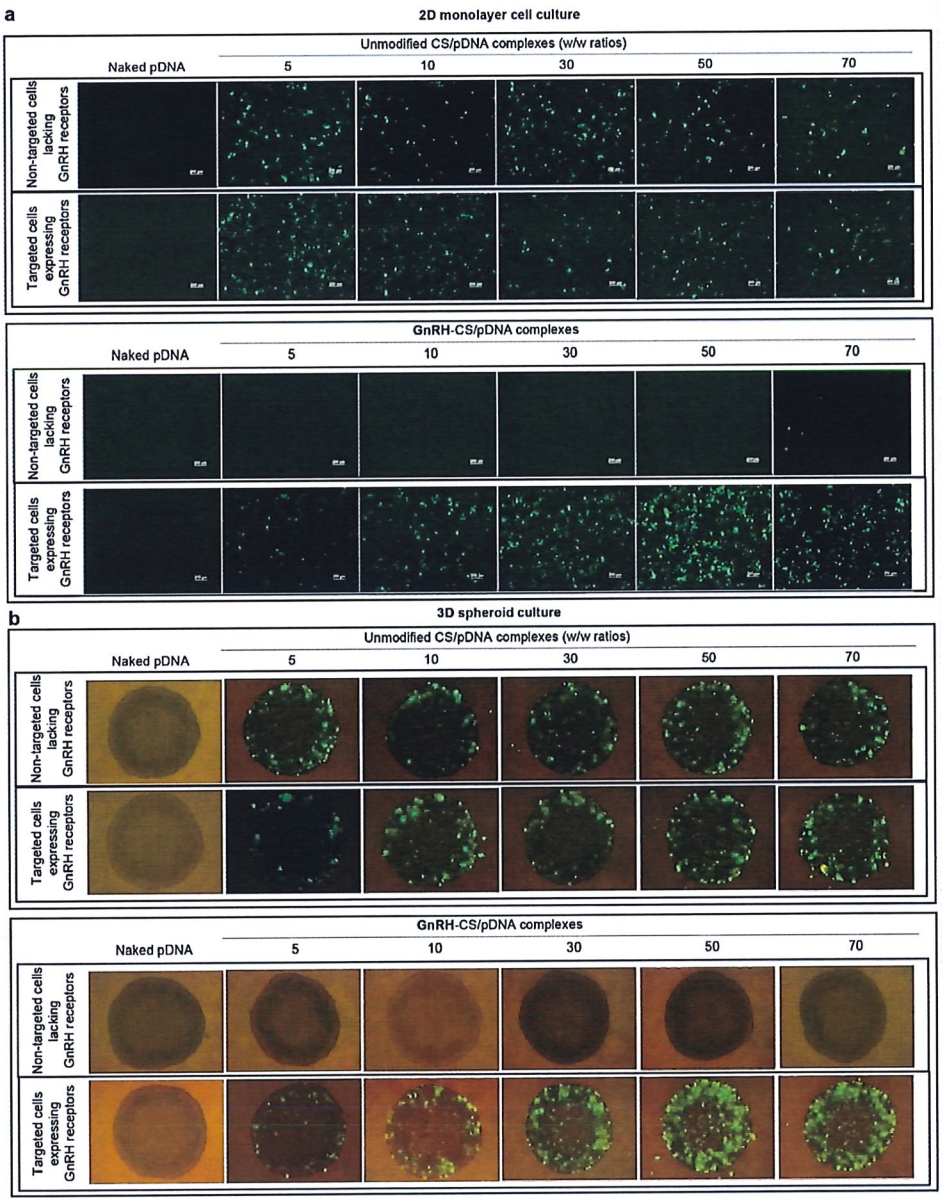


Figure 6. Evaluation of the specificity of targeted cell transfection by GnRH-CS/pDNA complexes. a) Assessment of targeted gene transfer by GnRH-CS/pDNA compared to the unmodified CS/pDNA complexes carrying the GFP reporter gene using a range of polymer concentrations in the targeted cells expressing 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.

Transfection efficiency

Next, to further explore the superiority of the GnRH-CS, we treated targeted cells expressing GnRHR with GnRH-CS/pDNA com-plex 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 7a. 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 7b.).

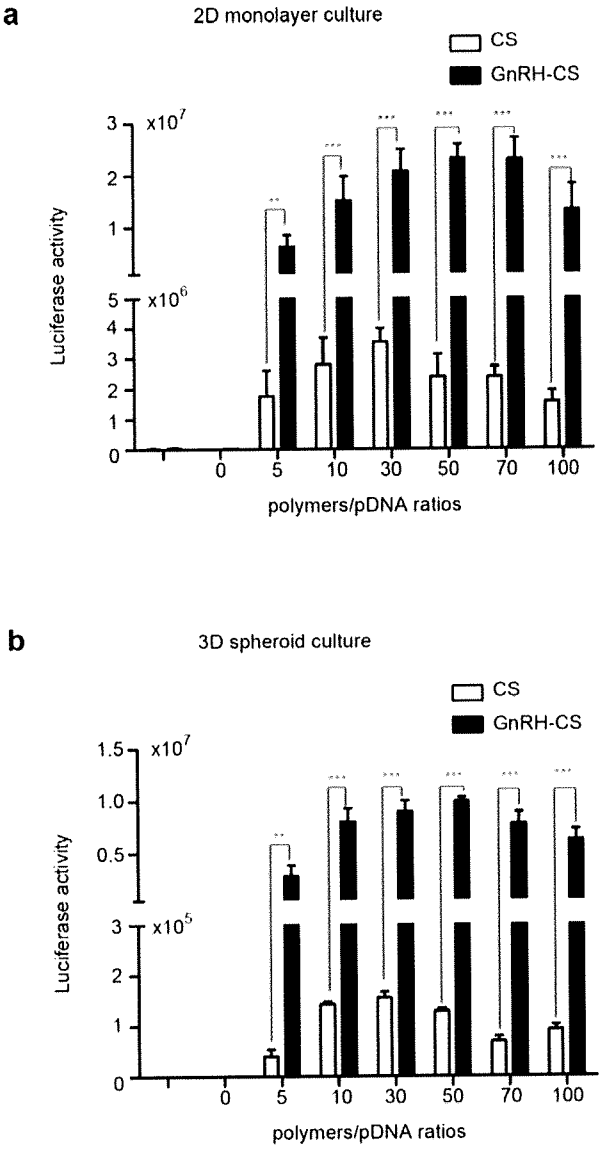


Figure 7. Comparison of transfection efficacies of GnRH-CS/pDNA and unmodified CS/pDNA complexes in GnRHR-expressing cells. a) 2D monolayer cultures b) 3Dmulticellular spheroids. Data represent the mean + SEM of triplicate samples from one representative experiments of three independent experiments, significant difference; n.s.-not significant, * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ (one way ANOVA with tukey's post hoc test).

In conclusion, 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.

Chapter II

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

2.1 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 (Sato 2006). 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 (Nagano, Shinohara et al. 2000, Takehashi, Kanatsu-Shinohara et al. 2007). The main limitations for this approach have been unfavorable immunological features and mutagenic integration for some viruses (Morille, Passirani et al. 2008). 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 (Saffari, Moghimi et al. 2016, Werfel and Duvall 2016).

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 (MacLaughlin, Mumper et al. 1998, Richardson, Kolbe et al. 1999, Roy, Mao et al. 1999). 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 (Saranya, Moorthi et al. 2011). However, the usefulness of this system is limited by its poor transfection efficiency (Prabaharan and Mano 2005). 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 (Jiang, Kwon et al. 2007).

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

(Boonthum, Namdee et al. 2017). 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 (Boonthum, Namdee et al. 2017).

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 (Boonthum, Namdee et al. 2017), 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.

2.2 Materials and methods

Preparation and characterization of polyplexes

The procedure for preparing and characterizing polyplexes was previously described (Boonthum, Namdee et al. 2017). 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 (Boonthum, Namdee et al. 2017).

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) (Burgess, Vigneron et al. 2010, McCloy, Rogers et al. 2014). 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 (Gavet and Pines 2010, Potapova, Sivakumar et al. 2011).

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 ***$p<0.001$.

2.3 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 8a). 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 8b).

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 9b). Cell death also was less evident in both controls (untreated or naked pDNA-treated cells) Figure 9b).

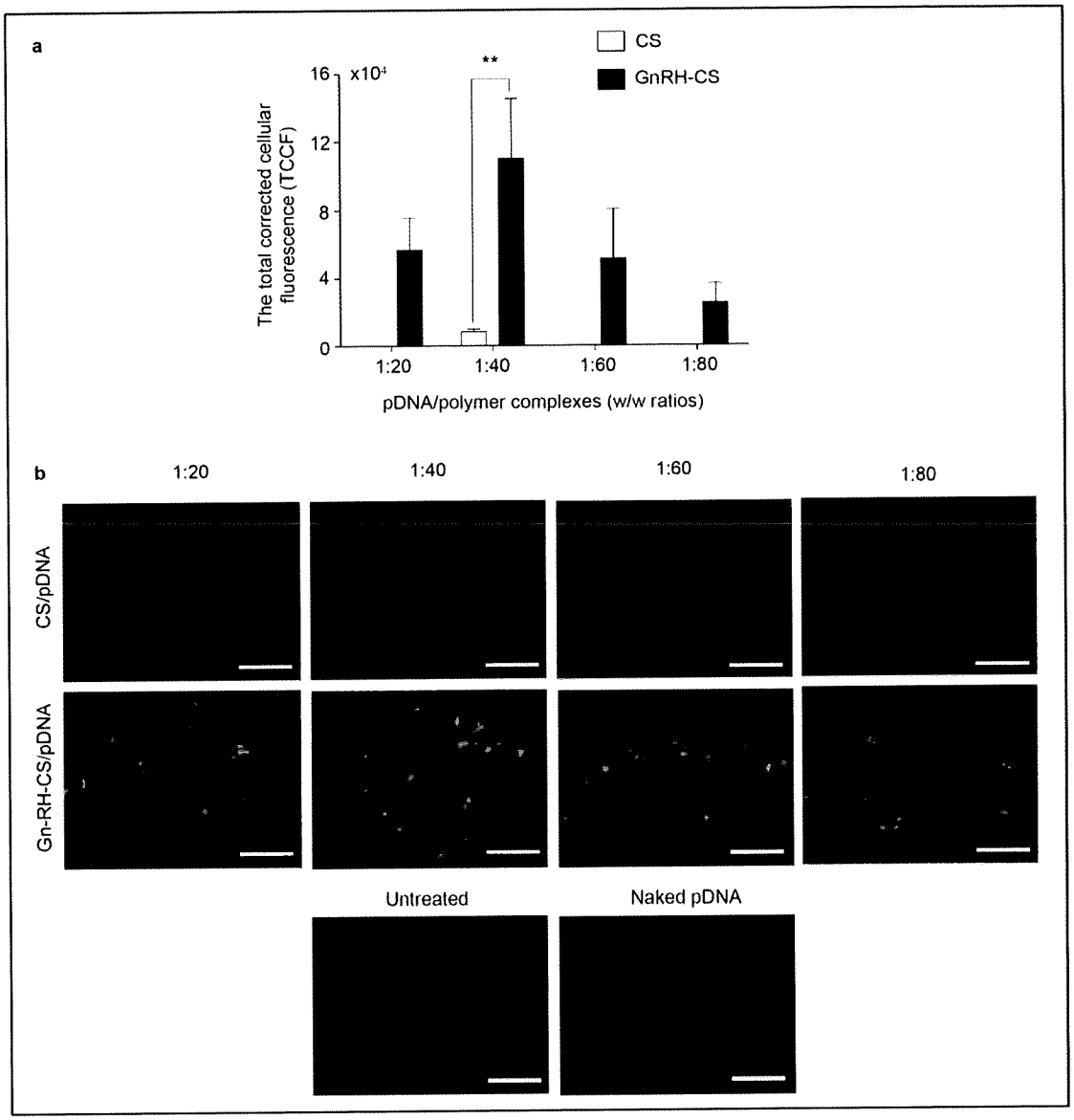


Figure 8. Evaluation of spermatogonia cell transfection efficacy by GnRH-CS/pDNA complexes.

