EFFECTS OF LYCOPENE ON THE OUTCOMES OF ASSISTED REPRODUCTIVE TECHNOLOGY

PATCHARADA AMATYAKUL

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
For the Doctor of Philosophy Degree in Medical Science
November 2017

Thesis entitled "Effects of Lycopene on the Outcome of Assisted Reproductive Technology" by Patcharada Amatyakul has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Medical Science of Naresuan University

Oral Defense Committee

..... Chair

(Assistant Professor Chainarong Tocharus, Ph.D.)

..... Advisor

(Assistant Professor Hathairat Kruevaisayawan, Ph.D.)

..... Co-Advisor

(Assistant Professor Onrawee Khongsombat, Ph.D.)

..... Co-Advisor

(Apinun Limmongkon, Ph.D.)

..... Internal Examiner

(Phrutthinun Surit, Ph.D.)

Approved

.....

(Associate Professor Paisarn Muneesawang, Ph.D.)

Dean of the Graduate School

Title	EFFECTS OF LYCOPENE ON THE OUTCOMES OF ASSISTED REPRODUCTIVE TECHNOLOGY
Author	Patcharada Amatyakul
Advisor	Assistant Professor Hathairat Kruevaisayawan, Ph.D.
Co-Advisor	Assistant Professor Onrawee Khongsombat, Ph.D. Apinun Limmongkon, Ph.D.
Academic Paper	Thesis Ph.D. in Medical Science, Naresuan University, 2017
Keywords	Lycopene, antioxidative effects, oxidative stress, *in vitro* fertilization, embryo developmental rate

ABSTRACT

Oxidative stress and reactive oxygen species have detrimental effects in sperm function and embryo development which can cause infertility. During *in vitro* fertilization process, ROS can be generated and success rate of the treatment might be decreased. Lycopene, a natural carotenoid is a very potent antioxidant. This study evaluated antioxidative effects of lycopene on sperm motility and apoptosis and oocyte in IVF culture system. In normal and oxidative stress-induced condition, sperm motility was examined after 4-hr incubation with lycopene at concentrations of 5, 10, 25 μM. Sperm apoptosis was assessed by flow cytometer and malondialdehyde levels were measured by HPLC. The results showed that lycopene had no protective effect on sperm motility. Late sperm apoptosis slightly decreased in the lycopene-treated groups but it was not statistically significant. The percentage of cleavage rate and good grade embryos when using lycopene-treated sperm to fertilize with oocytes were not statistically different. To study the effect of lycopene on oocytes and the fertilization outcomes, oocytes were incubated with lycopene 5, 10, 25 μM before insemination. The cleavage rate and embryo grading of lycopene-preincubated oocytes had no significant difference among all lycopene-treated groups. The higher concentration of lycopene, the higher degeneration rate of oocytes. The MDA levels of the Lycopene 25 μM- preincubated oocyte was quite high, but it was not statistic significant. In conclusion, lycopene is a strong antioxidant but it may have little antioxidative effects in sperm motility and apoptosis in *in vitro* fertilization system when incubated sperm

with lycopene 5, 10, 25 μM for 4 hours. These effects did not show the correlation in a dose dependent manner. Furthermore, oocytes preincubated with high concentration of lycopene may negatively affect the quality of oocyte and cause oocyte degeneration. This study would suggest that supplementation of lycopene in the *in vitro* fertilization system should be further studied for the most effective dose and incubation time.

LIST OF CONTENTS

LIST OF CONTENTS (CONT.)

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES

Figure **Page**

ABBREVIATIONS

ARE	=	Antioxidant Response Element
ART	=	Assisted Reproductive Technology
cAMP	=	Cyclic Adenosine monophosphate
CAT	=	Catalase
CG	=	Chorionic Gonadotropin
$Cl^{\cdot}$	=	Atomic chlorine
COCs	=	Cumulus-oocyte-complexes
CO_2	=	Carbondioxide
DMSO	=	Dimethyl Sulfoxide
DNA	=	Deoxyribonucleic acid
DNPH	=	Dinitrophenylhydrazine
DTT	=	Dithiothreitol
EDTA	=	Ethylenediaminetetraacetic acid
EGCG	=	Epigallocatechin gallate
FITC	=	Fluorescein isothiocyanate
g	=	Gram
GIFT	=	Gamete intrafallopian tube transfer
GR	=	Glutathione reductase
GPx	=	Glutathione Peroxidase
GSSG	=	Glutathione disulfide
G6PD	=	Glucose-6-phosphate dehydrogenase
hCG	=	Human chorionic gonadotropin
HPLC	=	High-performance liquid chromatography
HRP	=	Horse radish peroxidase
H_2O_2	=	Hydrogen peroxide
ICR	=	Imprinting control region
ICSI	=	Intracytoplasmic sperm injection
IUI	=	Intrauterine insemination
IVF	=	*In Vitro* Fertilization

ABBREVIATIONS (CONT.)

LDL	=	Low density lipoprotein
LH	=	Lutienizing hormone
LPS	=	Lipopolysaccharide
M	=	Million
MDA	=	Malondialdehyde
mg	=	Milligram
ml	=	Milliliter
mM	=	Millimolar
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NaOH	=	Sodium hydroxide
NF-kB	=	Nuclear factor kappa beta
nm	=	Nanometer
$NO^{\cdot}$	=	Nitric oxide
$NO_2^{\cdot}$	=	Nitrogen dioxide
$O^{\cdot}$	=	Singlet Oxygen
$O_2^{\cdot-}$	=	Superoxide anion radical
$OH^{\cdot}$	=	Hydroxyl Radical
OS	=	Oxidative Stress
PI	=	Propidium Iodide
PID	=	Pelvic inflammatory disease
PN	=	Pronuclei
PS	=	Phosphatidylserine
PUFA	=	Polyunsaturated fatty acid
RCS	=	Reactive Chloride Species
RNS	=	Reactive Nitrogen Species
ROS	=	Reactive Oxygen Species
$RO_2^{\cdot}$	=	Peroxyl
SOD	=	Superoxide dismutase
TBA	=	thiobarbituric acid

ABBREVIATIONS (CONT.)

TBARS	=	Thiobarbituric acid reactive substances
TMP	=	Tetramethoxypropane
TOH	=	alpha-tocopherol
TUNEL	=	Terminal deoxynucleotidyl transferase dUTP
WHO	=	World Health Organization
UV	=	Ultraviolet
μg	=	Microgram
μM	=	Micromolar
μL	=	Microliter

CHAPTER I

INTRODUCTION

Rationale and significant of the study

As now generally known, infertility becomes one of the big challenging reproductive problems in both developed and developing countries. Infertility is defined as couples that are unable to conceive a pregnancy after 1 year of regular sexual intercourse (2-3 times per week) and without using any method of contraception (Turek, 2009). The causes of infertility can be divided into 3 major groups which are female factors, male factors and unknown (unexplained) cause. From recent data, most of infertility causes are associated with oxidative stress which is referred to an unbalanced condition between reactive oxygen species (ROS) and antioxidants (Agarwal, et al., 2003, Agarwal, et al., 2005, Lavranos, et al., 2012). ROS or free radicals are formed in aerobic metabolism during the intermediate steps of oxygen reduction in mitochondria. They are superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^·$), and singlet oxygen ($O^·$) (Guerin, et al., 2001). However, these ROS are needed in the low levels in order to maintain normal physiology but at the high levels they can be harmful to cells and tissue causing apoptosis and DNA fragmentation. Therefore, under this condition of the excessive production of ROS, antioxidants known as scavenging molecules are very crucial for conversion of ROS to H_2O to decrease the serious consequence of the oxidative stress.

Several causes of male and female infertility are related to oxidative stress. Assisted Reproductive Technology (ART) may be a treatment of choice in some infertile couples, if oxidative stress has been presented in male/female and the ART setting, the pregnancy rate will be decreased undoubtedly.

In order to achieve better pregnancy rate per ART cycle, ROS production should be incaution and minimized by several techniques in the ART laboratory setting. Therefore, antioxidants become one of the interesting issues for decreasing the oxidative stress in the culture system. However, it is questionable whether IVF outcomes will be improved if ROS are reduced. The results of these antioxidants

supplementation such as vitamin E, vitamin C are still debatable. Other antioxidants found in natural products are also attractive and up to date information about their antioxidative effects remains unclear.

Carotenoids or Vitamin A are commonly known as one of antioxidants because of the conjugated double bonds structure in the molecule which act as antioxidative properties. Carotenoids are associated with the inhibition of peroxide formation and cancer prevention (Stahl and Sies, 2005). Lycopene is a member of carotenoids which is predominantly found in testes. In food products, lycopene is mainly presented in tomato paste and tomato juice. Antioxidative activities of lycopene has been shown to be as twice as higher than that of beta-carotene. Compared to alpha-tocopherol (vitamin E), lycopene contains approximately 100 times more potent antioxidative effects (Roldan-Gutierrez and Dolores, 2007). Therefore, it may have the highest antioxidative property among carotenoids (Breeman and Pajkovic, 2008). According to this information, lycopene may have an antioxidative effect which can improve the sperm and oocyte quality. Therefore, lycopene may be beneficial in ART setting by increased rate of fertilization and embryo development. However, there are not many of evidences demonstrating the effects of lycopene and its antioxidative properties on both male and female reproductive outcomes.

Purposes of the study

A general experiment objectives

This experiment was designed to investigate the effects of lycopene on the outcomes of assisted reproductive technology by using mouse model.

Specific objectives

1. To evaluate the effects of lycopene on sperm motility
2. To study the rate of early and late apoptosis in normal and H_2O_2-induced culture system
3. To study the effect of lycopene on the results of *in vitro* fertilization in lycopene-treated sperm
4. To examine the effects of lycopene on the results of *in vitro* fertilization in lycopene-preincubated oocytes

Scope of the study

ICR male and female mice at 5-10 weeks were used in this study in order to examine the antioxidative effects of lycopene on the outcomes of ART. In female mice, they were stimulated for superovulation and ovulation induction. In male mice, sperm were collected and treated with multiple concentrations of lycopene (5, 10, 25 μM) and evaluated the sperm motility in normal and H_2O_2-induced culture system. The sperm were stained by Fluorescein isothiocyanate conjugate (FITC) Annexin V and propidium iodide (PI) and investigated for the early apoptosis by flow cytometry. The lycopene-treated sperm were used for insemination with oocytes. On the other hand, another experiment was performed by using lycopene-preincubated oocytes to inseminate with untreated sperm. After the insemination, the fertilization and cleavage rate were morphologically evaluated by using embryo grading system. The antioxidant effects were studied by detection of glutathione peroxidase activity and malondialdehyde levels in the culture media.

Conceptual framework

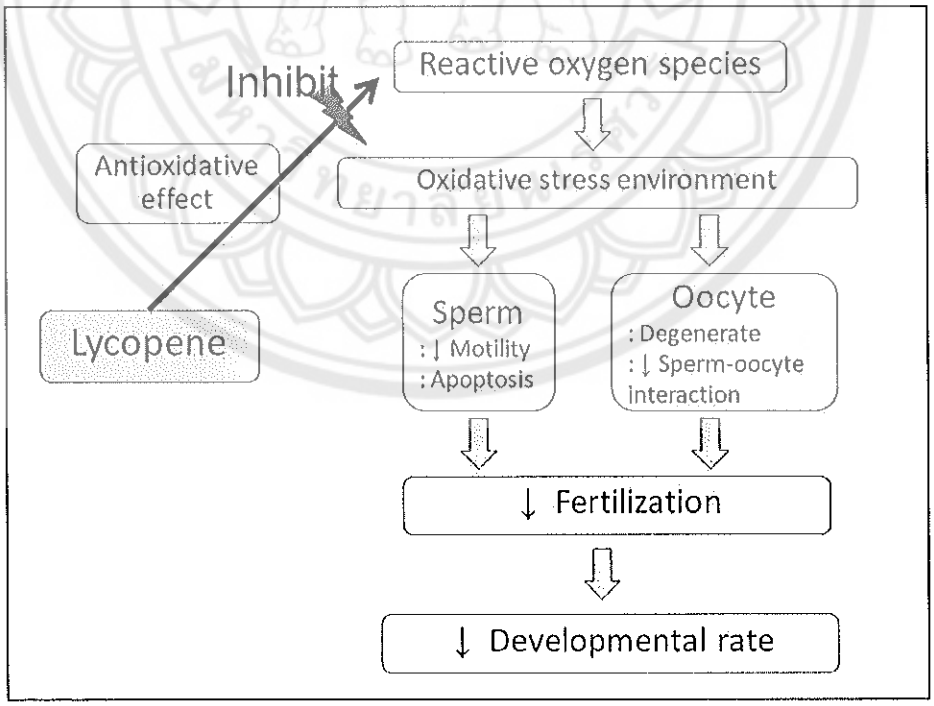

Figure 1 Conceptual research framework

Hypothesis

Lycopene is a natural product that has a lipophilic property with potent antioxidative effect. Supplementation of lycopene in the process of *in vitro* fertilization may prevent lipid peroxidation of sperm which is rich of polyunsaturated fatty acid (PUFA). Adding lycopene in the culture media may improve sperm motility and prevent early and late sperm apoptosis from oxidative stress. Using lycopene-treated sperm and lycopene-preincubated oocytes for *in vitro* fertilization may improve the fertilization and embryo developmental rate.

The outcomes of the study and application

The results from this study will demonstrate the effects of lycopene on sperm motility, apoptosis rate in normal and oxidative stress-induced culture system. The results include the effects of lycopene-treated sperm and lycopene-preincubated oocytes on the embryo development of *in vitro* fertilization. This knowledge may be useful for applying lycopene as an antioxidant in the culture system of ART for infertile couples.

CHAPTER II

LITERATURE REVIEW

Infertility

Infertility is defined as couples that are unable to conceive a pregnancy after 1 year of regular sexual intercourse (2-3 times per week) and without using any method of contraception (Turek, 2009). The incidence of infertility is approximately 15%. The causes of infertility can be divided into 3 major groups:

1. Female factors - Most common causes are ovarian dysfunction or anovulation and pelvic pathologies especially pelvic endometriosis (endometrial gland and stroma located at the ovaries and pelvic peritoneum), tubal obstruction and tumor(s) occupied in the uterine cavity such as leiomyoma and endometrial polyp.

2. Male factors – Common causes of male infertility are anatomical defects of male reproductive organs, abnormal production of androgens and spermatogenesis and abnormal male reproductive physiology, such as erectile dysfunction (Turek, 2009).

3. Unexplained infertility - If the infertile couples have been thoroughly investigated and shown that the semen analysis is normal, there is no tubal obstruction and ovulation can be documented, these couples will be diagnosed as unexplained infertility.

It is very important to identify the causes of infertility because the proper management depends on the causes. Some of infertility problems can be easily treated by ovulation induction with timing sexual intercourse and then following by intrauterine insemination (IUI). If these couples are unable to get pregnant within 3-6 cycles of treatment, the doctor will advise them about the assisted reproductive technology (ART) which is a complicated and expensive treatment. However, some couples with severe male and/or female factors may be appropriate to start the treatment with ART. The indications for ART are severe tubal damage (especially from pelvic inflammatory disease, chlamydial infection), moderate to severe pelvic adhesion from pelvic endometriosis, severe oligozoospermia (fewer than 5 million

motile sperm/ml), severe abnormal sperm morphology, bilateral obstruction of vas deferens (Turek, 2009, Haung and Rosenwaks, 2012). ART is referred to the process that manipulates male and female gamete *in vitro* and after that the gamete/embryo(s) will be transferred into the uterine cavity. ART includes gamete intrafallopian tube transfer (GIFT), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and cryopreservation of sperm and embryo (freezing). Although up to date ART is the most effective treatment for infertility, the success rate is only 30-40% depended on the causes of infertility in individual couple (Krausz, 2011). Factors that highly affect the pregnancy rate from ART are age of female patients, quality of sperm and oocyte, culture system in the ART laboratory, fertilization and embryo developmental rate, and technique of embryo transfer. Therefore, to increase the success rate, the detailed in every steps of ART has to be kept in mind.

Oxidative stress, free radicals and reactive oxygen species

Interestingly, bodies of evidence have shown that both male and female factors of infertility are associated with oxidative stress (OS) defined as excessive production of reactive oxygen species (ROS) compared to antioxidants that can cause potential damage to tissue or environment (Agarwal, et al., 2003, Agarwal, et al., 2005, Lavranos, et al., 2012). OS becomes an interesting topic in the field of health sciences including the reproductive system because OS has been shown to involve in pathophysiology of many diseases and disorders (Burton and Jauniaux, 2011). Free radicals can be defined as species containing one or more unpaired electrons at the shell and they are very highly reactivity and can react to other molecules initiating the chain reaction (Aprioku, 2013).

Free radicals can be divided into 3 major groups:

1. Reactive oxygen species (ROS): superoxide anion radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$) which were shown in Figure 2.

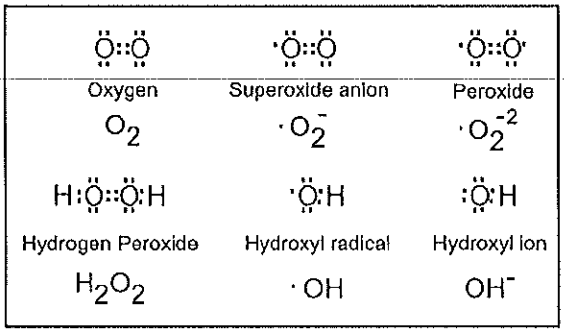

Figure 2 Chemical structures of reactive oxygen species

Source: www.biotek.com

Example: formation of superoxide anion (Aprioku, 2013):

$$O_2 + e^- \xrightarrow{\text{Xanthine}} \text{Hypozanthine} \xrightarrow{\text{Xanthine oxidase}} \text{Uric acid} + O^-_2 + H_2O_2$$

Formation of hydroxyl ions via Fenton reactions:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot-} + OH^-$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\cdot} + H^+$$

1. Reactive nitrogen species (RNS): nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$)

2. Reactive chloride species (RCS): atomic chlorine ($Cl^{\cdot}$)

The chain reaction of free radicals composes of 3 steps as following (Fridovich, 1975, Bus and Gibson, 1982, Cohen and d'Arcy Doherty, 1987, Ansari, 1997):

1. Chain initiation step

Free radicals or one electron intermediates are initially generated because of breaking of a covalent bond in the molecule and one atom becomes an unshared electron pair.

For example: the breakage of a covalent bond will produce 2 oxygen free radicals. This reaction is called "Homolysis".

R ----- O ----- O ----- R → R ----- O· + ·O ----- R

The initiation of free radicals may be occurred by ultraviolet exposure (photolysis), radiation (radiolysis), oxidation-reduction (redox) reaction.

1. Chain propagation step

This step is referred to a process that reactive intermediates are continuously generated during the chemical reaction creating at least a chain carrier. The new free radicals will be generated continuously by

1.1 Transferring of unpaired electron to another atom (atom transfer)

1.2 Transferring of electron from free radicals to the non-radical molecule (electron transfer)

1.3 Addition of radicals to another molecule

2. Chain termination step

At this step, a chain carrier is irreversibly converted into a non-propagating carrier. The termination of chain reaction will be occurred by

2.1 Generation of new molecules that are more stable by 2 molecules of free radicals (homolinking of free radicals)

2.2 Destroying of free radicals by scavengers or antioxidants (Radical scavenging)

2.3 Transferring of unpaired electron from the molecule of free radicals or receiving one electron from another molecule (electron transfer)

This equation is an example of free radicals reaction in lipid peroxidation.

Chain initiation:

L ---- H → L· + radical – H

Chain propagation

L· → L - O - O·

L - O - O· → L - O – O – H + L·

Chain termination

L· + L· → L --- L

Generally, ROS are formed in aerobic metabolism during the intermediate steps of oxygen reduction in mitochondria during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalyzed oxidation. However, these ROS are needed in the low levels in order to maintain normal physiology but at the high levels they can be harmful to cells and tissues causing apoptosis and DNA fragmentation (Guerin, et al., 2001). The rate of ROS formation depends on the numbers of unpaired electrons on the chain, especially under the conditions of hyperoxygenation and also hypoxia (Burton and Jauniaux, 2011).

