行政院國家科學委員會專題研究計畫 期末報告

氧化自由基及抗氧化物質於人類精子活化與染色質損壞的角色(第 3 年)

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中 華 民 國 102 年 10 月 31 日

中 文 摘 要 ： 背景：過多的氧化自由基(ROS)與精子活性氧相關損害，是男性不孕症的原因之一。飲食中補充抗氧化劑，則可改善試管嬰兒治療週期的結果。然而在精子活化獲能過程中，有限量的氧化自由基卻是觸發事件的信號。添加抗氧化劑對精子製備過程中，對精子功能的影響則是相對地未知。這項研究目的，旨在釐清在製備過程中，外源抗氧化劑對不明原因不孕夫婦的精子功能的影響。
研究方法:從不明原因不孕夫婦的男性伴侶收集三十精液樣本，製備培養液中添加抗氧化劑(谷胱甘肽和牛磺酸)，以釐清對精子獲能活化的效果。精子梯度離心後，針對精液運動特性進行了電腦輔助分析。由西方墨點法和螢光染色方法檢測到的酪氨酸磷酸化和頂體反應率水準。此外，30 對經歷過試管嬰兒治療週期的不明原因不孕夫婦，測量其男性伴侶的皮膚類胡蘿蔔素狀態、抗氧化能力、整體精液和清洗後精子中的氧化自由基量，作為氧化還原狀態的指標，用以釐清男性夥伴抗氧化狀態與受精及體外胚胎發育的相關性。
結果：谷胱甘肽，但不是牛磺酸，會減少精子的氧化自由基量，且減少酪氨酸磷酸化和頂體反應率水準；然而，只有皮膚類胡蘿蔔素的狀態與受精及體外胚胎發育相關。
結論： 精子與氧化自由基相關損壞可能發生在精子生成的過程。食物中補充抗氧化劑，可能會增加皮膚類胡蘿蔔素地位和試管嬰兒治療週期體外胚胎發育。

中文關鍵詞： 精子活化、氧化壓力、抗氧化物、氧化自由基

英 文 摘 要 ： Study question: This study aimed to elucidate the effects of exogenous antioxidants in the body or in preparation media on sperm function for couples with unexplained infertility (UI).
What is known already: Excessive ROS levels and ROS-related damage in spermatozoa are responsible for male infertility and UI. Oral supplementation of antioxidants improves the outcome of ART cycles. During sperm capacitation, a limited amount of ROS is the signal triggering event. The effect of adding antioxidants to sperm preparation media on sperm function is relatively unknown.
Study design, size, duration: Thirty semen samples from male partners of couples with UI were collected to determine the effect of adding antioxidants on capacitation in sperm preparation media. In addition,

30 couples with UI who underwent ART cycles were recruited to elucidate the relevance of antioxidant status of male partners to fertilization and embryo development in ART cycles.
Materials, setting, methods: The semen samples were analyzed for semen motion characteristics, and the aliquots of spermatozoa were divided into groups and incubated in various sperm preparation media with or without antioxidants (glutathione and taurine) after gradient centrifugation. The level of tyrosine phosphorylation and the acrosome reaction rate were detected by Western blot analysis and fluorescence staining methods. The skin carotenoid status, seminal antioxidant capacity, ROS levels in neat semen, and washed spermatozoa of male partners from couples with UI were measured as the redox index. The correlation coefficients between the four indices and fertilization and embryo development were determined.
Main results and the role of chance: The addition of GSH, but not taurine, reduced the ROS levels of spermatozoa in capacitating conditions, the level of tyrosine phosphorylation, and the acrosome reaction rates; however, only skin carotenoid status was correlated with fertilization rates and embryo development in ART cycles.
Wider implications of the findings: ROS-related damage to spermatozoa may occur in late spermatogenesis. Oral supplementation of antioxidants may increase skin carotenoid status and benefit embryo development in ART cycles.