(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).

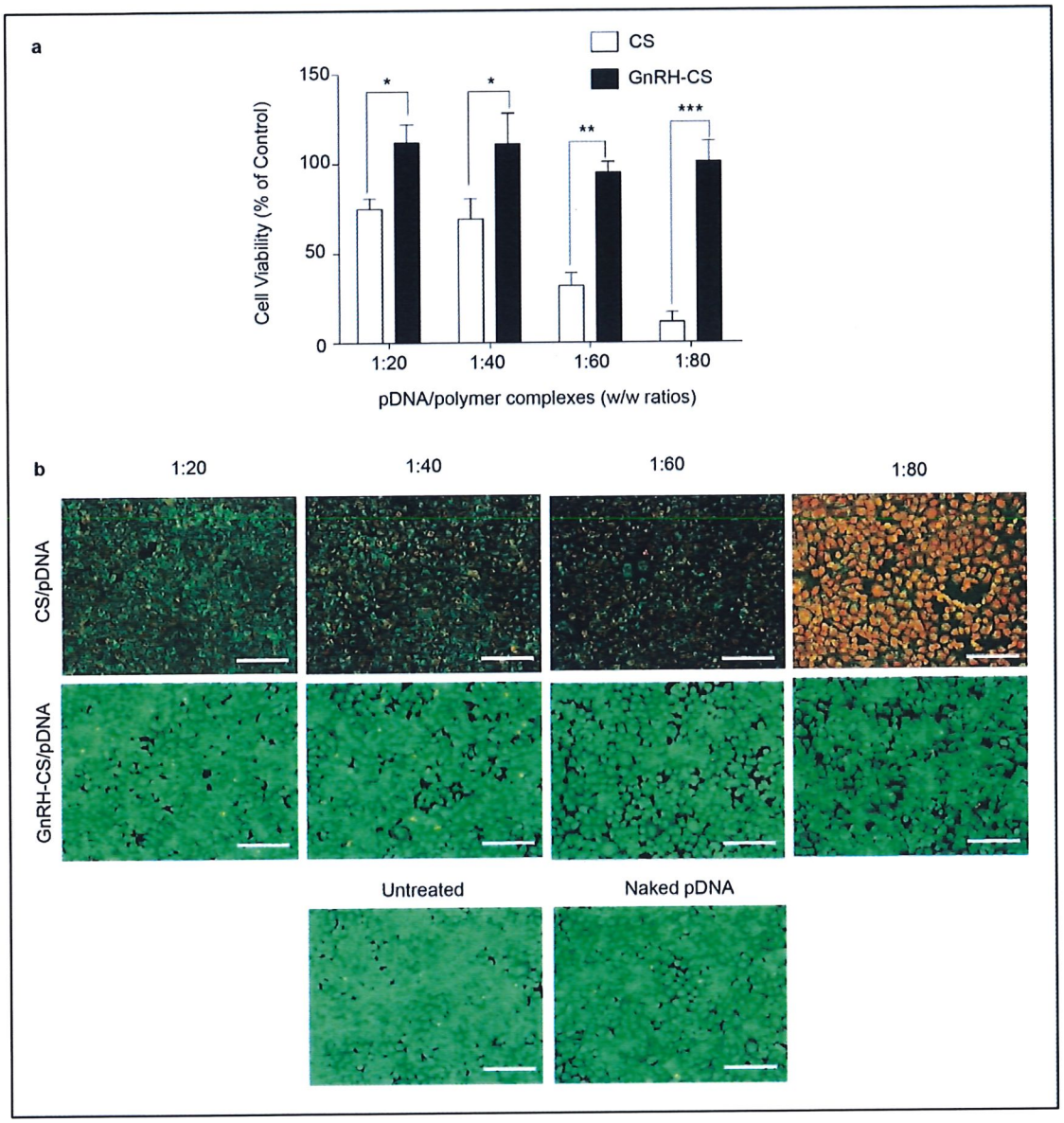


Figure 9. Spermatogonia cell viability after exposure to four different ratios of pDNA/polymer complexes. (a) Mean cell viability (% of control) between unmodified CS and GnRH-CS/pDNA complexes (*p<0.1, **p<0.01, ***p<0.001). (b) Representative figures of GC-1 cells were treated with pDNA/polymer complexes. Dead and live cells fluoresce red-orange and green, respectively.

2.4 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 (Tajik, Pajooh et al. 2017). 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* (Boonthum, Namdee et al. 2017). Similar results were observed in the current study.

Testes 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 (Nagano, Avarbock et al. 1998). 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 (Hofmann, Narisawa et al. 1992).

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 (Leroueil, Berry et al. 2008). 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 (Raina, Kumar et al. 2015). 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 (Ono and Harley 2013), as well as for contraception and fertility control (Dissen, Lomniczi et al. 2012). 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) (Cheung and Wong 2008), 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.

In 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.

Chapter III

Single bilateral intra-testicular injection of Gonadotropin Releasing Hormone-modified Chitosan mediated Tumor Necrosis Factor alpha gene as a nonsurgical sterilization in rats

3.1 Introduction

In the present time, nonsurgical sterilization has become a central issue for population control in animals. This alternative method has been studied by many researchers using chemical castration. A key aspect of chemical castration is to induce testicular cell necrosis. However some research study of chemical castration have indicated serious adverse effect in treated animal such as necrosuppurative orchitis and ulcerative dermatitis after administration (Tepsumethanon, Wilde et al. 2005, Levy, Crawford et al. 2008). These weak points remain a major problem to allow the user to convince this alternative method. The main challenge faced by many researchers is to reduce the severity of testicular necrosis and to remain the infertility effect after administration. One of the most important issues in the conventional sterilization method was the loss of the testosterone, the hormonal product of testis, may have the potential impact on dominance rank in humans, monkeys and other species (Rose, Holaday et al. 1971, Sapolsky 1991, Wickings and Dixson 1992, Czoty, Gould et al. 2009, Muehlenbein and Watts 2010).

Therefore this study set out to develop the new version of nonsurgical sterilization that shows low severity of testicular cell death, remains the serum testosterone level while is still effective in infertility. Previous studies, we successfully established our nanostrategy (Gonadotropin Releasing Hormone-modified Chitosan: GnRH-CS) to deliver gene of interested to GnRH receptor (GnRHR)-expressing cell model and spermatogonia cell in *in vitro* study (Boonthum, Namdee et al. 2017). From *in vitro* to *in vivo* study we tested our nanoparticle mediated apoptotic gene (Tumor Necrosis Factor alpha: TNF-α) to induce testicular cell death by using single intra-testicular injection in rat. The present study was designed to determine the effect of gene inducing testicular cell death delivered by GnRH-CS polymers, to generate the efficient method in order to address the animal overpopulation, to reduce the severe adverse reactions of commercial non-surgical sterilant and to remain the major hormone that has the impact on dominant social status in animals.

3.2 Materials and methods

Experimental design

Animal experimentation was performed according to procedures approved by the Chulalongkorn University Laboratory Animal Center (CULAC), Bangkok, Thailand. Twelve adult male Wistar rats (*Rattus norvegicus*) were randomly divided in 4 groups. Group A, B and C, were received with GnRH-CS/TNF-α (Nanoparticle/DNA), GnRH-CS (Naked nanoparticle) and TNF-α (Naked DNA) via intra-testicular injection each testicle, respectively. Group D was used as control group without treatment. Rats of each group were analyzed at 0, 7, 14, 28 and 35 days after testicular injection (de Macedo, de Lima et al. 2018). At the end point (Day 35), animals were euthanized, the testes were removed and then subjected to standard histological procedure.

Formulation of DNA nanoparticles

Detailed methodology for amplification of DNA and preparation of nanoparticles is reported in previous publications(Boonthum, Namdee et al. 2017). The nanoparticle and DNA solutions were mixed together under high-speed vortex for 1 minute. The ratio of nanoparticle/DNA complexes (weight/weight) were prepared depending on the experiment.

Intra-testicular injections

Before testicular injection, animals were anesthetized using Isoflurane (Attane™, USA) inhalation. The testes were exposed in scrotal sac and were fixed by a digital pressure, 70% alcohol pad applied on skin for aseptic technique (Amaral, Campos et al. 2011, de Macedo, de Lima et al. 2018). And then 700 µL (Kwak and Lee 2013) of each solution described previously was slowly injected into cranio-dorsal aspect of each testicle with 30G x ½" needle attached to 1-ml plastic disposable syringe at the 3-4 mm injection depth (de Macedo, de Lima et al. 2018). The needle was removed slowly after injection to avoid leakage of the solution to the scrotal sac. Both testes were injected.

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 (Nistal, Paniagua et al. 1985, Love, Garcia

et al. 1991). After euthanasia on day 35, the testicle of each animal was taken, weighed and mearsured in order to compare the testicular weight and volume between groups.

Serum testosterone

Blood samples were obtained by saphenous vein on day 0, 7, 14, 28 and 35 in all rats. The sample was centrifuged and then collected the supernatant for the hormone analysis. The testosterone levels were assayed via a chemiluminescent microparticle immunoassay (CMIA) (Xia, Xu et al. 2013).

Scrotal ultrasound

The testes were analyzed using a portable Mindray M9Vet® (Mindray Medical Limited, Thailand) ultrasound machine with 4–16 MHz linear transducer (Mindray L16-4Hs probe). Two planes (transverse and longitudinal) should be examined to compare the echotexture of testis (Edey and Sidhu 2008, Ammar, Sidhu et al. 2012).

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

After euthanasia with carbon dioxide: the testis was taken and was examined for gross analysis. And then the testis, 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 (Kwak and Lee 2013). The testicular degeneration was classified as normal, mild, moderate and severe. Classification of pathological changes was modified from previous studies (Mikuz 1985, Sağnak, Ersoy et al. 2007, Cerilli, Kuang et al. 2010, Teankum, Tummaruk et al. 2013) as shown in Table 2.

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 and denoted as follows: $*P<0.05$, $**P<0.01$ and $***P<0.001$.

Table 2. The severity of mature rat testicular degeneration after testicular injection

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

This table was modified from previous studies (Mikuz 1985, Sağnak, Ersoy et al. 2007, Cerilli, Kuang et al. 2010, Teankum, Tummaruk et al. 2013), the histopathology of 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.

3.3 Results

Body weight

Body weight is presented in Figure 12. At 0, 7, 14, 28 and 35 days after testicular injection, the body weight increased normally (Figure 10). No significant difference was found between groups ($P>0.05$).