Roles of ROS in biological processes

ROS play important roles in biological homeostasis and the process of inflammation leading to extensive cell damages and apoptosis by these mechanisms (Droge, 2002, Burton and Jauniaux, 2011)

1. Lipid peroxidation: Hydroxyl radicals directly affect the plasma membrane or any organelle that enriches of polyunsaturated fatty acid (PUFA). The cell membrane will be dysfunction and loss of membrane fluidity which can lead to apoptosis.

2. Activation of protein kinase: Superoxide anion can activate the phosphorylation pathway and the protein kinases resulting in cell death.

3. Activation of redox-sensitive transcription factors: Redox-sensitive transcription factors are p53, NF-kB which will be activated in the process of pro-inflammatory pathway. The activation of these factors will increase the tissue levels pro-inflammatory enzyme (such as cyclo-oxygenase enzyme) which affects the synthesis of prostaglandins, interleukin, tumor necrosis factor and activation of apoptotic process (Hensley, et al., 2000, Cindrova-Davies, et al., 2007).

4. Opening of ion channels: The effect of ROS will impair the regulation of intracellular calcium and cause releasing of calcium ions from the endoplasmic reticulum and mitochondrial dysfunction.

5. Protein modification: ROS may directly oxidize the side chain of amino acids and lead to the formation of carbonyl groups such as aldehydes and ketones. These carbonyl groups can be used as a marker of oxidation by ROS attack.

6. DNA oxidation: Hydroxyl radicals can attack the DNA bases or the deoxyribose sugar leading to DNA strand brake. Mitochondrial DNA is also susceptible to the superoxide radicals and it can cause DNA mutation (Richter, et al., 1988).

Free radicals can be harmful to the cells by inhibition or acceleration of degradation of proteins, lipids and nucleic acid. For example, lipid peroxides from the oxidation of phospholipids and fatty acids can initiate the lipid peroxidation in cellular membranes leading to dysfunction of membrane permeability. Up to date, lots of information support that free radicals are associated with many diseases as shown in Table 1 (Halliwell and Gutteridge, 1984, Valko, et al., 2007).

Table 1 Roles of ROS and free radical in several diseases

Diseases	Related free radicals
Atherosclerosis	Superoxide-mediated endothelial dysfunction
Diabetes Mellitus	ROS accerelated formation of advanced glycation end products
Myocardial infarction	ROS cause ischemic reperfusion injury, myocyte necrosis and induced apoptosis
Cancer	ROS cause gene mutation, post-translational modification leading to cellular damage
Alzheimer's disease	ROS can mediate neurotoxicity
Respiratory distress syndrome	ROS can mediate inflammation and endothelial dysfunction
Aging	ROS induce cell damage and abnormal metabolism
Autoimmune diseases	ROS mediate inflammation and tissue destruction

Source: Adapted from Halliwell B, et al. Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy, 1984.

Therefore, under this condition of the excessive production of ROS, antioxidants may be needed. An antioxidant is any substance that can counteract with free radicals and significantly delays or inhibits oxidation of that substrate. Major properties of antioxidants are preventing antioxidant, scavenging antioxidant and chain breaking antioxidant.

In the human body, antioxidants are divided into two major types (Agarwal, et al., 2005, Ratnam, et al., 2006).

1. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase.

2. Non-enzymatic antioxidants which are known as dietary supplements such as vitamin C, vitamin E, melatonin, zinc, selenium, glutathione, carotenoids, epigallocatechin-3-O-gallate, etc.

Mechanism of antioxidants

1. Enzymatic antioxidants

1.1 Superoxide dismutase (SOD) is an important antioxidant in human tissue. Superoxide molecules can be detoxified and becomes hydrogen peroxide which is less reactive by superoxide dismutase. Hydrogen peroxide will be detoxified to water by catalase and glutathione peroxidase (Burton and Jauniaux, 2011). SOD has three different forms: 1) manganese SOD (Mn-SOD) located in the mitochondria, 2) Copper/Zinc SOD (Cu/Zn-SOD) located in the cytoplasm and 3) extracellular SOD (EC-SOD) located in extracellular fluids, for example, plasma and lymph (Fridovich, 1975). Its antioxidative property can be shown by these reactions (Figure 3).

Cu/Zn-SOD

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Mn-SOD

$$Mn^{2+} + O_2^{\cdot-} \longrightarrow Mn^{3+} + H_2O_2$$

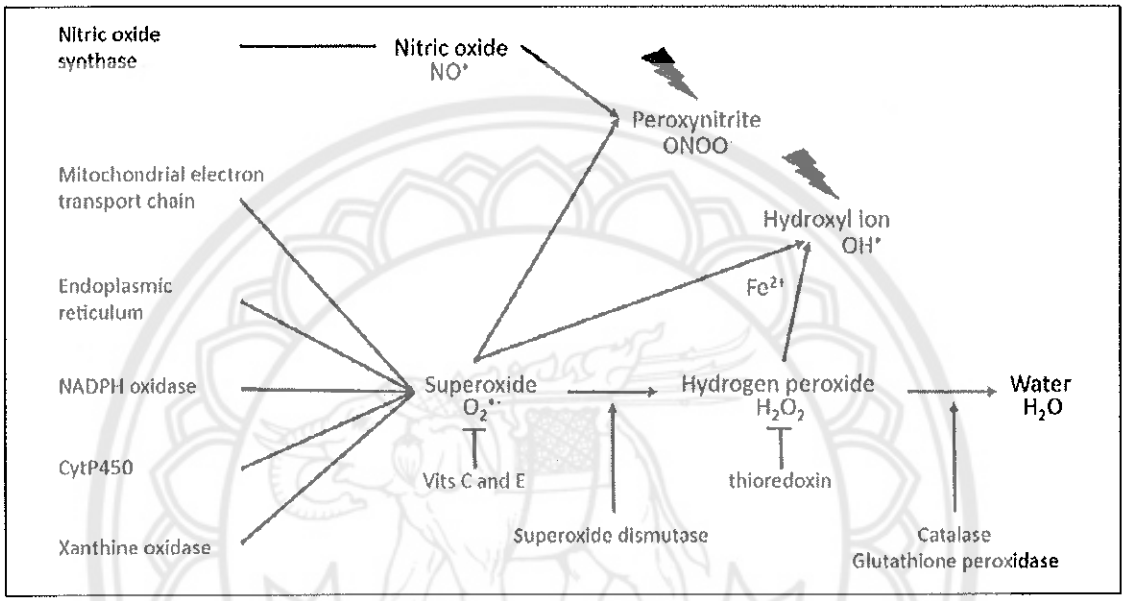

Figure 3 Common ROS and the detoxification pathways

Source: Burton, G. J. and Jauniaux, E.; Oxidative stress, 2011

SOD is in clinical interest because this antioxidative enzyme is associated with therapeutic use in neurological diseases, hypertension and lung injury (Schwedhelm, et al., 2003).

1.2 Catalase (CAT) can be found with the highest activity in the liver, erythrocytes and lungs. CAT converts H_2O_2 to water and oxygen as shown:

Catalase

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

1.3 Glutathione peroxidase (GPx) works together with CAT to prevent excessive production of H_2O_2 (Ratnam, et al., 2006). According to glutathione, it is a water soluble molecule and it can exist in reduced (GSH) and oxidized (GSSG) states. The reduced state can interact with hydroxyl radicals. Glutathione peroxidase is responsible to maintain glutathione in a reduced form (Aprioku, 2013).

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

2. Non-enzymatic antioxidants

2.1 Ascorbic acid (vitamin C): It is a hydrophilic antioxidant which can attack H_2O_2, hydroxyl radical and peroxyl radical. Supplementation of vitamin C has been shown to improve the endothelial function and reduce the risk of cardiovascular disease (Schwedhelm, et al., 2003). Ascorbic acid (AH^-) also has synergistic effect when added with alpha-tocopherol by removing free radical from alpha-tocopherol$^{\cdot}$ ($TO^{\cdot}$) to be alpha-tocopherol (TOH) which can be reused in the reaction again.

$$TO^{\cdot} + AH^- \rightarrow TOH + A^{\cdot}$$

2.2 Alpha-tocopherol (vitamin E) is a lipophilic antioxidant which acts as a radical chain-breaker in the lipid phase (Schwedhelm, et al., 2003). It can reduce lipid peroxidation by reaction with a second lipid-peroxyl radical to form a stable product, reaction with another alpha-tocopherol free radical to form a dimer and regeneration by synergistic effect with ascorbic acid. Supplementation of vitamin E has been shown to improve the endothelial function and can prevent myocardial infarction. The antioxidative effect of alpha-tocopherol can be shown with this equation.

$$\alpha - \text{tocopherol} + L - O - O^{\cdot} \rightarrow \alpha - \text{tocopherol}^{\cdot} + LOOH$$

α – tocopherol$^{\cdot}$ can react to peroxyl radicals and becomes LOO-alpha-tocopheral which is more stable.

The structure of vitamin C and alpha-tocopherol have been shown in Figure 4.

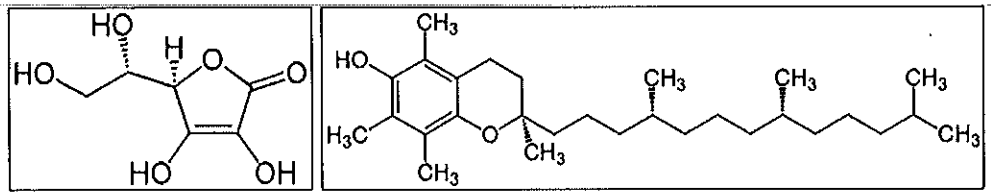

Figure 4 Left – structure of ascorbic acid, right – structure of alpha-tocopherol

Source: http://en.wikipedia.org/wiki/Ascorbic_acid
http://de.wikipedia.org/wiki/Tocopherol

2.3 Carotenoids (Vitamin A) are commonly known as one of antioxidants because of the conjugated double bonds structure in the molecule which acts as antioxidative properties. Carotenoids can inhibit of peroxide formation (Sies and Stahl, 1995, Jackson, et al., 2008). Supplementation of carotenoids may have beneficial effects on cancer prevention and cardiovascular diseases (Schwedhelm, et al., 2003, Stahl and Sies, 2005). They also affect gap junction communication, induced apoptosis of cancer cells, and promote gene activation.

2.4 Flavonoids family contains many phenolic compounds. One of the flavonoids family that is well known for its antioxidant property is catechins found in red wine, black and green tea. Catechins have been reported that it may reduce LDL oxidation and has protective effect on cardiovascular diseases (Schwedhelm, et al., 2003). Other flavonoids are isoflavone, flavones, anthocyanidins, gallocatechin, epigallocatechin gallate (EGCG) which can be mostly found in fruits and vegetables. Flavonoids will attack peroxyl radicals and inhibit the chain propagation step.

2.5 Selenium is hydrophilic and it is a part of active site or a co-factor of antioxidant enzymes such as selenocysteine is a component of glutathione peroxidase (Se-GPx). When this selenoprotein group breaks down, it will have an interaction with hydrogen peroxide. Supplementation of selenium has been shown to reduce the risk of cervical, lung and prostate cancer (Leung, 1998, Schwedhelm, et al., 2003).

2.6 Zinc has been shown to play a role in regulation of extracellular SOD and it may protect against nitric oxide and sulhydryl group and decrease the formation of hydroxyl radical group (Opara and Rockway, 2006).

The examples of antioxidants and their mechanism of action have been shown in Table 2.

Synthetic antioxidants

Most of synthetic antioxidants are adapted from the structure of alpha-tocopherol and polyphenol aimed at improving their chemical properties.

1. Trolox is an alpha-tocopherol derivative and it is water soluble so the action of trolox will be faster compared to alpha-tocopherol. Trolox is commonly used for detection of antioxidative effect.

2. Gallic acid is a derivative of tannin. It has antioxidative effect and can inhibit growth of fungi and virus.

3. Ethylenediaminetetraacetic acid (EDTA) is an important iron chelating agent which can bound to metal ion such as Fe, Zn, Cd and Mn. EDTA is usually added in the culture media for *in vitro* fertilization because of its antioxidative effect.

Table 2 Antioxidants and their mechanism of action

Antioxidants	Mechanism of action
Superoxide dismutase	Dismutation of superoxide to H_2O_2
Catalase	Decomposes of H_2O_2 to molecular oxygen and water
Glutathione	Intracellular reducing agent
Lycopene	Trapping of singlet oxygen
Vitamin C	Scavenging of superoxide anion
Vitamin E	Inhibit lipid peroxidation and direct scavenging of superoxide anion

Table 2 (cont.)

Antioxidants	Mechanism of action
N-acetyl cysteine	Scavenging of H_2O_2 and peroxide
Coenzyme Q10	Inhibit of lipid peroxidation and reduce mitochondrial oxidative stress

Source: Adapted from Halliwell B, et al. Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy, 1984.

Detection methods for evaluation of oxidative stress and ROS

It is difficult to detect oxidative stress, ROS and antioxidative effect in vivo and in vitro because the reaction is very fast and the product of the reaction may be unstable. Therefore, measurement of any antioxidant activity should not be based on a single test (Alam, et al., 2013). In human, the oxidative stress and antioxidant activity can be measured from serum, plasma, urine, tissue, follicular fluid, peritoneal fluid, semen, and seminal fluid. Several tests have been developed to detect particular substances and reactions occurring in the environment of oxidative stress (Devasagayam, et al., 2003, Gil-Villa, et al., 2009, Rhee, et al., 2010, Alam, et al., 2013). In this review, only some commonly used techniques will be mentioned.

1. Detection of lipid peroxidation

It is the most common test used as an indicators for oxidative stress. The principle of the test is that polyunsaturated fatty acid (PUFA) especially at the cell membranes are vulnerable to lipid peroxidation. As shown in the Figure 5, hydroxyl radical will capture a hydrogen moiety from an unsaturated carbon (double bond) to form water. This leaves an unpaired electron on the molecule of fatty acid. After that it will capture oxygen to form a peroxy radical which is unstable and transform to be reactive carbonyl compounds such as malondialdehyde (MDA). Therefore, in the process of peroxidation of lipids, MDA is formed as the end product or free oxygen radicals. MDA reacts with thiobarbituric acid (TBA) under acidic condition to generate a brown-red colored product.

The absorbance is measured at 532 nm against blank without the sample. The levels of lipid peroxide can be expressed as n moles of thiobarbituric acid reactive substances (TBARS)/mg protein or as μmol of MDA equivalent form/mg protein. This method is widely used and many of commercial assay kits are available. Furthermore, MDA levels can be detected by high-performance liquid chromatography (HPLC) technique which is highly sensitive (Nurten, et al., 2006).

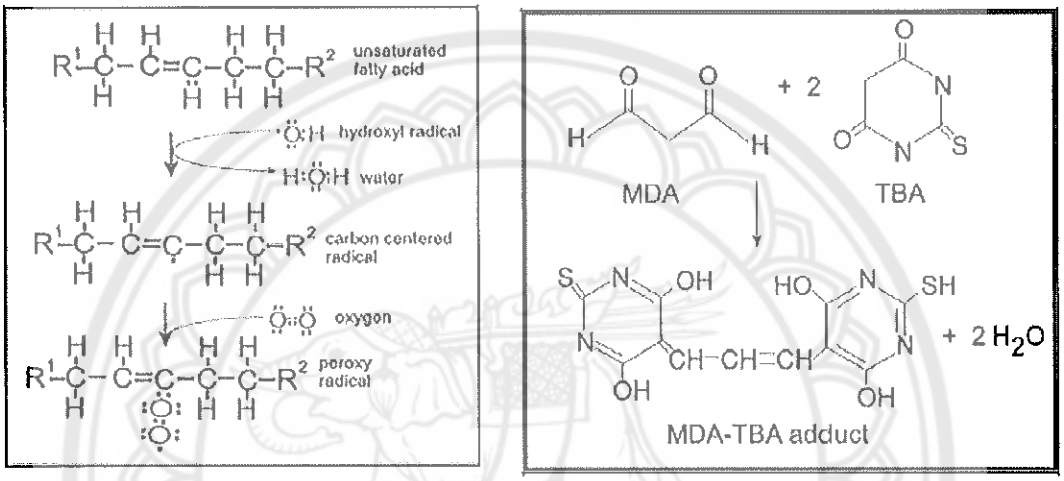

Figure 5 Reaciton of lipid peroxidation and detection of MDA with TBA

Source: www.biotek.com

2. Glutathione peroxidase (GPx) estimation

GPx can be found in many tissues and it catalyzes the reaction of hydroperoxides with reduced glutathione to form glutathione disulfide (GSSG). GPx measurement can determine the oxidative stress condition. The subunit of GPx contains a selenocysteine in the active site which participates directly in the two-electron reduction of peroxide substrate.

GPx activity is indirectly measured by a coupled reaction with glutathione reductase (GR). The reduction of hydrogenperoxide will form oxidized glutathione (GSSG) which is recycled to its reduced state by GR and NADPH (shown in Figure 6) . The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Therefore, the rate of decrease in the A_{340} is directly proportional to the GPx

activity in the sample. The glutathione peroxidase test is also commonly used to detect oxidative stress.

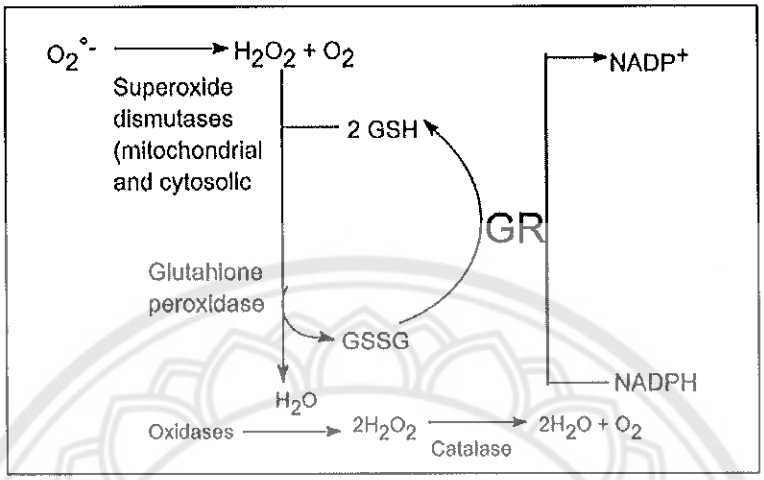

Figure 6 Reaction of glutathione peroxidase

Source: www.biotek.com

3. Superoxide dismutase method

SOD can be detected by several methods such as spectrophotometric method, chemiluminescence, immunochemical method, etc.

The spectrophotometric method is one of the commonly used assays. It is an indirect method based on a superoxide generator and a detector. In the absence of SOD, superoxide radicals react with the detector. If SOD is present, it competes with the detector for superoxide. The common superoxide generator is xanthine-xanthine oxidative system. The commonly used detectors are cytochrome C, nitroblue tetrazolium, pyrogallol, etc.

The chemiluminescent technique to detect SOD also needs a superoxide generator and a detector. The common superoxide generator is xanthine-xanthine oxidative system as well and the luminol-horseradish peroxidase system. The commonly used detectors are lucigenin and luminol. If SOD is present, the inhibition of the luminescent reaction is measured.

4. Catalase (CAT)

Catalase activity is measured by using lysate mixed with phosphate buffer and H_2O_2. The test will measure the absorbance at 240 nm by spectrophotometer. One unit of activity is equal to 1 mmol of H_2O_2 degraded per minute and is expressed as units per milligram of protein.

5. Hydrogen peroxide (H_2O_2) generation assay

This method uses mixture that contains phosphate buffer, and other flurogenic substrates which serve as hydrogen donors in conjunction with horse radish peroxidase (HRP) and then incubate with sample to produce fluorescent products. The commonly used substrates are Amplex red, diacetyldichloro-fluorescein. The reaction is terminated by NaOH and the absorbance is read by spectrophotometer to determine the quantity of H_2O_2. The figure 6 shows the use of Amplex Red for the substrate, after incubation with HRP and H_2O_2, resorufin is the fluorescent product for detection.