英文關鍵詞： sperm capacitation, oxidative stress, reactive oxygen species, antioxidants,

行政院國家科學委員會補助專題研究計畫 □期中進度報告 ■期末報告

氧化自由基及抗氧化物質於人類精子活化與染色質損壞的角色

The role of reactive oxygen species and antioxidants in human sperm capacitation and chromatin damage

計畫類別：■個別型計畫　□整合型計畫
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中　華　民　國 102 年 10 月 28 日

中文摘要

背景：過多的氧化自由基(ROS)與精子活性氧相關損害，是男性不孕症的原因之一。飲食中補充抗氧化劑，則可改善試管嬰兒治療週期的結果。然而在精子活化獲能過程中，有限量的氧化自由基卻是觸發事件的信號。添加抗氧化劑對精子製備過程中，對精子功能的影響則是相對地未知。這項研究目的，旨在釐清在製備過程中，外源抗氧化劑對不明原因不孕夫婦的精子功能的影響。

研究方法:從不明原因不孕夫婦的男性伴侶收集三十精液樣本，製備培養液中添加抗氧化劑(谷胱甘肽和牛磺酸)，以釐清對精子獲能活化的效果。精子梯度離心後，針對精液運動特性進行了電腦輔助分析。由西方墨點法和螢光染色方法檢測到的酪氨酸磷酸化和頂體反應率水準。此外，30 對經歷過試管嬰兒治療週期的不明原因不孕夫婦，測量其男性伴侶的皮膚類胡蘿蔔素狀態、抗氧化能力、整體精液和清洗後精子中的氧化自由基量，作為氧化還原狀態的指標，用以釐清男性夥伴抗氧化狀態與受精及體外胚胎發育的相關性。

結果：谷胱甘肽，但不是牛磺酸，會減少精子的氧化自由基量，且減少酪氨酸磷酸化和頂體反應率水準；然而，只有皮膚類胡蘿蔔素的狀態與受精及體外胚胎發育相關。

結論： 精子與氧化自由基相關損壞可能發生在精子生成的過程。食物中補充抗氧化劑，可能會增加皮膚類胡蘿蔔素地位和試管嬰兒治療週期體外胚胎發育。

關鍵詞：精子活化、氧化壓力、抗氧化物、氧化自由基

Abstract

Study question: This study aimed to elucidate the effects of exogenous antioxidants in the body or in preparation media on sperm function for couples with unexplained infertility (UI).

What is known already: Excessive ROS levels and ROS-related damage in spermatozoa are responsible for male infertility and UI. Oral supplementation of antioxidants improves the outcome of ART cycles. During sperm capacitation, a limited amount of ROS is the signal triggering event. The effect of adding antioxidants to sperm preparation media on sperm function is relatively unknown.

Study design, size, duration: Thirty semen samples from male partners of couples with UI were collected to determine the effect of adding antioxidants on capacitation in sperm preparation media. In addition, 30 couples with UI who underwent ART cycles were recruited to elucidate the relevance of antioxidant status of male partners to fertilization and embryo development in ART cycles.

Materials, setting, methods: The semen samples were analyzed for semen motion characteristics, and the aliquots of spermatozoa were divided into groups and incubated in various sperm preparation media with or without antioxidants (glutathione and taurine) after gradient centrifugation. The level of tyrosine phosphorylation and the acrosome reaction rate were detected by Western blot analysis and fluorescence staining methods. The skin carotenoid status, seminal antioxidant capacity, ROS levels in neat semen, and washed spermatozoa of male partners from couples with UI were measured as the redox index. The correlation coefficients between the four indices and fertilization and embryo development were determined.

Main results and the role of chance: The addition of GSH, but not taurine, reduced the ROS levels of spermatozoa in capacitating conditions, the level of tyrosine phosphorylation, and the acrosome reaction rates; however, only skin carotenoid status was correlated with fertilization rates and embryo development in ART cycles.

Wider implications of the findings: ROS-related damage to spermatozoa may occur in late spermatogenesis. Oral supplementation of antioxidants may increase skin carotenoid status and benefit embryo development in ART cycles.