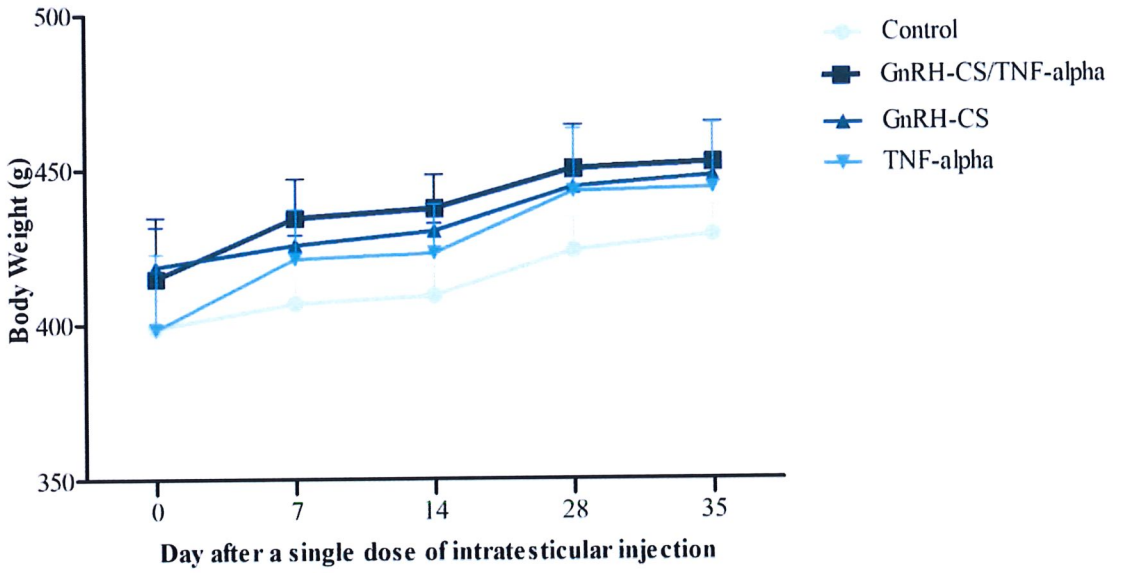


Figure 10. All of rats had no significant difference in body weight after testicular injection. Data represent the mean ± SEM of 4 rats, **P*<0.05, ***P*<0.01 and ****P*<0.001, two-way ANOVA followed with Bonferroni post-test.

Testicular volume

Figure 11 compares the testicular volume during experiment. Control group showed no significant difference. On the other hand, three treatment groups showed significantly decrease in testicular volume during the first week post testicular injection (*P*<0.001), especially in GnRH-CS/TNF-α and GnRH-CS groups, had a 39.7% ± 15.45% and 33.4% ± 10.50% reduction in starting testicular volume, respectively. The testicular volume continued to reduce on day 14 (*P*<0.05) in GnRH-CS/TNF-α group and day 28 (*P*<0.05) in GnRH-CS groups. The results at 28 days after testicular injection showed 67.47% ± 11.40%, 59.00% ± 8.38% and 33.67% ± 22.49% reduction in starting testicular volume in GnRH-CS/TNF-α, GnRH-CS and TNF-α groups, respectively.

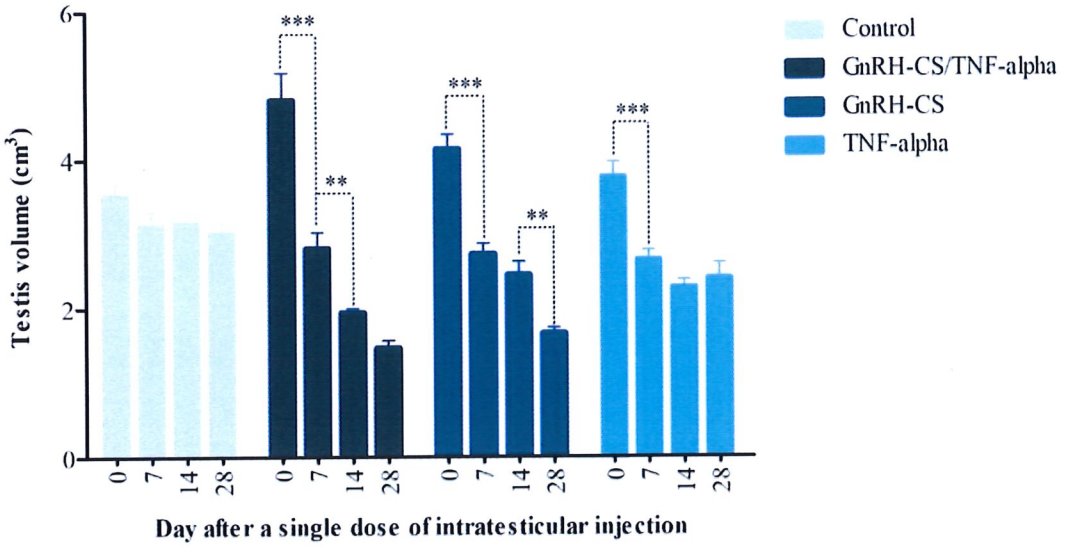


Figure 11. Testicular volume after intratesticular injection. The treatment groups showed significant differences in a reduction in testicular volume on day 7 ($P<0.001$). The testicular volume continued to reduce on day 14 ($P<0.05$) and 28 ($P<0.05$) in GnRH-CS/TNF-α and GnRH-CS groups, respectively. Data represent the mean ± SEM of 4 rats, *$P<0.05$, **$P<0.01$ and ***$P<0.001$, two-way ANOVA followed with Bonferroni post-test.

Testicular weight and volume of dissected testis

Testicular weight and volume of dissected testis can be compared in Figure 12. These parameters were significantly reduced ($P<0.001$) in GnRH-CS/TNF-α and GnRH-CS groups.

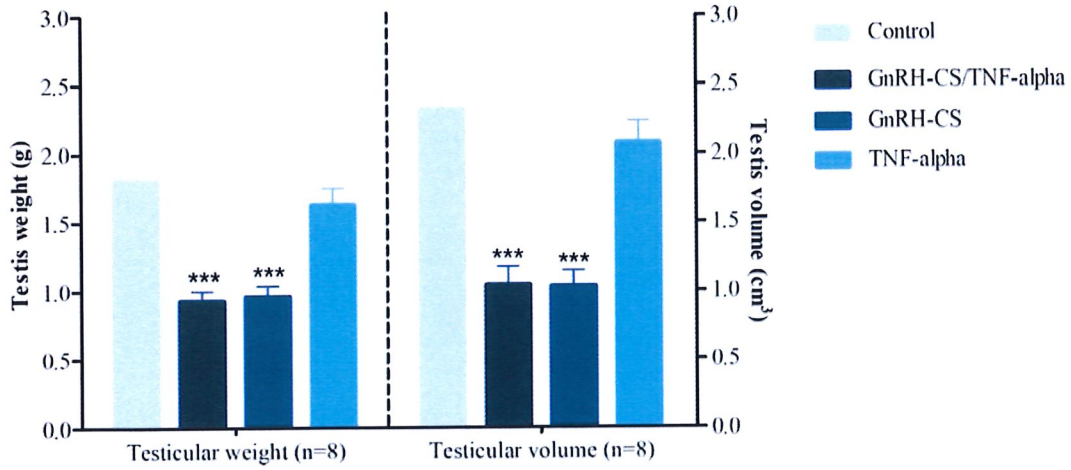


Figure 12. Testicular weight and volume in *in vitro* after castration on day 35. There were significantly decreses in GnRH-CS/TNF-α and GnRH-CS groups ($P<0.001$). Data represent the mean ± SEM of 4 rats, *$P<0.05$, **$P<0.01$ and ***$P<0.001$, two-way ANOVA followed with Bonferroni post-test.

Serum testosterone level

The data of serum testosterone level in 16 rats are highly variable, as shown in Figure 13. However, this result did not show a significant difference between groups (P>0.05).

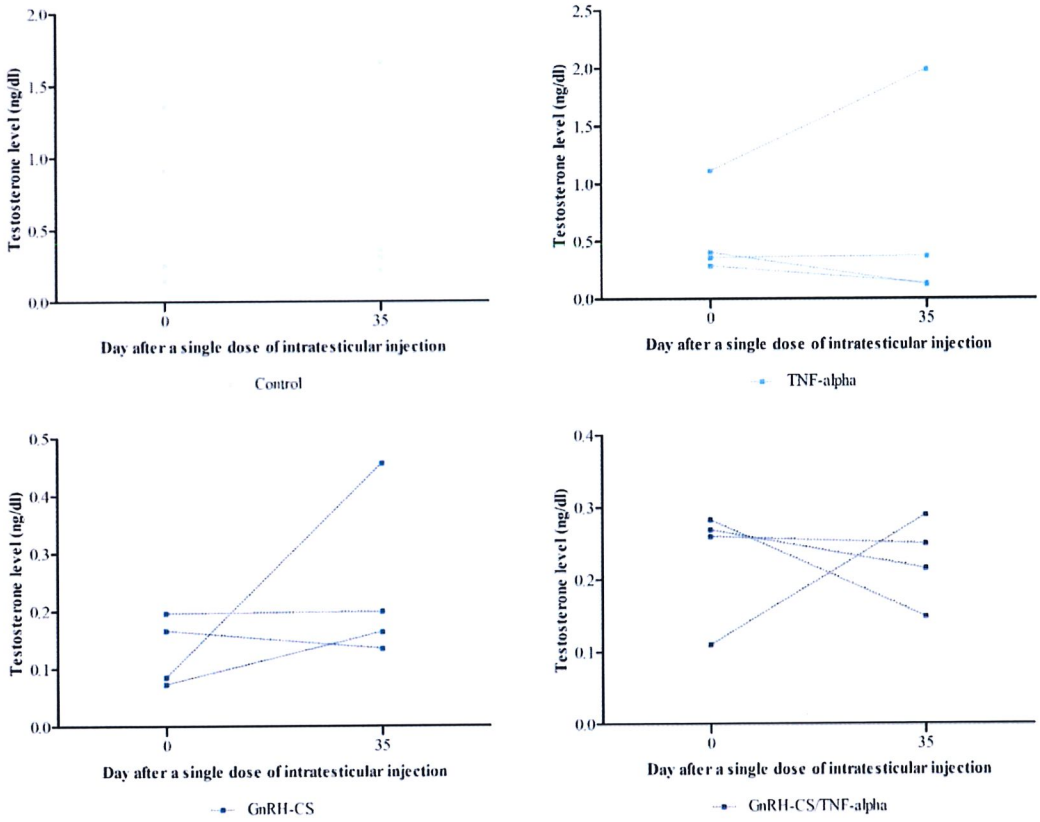


Figure13. Testosterone level (ng/dl) measured from 0 and 35 days after testicular injection in male rats. No significant difference was found between groups. Data represent the mean ± SEM of 4 rats, *P<0.05, **P<0.01 and ***P<0.001, two-way ANOVA followed with Bonferroni post-test.

Scrotal ultrasound

As shown in Figure 14, the ultrasonography results during experiment, control rat demonstrated a uniform texture of testis. The dilatation of testicular vessel with mild heterogeneity of the surrounding parenchyma was showed in three treatment groups at the first week post testicular injection. GnRH-CS/TNF-α and GnRH-CS groups showed irregular shaped hypoechoic nodule with/without hyperechoic edge, calcification within the testicular parenchyma, a global reduction in testicular volume and reflectivity on day 35.