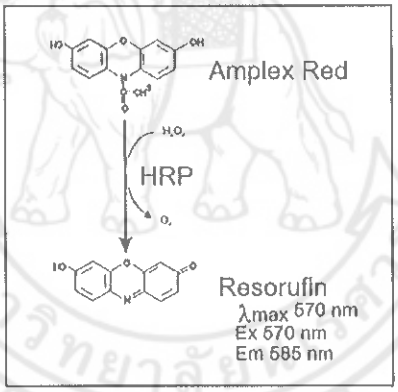

Figure 7 Detection of H_2O_2 production by horse radish peroxidase

Source: www.biotek.com

6. Superoxide measurement by chemiluminescence assay

Superoxide measurement is base on the interaction of superoxide with other compounds to create a measurable substance. Several ROS can be measured by chemiluminescence using luminol or lucigenin as a chemiluminescent substrate. Luminol probe can be used to measure hydrogen peroxide, hydroxyl, superoxide free radicals while lucigenin can be used to measure hydroxyl and superoxide free radicals. After incubation with the sample, the emission of light by that such reaction can be

measured. However, the limitations of the chemiluminescence assay are that it needs high volume of specimen (in case of sperm, it needs high concentration) and iron and copper in the culture media can interfere the result (Mahfouz, et al., 2009).

7. Flow cytometry

The principle of this method is to detect the amount of one or more fluorescent stain, and in oxidative stress it can detect early apoptosis of the cell. The early sign of apoptosis is the translocation of a negative charged phospholipid called phosphatidylserine (PS) which is located at the inner leaflet of the cell membrance to the outer side (externalization). PS highly reacts with annexin V staining. In order to separation with the cell death from other processes, it is suggested to stain with annexin V in the conjunction with propidium iodide (PI) which is a vital dye. The dead cells will stain with both annexin V and PI while the cells with early apoptosis will stain only annexin V in which represents membrane integrity (Hoogendijk, et al., 2009, Hossain, et al., 2011). The stain of annexin V and PI can be evaluated by the technique of flow cytometry. Furthermore, flow cytometry can also be used to detect sperm nuclear DNA fragmentation by staining of acridine orange (Cordelli, et al., 2005). Other methods that can detect sperm DNA fragmentation are comet assay and TUNEL but both of these methods will detect apoptotic change at the late stage (Mahfouz, et al., 2010, Hossain, et al., 2011).

Effects of ROS on reproductive system

Male reproductive system:

In normal physiology, ROS are mainly generated by sperm and leukocytes in the seminal plasma. The sources of ROS in sperm are: (1) nicotinamide adenine dinucleotide phosphate NADPH oxidase system at the plasma membrane, (2) the NADH-dependent oxido-reductase in sperm mitochondria and (3) the aromatic –L-amino acid oxidase (Agarwal, et al., 2006, Aitken, et al., 2014). Furthermore, sperm with abnormal morphology will highly produce ROS in human ejaculation. It has been reported that leukocytes in semen may affect the calcium - dependent signal (Musset, et al., 2012).

Leukocytes can be found in every ejaculation and they are a major source of ROS generation, especially peroxidase-positive leukocytes which are usually

presented in the prostate gland and seminal vesicles. The excessive amount of leukocytes $\geq 1x10^6$ /ml in semen is called "leukocytospermia" by the definition of the World Health Organization (Organization, 1999, WHO, 1999) and leukocytospermia which can be demonstrated in the conditions of male genital tract infection is associated with reduced sperm hyperactivation and poor sperm quality (Lavranos, et al., 2012).

The anatomical structure and biochemical property of the sperm make sperm vulnerable to oxidative stress. The structure of sperm has been shown in Figure 8. At the sperm plasma membrane which enriches of PUFA (such as arachidonic acid and docosahexaenoic acid), it contains more than 2 carbon-carbon double bonds and a methylene group. The covalent bond of the methylene carbon-hydrogen bonds is weak and will be susceptible to abstraction. Therefore, hydroxyl radical can react at this point and the initiation and propagation of lipid peroxidation will be occurred (Agarwal, et al., 2003). Furthermore, spermatozoa have very little cytoplasmic space limited to the midpiece which contains superoxide dismutase and gluthathione peroxidase. During sperm differentiation and maturation if the cytoplasmic remnants are excessive, there will be a defect on sperm function and can generate more oxidative stress (Gomez, et al., 1996, Aitken, et al., 2014).

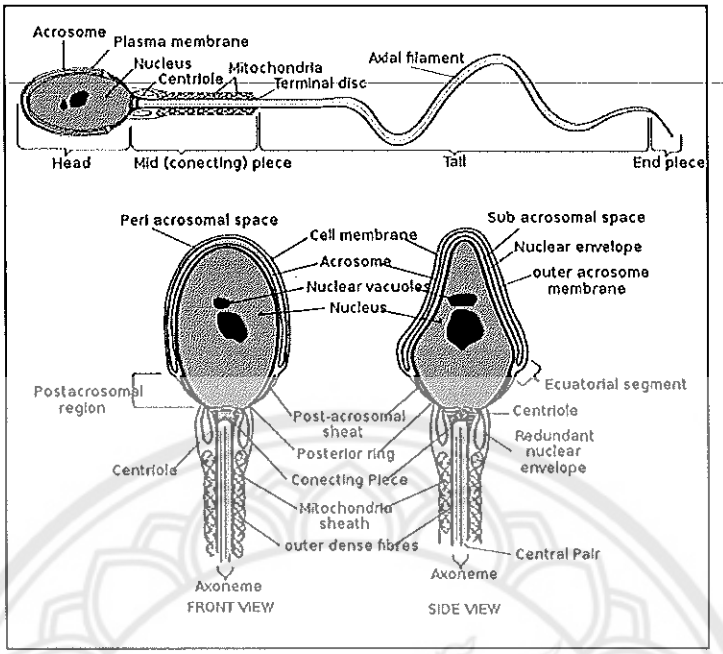

Figure 8 Structure of spermatozoa

Source: http://humanreproduction11.wordpress.com/fertilization/

However, PUFA at sperm membrane and small amount of ROS are important to promote activity of normal physiological enzymes such as plasma membrane ATPase in order to stimulate the fusion of sperm-oocyte interaction during the process of fertilization (Lavranos, et al., 2012, Aprioku, 2013, Aitken, et al., 2014). Another role of PUFA is to control plasma membrane fluidity which has a specific function for acrosome reaction (Lanzafame, et al., 2009). ROS also have a role in sperm capacitation by stimulation of cholesterol transporter from the plasma membrane, regulation of tyrosine phosphorylation and activated cAMP at the sperm tail to induce the sperm hyperactivated motility (Si and Okuno, 1999). Another mechanism is believed that superoxide anion and H_2O_2 can stimulate adenylyl cyclase activity which also has a positive effect on tyrosine phosphorylation (Zhang and Zheng, 1996). Interestingly, if there is no fertilization, these fully capacitated sperm will be involved in apoptotic process, because of the continuation of ROS generation in the sperm environment leading to the leakage of electrons from mitochondria and generation of superoxide anion (Agarwal, et al., 2003, Aitken, et al., 2014). However, the sperm

apoptosis is needed during normal spermatogenesis which will control excess immature and abnormal sperm cells (Aprioku, 2013). The negative effects of oxidative stress on spermatozoa have been shown in Figure 9.

The target of oxidative stress in sperm is not only the PUFA at sperm plasma membrane, but also the DNA in the sperm nucleus. Even though the nuclear DNA of spermatozoa is highly compacted in the sperm head and maybe more resistant to ROS, it has been shown that ROS will induce cascade activity in cytoplasm and mitochondria of the sperm midpiece and then apoptosis is initiated leading to single and double DNA strand breaks and DNA fragmentation in the sperm head (Aitken, et al., 2014). The excessive formation of ROS is also associated with a decrease in axonemal protein phosphorylation and decrease in membrane fluidity which can cause sperm immobilization.

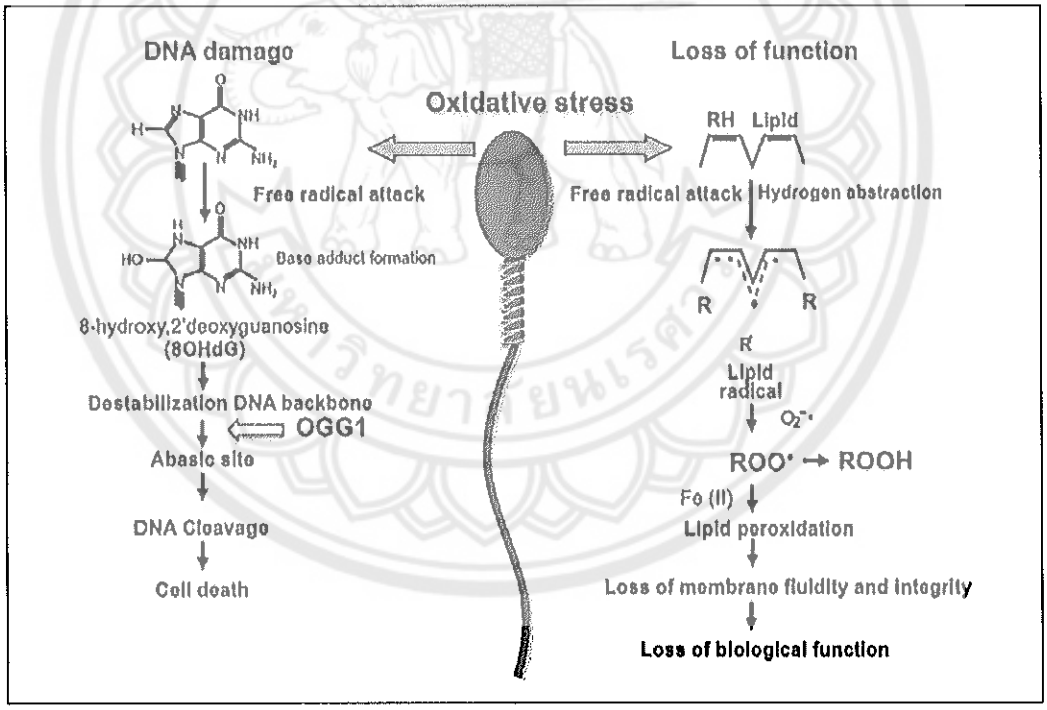

Figure 9 Negative impact of oxidative stress on mammalian spermatozoa

Source: Aitken J, et al. Aspect of sperm physiology – Oxidative stress and the functionality of stallion spermatozoa, 2014

Naturally, the mechanism of sperm protection against excessive oxidative stress is numerous antioxidants that presented in seminal plasma and spermatozoa such as glutathione, glutathione peroxidase, glutathione reductase, SOD, glucose-6-phosphate dehydrogenase (G6PD), ascorbate, alpha-tocopherol, coenzyme Q10 (Lanzafame, et al., 2009). However, many causes of male infertility have been reported to be associated with excessive amount of ROS production and oxidative stress (Aitken and Roman, 2008, Lanzafame, et al., 2009, Abd-Elmoaty, et al., 2010, Lavranos, et al., 2012). Conditions associated with ROS and effects on male reproduction have been shown in Figure 10.

For example:

1. Exposure to excessive heat and cold, radiation

2. Exposure to chemical toxins: chemotherapy, ethylene glycol, dioxin, including both heavy active and passive smoking

3. Medical conditions: diabetes mellitus, chronic infection, male genital tract infection

4. Varicocele which is characterized by dilation of venous plexus in scrotums which can increase testicular temperature.

5. Cryptorchidism or undescended testis and other causes of testicular damage

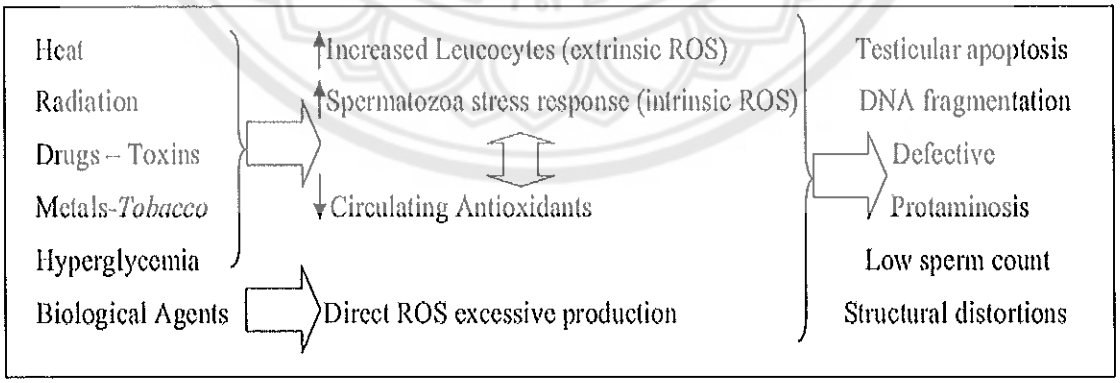

Figure 10 Conditions associated with ROS and effects on male reproduction

Source: Lavranos et al., Investigating ROS sources in male infertility: a common end for numerous pathways, 2012

Female reproductive system:

ROS have been reported to play several roles in normal female reproduction (Agarwal, et al., 2005, Agarwal, et al., 2006). During folliculogenesis and steroidogenesis, ROS may stimulate the proliferation of theca and interstitial cells of the ovary but the high levels of ROS may inhibit this action. According the maturation of oocytes, ROS may have an effect on chromosome segregration and gene expression of the oocyte leading to oocyte maturation arrest (Combelles, et al., 2009). Physiologically, follicular fluid is a house for both ROS and antioxidants for the oocyte which may be similar to seminal plasma and sperm.

For the luteal phase of the ovarian cycle, Cu-Zn SOD found in corpus luteum may have a role to maintain lifespan of corpus luteum for approximately 14 days. If there is no fertilization and implantation, high amount of ROS will induce regression of corpus luteum. The luteolysis will occur and the levels of progesterone and estrogen will be declined causing menstruation or withdrawal bleeding (Taylor, 2001, Agarwal, et al., 2005). Just before the time of menstruation, the endometrial microvasculature has been controlled by nitric oxide that causes vasodilatation. The hypervascularization of the endometrium is important for embryo implantation; therefore, it has been reported that nitric oxide synthetase (NOS) has been detected in the decidual endometrium (Taguchi, et al., 2000, Agarwal, et al., 2005).

High levels of ROS have been demonstrated in several female infertility conditions (Agarwal, et al., 2003, Agarwal, et al., 2005, Jana, et al., 2010). For example:

1. Pelvic endometriosis is a common gynecologic disease that causes painful menstruation and infertility. The definition of endometriosis is the presentation of endometrial tissue and stroma outside the uterine cavity and it is mimic the inflammatory process. This ectopic tissue can induce leukocyte and macrophage activity which is the source of ROS production (Liu, et al., 2001). It has been shown that the peritoneal fluid collected from patient with endometriosis had high levels of lipid peroxidation and nitric oxide. In the case of pelvic endometriosis, the sperm-oocyte interaction, fertilization, abortion and pregnancy rate are significantly decreased compare to normal woman (Agarwal, et al., 2005, Agarwal, et al., 2005, Agarwal, et al., 2006, Combelles, et al., 2009).

2. Polycystic ovarian syndrome that referred to a woman with chronic anovulation, hyperandrogenism and irregular menstruation is associated with infertility and abnormal carbohydrate metabolism. Some of these patients face with diabetes mellitus at young of age. Anovulation occurs because follicles become apoptosis which may be associated with excessive ROS.

3. Hydrosalpinx is defined as a fallopian tube obstruction with fluid collection which is a consequence of pelvic inflammatory disease (PID) and chlamydial infection of female genital tract. There was evidence that fluid from hydrosalpinx contained high levels of lipid peroxidation and total antioxidant capacity which could be impaired blastocyst developmental rate (Bedaiwy, et al., 2002).

4. Detrimental effects of ROS on female infertility are impaired oocyte-sperm interaction, failed fertilization, and impaired embryo development leading to fragmented embryos (Guerin, et al., 2001, Agarwal, et al., 2006, Bedaiwy, et al., 2006, Goud, et al., 2008, Burton and Jauniaux, 2011). Furthermore, ROS also negatively affects embryo implantation causing pregnancy loss or miscarriage (Burton and Jauniaux, 2011).

Oxidative stress during pregnancy

Oxidative stress and ROS also play a dominant role during pregnancy (Burton and Jauniaux, 2011). The oxygen levels in the uterus during the first trimester are low. In this phase of early organogenesis, it has been found that the fetal – placental unit utilizes nonphosphorylated sugar pathway which does not require high oxygen levels. During this period, the antioxidant levels are also low. For the second and third trimester, the accelerated fetal growth and uteroplacental unit need high oxygen levels to support the aerobic metabolism. At this time, antioxidant levels are increased (Dennery, 2010).

During the embryogenesis, elevation of ROS will affect the embryo development by inducing embryonic cytoplasmic fragmentation and apoptosis which can cause miscarriage (Agarwal, et al., 2005, Gil-Villa, et al., 2009).

In many abnormal conditions during pregnancy such as diabetes, intra-uterine growth restriction of the fetus, preterm premature rupture of the membranes, preeclampsia (pregnancy induced hypertension with loss of protein in urine), oxidative

stress and ROS is much higher (Dennery, 2010, Burton and Jauniaux, 2011). Especially the conditions that involve placental vascular function like diabetes, protein C kinase activation and glucose oxidation leading to impaired mitochondrial function and cell death. Hyperglycemic condition also increases mitochondrial ROS production (Rolo, 2006).

Long term fetal exposure to oxidative stress will upregulate the endothelial nitric oxide synthetase enzyme. It has been reported that fetal with growth restriction display high level of lipid peroxidation (Franco, et al., 2007). Exposure of high amount of ROS may also cause abnormal fetal pancreatic function leading to development of diabetes in adult (Dennery, 2010). The effects of ROS on syncytiotrophoblast have been shown in Figure 11.

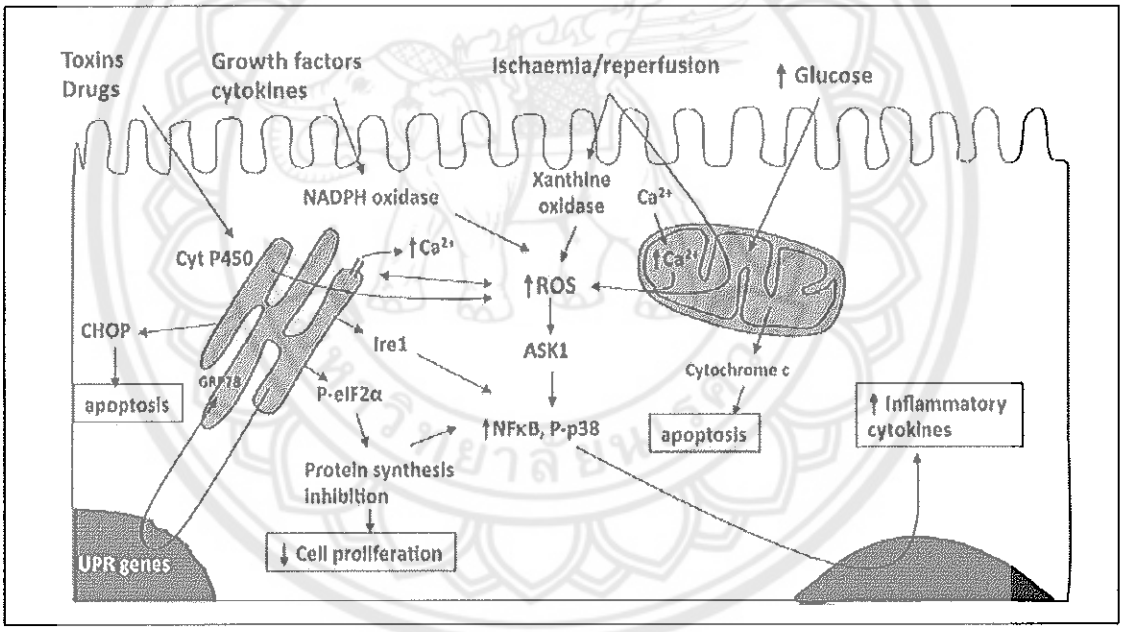

Figure 11 Generation of ROS within the syncytiotrophoblast

Source: Burton, G. J. and Jauniaux, E.; Oxidative stress, 2011

Assisted reproductive technology and ROS

Although ART is an expensive and high technology process, it wildly becomes an acceptable treatment for most infertile couples because the pregnancy rate is the highest, approximately 30-40%, among other methods of treatment (timing

sexual intercourse, follicle stimulation, intrauterine insemination) (Turek, 2009). The ART technique that is most commonly used in infertility centers is *in vitro* fertilization (IVF). The steps of IVF have been shown in Figure 12. In details, the process of IVF will be:

1. Stimulation of multiple follicular growth in female by injection of gonadotropins for approximately 7-10 days

2. Triggering of ovulation when at least one follicle reaches 18 mm by shooting luteinizing hormone (LH) or human chorionic gonadotropin (hCG)

3. Collection of oocytes by transvaginal ultrasound guidance to aspirate follicular fluid

4. Collection of cumulus-oocyte-complexes (COCs) from the follicular fluid under the stereoscope and then culture in the 5% CO_2 incubator until the time of insemination

5. Sperm preparation by gradient centrifugation or swim up method to harvest motile sperm for insemination

6. Insemination between sperm and COCs in the fertilization media and leave them at least for 4-6 hours

7. Removal of oocytes from the inseminated well in order to minimize the exposure of oocytes to ROS from sperm and cumulus cells (denude).