Key words: sperm capacitation, oxidative stress, reactive oxygen species, antioxidants,

Oxidative stress-mediated damage to spermatozoa is a significant contributing pathology to male infertility (Said *et al.*, 2004; Agarwal *et al.*, 2006; Tremellen, 2008) and unexplained infertility (Pasqualotto *et al.*, 2001). High levels of reactive oxygen species (ROS) in seminal fluid is responsible for impairing the sperm DNA integrity and then inhibiting sperm cell function (Moustafa *et al.*, 2004). Furthermore, several reports indicated a reduction of non-enzymatic antioxidant activity in seminal plasma of infertile compared to fertile men (Mostafa *et al.*, 2006; Song *et al.*, 2006). These finding suggest that supplementation of antioxidants to male infertility and unexplained infertility (UI) couples may be beneficial for fertilization or natural conception.

Oral supplementation of antioxidants to male partners of couples with male infertility did improve the outcome of ART cycles (Greco *et al.*, 2005a). Furthermore, a randomized clinical trial proved that oral antioxidants supplementation prior to assisted reproduction technology (ART) cycles is beneficial for pregnancy outcome (Tremellen *et al.*, 2007). A few commercial media for sperm preparation also emphasize the benefit of antioxidant addition. However, the investigation for the effect of antioxidant addition into sperm preparation media on sperm function is relatively rare (Tremellen, 2008).

The human spermatozoa have to undergo a physiological process, capacitation, to get the capability for fertilizing oocytes. During capacitation, various cellular changes occurred, including influx of calcium anion in the sperm head and flagellum, generation of limited amounts of ROS, and phosphorylation of proteins on tyrosine resides (Liguori *et al.*, 2005). Subsequent to the set of alternation during capacitation, the spermatozoa undergo acrosome reaction, which hydrolytic enzymes are released to allow spermatozoa to fertilized the oocytes (O'Flaherty *et al.*, 2003; Olds-Clarke, 2003).

Several reports indicated that ROS is a positive trigger of capacitation-related modification (Aitken *et al.*, 1998b; O'Flaherty *et al.*, 2005; de and Lamothe, 2009). A hypothesis has been proposed that a switch of ‘on’ and ‘off’ mechanism of sperm capacitation by ROS production and antioxidant enzymes, such as superoxide dismutase (SOD), in semen (de Lamirande *et al.*, 1997). In addition, Dona et al. reported that sperm ROS content directly influence the level and location of tyrosine phosphorylation pattern and then allow the spermatozoa to undergo acrosome reaction (Dona *et al.*, 2011). Taking together, the ROS content in spermatozoa has a dual role in the process of capacitation or fertilization (Aitken *et al.*, 1998a).

In ART cycles, the spermatozoa are prone to exposure of high levels of ROS due to centrifugation and removal of seminal plasma. Therefore, antioxidants are added into commercial sperm preparation media to decrease the oxidative stress. The modified human tubular fluid (HTF) and/or commercial media are utilized for sperm preparation for ART cycles in worldwide reproductive centers. The exact status of spermatozoa function during preparation process is really our concern. Therefore, we choose two contents, glutathione and taurine, which is abundant in male reproductive tract fluid (Lewis *et al.*, 1997; Donnelly *et al.*, 2000) to measure the effect of antioxidant supplementation on sperm capacitation.

The aim of this study is to elucidate the effect of exogenous antioxidant addition into sperm preparation media on capacitation. The relevance of the redox status in the body and semen to the fertilization rate and embryo development in ART cycles for UI couples was explored. Our data indicated that media with the addition of glutathione, but not taurine, might decrease the percentage of capacitation. However, the carotenoid levels in the body, instead of the total antioxidant capacity in the semen, were correlated to fertilization rates and good embryo rate in ART cycles for UI couples.

Patient selection and semen collection

All of the experimental procedures were approved by the Institution Review Boards of Chung Shan Medical University Hospital, Taichung, Taiwan (CS07162). Informed consents were obtained from unexplained infertile (UI) couples from July, 2010 to June, 2012. Semen samples were obtained from 30 male partners of UI couples for semen analysis to participating in part I study. Another 30 male partners of UI couples undergoing ART cycles were recruited for part II study.