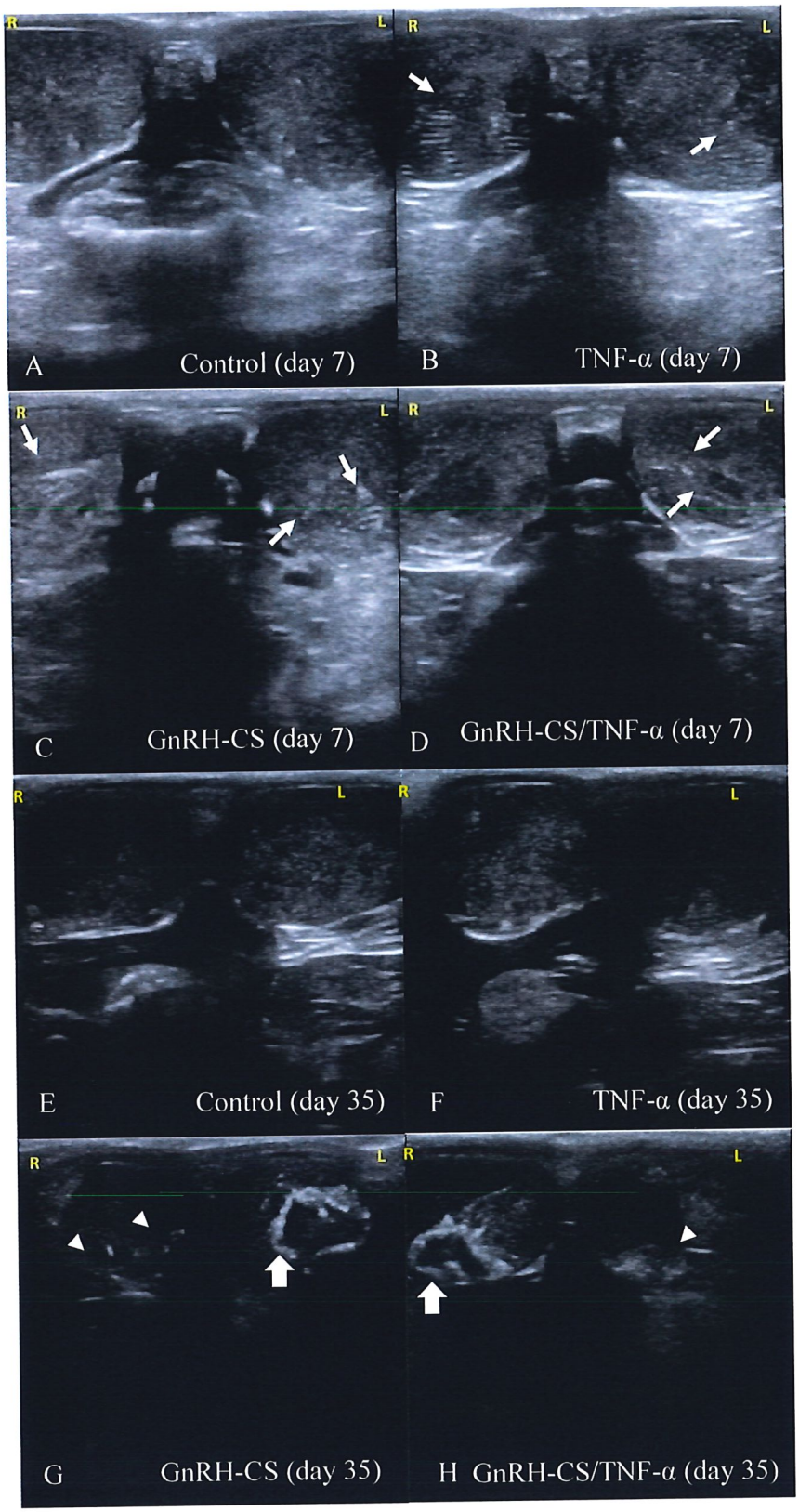


Figure 14. A scrotal ultrasound examination. Normal ultrasound anatomy, in control (A and E) and TNF-α (day 35, F) groups. The dilatation of testicular vessel (thin arrows) with mild heterogeneity of the surrounding parenchyma at 7 days after testicular injection, in three treatment groups (B, C and D).

GnRH-CS/TNF-α and GnRH-CS groups showed irregular shaped hypoechoic nodule with/without hyperechoic edge (broad arrows), calcification (arrowheads) and a reduction in testicular volume (G and H).

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

Macroscopic finding can be seen in Figure 15. In control and TNF-α groups showed normal testicular size. GnRH-CS and GnRH-CS/TNF-α groups showed a slightly small testicular size. As shown in Figure 16, H&E (40x), in control and TNF-α groups showed well organized seminiferous tubules and interstitial tissue (Leydig cells). Histopathological analysis of the testicle showed >75% and >50% of the entire biopsy had germ cell abnormalities in GnRH-CS/TNF-α and GnRH-CS groups, respectively. Leydig cells are present in all group. Figure 17 presents normal testicular tubules in control and TNF-α groups, H&E (200x). Moderate degeneration in the germinal epithelium, extensive intratubular hyalinization due to fibrosis, coagulative necrosis, lymphocytic infiltration, collapsed seminiferous tubules, mineralization and spermatid retention were found in animal treated with GnRH-CS/TNF-α. 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.

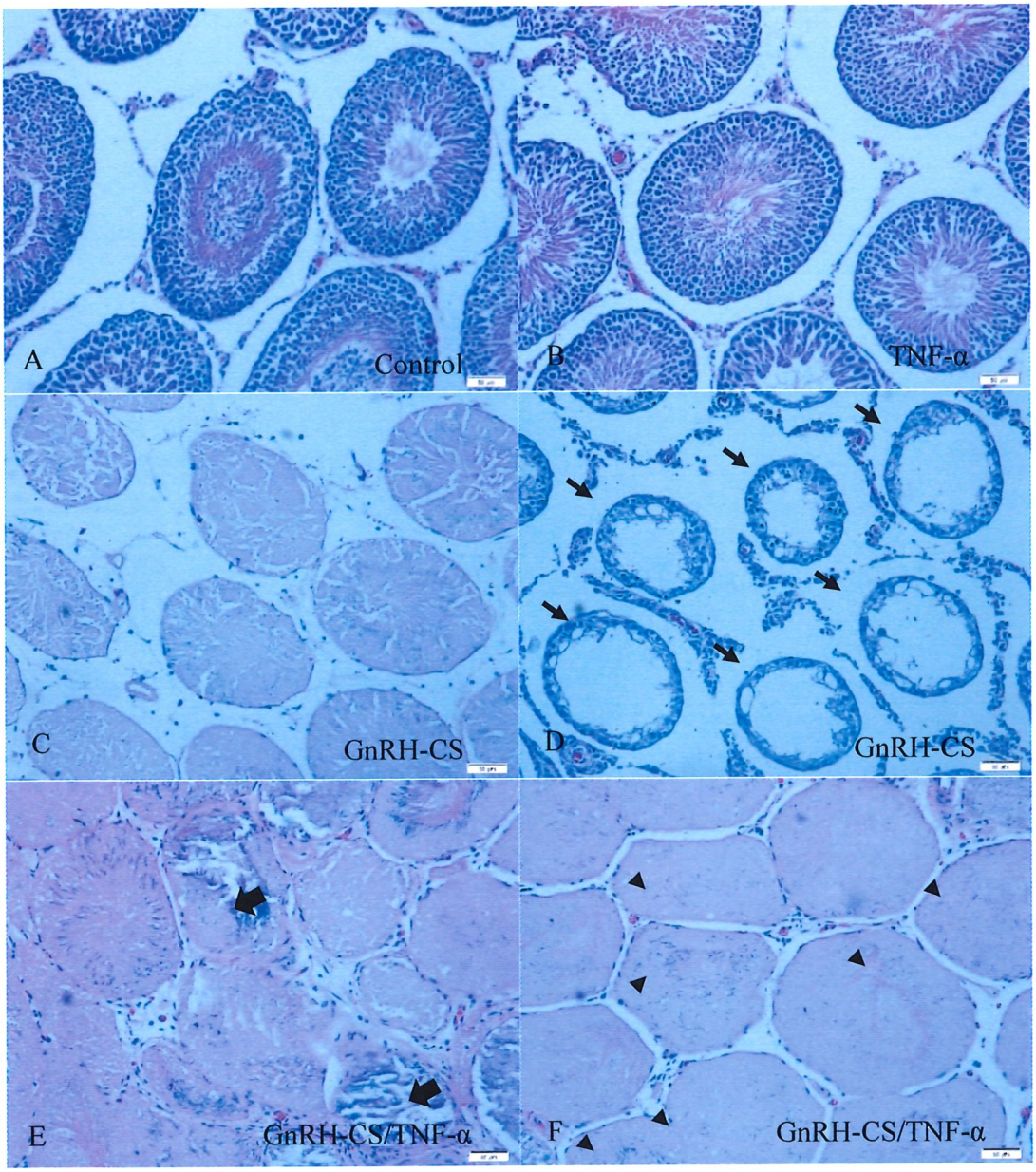


Figure 17. Microscopic appearance of testes (× 200 magnification, H&E). Testicular histological section of control rat (A) demonstrated normal seminiferous tubules and interstitial tissue. Seminiferous tubules in testis from GnRH-CS group (C and D) appeared normal interstitial tissue, intratubular coagulative necrosis (C), tubules lined by Sertoli cells remain (thin arrows) and tubular vacuolization (D). GnRH-CS/TNF-α (E and F) induced extensive intratubular hyalinization, coagulative necrosis, lymphocytic infiltration, collapsed seminiferous tubules, mineralization (broad arrows) and spermatid retention (arrowheads).

3.4 Discussion

Intra-testicular chemosterilant has been used as a fast and low cost method to control population in large scale animal. Non-surgical sterilants have been developed in several laboratories such as zinc-based solution in dogs (Fahim, Wang et al. 1993, Oliveira, Moura et al. 2007), cat (Oliveira, Fagundes et al. 2013) and bears (Brito, Sertich et al. 2011); 20% hypertonic saline in rats (Emir, Dadalı et al. 2008, Emir, Sunay et al. 2011) and cattle (Neto, Gasperin et al. 2014); chlorhexidine in bovines (Pineda and Hepler 1981); calcium chloride ($CaCl_2$) in rats (Jana, Samanta et al. 2002, Jana and Samanta 2006), cats (Jana and Samanta 2011), dogs (Jana and Samanta 2007), goats (Jana, Samanta et al. 2005) and cattle (Koger 1978); $CaCl_2$ with DMSO in cats (Paranzini, Sousa et al. 2018); cadmium chloride ($CdCl_2$) in lamps (Lymberopoulos, Kotsaki-Kovatsi et al. 2000) and clove oil in dogs (Abu-Ahmed 2015). The main point of chemical castration was either safe but not effective or effective but not safe(Oliveira, Moura et al. 2007). The challenges are to minimize side effect by using the natural compound for reducing the severity of testicular toxicity while is still effective in infertility.