8. Oocyte culture in the cleavage media in the 5% CO_2 incubator until the time to check fertilization and embryo development

9. Transfer embryo(s) into the uterine cavity and supplement the luteal phase with progesterone for 14 days until the time to check pregnancy

Before the process of ART, ROS can be naturally found in infertile patients as previously described. Furthermore, during the ART process, several factors may induce ROS generation, for example (Guerin, et al., 2001, Henkel and Schill, 2003, Agarwal, et al., 2006)

1. Sperm damage during sperm preparation for insemination especially from high speed centrifugation

2. High oxygen concentration in the medium or culture system which enhance oxidative activity

3. Exposure of dead sperm and degenerated cumulus cells: the levels of ROS in the culture system with cumulus-enclosed oocytes have been reported to be significantly higher than that of cumulus-denuded oocytes (Combelles, et al., 2009).

4. Exposure to metalic cations such as Fe and Cu in the culture media

5. Exposure to low or high temperature: all the IVF process should be performed under the condition of 37°C

6. Exposure to visible light that increases H_2O_2 production in the culture environment and embryos

7. Freeze-thaw process (cryopreservation) for both sperm and embryo which reduces glutathione concentration (enzymatic antioxidants)

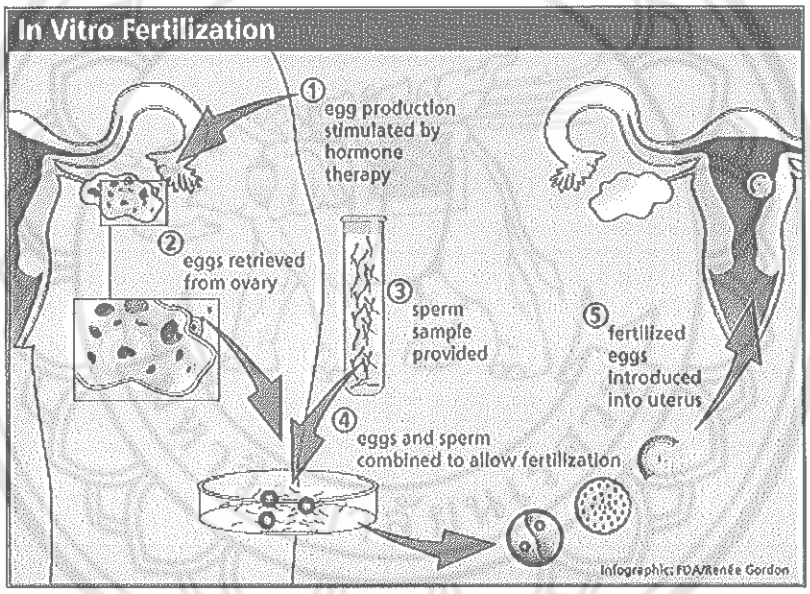

Figure 12 Steps of *in vitro* fertilization

Source: http://humanreproduction11.files.wordpress.com/2011/02/vitro.gif

The excessive production of ROS, both from the natural levels and during the ART process, can cause the negative effects on pregnancy outcome in the couples treated with ART such as lower fertilization and blastocyst developmental rate (Agarwal, et al., 2006). In order to achieve better pregnancy rate per ART cycle, ROS production should be incaution and minimized by several technique in the ART

laboratory setting. Therefore, antioxidants become one of the interesting issues for decreasing the oxidative stress in the culture system.

Antioxidants and reproductive outcomes

Supplementation of antioxidants such as vitamin C, vitamin E, glutathione, in patients and/or in culture media becomes more interesting. In mouse embryos, vitamin C and E supplementation in the culture system was associated with increasing of blastocyst developmental rates (Taylor, 2001, Wang, et al., 2002). It has been shown that oral administration of vitamin C and E to males with high levels of sperm DNA fragmentation was shown to reduce sperm DNA fragmentation and also increase pregnancy rates (Wang, et al., 2002, Gil-Villa, et al., 2009). There was a study showed that supplementation of vitamin C and E in sperm preparation could reduce ROS levels but sperm motility was not improved (Donnelly, et al., 1999). However, the results of these antioxidants are still debatable. Other antioxidants found in natural products are also attractive and up to date information about their antioxidative effects is of interest.

Carotenoids and lycopene

Carotenoids are synthesized by plants and microorganism, but not animals and they are a family of pigmented compounds. Yellow, orange or red colored fruits and vegetables are major sources of carotenoids for human. More than 600 of carotenoids have been demonstrated, but only 20 carotenoids have been identified in human blood and tissue (Rao and Rao, 2007). Carotenoids are lipophilic compounds and usually accumulate in lipophilic compartments such as cell membranes and lipoproteins. Their antioxidative effects are associated with the inhibition of peroxide formation (Stahl and Sies, 2005). They also affect gap junction communication, induced apoptosis of cancer cells, and promote gene activation. The efficacy of carotenoids quenching is associated with the number of conjugated double bonds presented in the molecule. Carotenoids most likely scavenge the singlet molecular oxygen ($^1O_2$), and peroxyl radicals. The energy of $^1O_2$ will be transferred to the carotenoid molecule and as a consequence it becomes ground state oxygen and triplet excited carotene. After this quenching reaction, carotenoids can be reused several fold

in such quenching cycles. Because of their lipophilicity, carotenoids may have a role to protect the cellular membranes and lipoproteins from oxidative damage (Stahl and Sies, 2005, Rao and Rao, 2007).

Even though carotenoids can be found in natural products, the bioavailability of the dietary carotenoids depends on several factors. The absorption of carotenoids may be affected by heating of food product, fat content and type of fat. For example, the bioavailability of lycopene is enhanced by heating process of tomato products and it will be absorbed better if consuming with high-fat diet. After ingestion, carotenoids are absorbed by passive diffusion into the gastrointestinal mucosa in the form of incorporation with micelles and transported via lymphatic system. Most of carotenoids will be stored in the adipose tissue (Rao and Rao, 2007). In terms of carotenoids degradation, carotenoids are quite instable and unsaturated because of they have many double bonds in their structures. Therefore, carotenoids are vulnerable to oxidation and also susceptible to temperature, light and pH (Ratnam, et al., 2006).

Carotenoids can be divided into 2 groups (Stahl and Sies, 2003)

1. Carotenes which are pure hydrocarbons such as beta-carotene, alpha-carotene, and lycopene (carotene and lycopene are the important parts of carotenoids)

2. Xanthophylls which are oxygenated derivatives such as zeaxanthine and lutein

The chemical structure of carotenoids is composed of a 40-carbon basal structure in long conjugated chain of double bonds called "polyisoprenoid" with a near bilateral symmetry around the central double bond (Britton, 1995). Each type of carotenoids has different base structure by cyclization of the end groups which have been shown in figure 13. The conjugated double bonds in carotenoids can undergo isomerization to cis and trans isomers. The trans isomer are commonly found in food and more stable than the cis isomers.

Carotenoids can also be divided into provitamin A and non-provitamin A compounds; for example, beta and alpha-carotene can contribute to vitamin A. Vitamin A is important for promotion of cell growth and gene activation, embryo development and especially visual function (Jackson, et al., 2008).

Beta-carotene is the major carotenoids in human and can be found in liver, adrenal gland, kidney, ovary and fat. It can prevent photooxidative damage, erythrema

formation, premature aging of skin and sunburn for UV light, especially in the combination with alpha-tocopherol. It has been reported that ingestion of tomato paste (approximately 16 mg of lycopene/day) for over 10 weeks is associated with increase serum lycopene and decrease erythrema formation. Beta-carotene also has an protective effect on age-related macular degeneration which is the common cause of irreversible blindness among elderly (Stahl and Sies, 2005). However, it should be noted that the high dose supplementation of beta-carotene may have pro-oxidative effects which increase the production of ROS, and may be associated with the development of lung cancer especially at high oxygen tension (Palozza, et al., 2003, Stahl and Sies, 2003). The mechanism has not been clearly identified yet, but in animal models, it has been shown that high doses of beta-carotene may affect the expression of a retinoic acid receptor subtype and may be a carcinogen.

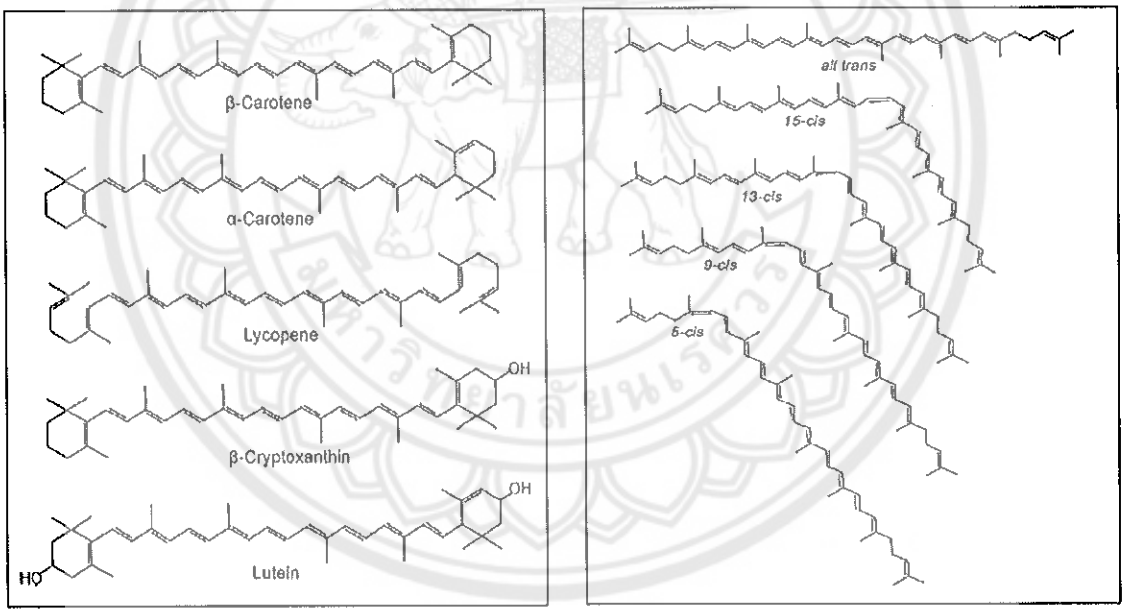

Figure 13 Chemical structures of carotenoids and lycopene

Source: Rao AV, Rao LG. Carotenoids and human health, 2007 and http://en.wikipedia.org/wiki/Lycopene_(data_page)

Most of recent researchers have been pointed to the effects of lycopene on human health and diseases. In the reproductive system, not much of evidence of other carotenoids has been reported.

Lycopene

Lycopene came from a latin word "**Lycopersicum**" which is referred to the tomato species. Like other carotenoids, it is a polyunsaturated hydrocarbon. The chemical structure of lycopene is acyclic and contains 11 conjugated and 2 non-conjugated double bonds. The chemical name of lycopene is 2,6,10,14,19,23,27,31-octamethyl-2,6,8,10,12,14,16,18,20,22,24,26,30-dotriacontatridecaene. The chemical formula is $C_{40}H_{56}$ with the molecular weight of 536.87 Da. It is the only one of carotenoids that contains acyclic end groups; therefore, there is no provitamin A property. In fruits, lycopene is commonly found in tomatoes, watermelon, pink grapefruit and papaya. In food products, lycopene is predominately presented in tomato paste, ketchup and tomato juice (shown in Table 3 and 4). Processing of tomatoes will increase the concentration of bioavailability of lycopene. The lycopene content in tomato paste is four times more available than in the fresh tomatoes (Clinton, 1998). Only 10-30% of dietary lycopene is absorbed after ingestion. In nature, most of lycopene presents primarily in trans-isomeric form. In human, lycopene is the most predominant carotenoids in plasma, prostate gland, adrenal gland and testes. The cis-isomeric form can be occurred by light, thermal energy, oxygen and chemical reactions. The most stable isomer is 5-cis lycopene which has also been shown to have the highest antioxidant properties follow by 9-cis, 7-cis, 13-cis and 11-cis (Rao and Rao, 2007). Lycopene is quite unstable and its half-life is approximately 2-3 days. Synthetic lycopene is also unstable and it should be kept under inert gas and lightproof containers and stored in a cool place to prevent isomerization and oxidation. The half – life of lycopene in organic-aquous solutions such as dimethylsulfoxide (DMSO) may be slightly increased and this property makes lycopene difficult to use in cell culture (Roldán-Gutiérrez and Luque de Castro, 2007).

In case of allergic reaction to lycopene, the patient will present with diarrhea, nausea, stomach pain, vomiting and loss of appetite. Excessive ingestion of lycopene will cause the discolorization of the skin known as lycopenodermia which is non-toxic and be spontaneously resolved. However, the safety of synthetic lycopene has been

studied (McClain and Bausch, 2003). Lycopene has no significant hepatotoxicity when studied in rats with the very high dose of up to 500 mg/kg BW/day for 14 weeks and 1000 mg/kg BW/day for 4 weeks. With this high dose, there is no teratogenic effect in a rat-two generation study. The accumulation of lycopene is presented orange-brown pigment deposits in the hepatocytes and elevated liver enzyme. This effect is reversible and the pigment deposit is no longer present after 13 weeks of depletion. From this study, it can be documented the large margin of safety of lycopene-formulated form (McClain and Bausch, 2003).

Table 3 Lycopene content of common fruits and vegetable

Fruits and vegetables	Lycopene (ug/g wet weight)
Tomatoes	8.8 – 42.0
Pink guava	54.0
Watermelon	23.0 – 72.0
Papaya	20.0 – 53.0
Apricot	< 0.1

Source: Rao AV and Rao LG. Carotenoids and human health, 2007

Table 4 Lycopene content of various food products

Food	Lycopene content (mg/100 g)
Tomatoes, cooked	3.70
Tomato sauce	6.20
Tomato paste	5.40 – 150.00
Tomato soup	7.99

Table 4 (cont.)

Food	Lycopene content (mg/100 g)
Tomato juice	5.00 – 11.60
Sun-dried tomato in oil	46.50

Source: Adapted from Clinton SK. Lycopene: chemistry, biology, and implications for human health and disease, 1998.

Lycopene and antioxidative effects

Lycopene has a hydrophobic property and mostly located within the cell membrane. It is believed that lycopene may react to ROS at the inner core of membrane unless lycopene may have specific protein extending to the membrane surface and interacting the aqueous environment (Clinton, 1998). Therefore, lycopene scavenging properties are more profound in a lipophilic environment.

The mechanism of lycopene on quenching singlet oxygen can be shown by the following equation (Breeman and Pajkovic, 2008).

Lycopene + $^1O_2$ → ^{3}lycopene + $^3O_2$

$^1O_2$ + Lycopene → $^3O_2$ + ^{3}lycopene

^{3}lycopene is a lycopene in the excited state and it has enough energy to cause excitation of other molecules and generate reactive species. After that the regenerated lycopene can be quenched additional singlet oxygen molecules. The quenching capacity of any carotenoid molecule will be depended on the number of conjugated double bonds. By these mechanisms, a single molecule of lycopene can quench thousands of singlet oxygen molecules (Breeman and Pajkovic, 2008).

For example:

Lycopene + $ROO^{\cdot}$ → ROO --- $Lycopene^{\cdot}$

ROO --- $Lycopene^{\cdot}$ + $ROO^{\cdot}$ → ROO --- Lycopene – OOR

Furthermore, lycopene may have a synergistic effect to repair vitamin E and vitamin C radicals as following equation.

Lycopene + alpha - $TO^{\cdot}$ + H^+ → $Lycopene^{+\cdot}$ + alpha - TOH

Antioxidative activities of lycopene has been shown to be as twice as higher than that of beta-carotene. Compared to alpha-tocopherol (vitamin E), lycopene contains approximately 100 times more potent antioxidative effects (Roldan-Gutierrez and Dolores, 2007). According to the efficiency of quenching singlet oxygen, lycopene has been shown to be more powerful 2- to 10- fold than that of beta-carotene and alpha-tocopherol (Erdman, et al., 2009). Therefore, it may have the highest antioxidative property among carotenoids (Breeman and Pajkovic, 2008). The antioxidative mechanisms of lycopene may be 1) quenching singlet oxygen and free radicals, 2) upregulation of the antioxidant response element (ARE), 3) stimulation of the enzymatic antioxidant production such as superoxide dismutase (Khachik, et al., 1991, Clinton, 1998, Breeman and Pajkovic, 2008). Several reports have been shown that lycopene may modulate the biomarkers in many chronic diseases by these mechanisms: increase gap junction communication, decrease inflammation, decrease cell cycle progression, induce cell cycle arrest, induce detoxification enzymes, decrease serum cholesterol, and C reactive protein (Erdman, et al., 2009).

Because of its antioxidative effects, many of evidences have demonstrated the beneficial effects of lycopene on prevention of several diseases (Clinton, 1998, Rao and Rao, 2007, Erdman, et al., 2009). Lycopene is predominately found in prostate gland; therefore, many evidences suggest that lycopene may decrease risk of benign prostatic hyperplasia and prostate cancer. The relative risk is 0.6 for men who consume tomatoes more than five times a week. The levels of prostate specific antigen is also decreased (Rao and Rao, 2007, Breeman and Pajkovic, 2008, Ilic and Misso, 2012, Lee and Foo, 2013). Recently, evidences have been shown that lycopene can inhibit the growth of human prostate carcinoma cell lines. Lycopene at 0.3 – 3.0 μM has been reported that it can induce apoptosis in the androgen sensitive human prostate cancer cell line (Hantz, et al., 2005).

Taking into account with other cancers, weekly tomato consumption has been reported to decrease the risk of esophageal cancer by 40%. High concentration of serum lycopene has been shown to be associated with a reduce risk of gastric cancer (Clinton, 1998). A cell culture study of human mammary cancer cells has shown that lycopene can inhibit proliferation of this cell line. However, more data on breast cancer prevention of lycopene is still needed.

Lycopene has been shown to significantly reduce the levels of total cholesterol and low density lipoprotein (LDL). Therefore, it may decrease the risk of myocardial infarction and cardiovascular diseases. The mechanism is that lycopene may oxidize the LDL and reduce the atherosclerosis (Rao and Rao, 2007, Breeman and Pajkovic, 2008). Moreover, lycopene can cross blood-brain barrier and may have an effect to prevent lipid peroxidation in Alzheimer's disease, vascular dementia and Parkinson's disease. The relationship between lycopene and neurodegenerative disease prevention should be investigated in the future (Rao and Rao, 2007).

Lycopene and reproductive system

As previously mentioned, high amount of ROS can be found in the infertility-related problems and the tissue concentrations of lycopene in testis are higher than that in the ovary; therefore, lycopene may play a crucial role as an antioxidant in male reproductive system.

It has been reported that infertility men have lower resume lycopene levels compared to fertile men (Palan and Naz, 1996). In a recent study, preincubation of sperm with lycopene 5 μM may have protective effects on sperm DNA damage and increasing sperm motility (Zini, et al., 2010). The study by Aly, et al was to investigate the effect of lycopene on sperm count and motility in the Wistar rats received lycopene gavage at the dose of 4 mg/kg/day. These rats were treated with lypopolysaccharide (LPS) intraperitoneal injection to induce lipid peroxidation. The results were shown that lycopene pretreatment may reduce the oxidative stress and decrease the damage of testicular mitochondria (Aly, et al., 2012).