The present study used chitosan that is a natural polysaccharide derived from chitin. Chitosan has anticancer and anti-inflammatory properties (Hasegawa, Yagi et al. 2001, Azuma, Osaki et al. 2015). 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 (Bahk, Kim et al. 2008, Cheung and Wong 2008, Anjum, Krishna et al. 2012). A gene of interested in this study is TNF-α as a gene inducing apoptosis. Moreover under normal physiological conditions, TNF-α are produced in the testis and play a role in maintaining testicular function (Lysiak 2004). Previous study concluded this version of chitosan was able to condense DNA and specifically deliver DNA to GnRHR-expressing cells (Boonthum, Namdee et al. 2017) and spermatogonia cells. To examine in animal model, GnRH-CS was used to deliver TNF-α gene to induce testicular cell death in male rat.

The most interesting finding was a progressive decrease in testicular volume post testicular injection with GnRH-CS/TNF-α and GnRH-CS. Control and TNF-α groups showed no significant decrease in testicular volume during experiment. Moreover the testicular volume by using sliding caliper in *in vitro* after castration and testicular weight of dissected testicle were significantly less in GnRH-CS/TNF-α and GnRH-CS groups than control and TNF-α groups. Testicular gross weight and volume were on the same pace. This also accords with earlier observations, which showed that the testis weight should considered equal to volume because of its density (1.03–1.04). In addition to the reduced total testicular volume (*In vivo* over the scrotal skin and *In vitro* after castration) and testicular weight, it might

be affected by testicular germ cell degeneration, a loss in germ cell number and seminiferous tubular fluid (STF) (Haschek, Rousseaux et al. 2010). However many research studies of chemical sterilant revealed individual variation in testicular size after testicular injection, no changes were observed in some animals while others showed small testis (Soto, Viana et al. 2009, Brito, Sertich et al. 2011). The presence of testis in animal treated with some chemical sterilant that might be blind observer to indicate castrated or non-castrated animal. Therefore the reduction of testicle after treatment might show the advantage for the identification of castrated animal in large scale.

Body weight was measured to monitor weight loss due to stress after treatment. No significant changes in body weight were detected. It is possible that treated animals did not suffer and could eat normally. 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 animals may feel pain due to capsular distension within 24 hours and may remain for 7-15 days (Kutzler and Wood 2006). This study showed no change that indicated pain after recovering from anesthesia, especially in body weight was no statistically significant differences between groups due to normal food intake.

Concerning serum testosterone, surgical sterilization eliminate gonadal sources of testosterone. Many research studies examined testosterone is needed for secondary sexual characteristics, including male dominance rank (Rose, Holaday et al. 1971, Sapolsky 1991, Wickings and Dixson 1992, Czoty, Gould et al. 2009, Muehlenbein and Watts 2010). Unlike the conventional method, non-surgical sterilization does not eliminate Leydig cell that is the sources of testosterone (Kutzler and Wood 2006, Jana and Samanta 2011). The present study supported the finding of the previous work in testosterone concentration, revealed no significant change in serum testosterone level in all groups. 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 (Kobyliak, Falalyeyeva et al. 2015). Normal ultrasound image of the testis, revealed a uniform texture and reflectivity as shown in control group. At 7 days after injection, three treatment groups showed the dilatation of testicular vessel due to atrophy (Kobyliak, Falalyeyeva et al. 2015), this finding broadly supports the results of testicular volume. However the color Doppler ultrasound should be used to confirm dilated testicular vessel (Belay, Huang et al. 2016). A scrotal ultrasound examination of GnRH-CS/TNF-α and GnRH-CS groups, on day 14, showed irregular shaped hypoechoic nodule with/without hyperechoic edge. Day 28 and 35, calcifications within the testicular parenchyma and hypoechoic nodule with/without irregular hyperechoic margins were demonstrated. A global reduction in testicular volume

and reflectivity were found especially on day 35, these findings refer to testicular atrophy (Ammar, Sidhu et al. 2012). The hypoechoic nodule might relate to the site of injection that showed more heterogeneity of echo texture than other sites. Interestingly, the abnormalities expressed only part of the injection site or not the entire testicle. The challenge is to develop the solution property to diffuse through whole tissue or adjust the technique of injection to cover total testicle.

An ideal chemosterilant is safe, effective, inexpensive, permanent and required single injection (Jana and Samanta 2006, Jana and Samanta 2007). Permanent sterilization needs irreversible damage of testicular cells. In this study, GnRH-CS/TNF-α group showed more severity of testicular cell death than others. The severity of testicular degeneration in this study divided in to normal, mild, moderate and severe. According to this, normal grade shows normal size of testicle in gross examination and regular structure demonstrating normal spermatogenesis of seminiferous tubule in histopathological analysis. Mild grade exhibited one or more criterion including slightly small testicular size, $\geq$20-40% reduction in starting testicular volume and >50% of the entire biopsy had germ cell abnormalities. Small testis, $\geq$40-60% reduction in starting testicular volume or >75% germ cell abnormalities were found in moderate grade. Severe grade shows marked small testicular size, $\geq$60-80% reduction in starting testicular volume and 100% testicular cell death. The current study found that GnRH-CS/TNF-α 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 (Azuma, Osaki et al. 2015). While control and TNF-α groups did not affect testis. Because naked TNF-α was degraded by the body enzyme before showing any effects. As for 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) (Cerilli, Kuang et al. 2010). Testicular degeneration is 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 (Haschek, Rousseaux et al. 2010). Coagulative necrosis can be seen as a big area of pale eosinophilic region due to infarction. These effects are agree with previous studies using other chemical sterilants (Immegart and Threlfall 2000). Testicular necrosis is not reversible and the affected area is replaced by fibrous tissue. According to testicular necrosis, spermatids fail to be moved and are still remained at the lumen. Concerning safe, no pathology was found in liver and kidney that are important organs of metabolism, detoxification, storage and excretion (Brzóska, Moniuszko-Jakoniuk et al. 2003). Moreover all treatments did not affect urinary bladder that represents cells expressing GnRHR (Bahk, Kim et al. 2008).

In 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. From the results, GnRH-CS/TNF-α acts as a non-surgical sterilization resulting in seminiferous tubules necrosis (>75% of the entire biopsy) without affecting stress and serum hormone levels that is the essential hormone of the social dominance status. Therefore it can be used as gentle non-surgical sterilization to reduce the severe adverse reactions of chemosterilant and to remain the major hormone that has the impact on dominant social status in some species. Concerning permanent sterilization needs an in-depth study of long-term effects and mating fertility that should be explored in further study.

References

1. Abu-Ahmed, H. (2015). Chemical Sterilization of Dogs using Single Bilateral Intra-testicular Injection of Calcium Chloride or Clove Oil.
2. ACC&D (2012). "Esterilsol™ ("Zinc Neutering")." Alliance for Contraception in Cats & Dogs (ACC&D).
3. Aframian, D. J., C. Zheng, C. M. Goldsmith, J. Nikolovski, E. Cukierman, K. M. Yamada, D. J. Mooney, H. Birkedal-Hansen and B. J. Baum (2001). "Using HSV-thymidine kinase for safety in an allogeneic salivary graft cell line." Tissue Eng 7(4): 405-413.
4. Agirre, M., J. Zarate, E. Ojeda, G. Puras, J. Desbrieres and J. L. Pedraz (2014). "Low molecular weight chitosan (LMWC)-based polyplexes for pDNA delivery: from bench to bedside." Polymers 6(6): 1727-1755.
5. Al-Dosari, M. S. and X. Gao (2009). "Nonviral Gene Delivery: Principle, Limitations, and Recent Progress." The AAPS Journal 11(4): 671.
6. Alikhani, M., Z. Alikhani, M. Raptis and D. T. Graves (2004). "TNF-alpha in vivo stimulates apoptosis in fibroblasts through caspase-8 activation and modulates the expression of pro-apoptotic genes." J Cell Physiol 201(3): 341-348.
7. Amaral, M. G., V. F. Campos, F. K. Seixas, P. V. Cavalcanti, L. P. Selau, J. C. Deschamps and T. Collares (2011). "Testis-mediated gene transfer in mice: comparison of transfection reagents regarding transgene transmission and testicular damage." Biological research 44(3): 229-234.
8. Amaral, M. G., V. F. Campos, F. K. Seixas, P. V. Cavalcanti, L. P. Selau, J. C. Deschamps and T. Collares (2011). "Testis-mediated gene transfer in mice: comparison of transfection reagents regarding transgene transmission and testicular damage." Biol Res 44(3): 229-234.
9. Ammar, T., P. S. Sidhu and C. J. Wilkins (2012). "Male infertility: the role of imaging in diagnosis and management." Br J Radiol 85 Spec No 1: S59-68.
10. Ammar, T., P. S. Sidhu and C. J. Wilkins (2012). "Male infertility: the role of imaging in diagnosis and management." The British Journal of Radiology 85(Spec Iss 1): S59-S68.
11. Anjum, S., A. Krishna, R. Sridaran and K. Tsutsui (2012). "Localization of gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin and GnRH receptor and their possible roles in testicular activities from birth to senescence in mice." J Exp Zool A Ecol Genet Physiol 317(10): 630-644.

12. Atwood, B. K., J. Lopez, J. Wager-Miller, K. Mackie and A. Straiker (2011). "Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis." BMC Genomics 12(1): 14.

13. Azuma, K., T. Osaki, S. Minami and Y. Okamoto (2015). "Anticancer and anti-inflammatory properties of chitin and chitosan oligosaccharides." J Funct Biomater 6(1): 33-49.

14. Babu, A. and R. Ramesh (2017). "Multifaceted Applications of Chitosan in Cancer Drug Delivery and Therapy." Marine Drugs 15(4): 96.

15. Badiga, A. V., C. Chetty, D. Kesanakurti, D. Are, M. Gujrati, J. D. Klopfenstein, D. H. Dinh and J. S. Rao (2011). "MMP-2 siRNA inhibits radiation-enhanced invasiveness in glioma cells." PLoS One 6(6): e20614.

16. Bahk, J. Y., M. O. Kim, M. S. Park, H. Y. Lee, J. H. Lee, B. C. Chung and S. K. Min (2008). "Gonadotropin-releasing hormone (GnRH) and GnRH receptor in bladder cancer epithelia and GnRH effect on bladder cancer cell proliferation." Urol Int 80(4): 431-438.

17. Barua, S. and S. Mitragotri (2014). "Challenges associated with Penetration of Nanoparticles across Cell and Tissue Barriers: A Review of Current Status and Future Prospects." Nano Today 9(2): 223-243.

18. Bauer, K. R., M. Brown, R. D. Cress, C. A. Parise and V. Caggiano (2007). "Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype." Cancer 109(9): 1721-1728.

19. Bauer, K. R., M. Brown, R. D. Cress, C. A. Parise and V. Caggiano (2007). "Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry." Cancer 109(9): 1721-1728.

20. Belay, R., G. Huang, J. Shen and E. Ko (2016). "Diagnosis of clinical and subclinical varicocele: how has it evolved?" Asian Journal of Andrology 18(2): 182-185.

21. 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.

22. Bravo-Anaya, L. M., J. F. A. Soltero and M. Rinaudo (2016). "DNA/chitosan electrostatic complex." International Journal of Biological Macromolecules 88: 345-353.

23. Brito, L. F., P. L. Sertich, W. Rives, M. Knobbe, F. Del Piero and G. B. Stull (2011). "Effects of intratesticular zinc gluconate treatment on testicular dimensions, echodensity, histology, sperm

production, and testosterone secretion in American black bears (Ursus americanus)." Theriogenology 75(8): 1444-1452.

24. Brito, L. F. C., P. L. Sertich, W. Rives, M. Knobbe, F. D. Piero and G. B. Stull (2011). "Effects of intratesticular zinc gluconate treatment on testicular dimensions, echodensity, histology, sperm production, and testosterone secretion in American black bears (Ursus americanus)." Theriogenology 75(8): 1444-1452.
25. Brus, L., C. Lambalk, J. De Koning, M. Helder, R. Janssens and J. Schoemaker (1997). "Specific gonadotrophin-releasing hormone analogue binding predominantly in human luteinized follicular aspirates and not in human pre-ovulatory follicles." Human reproduction 12(4): 769-773.
26. Brzóska, M. M., J. Moniuszko-Jakoniuk, B. Pi‡at-Marcinkiewicz and B. Sawicki (2003). "LIVER AND KIDNEY FUNCTION AND HISTOLOGY IN RATS EXPOSED TO CADMIUM AND ETHANOL." Alcohol and Alcoholism 38(1): 2-10.
27. Buckley, R. H. (2002). "Gene therapy for SCID--a complication after remarkable progress." Lancet 360(9341): 1185-1186.
28. Burgess, A., S. Vigneron, E. Brioudes, J. C. Labbe, T. Lorca and A. Castro (2010). "Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance." Proc Natl Acad Sci U S A 107(28): 12564-12569.
29. Cerilli, L. A., W. Kuang and D. Rogers (2010). "A practical approach to testicular biopsy interpretation for male infertility." Arch Pathol Lab Med 134(8): 1197-1204.
30. Cheng, C. Y. and D. D. Mruk (2012). "The blood-testis barrier and its implications for male contraception." Pharmacol Rev 64(1): 16-64.
31. Cheng, Y.-Y. and H. Yuan (2006). "Quantitative study of electrostatic and steric effects on physicochemical property and biological activity." Journal of Molecular Graphics and Modelling 24(4): 219-226.
32. Cheung, L. W. and A. S. Wong (2008). "Gonadotropin-releasing hormone: GnRH receptor signaling in extrapituitary tissues." FEBS J 275(22): 5479-5495.
33. Cheung, L. W. T. and A. S. T. Wong (2008). "Gonadotropin-releasing hormone: GnRH receptor signaling in extrapituitary tissues." The FEBS Journal 275(22): 5479-5495.
34. Choy, C. J., X. Ling, J. J. Geruntho, S. K. Beyer, J. D. Latoche, B. Langton-Webster, C. J. Anderson and C. E. Berkman (2017). "(177)Lu-Labeled Phosphoramidate-Based PSMA Inhibitors: The Effect of an Albumin Binder on Biodistribution and Therapeutic Efficacy in Prostate Tumor-Bearing Mice." Theranostics 7(7): 1928-1939.

35. Coleman, P., H. Veleanu and S. K. Wolkov (2010). "It's training cats and dogs... Government lawyers take note: Different licensing laws generate revenue, reduce costs, protect citizens, and save lives." Stetson L. Rev. 40: 393.

36. Cross, D. and J. K. Burmester (2006). "Gene therapy for cancer treatment: past, present and future." Clin Med Res 4(3): 218-227.

37. Czoty, P. W., R. W. Gould and M. A. Nader (2009). "Relationship Between Social Rank and Cortisol and Testosterone Concentrations in Male Cynomolgus Monkeys (Macaca fascicularis)." Journal of Neuroendocrinology 21(1): 68-76.

38. de Macedo, S. R. B., L. A. R. de Lima, S. M. de Torres, V. V. G. de Oliveira, R. N. de Morais, C. A. Peixoto, B. M. Tenorio and V. Junior (2018). "Effects of intratesticular injection of zinc-based solution in rats in combination with anti-inflammatory and analgesic drugs during chemical sterilization." Vet World 11(5): 649-656.

39. Demeneix, B. and J. P. Behr (2005). Polyethylenimine (PEI). Advances in Genetics, Academic Press. 53: 215-230.

40. Dharap, S. S., Y. Wang, P. Chandna, J. J. Khandare, B. Qiu, S. Gunaseelan, P. J. Sinko, S. Stein, A. Farmanfarmaian and T. Minko (2005). "Tumor-specific targeting of an anticancer drug delivery system by LHRH peptide." Proceedings of the National Academy of Sciences of the United States of America 102(36): 12962-12967.

41. Dissen, G., A. Lomniczi, R. Boudreau, Y. Chen, B. Davidson and S. Ojeda (2012). "Applying Gene Silencing Technology to Contraception." Reproduction in Domestic Animals 47(s6): 381-386.

42. Dissen, G. A., A. Lomniczi, R. L. Boudreau, Y. H. Chen, B. L. Davidson and S. R. Ojeda (2012). "Applying gene silencing technology to contraception." Reprod Domest Anim 47 Suppl 6: 381-386.

43. Edey, A. J. and P. S. Sidhu (2008). "Male infertility: role of imaging in the diagnosis and management." Imaging 20(2): 139-146.

44. Ehlers, K. and L. M. Halvorson (2013). "Gonadotropinreleasing Hormone (GnRH) and the GnRH Receptor (GnRHR)." The Global Library of Women's Medicine: 1-23.

45. Emir, L., M. Dadalı, M. Sunay, D. Erol, M. Çaydere and H. Üstün (2008). "Chemical castration with intratesticular injection of 20% hypertonic saline: A minimally invasive method." Urologic Oncology: Seminars and Original Investigations 26(4): 392-396.

46. Emir, L., M. Sunay, O. Yalbuzdag, Y. Karakaya and D. Erol (2011). "Hormonal and pathologic changes after chemoablation of testes with hypertonic saline solution as a treatment method alternative to orchiectomy in patients with hormone sensitive metastatic prostatic cancer." Urol Oncol 29(2): 212-217.

47. Fahim, M. S., M. Wang, M. F. Sutcu, Z. Fahim and R. S. Youngquist (1993). "Sterilization of dogs with intra-epididymal injection of zinc arginine." Contraception 47(1): 107-122.

48. Fischer, D., T. Bieber, Y. Li, H. P. Elsasser and T. Kissel (1999). "A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity." Pharm Res 16(8): 1273-1279.

49. Forzan, M. J., E. Garde, G. E. Perez and R. V. Vanderstichel (2014). "Necrosuppurative orchitis and scrotal necrotizing dermatitis following intratesticular administration of zinc gluconate neutralized with arginine (EsterilSol) in 2 mixed-breed dogs." Vet Pathol 51(4): 820-823.

50. Gavet, O. and J. Pines (2010). "Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis." Developmental cell 18(4): 533-543.

51. Gillett, T. (2014). "Pet Overpopulation: A Global Crisis." International Animal Health Journal 1(2): 38-42.

52. Guazzone, V. A., P. Jacobo, M. S. Theas and L. Lustig (2009). "Cytokines and chemokines in testicular inflammation: A brief review." Microsc Res Tech 72(8): 620-628.

53. Haschek, W. M., C. G. Rousseaux and M. A. Wallig (2010). Chapter 18 - Male Reproductive System. Fundamentals of Toxicologic Pathology (Second Edition). W. M. Haschek, C. G. Rousseaux and M. A. Wallig. San Diego, Academic Press: 553-597.

54. Hasegawa, M., K. Yagi, S. Iwakawa and M. Hirai (2001). "Chitosan induces apoptosis via caspase-3 activation in bladder tumor cells." Jpn J Cancer Res 92(4): 459-466.

55. He, C.-X., Y. Tabata and J.-Q. Gao (2010). "Non-viral gene delivery carrier and its three-dimensional transfection system." International Journal of Pharmaceutics 386(1): 232-242.

56. Hofmann, M.-C., S. Narisawa, R. A. Hess and J. L. Millán (1992). "Immortalization of germ cells and somatic testicular cells using the SV40 large T antigen." Experimental Cell Research 201(2): 417-435.

57. Horta, R. S., M. S. Figueiredo, G. E. Lavalle, M. P. Costa, R. M. C. Cunha and R. B. Araújo (2015). "Surgical stress and postoperative complications related to regional and radical mastectomy in dogs." Acta Veterinaria Scandinavica 57(1): 34.

58. Hou, S., L. Xian, P. Shi, C. Li, Z. Lin and X. Gao (2015). "The Magea gene cluster regulates male germ cell apoptosis without affecting the fertility in mice." Scientific reports 6: 26735-26735.

59. Hsu, H., H. B. Shu, M. G. Pan and D. V. Goeddel (1996). "TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways." Cell 84(2): 299-308.

60. Hsu, Y., L. L. Severinghaus and J. A. Serpell (2003). "Dog keeping in Taiwan: its contribution to the problem of free-roaming dogs." J Appl Anim Welf Sci 6(1): 1-23.

61. Huang, J., X. Wang, Z. Li, R. Ma and W. Xiao (2015). "Effects of GnRH agonists on the expression of developmental follicular anti-mullerian hormone in varying follicular stages in cyclic mice in vivo." Mol Med Rep 12(3): 4305-4313.

62. Hunt, K. K., S. A. Vorburger and S. G. Swisher (2007). "Gene therapy for cancer." Cancer Drug Discovery and Development: 3-460.

63. Illum, L. (1998). "Chitosan and its use as a pharmaceutical excipient." Pharm Res 15(9): 1326-1331.

64. Immegart, H. M. and W. R. Threlfall (2000). "Evaluation of intratesticular injection of glycerol for nonsurgical sterilization of dogs." Am J Vet Res 61(5): 544-549.

65. Jana, K. and P. K. Samanta (2006). "Evaluation of single intratesticular injection of calcium chloride for nonsurgical sterilization in adult albino rats." Contraception 73(3): 289-300.

66. Jana, K. and P. K. Samanta (2007). "Sterilization of male stray dogs with a single intratesticular injection of calcium chloride: a dose-dependent study." Contraception 75(5): 390-400.

67. Jana, K. and P. K. Samanta (2011). "Clinical Evaluation of Non-surgical Sterilization of Male Cats with Single Intra-testicular Injection of Calcium Chloride." BMC Veterinary Research 7(1): 39.

68. Jana, K., P. K. Samanta and D. Ghosh (2002). "Dose-dependent response to an intratesticular injection of calcium chloride for induction of chemosterilization in adult albino rats." Vet Res Commun 26(8): 651-673.

69. Jana, K., P. K. Samanta and D. Ghosh (2005). "Evaluation of single intratesticular injection of calcium chloride for nonsurgical sterilization of male Black Bengal goats (Capra hircus): a dose-dependent study." Animal Reproduction Science 86(1): 89-108.

70. Jiang, H. L., J. T. Kwon, Y. K. Kim, E. M. Kim, R. Arote, H. J. Jeong, J. W. Nah, Y. J. Choi, T. Akaike, M. H. Cho and C. S. Cho (2007). "Galactosylated chitosan-graft-polyethylenimine as a gene carrier for hepatocyte targeting." Gene Therapy 14(19): 1389-1398.

71. Jiang, H. L., J. T. Kwon, Y. K. Kim, E. M. Kim, R. Arote, H. J. Jeong, J. W. Nah, Y. J. Choi, T. Akaike, M. H. Cho and C. S. Cho (2007). "Galactosylated chitosan-graft-polyethylenimine as a gene carrier for hepatocyte targeting." Gene Ther 14(19): 1389-1398.