To study the effect of lycopene on female gamete, from the intensive search, one published paper has been found. Chinese hamster ovary cell was treated by hydrogen peroxide and the various concentrations of lycopene (10, 25, 50 μM) with the pretreatment, simultaneous and post-treatment groups. The level of DNA damage was investigated. The results were shown that three concentrations of lycopene reduced the DNA damage, especially in the group with simultaneous treatment. From this study, lycopene may have chemopreventive activity on oxidative stress in Chinese hamster ovary cell (Scoloastici, et al., 2007).

CHAPTER III

RESEARCH METHODOLOGY

Instruments and materials

1. Instruments

Benchtop incubator (MINC-1000; Cook, Australia)

CO_2 incubator (CO_2cell 170, supreme)

Digital dry bath/Heated block (Accublock, Labnet)

Flow cytometry (Fach caliber, Becton Dickinson, United States)

HPLC (SHIMADZU apparatus, Japan)

HPLC analytic column Prodigy ODS3 5 uM 100A°, 250 x 4.6 mm *(Phenomenex®* USA)

Inverted microscope (Olympus 1X71, United states)

IVF chamber (Origio, Coopersurgical company, Denmark)

Light microscope (Olympus CH30, Japan)

Makler counting chamber (Sefi-medical instruments LTD, United States)

Microcentrifuge (MX-301, Tomy, Japan)

Spectrophotometer (Synergy HT, BioTex, United States

Stereoscope (Olympus SZ51, Japan)

2. Materials

30% hydrogen peroxide (Merck, United States)

Acetic acid (Sigma-Aldrich Corp., MI, United States)

Acetonitrile (Sigma-Aldrich Corp., MI, United States)

Chorionic gonadotropin (Merck, United states)

Cleavage media for zygote to blastocyst (Life Global group, United States)

: composes of bicarbonate- buffered protein-supplemented replete with glucose, lactate, pyruvate, human serum albumin, human alpha and beta-globulins, 20 amino acid and gentamicin sulfate

Dimethyl sulfoxide or DMSO (Sigma-Aldrich Corp., MI, United States)

Dinitrophenylhydrazine (DNPH) solution HPLC grade (Sigma-Aldrich Corp., MI, United States)

Distilled water

Ethanol (Sigma-Aldrich Corp., MI, United States)

Flushing media (FertiPro N.V., Belgium)

: composes of HEPES buffer with 0.4% human serum albumin

Fertilization media for in vitro fertilization (Life Global group, United States)

: composes of bicarbonate- buffered protein-supplemented replete with glucose, lactate, pyruvate, human serum albumin, human alpha and beta-globulins, 20 amino acid and gentamicin sulfate

Fluorescein isothiocyanate conjugate (FITC) Annexin V apoptosis detection kit (BD biosciences, United States)

: composes of FITC Annexin V, Propidium Iodide (PI), and 10% Annexin V binding buffer

Glutathione peroxidative assay kit (Cayman Chemical Company, United States)

: composes of GPx assay buffer, GPx sample buffer, glutathione peroxidase (control), GPx co-substrate mixture, GPx Cumene hydroxyperoxide.

Hydrogen chloride (Fisher Scientific, England)

Lycopene (Sigma-Aldrich Corp., MI, United States)

Mineral oil (Life Global group, United States)

Perchloric acid (Fisher Scientific, England)

Pregnant mare serum gonadotropin (PMSG) (Sigma-Aldrich Corp., MI, United States)

Sulfuric acid (Fisher Scientific, England)

Tetramethoxypropane (TMP) (Sigma-Aldrich Corp., MI, United States)

Animals

Four-week-old male and female ICR mice were purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand. The mice were acclimatized at the Center for Animal Research, Naresuan University at least one week before setting the experiment. The temperature was controlled for

22±1°C with light:dark standard cycle (12:12 hours). All mice were fed with food and water *ad libitum*.

Methods

Preparation of lycopene

Lycopene was dissolved in DMSO (Sigma-Aldrich Corp.) and made up to 1 ml with distilled water preheated to 37°C. The stock concentration was 1 mg/ml. Before the experiment, lycopene was freshly prepared for various concentrations with adding to the culture media. When adding to the culture medium, the final concentration of DMSO was less than 0.01% W/V which has no effect on embryo development (Srinivasan, et al., 2007).

Superovulation and oocyte collection

Three days before the experiment on IVF, female mice had to be prepared for superovulation and ovulation induction. Pregnant mare serum gonadotropin or PMSG (Sigma-Aldrich) 10 IU (0.1 ml) was injected intraperitoneally to stimulate the oocyte growth. At 48-hour interval 10 IU (0.1 ml) of chorionic gonadotropin (CG) was intraperitoneally injected to induce ovulation.

For oocyte collection, 10-13 hours after CG injection female mice were sacrificed by cervical dislocation and then the lower abdominal wall was opened to approach the lower abdominal cavity. The upper end of the uterine horn was identified and followed to the fat pad. The oviduct was dissected separately from the ovary and cut at the distal end of the uterine horn. The oviduct was placed in a Petri dish with small amount of flushing medium that overnight preincubated in 37°C with 5% CO_2. The oviduct was grasped firmly on the bottom of the dish by small forceps. The needle gauze 27 was gently applied to tear the oviduct near the distended area of the oviduct where the oocytes were located. Then the oocytes were flowed out spontaneously. The cumulus-oocyte-complex was transferred to the 0.8 ml of fertilization medium in the 4-well plate and kept in the benchtop incubator (MINC-1000, Cook) supplied with a humidified triple gas mixture of 6% CO_2, 5% O_2 and 89% N_2 at 37°C for 3-4 hours for oocyte maturation until the time of insemination.

The experimental designs were divided into three experiments as shown in Figure 14.

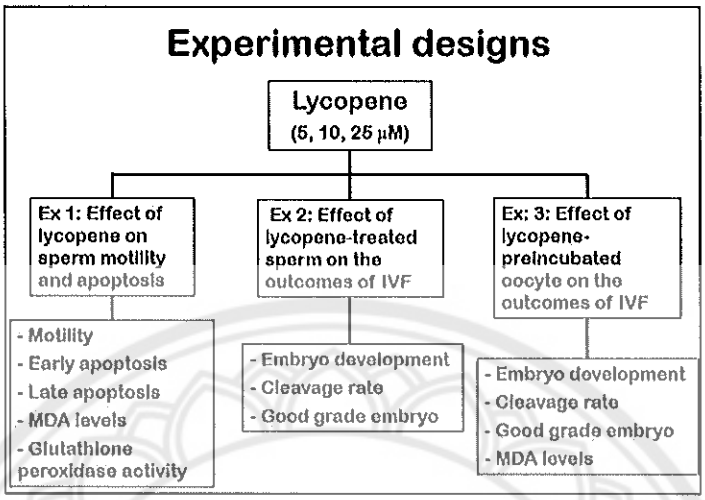

Figure 14 Experimental designs

Experiment 1: To examine the effects of lycopene on sperm motility and apoptosis

Sperm preparation and evaluation for concentration and motility

Flushing medium was prepared overnight by incubation at 37°C with 5% CO_2 before the day of sperm collection. Five male mice were sacrificed by neck dislocation and then the abdominal wall was opened to approach the lower abdominal cavity. The epididymis was dissected and placed into a 700 µl drop of flushing medium under mineral oil. Needle gauze 27 was applied to puncture the epididymis and sperm were actively swum out. The sperm collected from 5 males were pooled together. The pooled specimen was evaluated for the concentration and motility by loading 10 µl of sample on the Makler counting chamber (Sefi-medical instruments LTD) and examined under the light microscope (Olympus). The motility parameters were measured as motile and non-motile sperm (Grunewald and Paasch, 2013). The sperm concentration and motility were evaluated by two examiners independently and then the mean of concentration and motility was calculated.

The pooled sperm sample was divided into 8 groups (500 µl /sample) as following:

1. Control group with no treatment
2. Treatment group with lycopene 5 µM
3. Treatment group with lycopene 10 µM
4. Treatment group with lycopene 25 µM
5. Control group with hydrogen peroxide 10 µM
6. Treatment group with lycopene5 µM and hydrogen peroxide 10 µM
7. Treatment group with lycopene 10 µM and hydrogen peroxide 10 µM
8. Treatment group with lycopene 25 µM and hydrogen peroxide 10 µM

The sperm in all groups were incubated for sperm capacitation in the 5% CO_2 incubator, 37°C for 4 hour and all the samples were reevaluated for sperm motility under light microscope. The sperm with a concentration of $1x10^6$ cells/ml, 100 µl were immediately prepared for evaluation of sperm apoptosis by flow cytometry. The 200 µl of the specimen from each group were collected for detection of oxidative stress and antioxidant effect by the glutathione peroxidase assay and detection of malondialdehyde levels which was described later. The experiment 1 was shown in Figure 15.

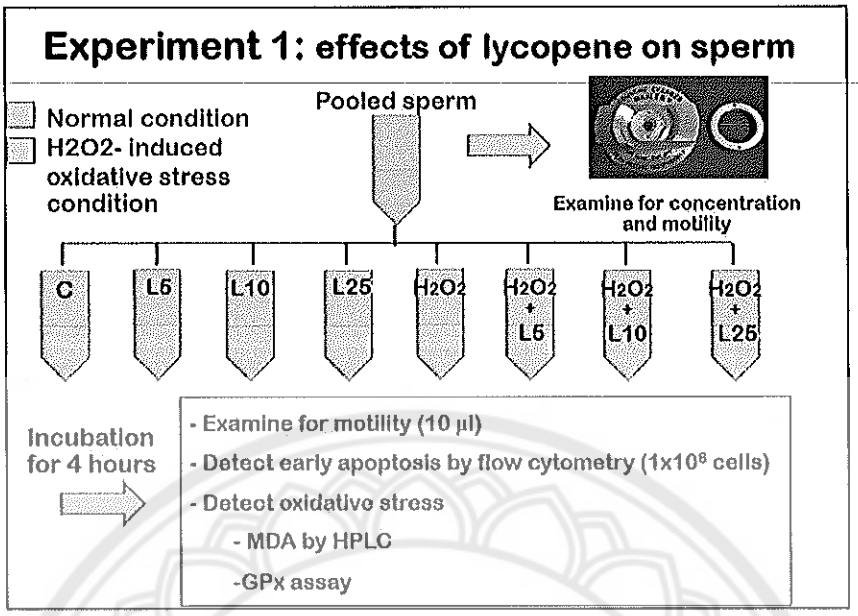

Figure 15 **Experiment 1 – Effect of lycopene on sperm motility and apoptosis Detection of sperm apoptosis by FITC annexin V and PI with flow cytometry**

Sperm from eight groups were washed twice with PBS and resuspended in 1X binding buffer. Approximately $1x10^5$ cells were incubated with 2 µl of annexin V fluorescein isothiocyanate conjugate (FITC) and 1 µl of propidium iodide. The sperm were vortexed and incubated for 15 minutes at room temperature in the dark. After that 400 µl of 1X Binding Buffer was added and the samples were analyzed for early and late apoptosis by flow cytometry.

Experiment 2 To examine the effects lycopene-treated sperm on the outcomes of *in vitro* fertilization

This experiment was divided into 4 groups as following:

1. Control group with no treatment
2. Sperm treated with lycopene 5 µM
3. Sperm treated with lycopene 10 µM
4. Sperm treated with lycopene 25 µM

***In vitro* fertilization and culture**

The fertilization medium was prepared overnight by incubation at 37°C with 5% CO_2. Approximately 5-10 cumulus-oocyte-complexes were placed in each well of the 4-well plate filled with fertilization medium 0.8 ml. The sperm from each group were used for *in vitro* fertilization process. Total $5x10^5$ sperm were inseminated in each well and then the plate was incubated in benchtop incubator with triple gas mixture of 6% CO_2, 5% O_2 and 89% N_2 at 37°C. After 4 hours, the oocytes were denuded to remove the remained cumulus cells and then transferred to culture in cleavage medium which each drop was composed of 30 µL of the culture media under mineral oil and incubated in the benchtop incubator. Fertilization was checked by presenting of 2 pronuclei at 16-19 hours or 2-cell embryos at 34-36 hours post-insemination under Inverted microscope (Olympus 1X71, United States). The experiment was performed in duplicate and at least in 2 independent experiments.

Evaluation of embryo development and embryo grading

After the process of *in vitro* fertilization, the oocytes were cultured in the cleavage media in benchtop incubator with triple gas mixture of 6% CO_2, 5% O_2 and 89% N_2 at 37°C. The rate of embryo development and embryo grading was examined as following.

On day 1, after 16-19 hours post-insemination, oocytes were checked for fertilization by presenting of two pronuclei (2 PN from male and female). The fertilization rate was recorded and presented in percentage.

On day 2 and day 3, embryo development was evaluated by the number, size, shape of blastomeres and degree of fragmentation. The embryos were supposed to be 4-8 blastomere on day 2 and become morula or early blastocyst on day 3. The cleavage rate was recorded and presented in percentage.

The grading of embryo can be classified as following (Figure 16):

Grade 1: Good - < 10% fragmentation, stage-specific cell size, equal blastomeres

Grade 2: Fair - 10-25% fragmentation, stage-specific cell size for majority of cells and no evidence of multinucleation

Grade 3: Poor - > 25% fragmentation, cell size not stage specific and evidence of multinucleation

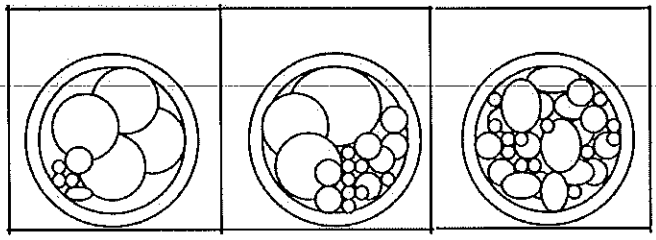

Figure 16 Embryo grading: Good, fair, poor

Source: http://fertilitycenter-uconn.org/how-do-we-choose-embryos-for-transfer

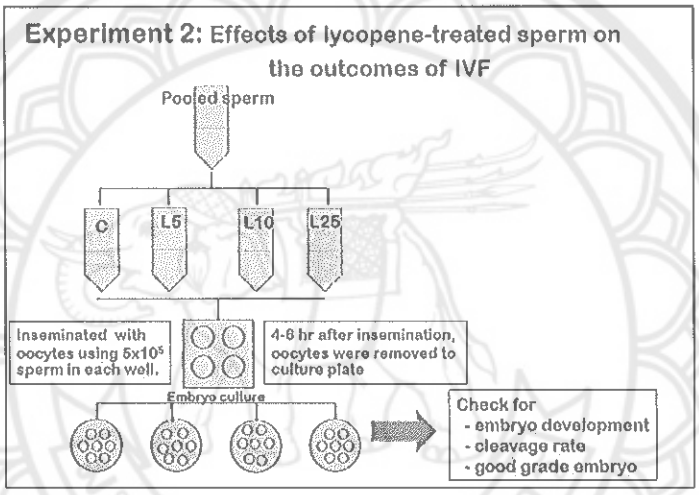

Figure 17 Experiment 2 – Effect of lycopene-treated sperm on the outcomes of IVF

Experiment 3 To examine the effects lycopene-preincubated oocytes on the outcomes of *in vitro* fertilization

The sperm freshly collected from 3 male mice (with the same technique as previously described) were pooled together and evaluation for concentration and motility. The sperm was stored in the 5% CO_2 incubator, 37°C for 4 hour in the flushing medium to be capacitated until the time of insemination.

Cumulus-oocyte complexes were collected (with the same technique as previously described) and divided into 4 groups as following:

1. Control group with no treatment
2. Treatment group with lycopene 5 μM added in fertilization media

3. Treatment group with lycopene 10 μM added in fertilization media
4. Treatment group with lycopene 25 μM added in fertilization media

Preparing of lycopene-preincubated oocytes

Five cumulus-oocyte-complexes were placed in each well of the 4-well plate filled with fertilization medium 0.8 ml that lycopene was added at different concentrations. The 4-well plates were kept in the benchtop incubator (MINC-1000, Cook) supplied with a humidified triple gas mixture of 6% CO_2, 5% O_2 and 89% N_2 at 37°C for 3-4 hours waiting for oocyte maturation until the time of insemination.

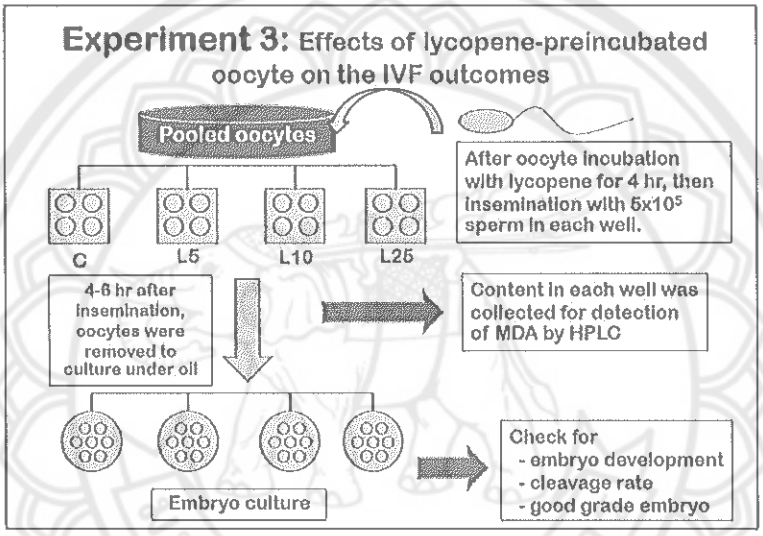

Figure 18 Experiment 3 – Effects of lycopene-preincubated oocytes on the outcomes of IVF

***In vitro* fertilization and culture**

Total $5x10^5$ sperm were inseminated in each well and then the plate was incubated in benchtop incubator with triple gas mixture of 6% CO_2, 5 of 5% O_2 and 89% N_2 at 37°C. After 4 hours, the oocytes were denuded to remove the remained cumulus cells and then transferred to culture in cleavage medium which each drop was composed of 30 μL of the culture media under mineral oil and incubated in the benchtop incubator. The fertilization, cleavage rate, embryo grading and blastocyst staging were evaluated with the same protocol as previously described.

Detection of oxidative stress and anti-oxidative effects

After removal of the oocytes, the culture media from the inseminated well of each group was collected for detection of oxidative stress and antioxidant effect by the glutathione peroxidase assay and detection of malondialdehyde levels which was described later.

Glutathione peroxidase assay

The specimen from culture media was stored at -80°C until the time of the assay. Each specimen was centrifuged at 1000 x g for 10 minutes at 4°C and homogenized in cold buffer which was composed of 50 µM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT and then centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was removed for the assay and stored on ice.

For the background or non-enzymatic wells, 120 µl of assay buffer and 50 µl of co-substrate mixture was added to 3 wells. For the positive control wells, 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of diluted GPx (control) were added to 3 wells. For the sample wells, 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of sample were added to 3 wells. To initiate the reaction, 20 µl of cumene hydroperoxide was added to all wells as quickly as possible. The plate was shaken to mix all reagents for a few seconds and then read the absorbance once every minute at 340 nm.

After the assay, the change in absorbance (ΔA_{340}) per minute will be recorded and the rate of ΔA_{340} /min will be calculated.

Lipid peroxidation measurement by detection of MDA with High Performance Liquid Chromatography (HPLC)

This technique was modified from Tüközkan's method (Nurten, et al., 2006). Cells in the culture media (sperm, cumulus cell or embryos) were homogenized by Wise Tis® HG-15D homogenizer (Daihan Scientific, Korea) in 200 µl of 0.1 M perchloric acid. After centrifugation (13,000 g for 10 min at 4°c), supernatant was removed into a 1.5 ml microcentrifuge tube. The equal volume of acetonitrile (HPLC grade) was added to the aliquot of tissue homogenate in order to precipitate proteins. The suspension was mixed for 30 seconds and centrifuged at 13,000 g, 10 min at 4°c. The upper clear supernatant was filtered through a 0.45 µm nylon membrane. The filtered sample (250 µl) was then mixed with 25 µl dinitrophenylhydrazine (DNPH)

solution (5 mM in 2 M HCl) and then incubated for 10 min at room temperature. Later on, the sample aliquots of at least 20 µl were injected onto HPLC system. An aliquot of sample was measured protein levels of tissue by Bradford method (Bio-Rad).