72. Jiang, H. L., J. T. Kwon, Y. K. Kim, E. M. Kim, R. Arote, H. J. Jeong, J. W. Nah, Y. J. Choi, T. Akaike, M. H. Cho and C. S. Cho (2007). "Galactosylated chitosan-graft-polyethylenimine as a gene carrier for hepatocyte targeting." Gene Therapy 14: 1389.

73. Kafil, V. and Y. Omidi (2011). "Cytotoxic impacts of linear and branched polyethylenimine nanostructures in A431 cells." BioImpacts 1(1): 23-30.

74. Kay, M. A. (2011). "State-of-the-art gene-based therapies: the road ahead." Nature Reviews Genetics 12: 316.

75. Khandare, J. J., P. Chandna, Y. Wang, V. P. Pozharov and T. Minko (2006). "Novel Polymeric Prodrug with Multivalent Components for Cancer Therapy." Journal of Pharmacology and Experimental Therapeutics 317(3): 929-937.

76. Kobyliak, N., T. Falalyeyeva, O. Kuryk, T. Beregova, P. Bodnar, N. Zholobak, A. Shcherbakov, R. Bubnov and M. Ya Spivak (2015). Antioxidative effects of cerium dioxide nanoparticles ameliorate age-related male infertility: Optimistic results in rats and the review of clinical clues for integrative concept of men health and fertility.

77. Koger, L. M. (1978). "Calcium chloride castration." Modern veterinary practice 59(2): 119-121.

78. Kumar, M. N. R. (2000). "A review of chitin and chitosan applications." Reactive and functional polymers 46(1): 1-27.

79. Kustritz, R. (2012). "Effects of surgical sterilization on canine and feline health and on society." Reproduction in domestic animals 47(s4): 214-222.

80. Kutzler, M. and A. Wood (2006). "Non-surgical methods of contraception and sterilization." Theriogenology 66(3): 514-525.

81. Kwak, B. K. and S.-H. Lee (2013). "Intratesticular Injection of Hypertonic Saline : Non-Invasive Alternative Method for Animal Castration Model." Development & Reproduction 17(4): 435-440.

82. Lavertu, M., Z. Xia, A. N. Serreqi, M. Berrada, A. Rodrigues, D. Wang, M. D. Buschmann and A. Gupta (2003). "A validated 1H NMR method for the determination of the degree of deacetylation of chitosan." Journal of Pharmaceutical and Biomedical Analysis 32(6): 1149-1158.

83. Lee, M., J. W. Nah, Y. Kwon, J. J. Koh, K. S. Ko and S. W. Kim (2001). "Water-soluble and low molecular weight chitosan-based plasmid DNA delivery." Pharm Res 18(4): 427-431.

84. Leroueil, P. R., S. A. Berry, K. Duthie, G. Han, V. M. Rotello, D. Q. McNerny, J. R. Baker, Jr., B. G. Orr and M. M. Holl (2008). "Wide varieties of cationic nanoparticles induce defects in supported lipid bilayers." Nano Lett 8(2): 420-424.

85. Levy, J. K., P. C. Crawford, L. D. Appel and E. L. Clifford (2008). "Comparison of intratesticular injection of zinc gluconate versus surgical castration to sterilize male dogs." Am J Vet Res 69(1): 140-143.

86. Li, M. W., W. Xia, D. D. Mruk, C. Q. Wang, H. H. Yan, M. K. Siu, W. Y. Lui, W. M. Lee and C. Y. Cheng (2006). "Tumor necrosis factor {alpha} reversibly disrupts the blood-testis barrier and impairs Sertoli-germ cell adhesion in the seminiferous epithelium of adult rat testes." J Endocrinol 190(2): 313-329.

87. Limonta, P., M. M. Marelli, S. Mai, M. Motta, L. Martini and R. M. Moretti (2012). "GnRH Receptors in Cancer: From Cell Biology to Novel Targeted Therapeutic Strategies." Endocrine Reviews 33(5): 784-811.

88. Limonta, P., M. Montagnani Marelli, S. Mai, M. Motta, L. Martini and R. M. Moretti (2012). "GnRH receptors in cancer: from cell biology to novel targeted therapeutic strategies." Endocr Rev 33(5): 784-811.

89. Lin, Y.-C., M. Boone, L. Meuris, I. Lemmens, N. Van Roy, A. Soete, J. Reumers, M. Moisse, S. Plaisance, R. Drmanac, J. Chen, F. Speleman, D. Lambrechts, Y. Van de Peer, J. Tavernier and N. Callewaert (2014). "Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations." Nature Communications 5: 4767.

90. Love, C. C., M. C. Garcia, F. R. Riera and R. M. Kenney (1991). "Evaluation of measures taken by ultrasonography and caliper to estimate testicular volume and predict daily sperm output in the stallion." J Reprod Fertil Suppl 44: 99-105.

91. Lymberopoulos, A. G., V. P. Kotsaki-Kovatsi, A. Taylor, N. Papaioannou and P. Brikas (2000). "Effects of cadmium chloride administration on the macroscopic and microscopic characteristics of ejaculates from chios ram-lambs." Theriogenology 54(7): 1145-1157.

92. Lysiak, J. J. (2004). "The role of tumor necrosis factor-alpha and interleukin-1 in the mammalian testis and their involvement in testicular torsion and autoimmune orchitis." Reprod Biol Endocrinol 2: 9.

93. MacLaughlin, F. C., R. J. Mumper, J. Wang, J. M. Tagliaferri, I. Gill, M. Hinchcliffe and A. P. Rolland (1998). "Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery." Journal of Controlled Release 56(1): 259-272.

94. Marshall, E. (2002). "Clinical research. Gene therapy a suspect in leukemia-like disease." Science 298(5591): 34-35.

95. McCloy, R. A., S. Rogers, C. E. Caldon, T. Lorca, A. Castro and A. Burgess (2014). "Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events." Cell Cycle 13(9): 1400-1412.

96. Mealy, K., B. Robinson, C. F. Millette, J. Majzoub and D. W. Wilmore (1990). "The testicular effects of tumor necrosis factor." Ann Surg 211(4): 470-475.

97. Mikuz, G. (1985). "Testicular torsion: simple grading for histological evaluation of tissue damage." Appl Pathol 3(3): 134-139.

98. Millar, R. P. (2005). "GnRHs and GnRH receptors." Anim Reprod Sci 88(1-2): 5-28.

99. Millar, R. P. (2005). "GnRHs and GnRH receptors." Animal Reproduction Science 88(1): 5-28.

100. Moreno, R. D., C. Lizama, N. Urzua, S. P. Vergara and J. G. Reyes (2006). "Caspase activation throughout the first wave of spermatogenesis in the rat." Cell Tissue Res 325(3): 533-540.

101. Moretti, R. M., M. M. Marelli, D. M. Taylor, P. G. V. Martini, M. Marzagalli and P. Limonta (2014). "Gonadotropin-Releasing Hormone Agonists Sensitize, and Resensitize, Prostate Cancer Cells to Docetaxel in a p53-Dependent Manner." PLoS One 9(4).

102. Morgan, K., A. J. Stewart, N. Miller, P. Mullen, M. Muir, M. Dodds, F. Medda, D. Harrison, S. Langdon and R. P. Millar (2008). "Gonadotropin-Releasing Hormone Receptor Levels and Cell Context Affect Tumor Cell Responses to Agonist In vitro and In vivo." Cancer Research 68(15): 6331-6340.

103. Morille, M., C. Passirani, A. Vonarbourg, A. Clavreul and J.-P. Benoit (2008). "Progress in developing cationic vectors for non-viral systemic gene therapy against cancer." Biomaterials 29(24): 3477-3496.

104. Morille, M., C. Passirani, A. Vonarbourg, A. Clavreul and J. P. Benoit (2008). "Progress in developing cationic vectors for non-viral systemic gene therapy against cancer." Biomaterials 29(24-25): 3477-3496.

105. Muehlenbein, M. P. and D. P. Watts (2010). "The costs of dominance: testosterone, cortisol and intestinal parasites in wild male chimpanzees." BioPsychoSocial Medicine 4(1): 21.

106. Muruve, D. A., M. J. Barnes, I. E. Stillman and T. A. Libermann (1999). "Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury in vivo." Hum Gene Ther 10(6): 965-976.

107. Nagano, M., M. R. Avarbock, E. B. Leonida, C. J. Brinster and R. L. Brinster (1998). "Culture of mouse spermatogonial stem cells." Tissue & cell 30(4): 389-397.

108. Nagano, M., T. Shinohara, M. R. Avarbock and R. L. Brinster (2000). "Retrovirus-mediated gene delivery into male germ line stem cells." FEBS letters 475(1): 7-10.

109. Neto, O. A., B. G. Gasperin, M. T. Rovani, G. F. Ilha, J. E. Nobrega, Jr., R. G. Mondadori, P. B. Goncalves and A. Q. Antoniazzi (2014). "Intratesticular hypertonic sodium chloride solution treatment as a method of chemical castration in cattle." Theriogenology 82(7): 1007-1011.e1001.

110. Nistal, M., R. Paniagua and A. Queizan (1985). "Histologic lesions in undescended ectopic obstructed testes." Fertil Steril 43(3): 455-462.

111. Oliveira, E. C. S., A. K. F. Fagundes, C. C. S. Melo, L. T. B. Nery, R. G. Rêvoredo, T. F. G. Andrade, K. Oliveira-Esquerre, J. P. Kastelic and V. A. Silva (2013). "Intratesticular injection of a zinc-based solution for contraception of domestic cats: A randomized clinical trial of efficacy and safety." The Veterinary Journal 197(2): 307-310.

112. Oliveira, E. C. S., M. R. Moura, V. A. Silva, C. A. Peixoto, K. L. A. Saraiva, M. J. C. de Sá, R. H. Douglas and A. de Pinho Marques (2007). "Intratesticular injection of a zinc-based solution as a contraceptive for dogs." Theriogenology 68(2): 137-145.

113. Ono, M. and V. R. Harley (2012). "Disorders of sex development: new genes, new concepts." Nature Reviews Endocrinology 9: 79.

114. Ono, M. and V. R. Harley (2013). "Disorders of sex development: new genes, new concepts." Nat Rev Endocrinol 9(2): 79-91.

115. Opalka, B., A. Dickopp and H. C. Kirch (2002). "Apoptotic genes in cancer therapy." Cells Tissues Organs 172(2): 126-132.

116. Pack, D. W., A. S. Hoffman, S. Pun and P. S. Stayton (2005). "Design and development of polymers for gene delivery." Nature Reviews Drug Discovery 4: 581.

117. Paranzini, C. S., A. K. Sousa, G. S. Cardoso, F. M. Perencin, L. G. C. Trautwein, A. P. F. R. L. Bracarense and M. I. M. Martins (2018). "Effects of chemical castration using 20% CaCl2 with 0.5% DMSO in tomcats: Evaluation of inflammatory reaction by infrared thermography and effectiveness of treatment." Theriogenology 106: 253-258.

118. Park, T. G., J. H. Jeong and S. W. Kim (2006). "Current status of polymeric gene delivery systems." Adv Drug Deliv Rev 58(4): 467-486.