Preparation of the standard curve:

To prepare MDA standard, 4.167 µl of 1,1,3,3 tetramethoxypropane (TMP) was dissolved in 50 ml ultrapure water to give a 500 µM stock solution. Working standard was prepared by hydrolysis of 1 ml TMP stock solution in 49 ml 1% sulfuric acid and incubated for 2 hours at room temperature. The resulting MDA standard was diluted with 1% sulfuric acid to produce the final concentration of 0.0125, 0.025, 0.05, 0.1, 0.2, and 0.5 µM to get the standard curve for the estimation of total MDA. Each concentration of MDA standard was filtered through 0.45 µm nylon membrane and 250 µl of standards was mixed with 25 µl of DNPH solution and incubated for 10 min. At least 20 µl of the reaction mixture was injected onto HPLC system.

HPLC analysis

The samples were analysed on SHIMADZU apparatus (Japan). The analytical column Prodigy ODS3 5 µm 100A°, 250 x 4.6 mm *(Phenomenex®* USA) was used. The retention time of the MDA-hydra zone averaged 18.3 min for daily runs. Acetonitrile-ultra pure water (38: 62, v/v) containing 0.2% (v/v) acetic acid was used in the mobile phase. HPLC apparatus was isocratic a condition at a flow rate 1 ml/min and UV detector was set at 310 nm, with the temperature of 30°C. MDA peaks were determined according to its retention time and confirmed by spiking with added exogenous standard. Level of MDA was calculated from standard curve prepared from TMP and expressed as µM for the culture media and µM/mg protein for the sperm and cumulus cells.

Statistic analysis

Sperm motility, sperm apoptosis and cleavage rate were presented as a percentage. All assays were carried out in duplicate. Continuous and ordinal data were calculated as mean $\pm$ SD unless otherwise specified. Statistic analysis was performed using SPSS software version 17 for Windows (SPSS Inc., Chicago, IL, USA). Kruskal- Wallis nonparametric tests were used to analyze the difference

between groups. Mann-Whitney test was used to analyze the difference between 2 independent samples. A P-value of ≤ 0.05 was considered statistically significant.

Ethics consideration

The experimental protocols were approved by the Animal Ethics Committee of Naresuan University before the experiments were performed.

CHAPTER IV

RESULT OF THE STUDY

Experiment 1: Effect of lycopene on sperm motility and apoptosis

The average concentration of pooled sperm was $26x10^5$ cells/ml. At the beginning, sperm motility was 59.5%. After a 4-hour incubation, sperm motility decreased in all groups compared to the untreated control. The treatment group of lycopene 10 μM showed that sperm motility was comparable to the untreated control. However, treatment of lycopene with three concentrations (5, 10, 25 μM) did not have significant effect on protection sperm from the decline in motility. Compared between motility of lycopene-treated sperm with various concentrations in H_2O_2-induced oxidative stress condition to the sperm treated with H_2O_2 group, the difference of sperm motility confirmed by statistical non-significant (P-value = 0.36).

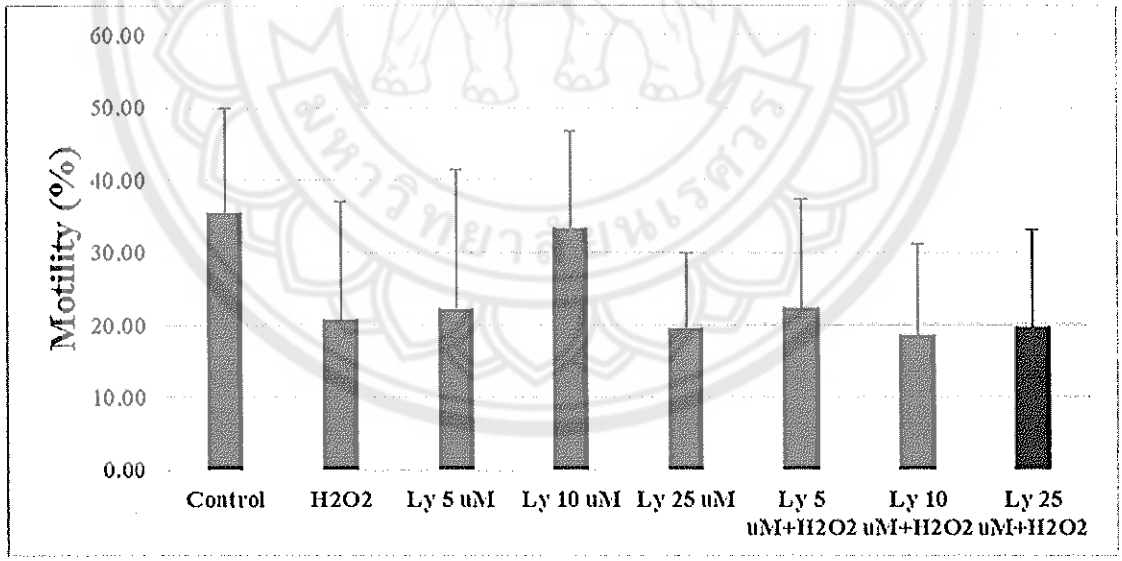

Figure 19 Effect of lycopene with and without H_2O_2-induced oxidative stress on percentage of sperm motility after incubation for 4 hours. The sperm motility was statistical non-significant

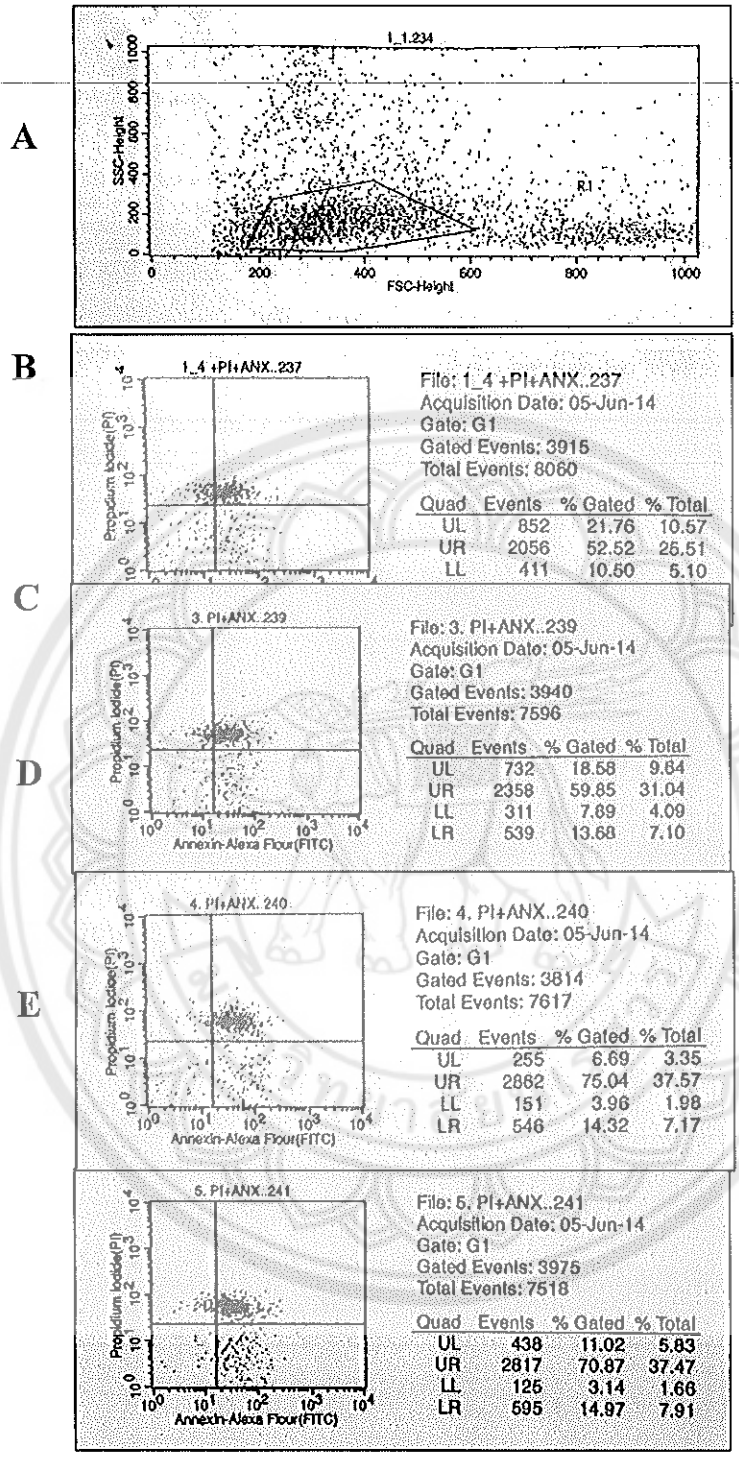

Figure 20 Flow cytometry showed sperm viability, early apoptosis, late apoptosis and necrosis in normal condition

Note: **A =** area of cell analysis, B = control group, C = lycopene 5 μM, D = lycopene 10 μM, E = lycopene 25 μM

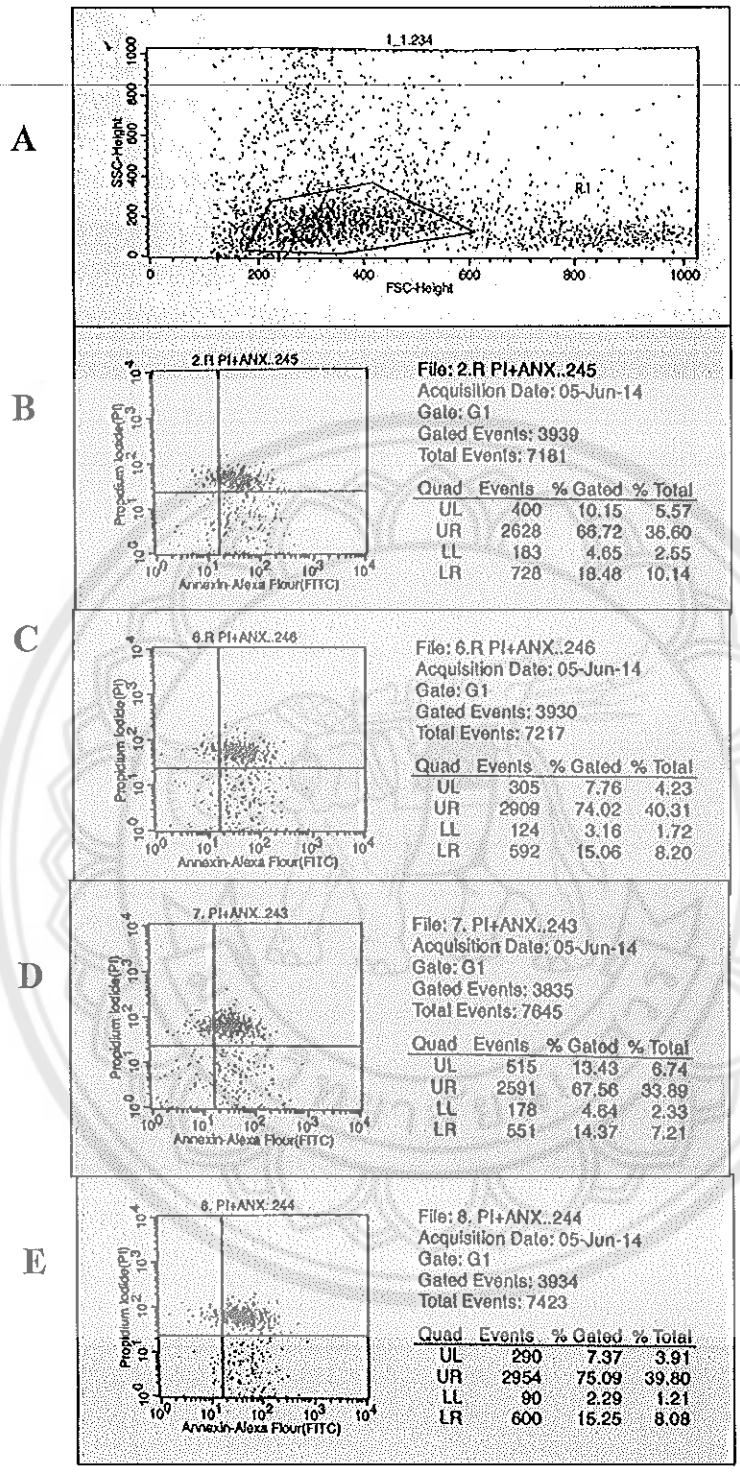

Figure 21 Flow cytometry showed sperm viability, early apoptosis, late apoptosis and necrosis in H_2O_2-induced condition

Note: **A** = area of cell analysis, B = H_2O_2, C = lycopene 5 μM + H_2O_2, D = lycopene 10 μM + H_2O_2, E = lycopene 25 μM + H_2O_2

The effect of lycopene on the percentage of early and late sperm apoptosis detected by flow cytometry showed that the rate of early apoptosis slightly increased in the normal condition. There was a trend that lycopene may decrease the rate of late apoptosis in the group of lycopene 5 and 25 μM compared to untreated control. However, it did not reach statistically significance. Among the groups with H_2O_2 - induced oxidative stress, the percentage of both early and late apoptosis was slightly decreased in all three concentrations of lycopene. However, it was not statistically significant (P-value > 0.05).

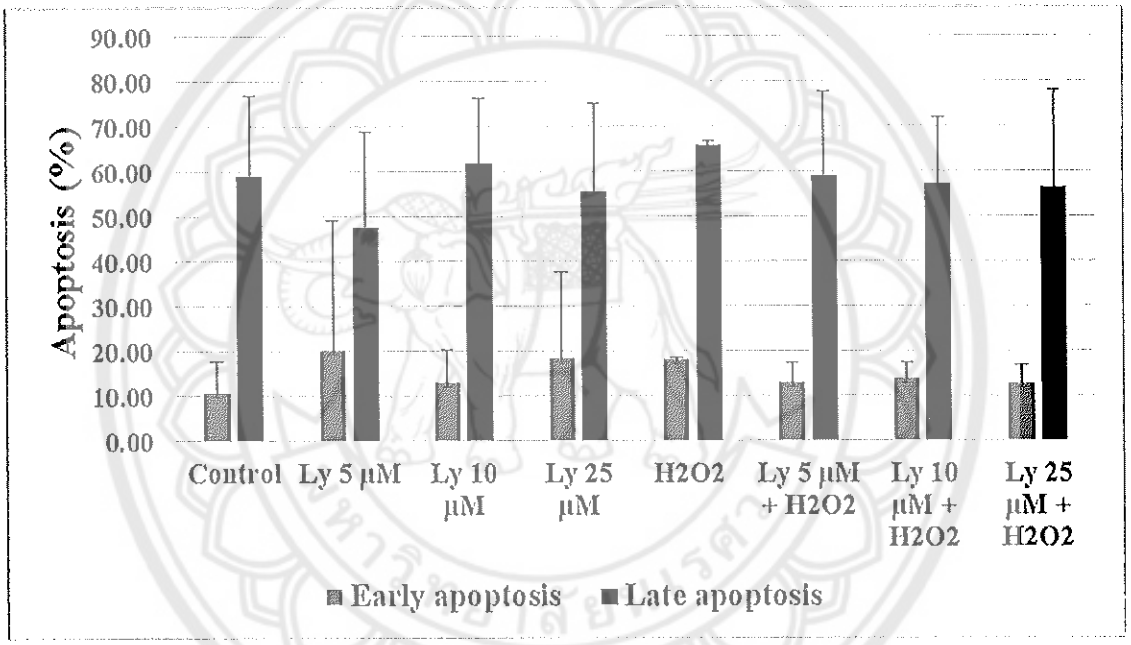

Figure 22 Effect of lycopene on the percentage of early and late sperm apoptosis which was not significantly different among all groups

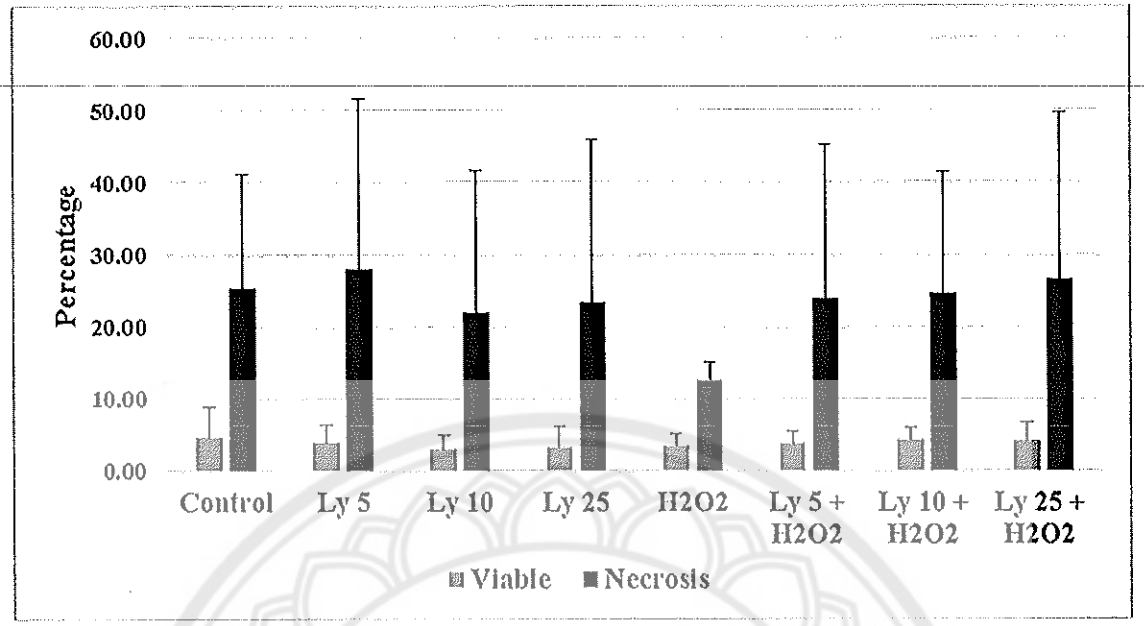

Figure 23 Effect of lycopene on the percentage of viable and necrotic sperm compared between in normal condition and H_2O_2-induced condition which was not significantly different among all groups

In the normal condition, the percentage of viable sperm in all groups of lycopene-treated sperm was comparable to the controlled group. The percentage of necrotic sperm was slightly decreased in the groups of lycopene 10 and 25 μM. However, this difference was statistical non-significant (P-value > 0.05). Among the groups with H_2O_2 -induced oxidative stress, the percentage of viable sperm was not different. The percentage of necrotic sperm in all groups of lycopene-treated sperm was increased when compared to the H_2O_2-controlled group (P-value > 0.05).

Detection of oxidative stress and anti-oxidative effects in lycopene-treated sperm

To detect MDA levels by HPLC technique, MDA peak was determined according to its retention time which was approximately at 19 minutes of injection (Figure 24). The levels of MDA from lycopene-treated sperm in normal and oxidative stress conditions were calculated and shown as Table 5.

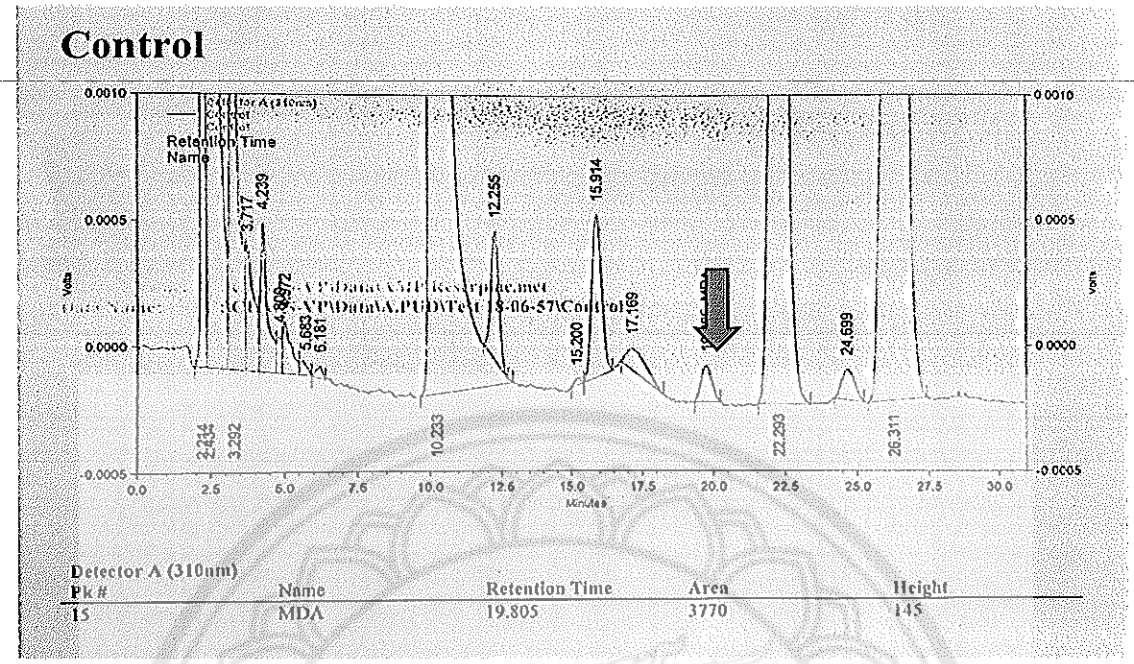

Figure 24 MDA peak at its retention time

In normal condition, the MDA levels of all lycopene concentrations slightly decreased compared to the control group but the decline levels were not statistically different (P-value > 0.05). In the oxidative stress-induced condition, the MDA levels were comparable in all groups (Table 5).

Table 5 MDA levels in lycopene-treated sperm

Group	MDA levels ± SD (nM/mg)	P-value Kruskal- Wallis
Control	1.57 ± 0.99	> 0.05
Lycopene 5 μM	1.07 ± 0.79	
Lycopene 10 μM	1.01 ± 1.10	
Lycopene 25 μM	1.13 ± 1.42	
H_2O_2 10 μM	0.63 ± 0.22	
Lycopene 5 μM + H_2O_2	0.82 ± 0.18	
Lycopene 10 μM + H_2O_2	0.81 ± 0.57	
Lycopene 25 μM + H_2O_2	0.57 ± 0.39	

The results of glutathione peroxidase activity in all groups were not statistically different compared in normal condition and H_2O_2-induced condition (P-value > 0.05) as shown in Table 6.

Table 6 Glutathione peroxidase activity (GPx) in lycopene-treated sperm

Group	GPx ± SD (nmol/min/ml)	P-value Kruskal- Wallis
Control	9.34 ± 1.04	> 0.05
Lycopene 5 μM	8.91 ± 1.14	
Lycopene 10 μM	8.91 ± 0.81	
Lycopene 25 μM	9.34 ± 1.04	
H_2O_2 10 μM	8.15 ± 0.69	
Lycopene 5 μM + H_2O_2	7.89 ± 1.39	
Lycopene 10 μM + H_2O_2	10.19 ± 1.59	
Lycopene 25 μM + H_2O_2	8.15 ± 0.88	

Experiment 2 Effects of lycopene-treated sperm on the outcomes of *in vitro* fertilization

This experiment was performed in normal condition with control group and lycopene-treated sperm at concentration of 5, 10 and 25 μM. The process of IVF was composed of sperm and oocyte insemination. The day after, embryos were evaluated for fertilization and cleavage rate (shown as Figure 25). The number of good grade embryos and degenerated oocytes were recorded separately.

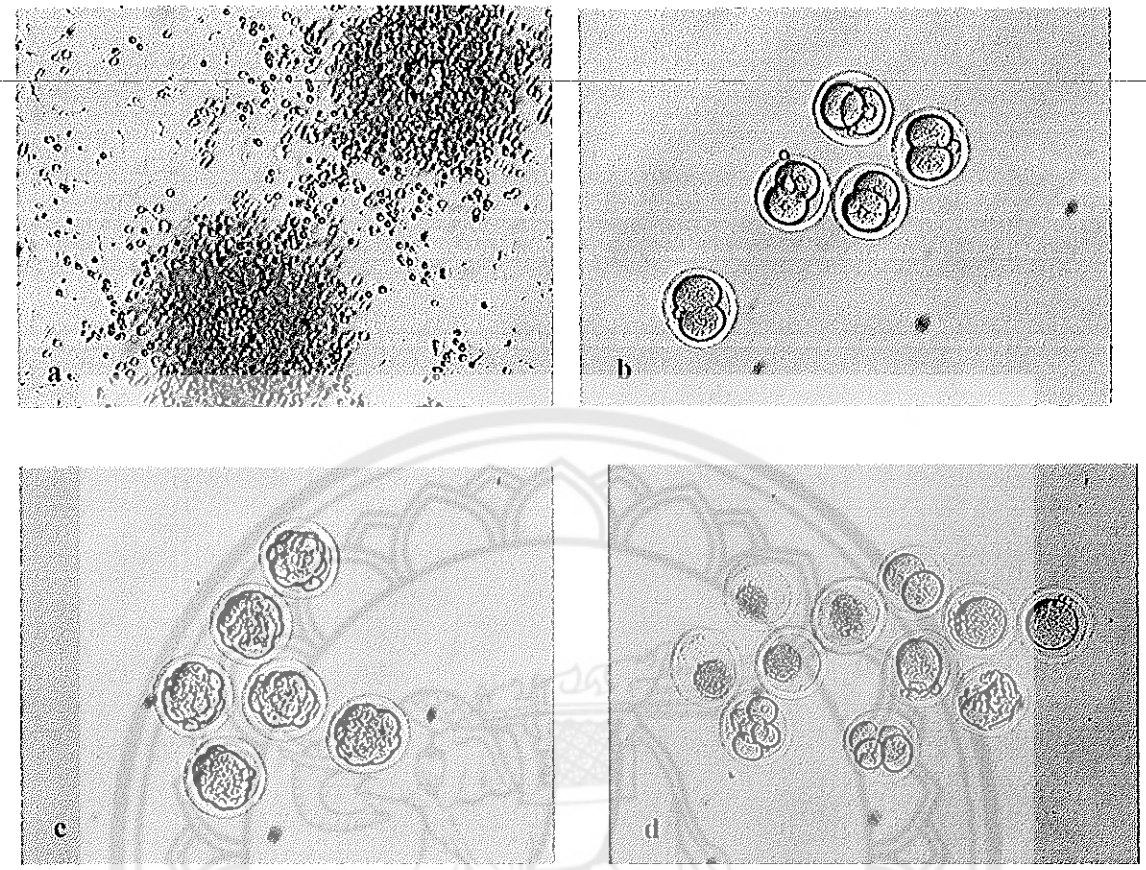

Figure 25 *In vitro* fertilization process and embryo evaluation

Note: a = Cumulus-oophorus complex and sperm in the inseminated well,
b = Two-cell embryos with good quality
c = Morula stage,
d = Multiple stages of embryo with degenerated oocytes (dark, brown and clumping ooplasm)

Lycopene-treated sperm of three concentrations were incubated with oocytes in the *in vitro* fertilization culture system (numbers of oocytes in each group were 46, 34, 37 and 34, respectively). The cleavage rate and good grade embryo quality were slightly decreased in the group which sperm were treated with lycopene 5 μM. On the other hand, the cleavage rate in the group which sperm were treated with lycopene 10 and 25 μM was slightly increased but in these 2 groups the rate of good grade embryos decreased. However, this difference of cleavage rate among all groups was statistically

non-significant (P-value = 0.16). The difference of good grade embryo rate among all groups did not reach the statistic significance (P-value = 0.63).

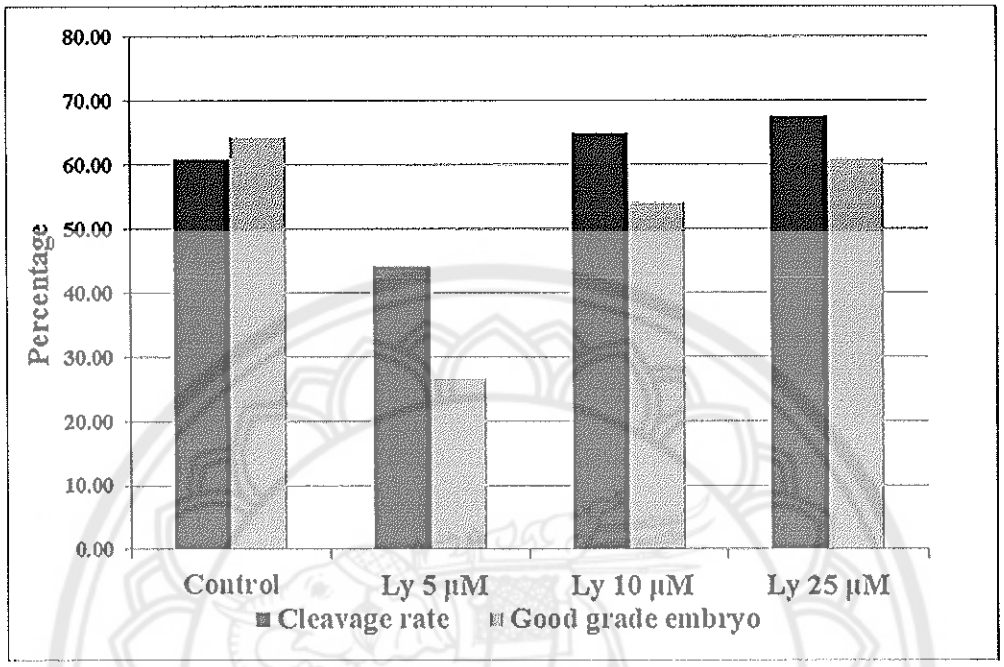

Figure 26 Percentage of cleavage and good grade embryo rate in IVF with lycopene-treated sperm

Experiment 3 Effects of lycopene-preincubated oocytes on the outcomes of *in vitro* fertilization

This experiment was performed in normal condition with control group and lycopene-preincubated oocytes at concentration of 5, 10 and 25 µM. The fertilization and embryo development were evaluated. Data was shown that the higher lycopene concentration, the lower cleavage rate of embryos was observed. However, this decline in cleavage rate was not significantly different. The oocyte degeneration rate seemed to be quite high in the group of oocyte-preincubated with lycopene 25 µM (62.5%) but it did not reach statistical significance when compared to control group. When evaluated the embryo grading among cleaved embryos, it was shown that the percentage of good-grade embryo was comparable among various lycopene concentrations (Table 7).

Table 7 Percentages of cleavage, oocyte degeneration rate and good-grade embryos in the lycopene-preincubated oocytes

Group (No. of oocytes)	Cleavage rate (%)[a]	Degeneration rate (%)[b]	Good grade embryo (%)[c]
Control (30)	50.0	16.7	66.7
Lycopene 5 μM (49)	48.9	14.3	62.5
Lycopene 10 μM (57)	36.8	26.3	57.1
Lycopene 25 μM (40)	17.5	62.5	71.4

a – P-value = 0.97
b – P-value = 0.68 } (compared to control group)
c – P-value = 0.94

The MDA levels of cumulus cells and sperm in each inseminated well were increased in all groups that preincubated with lycopene. The group of oocyte-preincubated with lycopene 25 μM had the highest levels but the levels were not significantly different. This highest levels of MDA may be associated with the percentage of oocyte degeneration (Figure 27).

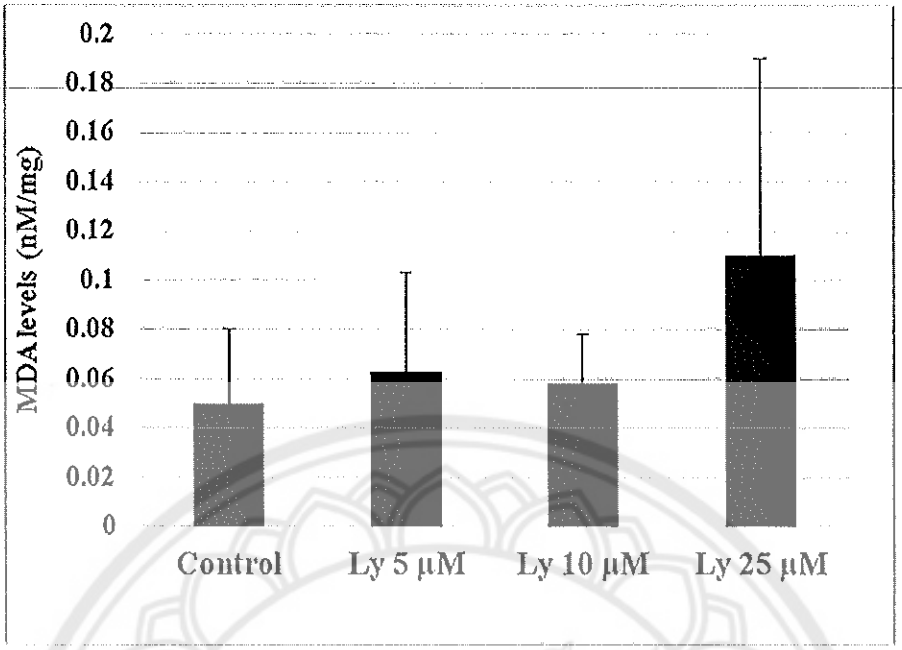

Figure 27 The MDA levels from the inseminated well of the lycopene treated – oocytes in IVF slightly increased with the lycopene concentration compared to the control which was correlated with the percentage of oocyte degeneration (P-value > 0.05)

CHAPTER V

DISCUSSION AND CONCLUSIONS

Lycopene known as a potent antioxidative properties has been studied of its beneficial effects in many health conditions such as prostate cancer. Lycopene is one of carotenoids but lycopene is different from other vitamin A derivatives because it contains acyclic end groups which make lycopene has no provitamin A property (McClain and Bausch, 2003). This is the strength of lycopene. It has no teratogenic effect; therefore, reproductive persons can consume lycopene. Furthermore, it can be used in the study of reproductive system. In both male and female infertility, several conditions have been studied and are related to high levels of ROS such as pelvic endometriosis, infection of genital tracts. The oxidative stress in vivo may interfere with oocyte and sperm function. Not only in the natural condition of infertile couples, but also in the steps of infertile treatment can generate high ROS. In the process of assisted reproductive technology, several factors can induce ROS production such as sperm damage during sperm preparation by high speed centrifugation, dead sperm and degenerated cumulus cells, high oxygen concentration in the culture media and system, exposure to low or high temperature and visible light (Guerin, et al., 2001, Henkel and Schill, 2003, Agarwal, et al., 2006). The author hypothesized that adding lycopene in the culture system for sperm and oocyte might have positive effect to improve sperm parameters and embryo developmental rate. Lycopene may prevent or reduce oxidative stress in the culture media in IVF process. According to thoroughly searching of literature review, there is little evidence about using lycopene adding in the IVF culture system. According to previous study by Zini, et al., they used lycopene 2 and 5 μM for human sperm preincubation 30 minutes at 25°C and in this study, oxidative stress was induced by H_2O_2 100 μM. The result showed that these concentrations of lycopene did not protect sperm from the decline in motility (Zini, et al., 2010). From the hypothesis, if the higher concentration was used, the protective effect might be demonstrated. Therefore, this study chose lycopene with the concentrations of 5, 10, 25 μM. To induce oxidative stress in the IVF culture system,

H_2O_2 10 μM was used. This dose was much less than the dose of Zini's study. From our preliminary study, it was shown that mouse sperm could not tolerate the high concentration of H_2O_2 as human sperm. In this study, there was no positive control group because up to date there were no definite data to confirm the antioxidative effect of any antioxidant in IVF culture system.

Naturally, sperm motility decreases as time goes by. In the process of IVF, sperm will be prepared by gradient centrifugation to collect only active sperm for insemination. This specimen will be kept in the 37^0C incubator for 3-4 hours to wait for sperm capacitation and oocyte maturation. In this experiment, after incubating sperm in lycopene 5, 10, 25 μM, the sperm motility was decreased in both normal condition and H_2O_2-induced oxidative stress condition. This result was quite similar to the previous study that preincubation of sperm with lycopene 2 and 5 μM did not protect decline in sperm motility (Zini, et al., 2010). These results might confirm that even lycopene concentrations higher than 5 μM and longer time of incubation, it still had no protective effect on sperm motility. However, in the normal condition, sperm treated with lycopene 10 μM showed the highest motility rate compared to lycopene 5 and 25 μM. It may imply that the protective effect of lycopene on sperm motility was not a dose dependent manner and it was not statistical significance. The results of this study may be supported by the study of Akalin et al about the effect of lycopene on the rat sperm motility during liquid storage at 5^oC Supplementation of lycopene at 0.5 mM dose for 24, 48 and 72 hours resulted in higher sperm motility rate compared to 1 and 2 mM lycopene (Akalin, et al., 2016). Therefore, probably the higher dose of lycopene does not improve sperm motility.

The sperm motility in this experiment was impaired after incubating sperm for 4 hours in the culture system for capacitation. It may be explained that human sperm capacitation needs 4 hours but mouse sperm capacitation needs 3 hours. Therefore, after 4 hours mouse sperm may agglutinate resulting in decreased sperm motility.

According to oxidative stress and sperm DNA damage, it can lead to sperm apoptosis and it may have effect on failed or impaired fertilization which will decrease number of good quality embryos. The results by Zini, et al revealed that preincubation lycopene 5 μM for 30 minutes had a protective effect against oxidative sperm DNA

damage even though the motility was not improved (Zini, et al., 2010). The mechanism of protective effect on sperm DNA damage was described by lycopene may reduce lipid peroxidation at the sperm plasma membrane which is rich of PUFA and prevent oxidative DNA injury.

In this study, sperm apoptosis was detected by flow cytometry. The results in the normal condition showed that the percentage of early apoptotic rate slightly increased in all three lycopene concentrations. On the contrary, the percentage of late apoptosis in the normal condition has a trend to be slightly decreased in the group of lycopene 5 and 25 μM. In the H_2O_2-induced oxidative stress condition, the percentage of both early and late apoptosis was decreased in all groups of sperm treated lycopene. The statistical analysis was not significant. Lycopene effect on reversible sperm apoptosis is still questionable. Probably, the process of sperm apoptosis is irreversible. In both normal and oxidative stress-induced condition, the percentage of viable sperm was quite similar compared to each control group. From these data, lycopene may possibly have positive effect to prevent further sperm damage because of the decreased percentage of late apoptosis and increased percentage of early apoptosis. However, it was not in a dose dependent manner.

MDA is commonly used for evaluation of lipid peroxidation process because it is one the end product. In this study, the MDA levels which indicate oxidative stress were shown that lycopene treated groups had lower MDA levels even though it was not statistically significant. The decreased MDA levels and late apoptotic rate could represent an antioxidative property of lycopene but the *in vitro* effect of lycopene may be a little. In vivo study, it has been shown that Wistar rats received lycopene gavage at the dose of 4 mg/kg/day and later treated with lypopolysaccharide (LPS) intraperitoneal injection to induce lipid peroxidation show a protective effect on testicular mitochondria damage and increase sperm count and motility (Aly, et al., 2012).

Most of scientific data about antioxidative effect of lycopene on anticancer in prostate gland were conducted with lycopene enriched-food consumption such as tomatoes. Supplementation of other antioxidants in the culture system was studied in mouse by Donnelly et al. The study showed that supplementation of vitamin C and E in sperm preparation could reduce ROS levels but sperm motility was not improved

(Donnelly, et al., 1999). Therefore, it may be implied that adding any antioxidant in the culture medium may not be clearly affected sperm parameters and MDA levels.

The results of glutathione peroxidase activity were not different in all groups, both in normal condition and oxidative stress-induced condition. There are several methods for *in vivo* measurement of oxidative stress such as glutathione peroxidase activity, superoxide dismutase, catalase (Alam, et al., 2013). No recent data can demonstrate which pathway that lycopene acts. Because of the limitation of the budget, this study chose to measure only glutathione peroxidase activity which was one method of enzymatic tests. The result of glutathione peroxidase activity in this study was comparable in all groups. Unfortunately, glutathione peroxidase activity may not be the main of antioxidative mechanism of lycopene. Another reason was that probably the change of the levels of glutathione peroxidase activity in this experiment was too less to be detected for the difference. If it is possible, study of antioxidative pathway of lycopene should be carried out by testing of catalase and superoxide dismutase activity.