119. Peng, Y. S., P. L. Lai, S. Peng, H. C. Wu, S. Yu, T. Y. Tseng, L. F. Wang and I. M. Chu (2014). "Glial cell line-derived neurotrophic factor gene delivery via a polyethylene imine grafted chitosan carrier." International Journal of Nanomedicine 9(1): 3163-3174.

120. Peng, Y. S., P. L. Lai, S. Peng, H. C. Wu, S. Yu, T. Y. Tseng, L. F. Wang and I. M. Chu (2014). "Glial cell line-derived neurotrophic factor gene delivery via a polyethylene imine grafted chitosan carrier." Int J Nanomedicine 9: 3163-3174.

121. Pineda, M. H. and D. I. Hepler (1981). "Chemical vasectomy in dogs. Long-term study." Theriogenology 16(1): 1-11.

122. Potapova, T. A., S. Sivakumar, J. N. Flynn, R. Li and G. J. Gorbsky (2011). "Mitotic progression becomes irreversible in prometaphase and collapses when Wee1 and Cdc25 are inhibited." Mol Biol Cell 22(8): 1191-1206.

123. Prabaharan, M. and J. F. Mano (2005). "Chitosan-based particles as controlled drug delivery systems." Drug Deliv 12(1): 41-57.

124. Prabaharan, M. and J. F. Mano (2005). "Chitosan-based particles as controlled drug delivery systems." Drug Delivery: Journal of Delivery and Targeting of Therapeutic Agents 12(1): 41-57.

125. Pucci, B., M. Kasten and A. Giordano (2000). "Cell cycle and apoptosis." Neoplasia 2(4): 291-299.

126. Raina, A., S. Kumar, R. Shrivastava and A. Mitra (2015). "Testis mediated gene transfer: in vitro transfection in goat testis by electroporation." Gene 554(1): 96-100.

127. Ramamoorth, M. and A. Narvekar (2015). "Non Viral Vectors in Gene Therapy- An Overview." Journal of Clinical and Diagnostic Research : JCDR 9(1): GE01-GE06.

128. Richardson, S. W., H. J. Kolbe and R. Duncan (1999). "Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA." International journal of pharmaceutics 178(2): 231-243.

129. Richburg, J. H. and A. Nanez (2003). "Fas- or FasL-deficient mice display an increased sensitivity to nitrobenzene-induced testicular germ cell apoptosis." Toxicol Lett 139(1): 1-10.

130. Rinaudo, M. (2006). "Chitin and chitosan: Properties and applications." Progress in Polymer Science 31(7): 603-632.

131. Riss, T. L., R. A. Moravec, A. L. Niles, S. Duellman, H. A. Benink, T. J. Worzella and L. Minor (2004). Cell Viability Assays. Assay Guidance Manual. G. S. Sittampalam, N. P. Coussens, K. Brimacombe et al. Bethesda (MD), Eli Lilly & Company and the National Center for Advancing Translational Sciences.

132. Rose, R. M., J. W. Holaday and I. S. Bernstein (1971). "Plasma Testosterone, Dominance Rank and Aggressive Behaviour in Male Rhesus Monkeys." Nature 231: 366.

133. Roy, K., H.-Q. Mao, S.-K. Huang and K. W. Leong (1999). "Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy." Nature medicine 5(4): 387-391.

134. Rucker, E. B., 3rd, P. Dierisseau, K. U. Wagner, L. Garrett, A. Wynshaw-Boris, J. A. Flaws and L. Hennighausen (2000). "Bcl-x and Bax regulate mouse primordial germ cell survival and apoptosis during embryogenesis." Mol Endocrinol 14(7): 1038-1052.

135. Russell, L. D., H. Chiarini-Garcia, S. J. Korsmeyer and C. M. Knudson (2002). "Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis." Biol Reprod 66(4): 950-958.

136. Saad, M., O. B. Garbuzenko, E. Ber, P. Chandna, J. J. Khandare, V. P. Pozharov and T. Minko (2008). "Receptor targeted polymers, dendrimers, liposomes: Which nanocarrier is the most efficient for tumor-specific treatment and imaging?" Journal of Controlled Release 130(2): 107-114.

137. Saffari, M., H. R. Moghimi and C. R. Dass (2016). "Barriers to liposomal gene delivery: from application site to the target." Iranian journal of pharmaceutical research: IJPR 15(Suppl): 3.

138. Sağnak, A. L., H. Ersoy, B. Burgu and R. Haziroglu (2007). Histopathological changes and the effects of prednisolone treatment in contralateral testis in rats subjected to unilateral testicular torsion.

139. Saleh, S. and P. Pecina (2014). CUNI at the ShARe/CLEF eHealth Evaluation Lab 2014. CEUR Workshop Proceedings.

140. Sapolsky, R. M. (1991). "Testicular function, social rank and personality among wild baboons." Psychoneuroendocrinology 16(4): 281-293.

141. Saranya, N., A. Moorthi, S. Saravanan, M. P. Devi and N. Selvamurugan (2011). "Chitosan and its derivatives for gene delivery." Int J Biol Macromol 48(2): 234-238.

142. Saranya, N., A. Moorthi, S. Saravanan, M. P. Devi and N. Selvamurugan (2011). "Chitosan and its derivatives for gene delivery." International Journal of Biological Macromolecules 48(2): 234-238.

143. Sato, M. (2006). "Direct gene delivery to murine testis as a possible means of transfection of mature sperm and epithelial cells lining epididymal ducts." Reproductive Medicine and Biology 5(1): 1-7.

144. Shaha, C., R. Tripathi and D. P. Mishra (2010). "Male germ cell apoptosis: regulation and biology." Philos Trans R Soc Lond B Biol Sci 365(1546): 1501-1515.

145. Somia, N. and I. M. Verma (2000). "Gene therapy: trials and tribulations." Nat Rev Genet 1(2): 91-99.

146. Soto, F. R., W. G. Viana, G. C. Mucciolo, F. Y. Hosomi, C. I. Vannucchi, C. P. Mazzei, A. R. Eyherabide, C. de Fatima Lucio, R. A. Dias and S. S. de Azevedo (2009). "Evaluation of efficacy and safety of zinc gluconate associated with dimethyl sulphoxide for sexually mature canine males chemical neutering." Reprod Domest Anim 44(6): 927-931.

147. Tajik, P., K. H. Pajooh, Z. F. Elahi, G. J. Shahedin and H. Ghasemzadeh-Nava (2017). "Transfection of bovine spermatogonial stem cells in vitro." Iranian journal of veterinary research 18(2): 113.

148. Takehashi, M., M. Kanatsu-Shinohara, K. Inoue, N. Ogonuki, H. Miki, S. Toyokuni, A. Ogura and T. Shinohara (2007). "Adenovirus-mediated gene delivery into mouse spermatogonial stem cells." Proceedings of the National Academy of Sciences 104(8): 2596-2601.

149. Tasker, L. (2007). "Non-surgical methods for controlling the reproduction of dogs and cats." 3-12.

150. Teankum, K., P. Tummaruk, S. Kesdangsakonwut, T. Antarasena, S. Lacharoj, J. Singlor, A. Kunavongkrit and R. Thanawongnuwech (2013). Testicular Atrophy and Its Related Changes in Culled Boars: A Pathological Investigation.

151. Tepsumethanon, V., H. Wilde and T. Hemachudha (2005). "Intratesticular injection of a balanced zinc solution for permanent sterilization of dogs." J Med Assoc Thai 88(5): 686-689.

152. Theas, M., C. Rival, S. J. Dietrich, V. Guazzone and L. Lustig (2006). "Death receptor and mitochondrial pathways are involved in germ cell apoptosis in an experimental model of autoimmune orchitis." Human Reproduction 21(7): 1734-1742.

153. Thomas, P. and T. G. Smart (2005). "HEK293 cell line: A vehicle for the expression of recombinant proteins." Journal of Pharmacological and Toxicological Methods 51(3): 187-200.

154. Toh, E. K.-W., H.-Y. Chen, Y.-L. Lo, S.-J. Huang and L.-F. Wang (2011). "Succinated chitosan as a gene carrier for improved chitosan solubility and gene transfection." Nanomedicine: Nanotechnology, Biology and Medicine 7(2): 174-183.

155. Uram, Ł., M. Szuster, K. Gargasz, A. Filipowicz, E. Wałajtys-Rode and S. Wołowiec (2013). "In vitro cytotoxicity of the ternary PAMAM G3-pyridoxal-biotin bioconjugate." International Journal of Nanomedicine 8: 4707-4720.

156. Werfel, T. and C. Duvall (2016). Polymeric nanoparticles for gene delivery. Polymers and Nanomaterials for Gene Therapy, Elsevier: 147-188.

157. Wickings, E. J. and A. F. Dixson (1992). "Testicular function, secondary sexual development, and social status in male mandrills (Mandrillus sphinx)." Physiology & Behavior 52(5): 909-916.

158. Wolins, N., J. Lozier, T. L. Eggerman, E. Jones, E. Aguilar-Cordova and J. G. Vostal (2003). "Intravenous administration of replication-incompetent adenovirus to rhesus monkeys induces thrombocytopenia by increasing in vivo platelet clearance." Br J Haematol 123(5): 903-905.

159. Xia, F., X. Xu, H. Zhai, Y. Meng, H. Zhang, S. Du, H. Xu, H. Wu and Y. Lu (2013). "Castration-induced testosterone deficiency increases fasting glucose associated with hepatic and extra-hepatic insulin resistance in adult male rats." Reproductive Biology and Endocrinology : RB&E 11: 106-106.

160. Xiangyang, X., L. Ling, Z. Jianping, L. Shiyue, Y. Jie, Y. Xiaojin and R. Jinsheng (2007). "Preparation and characterization of N-succinyl-N′-octyl chitosan micelles as doxorubicin carriers for effective anti-tumor activity." Colloids and Surfaces B: Biointerfaces 55(2): 222-228.

161. Xu, P., G. Bajaj, T. Shugg, W. G. Van Alstine and Y. Yeo (2010). "Zwitterionic Chitosan Derivatives for pH-Sensitive Stealth Coating." Biomacromolecules 11(9): 2352-2358.

162. Zeng, J., X. Wang and S. Wang (2007). "Self-assembled ternary complexes of plasmid DNA, low molecular weight polyethylenimine and targeting peptide for nonviral gene delivery into neurons." Biomaterials 28(7): 1443-1451.

163. Zheng, S., T. T. Turner and J. J. Lysiak (2006). "Caspase 2 activity contributes to the initial wave of germ cell apoptosis during the first round of spermatogenesis." Biol Reprod 74(6): 1026-1033.

Curriculum Vitae

1. Assistant Professor Dr. Suppawiwat Ponglowhapan

Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University

1997	DVM (Honor)	Faculty of Veterinary Science, Chulalongkorn University
2002	M.Sc. (Theriogenology)	Faculty of Veterinary Science, Chulalongkorn University
2003	M.Sc. (Animal Reproduction)	Swedish University of Agricultural Science, Sweden
2009	Ph.D. (Animal Reproduction)	Royal Veterinary College, University of London, UK

2. Dr. Teerapong Yata

Unit of Biochemistry, Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University

2007	B.Sc (Honor)	Faculty of Science, Chiang Mai University
2010	M.Sc. (Molecular medicine)	Imperial College, UK
2014	Ph.D. (Clinical Medicine Research)	Imperial College, UK