To evaluate the effects lycopene-treated sperm on the outcomes of *in vitro* fertilization, oocytes were inseminated and embryo developmental rate was observed. The cleavage rate was decreased when incubated sperm with lycopene 5 μM. When lycopene 10 and 25 μM of lycopene were used, the cleavage rate seemingly increased which related to a dose dependent manner compared to control group. However, grading of embryo may be problematic because good quality embryos decreased in all three concentrations of lycopene. In clinical situation, grading of embryo is also an important prognostic factor to predict the success rate in IVF treatment. Even though, there are several cleaved embryos, if the quality is not good, the pregnancy rate might be impaired. Data from this experiment is still questionable whether lycopene-incubated sperm will really improve the IVF outcome. From this study, the high dose of lycopene seemed to improve cleavage rate but not improve the quality of the embryos.

For the study of *in vitro* fertilization, number of oocytes in this experiment may be not enough to demonstrate the difference. This may be a limitation of this study. Female mice used for superovulation in this study did not respond well to the stimulation. Therefore, numbers of retrieved oocyte were less than expectation.

Unfortunately, data of antioxidative effect of lycopene on female gametes are quite scarce. From the intensive search of literature review, only one published paper about female gamete and lycopene effect was found. Chinese hamster ovary cells were treated by hydrogen peroxide and the various concentrations of lycopene (10, 25, 50 μM) with the pretreatment, simultaneous and posttreatment groups. The results were shown that these three concentrations of lycopene reduced the ovarian cell DNA damage, especially in the group with simultaneous treatment. From this study, they concluded that lycopene may have chemopreventive activity on oxidative stress in Chinese hamster ovary cells (Scoloastici, et al., 2007).

In our study, cumulus-oophorus complexes were incubated with lycopene supplementation in the culture medium. Interestingly, rate of oocyte degeneration was the highest if incubated oocyte with lycopene 25 μM and the cleavage rate in this group was also decreased. This result was correlated with the MDA levels in the inseminated well and may be interpreted that high concentration of lycopene may have negative effect on oocyte function and cleavage embryos. When analyzed the embryo quality from the cleaved embryo, percentage of good grade embryos was not different. However, in the IVF unit the number of cleaved embryo is also very important for the chance to achieve pregnancy. Therefore, the ideal substance for supplementation in the IVF culture system should improve both cleavage rate and good embryo quality.

In this study, the data were shown that lycopene may not significantly improve sperm motility and not significantly reduce early and late apoptosis. Furthermore, adding lycopene in the IVF culture system may also have detrimental effect on oocytes quality and cause oocyte degeneration. Probably, lycopene may have a pro-oxidative effect if adding too much amount of lycopene. Concerning about the animal ethics, further experiment should not be continued. Although lycopene has a very potent antioxidative effects but it has a limitation because it is expensive and quite unstable. Synthetic lycopene should be kept under lightproof containers and stored in a cool place to prevent isomerization and oxidation. Therefore, supplementation of lycopene in the culture system may be a problem because of its unfavorable property (Roldán-Gutiérrez and Luque de Castro, 2007).

For the perspective study of lycopene in the future, the optimal concentration of lycopene adding in the culture system and appropriate incubation time should be

studied. One more point is to extend time for embryo culture. Parthenogenesis can occur in mouse embryo that usually happens on day 2 and these embryos will be arrested. To avoid parthenogenesis, embryo culture should be carried out until day 5 to achieve blastocyst stage in order to exclude normal cleaved embryos from parthenogenesis.

Summary and conclusions

From this study, administration of lycopene 5, 10, 25 μM in sperm and oocyte culture media may have little antioxidative effects in *in vitro* fertilization system. The optimal concentration of antioxidant is also challenging and problematic because small amount of ROS are crucial in normal physiology. It is really difficult to balance between ROS and antioxidant that are needed in the physiologic homeostasis. It should be kept in mind that antioxidant therapy may indeed be a double-edged sword, with negative effects in a certain threshold concentration is exceeded. Therefore, supplementation of lycopene in the culture media of IVF system should be further studied for the most effective dose and incubation time. Also the exact antioxidative pathway of lycopene is still important to be identified.

REFERENCE

REFERENCE

Abd-Elmoaty, M. A., Saleh, R. A., Sharma, R., & Agarwal, A. (2010). Increased levels of oxidants and reduced antioxidants in semen of infertile men with varicocele. *Fertil Steril, 94*(4), 1531-1534.

Agarwal, A., Gupta, S., & Sharma, R. (2005). "Oxidative stress and its implications in female infertility - clinician's perspective. *Reproductive Biomedicine Online, 11*(5), 641-650.

Agarwal, A., Gupta, S., & Sharma, R. K. (2005). Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol, 3*, 28-49.

Agarwal, A., Gupta, S., & Sikka, S. (2006). The role of free radicals and antioxidants in reproduction. *Curr Opin Obstet Gynecol, 18*(3), 325-332.

Agarwal, A., Nandipati, K. C., Sharma, R. K., Zippe, C. D., & Raina, R. (2006). Role of oxidative stress in the pathophysiological mechanism of erectile dysfunction. *J Androl, 27*(3), 335-347.

Agarwal, A., Said, T. M., Bedaiwy, M. A., Banerjee, J., & Alvarez, J. G. (2006). Oxidative stress in an assisted reproductive techniques setting. *Fertil Steril, 86*(3), 503-512.

Agarwal, A., Saleh, R. A., & Bedaiwy, M. A. (2003). Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril, 79*(4), 829-843.

Aitken, R. J., Lambourne, S., & Gibb, Z. (2014). The John Hughes Memorial Lecture: Aspects of Sperm Physiology—Oxidative Stress and the Functionality of Stallion Spermatozoa. *Journal of Equine Veterinary Science, 34*(1), 17-27.

Aitken, R. J., & Roman, S. D. (2008). Antioxidant systems and oxidative stress in the testes. *Oxid Med Cell Longev, 1*(1), 15-24.

Akalin, P. P., Bucak, M. N., GÜngÖr, Ş., BaŞpinar, N., Çoyan, K., Dursun, Ş., & Aksoy, A. (2016). Influence of lycopene and cysteamine on sperm and oxidative stress parameters during liquid storage of ram semen at 5°c. *Small Ruminant Research, 137*, 117-123.

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal, 21*(2), 143-152.

Aly, H. A., El-Beshbishy, H. A., & Banjar, Z. M. (2012). Mitochondrial dysfunction induced impairment of spermatogenesis in LPS-treated rats: modulatory role of lycopene. *Eur J Pharmacol, 677*(1-3), 31-38.

Ansari, K. N. (1997). The free radicals--the hidden culprits--an update. *Indian J Med Sci, 51*(9), 319-336.

Aprioku, J. S. (2013). Pharmacology of Free Radicals and the Impact of Reactive Oxygen Species on the Testis. *J Reprod Infertil, 14*(4), 158-172.

Bedaiwy, M., Agarwal, A., Said, T. M., Goldberg, J. M., Sharma, R. K., Worley, S., & Falcone, T. (2006). Role of total antioxidant capacity in the differential growth of human embryos in vitro. *Fertil Steril, 86*(2), 304-309.

Bedaiwy, M. A., Goldberg, J. M., Falcone, T., Singh, M., Nelson, D., Azab, H., & et al. (2002). Relationship between oxidative stress and embryotoxicity of hydrosalpingeal fluid. *Hum Reprod, 17*, 601-604.

Breeman, R., & Pajkovic, N. (2008). Multitargeted therapy of cancer by lycopene. *Cancer Lett, 269*, 339-351.

Britton, G., & Faseb J. (1995). "Structure and properties of carotenoids in relation to function. *FASEB Journal, 9*, 1551-1558.

Burton, G. J., & Jauniaux, E. (2011). Oxidative stress. Best Pract Res Clin Obstet Gynaecol, 25(3), 287-299.

Bus, J. S., & Gibson, J. E. (1982). Mechanisms of superoxide radical-mediated toxicity. *J Toxicol Clin Toxicol, 19*(6-7), 689-697.

Cindrova-Davies, T., Spasic-Boskovic, O., & Jauniaux, E. (2007). Nuclear factor-kappa B, p38, and stress-activated protein kinase mitogen-activated protien kinase signaling pathways regulate proinflammatory cytokines and apoptosis in human placental explants in response to oxidative stress: effects of antioxidant vitamins. *Am J Pathol, 170*, 1511-1520.

Clinton, S. K. (1998). Lycopene: chemistry, biology, and implications for human health and disease. *Nutr Rev, 56*(2 Pt 1), 35-51.

Cohen, G. M., & d'Arcy Doherty, M. (1987). Free radical mediated cell toxicity by redox cycling chemicals. *Br J Cancer Suppl, 8*, 46-52.

Combelles, C. M., Gupta, S., & Agarwal, A. (2009). Could oxidative stress influence the in-vitro maturation of oocytes?. *Reprod Biomed Online, 18*(6), 864-880.

Cordelli, E., Eleuteri, P., Leter, G., Rescia, M., & Spano, M. (2005). Flow cytometry applications in the evaluation of sperm quality: semen analysis, sperm function and DNA integrity. *Contraception, 72*(4), 273-279.

Dennery, P. A. (2010). Oxidative stress in development: nature or nurture?. F*ree Radic Biol Med, 49*(7), 1147-1151.

Devasagayam, T. P. A., Boloor, K. K., & Ramasarma, T. (2003). methods for estimating lipid peroxidation: an analysis of merits and demerits. *Indian journal of Biochemistry & Biophysics, 40*, 300-308.

Donnelly, E., McClure, N. and Lewis, S. (1999). Antioxidant supplementation in vitro does not improve human sperm motility. *Fertil Steril, 72*(3), 484-495.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev, 82*, 47-95.

Erdman, J. W., Jr., Ford, N. A., & Lindshield, B. L. (2009). Are the health attributes of lycopene related to its antioxidant function?. *Arch Biochem Biophys, 483*(2), 229-235.

Franco, M. C., Kawamoto, E. M., Gorjao, R., Rastelli, V. M., Curi, R., Scavone, C., et al., (2007). Biomarkers of oxidative stress and antioxidant stutus in children born small for gestational age: evidence of lipid peroxidation. *Pediatr Res, 62*, 204-208.

Fridovich, I. (1975). Superoxide dismutase. *Annual Review Biochemistry, 44*, 147-159.

Gil-Villa, A. M., Cardona-Maya, W., Agarwal, A., Sharma, R., & Cadavid, A. (2009). Role of male factor in early recurrent embryo loss: do antioxidants have any effect?. *Fertil Steril, 92*(2), 565-571.

Gil-Villa, A. M., Cardona-Maya, W., Agarwal, A., Sharma, R., & Cadavid, A. (2009). Role of male factor in early recurrent embryo loss: do antioxidants have any effect?. *Fertil Steril, 92*(2), 565-571.

Gomez, E., Buckingham, D. W., Brindle, J., Lanzafame, F. M., Irvine, D. S.. & Aitken, R. J. (1996). Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl, 17*, 276-287.

Goud, A. P., Goud, P. T., Diamond, M. P., Gonik, B., & Abu-Soud, H. M. (2008). Reactive oxygen species and oocyte aging: role of superoxide, hydrogen peroxide, and hypochlorous acid. *Free Radic Biol Med, 44*(7), 1295-1304.

Grunewald, S., & Paasch, U. (2013). Basic diagnostics in andrology. *J Dtsch Dermatol Ges, 11*(9), 799-814; quiz 815.

Guerin, P., El Mouatassim, S., & Menezo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update, 7*(2), 175-189.

Halliwell, B., & Gutteridge, J. M. (1984). Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. *The Lancet, 323*(8391), 1396-1397.

Hantz, H. L., Young, L. F., & Martin, K. R. (2005). Physiologically attainable concentrations of lycopene induce mitochondrial apoptosis in LNCaP human prostate cancer cells. *Exp Biol Med, 230,* 171-179.

Haung, J., & Rosenwaks, Z. (2012). In vitro fertilisation treatment and factors affecting success. Best Pract Res Clin Obstet Gynaecol, 26, 777-788.

Henkel, R. R., & Schill, W. B. (2003). Sperm preparation for ART. Reprod Biol Endocrinol, 1, 108-130.

Hensley, K., Robinson, K. A., Gabbita, S. P., Salsman, S., & Floyd, R. A. (2000). Reactive oxygen species, cell signaling, and cell injury. *Free Radic Biol Med, 28*(10), 1456-1462.

Hoogendijk, C. F., Kruger, T. F., Bouic, P. J., & Henkel, R. R. (2009). A novel approach for the selection of human sperm using annexin V-binding and flow cytometry. *Fertil Steril, 91*(4), 1285-1292.

Hossain, M. S., Johannisson, A., Wallgren, M., Nagy, S., Siqueira, A. P., & Rodriguez-Martinez, H. (2011). Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian J Androl, 13*(3), 406-419.

Ilic, D., & Misso, M. (2012). Lycopene for the prevention and treatment of benign prostatic hyperplasia and prostate cancer: a systematic review. *Maturitas, 72*(4), 269-276.

Jackson, H., Braun, C. L., & Ernst, H. (2008). The chemistry of novel xanthophyll carotenoids. *Am J Cardiol, 101*(10A), 50D-57D.

Jana, S. K., K, N. B., Chattopadhyay, R., Chakravarty, B., & Chaudhury, K. (2010). Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. *Reprod Toxicol, 29*(4), 447-451.

Khachik, F., Beecher, G. R., Goli, M. B., & Lusby, W. R. (1991). Separation, identification, and quantification of carotenoids in fruits, vegetables, and human plasma by high performance liquid chromatography. *Pure and Applied Chemistry, 63*, 71-80.

Krausz, C. B. p. R. C. E. M.-. (2011). Male infertility: Pathogenesis and clinical diagnosis. *Best practice & Research Clinical Endocrinology & Metabolism 25*, 271-285.

Lanzafame, F. M., La Vignera, S., Vicari, E., & Calogero, A. E. (2009). Oxidative stress and medical antioxidant treatment in male infertility. *Reprod Biomed Online, 19*(5), 638-659.

Lavranos, G., Balla, M., Tzortzopoulou, A., Syriou, V., & Angelopoulou, R. (2012). Investigating ROS sources in male infertility: a common end for numerous pathways. *Reprod Toxicol, 34*(3), 298-307.

Lee, L. K., & Foo, K. Y. (2013). An appraisal of the therapeutic value of lycopene for the chemoprevention of prostate cancer: A nutrigenomic approach. *Food Research International, 54*(1), 1217-1228.

Leung, F. Y. (1998). Trace elements that act as antioxidants in parenteral micronutrition. J Nutr Biochem, 9, 304-307.

Liu, Y., Luo, L., & Zhao, H. (2001). Levels of lipid peroxides and superoxide dismutase in peritoneal fluid of patients with endometriosis. *Journal of Tongji Medical University, 21,* 166-167.

Mahfouz, R., Sharma, R., Lackner, J., Aziz, N., & Agarwal, A. (2009). Evaluation of chemiluminescence and flow cytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa. *Fertil Steril, 92*(2), 819-827.

Mahfouz, R., Sharma, R., Thiyagarajan, A., Kale, V., Gupta, S., Sabanegh, E., & Agarwal, A. (2010). Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species. *Fertil Steril, 94*(6), 2141-2146.

McClain, R. M., & Bausch, J. (2003). Summary of safety studies conducted with synthetic lycopene. *Regulatory Toxicology and Pharmacology, 37*, 274-285.

Musset, B., Clark, R. A., Decoursey, T. E., Petheo, G. L., Geiszt, M., & Chen, Y. (2012). NOX 5 in human spermatozoa: expression, function, and regulation. *J Biol Chem, 287*, 9376-9388.

Nurten, T., Hüsamettin, E., & Seven, I. (2006). Measurement of Total Malondialdehyde in Plasma and Tissues by High-Performance Liquid Chromatography and Thiobarbituric Acid Assay. *Fırat Tıp Dergisi, 11*(2), 88-92.

Opara, E. C., & Rockway, S. W. (2006). Antioxidants and micronutrients. *Dis Mon, 52*(4), 151-163.

Organization, W. H. (1999). *Laborsatory manual for the examination of human semen and sperm-cervical mucous interaction*. New york: Cambridge university.

Palan, P., & Naz, R. (1996). "Changes in various antioxidant levels in human seminal plasma related to immunoinfertility. *Archives of Andrology, 36*, 139-143.

Palozza, P., Serini, S., Torsello, A., & al., E. (2003). "Beta-carotene regulate NF-kappa B DNA-binding activity by a redox mechanisms in human leukemia and colon adenocarcinoma cells. *J Nutr, 133*, 381-388.

Rao, A. V., & Rao, L. G. (2007). Carotenoids and human health. *Pharmacol Res, 55*(3), 207-216.

Ratnam, D. V., Ankola, D. D., Bhardwaj, V., Sahana, D. K., & Kumar, M. N. (2006). Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. *J Control Release, 113*(3), 189-207.

Rhee, S. G., Chang, T., Jeong, W., & Kang, D. (2010). Methods for detection and measurement of hydrogen peroxide inside and outside of cells. *Molecules and cells, 29*, 539-549.

Richter, C., Park, J., & Arnes, B. N. (1988). Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Nat Acad Sci, 85*, 6465-6467.

Roldan-Gutierrez, J., & Dolores, M. (2007). Lycopene: the need for better methods for characterization and determination. *Trends in Analytical Chemistry, 26*(2), 163-170.

Roldán-Gutiérrez, J. M., & Luque de Castro, M. D. (2007). Lycopene: The need for better methods for characterization and determination. *Trends in Analytical Chemistry, 26*(2), 163-170.

Rolo, A. P. (2006). Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. *Toxicol Appl Pharmacol, 212*, 167-178.

Schwedhelm, E., Maas, R., Troost, R., & Boger, R. H. (2003). Clinical pharmacokinetics of antioxidants and their impact on systemic oxidative stress. *Clin Pharmacokinet, 42*(5), 437-459.

Scoloastici, C., Alves de Lima, R. O., Barbisan, L. F., Ferreira, A. L., Ribeiro, D. A., Salvadori, D. M. F. (2007). Lycopene activity against chemically induced DNA damage in Chinese hamster ovary cells. *Toxicology in Vitro, 21*, 840-845.

Si, Y., & Okuno, M. (1999). Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biol Reprod, 61*, 240-246.

Sies, H., & Stahl, W. (1995). Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. *Am J Clin Nutr, 62*(6 Suppl), 1315S-1321S.

Srinivasan, M., Sudheer, A. R., Pillai, K. R., Kumar, P. R., Sudhakaran, P. R., & Menon, V. P. (2007). Lycopene as a natural protector against gamma-radiation induced DNA damage, lipid peroxidation and antioxidant status in primary culture of isolated rat hepatocytes in vitro. *Biochim Biophys Acta, 1770*(4), 659-665.

Stahl, W., & Sies, H. (2003). Antioxidant activity of carotenoids. *Mol Aspects Med, 24*(6), 345-351.

Stahl, W., & Sies, H. (2005). Bioactivity and protective effects of natural carotenoids. *Biochim Biophys Acta , 1740*(2), 101-107.

Taguchi, M., Alfer, J., Chwalisz, K., & al., E. (2000). Endothelial nitric oxide synthetase is differently expressed in human endometrial vessels during the menstrual cycle. *Molecular Human Reproduction, 6,* 185-190.

Taylor, C. T. (2001). Antioxidants and reactive oxygen species in human fertility. *Environ Toxicol Pharmacol, 10*(4), 189-198.

Turek, P. J. (2009). Male infertility. Yen and Jaffe's Reproductive Endocrinology. J. F. Strauss, Barbieri RL. Philadelphia, Saunders.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol, 39*(1), 44-84.

Wang, X., Falcone, T., Attaran, M., Goldberg, J. M., Agarwal, A., & Sharma, R. K. (2002). Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. *Fertil Steril, 78*(6), 1272-1277.

WHO (1999). World Health Organization. *Laboratory manual for the examination of human semen and sperm-cervical mucous interaction.* New york: Cambridge university.

Zhang, H., & Zheng, R.-L. (1996). Promotion of human sperm capacitation by superoxide anion. *Free Radic Res, 24*, 261-268.

Zini, A., San Gabriel, M., & Libman, J. (2010). Lycopene supplementation in vitro can protect human sperm deoxyribonucleic acid from oxidative damage. *Fertil Steril, 94*(3), 1033-1036.