The definition of UI couples is the same as our previous reports (Lee *et al.*, 2010). In brief, the infertile couples with the following characteristics were included: ovulating women with bilateral patent oviducts, the presence of bilateral ovaries, no endocrine disorders (polycystic ovary syndrome or hyperprolactinemia) and a male partner with normal parameters on basic semen analysis. Exclusion criteria were as follows: females >38 years of age; baseline FSH >12 mIU/ml; endometriosis; and uterine synechiae.

Following a period of 3–5 days of sexual abstinence, semen analysis was performed according to the World Health Organization (WHO) guidelines (1999). All semen samples feature normal basic semen analysis (sperm count $>20x10^6$/ml, motility>50% and morphology>14%) and Endtz test $<1.0x10^6$ /ml.

Part I: The effects of antioxidants in preparation media on sperm capacitation

The semen samples were kept at 37^{o}C for liquefaction for an average of 1 h (range 0.5–1.5 h) prior to analyses. One aliquot of liquefied neat semen was used for ROS measurement and analysis of sperm motion characteristics. The residual semen was centrifuged at 650g for 15min.The supernatant seminal plasma was then carefully removed and was frozen at -80^{o}C until total antioxidant capacity (TAC) examination.

Then, the sperm samples were separated into three aliquots and incubated in a variant of preparation media under 37^{o}C, 5%CO_2 for 2 hours. The three different media are as following: 1) HTF with 2g/L pentoxyphyline (HTF+P); 2) HTF+P plus 5 mM glutathione (GSH); and 3) HTF+P plus 5 mM taurine (TAU). Pentoxifylline, which is an inhibitor of phosphodiesterase and improves sperm motility by prompting the cAMP-dependent tyrosine phosphorylation of sperm proteins (Yunes *et al.*, 2005), is utilized to make a capacitating condition for spermatozoa. The washed spermatozoa were also evaluated for ROS levels and analysis of sperm motion characteristics. After two hours of incubation, the spermatozoa were evaluated acrosome reaction rates and tyrosine phosphorylation levels.

Sperm motion characteristics

Sperm motion characteristics were analyzed using computer assisted sperm analysis (CASA; Hamilton Thorne, Inc., MA, USA) and is performed following the guidelines of the European Society of Human Reproduction and Embryology in 1998 (ESHRE Andrology Special Interest Group, 1998). The semen samples were kept at 37^{0}C for liquefaction for an average of 1 h (range 0.5–1.5 h) prior to analyses for motion characteristics. In brief, the setting parameters for analysis included the following: 80 Hz, image-acquisition rate; at least 200 spermatozoa sampled; and at least one microscopic field sampled at x200 magnification. The chamber utilized for sperm analysis was 0.01 mm^2 in surface area and 0.02 mm deep.

The CASA was used to determine various sperm parameters, including concentration, motility, average path velocity (VAP), straight line velocity (VSL), straightness of sperm motion (STR) and the amplitude of lateral head displacement (ALH). Other sperm parameters measured included the percentage of progressive motile spermatozoa, featuring a VAP > 25 μm/s.

Measurement of ROS levels

Levels of ROS were measured by a chemiluminescence assay using luminol (5-amino-2, 3, -dihydro-1,

4-phthalazinedione; Sigma, St. Louis, MO) as a probe. Samples were prepared as 100µl aliquots of $10X10^6$/ml sperm with 2.5 microliters of luminol, prepared as a 5-mM stock in dimethyl sulfoxide (DMSO; Sigma Chemical Co, St Louis, MI, USA). Each sample was scanned by a luminometer (FlexStation 3 Benchtop MultiMode Microplate Reader; Molecular Devices, LLC, USA). All of the samples were measured in duplicate. We further scanned the sperm samples of washed spermatozoa for 180 min to detect the dynamic changes of ROS levels. The ROS values were expressed as relative light units (RLUs).

Measurement of total antioxidant capacity (TAC)

Seminal plasma TAC measurement was done using the antioxidant assay kit (Cayman Chemical, Ann Arbor, MI). The principal of the assay is the ability of aqueous and lipid antioxidants in the seminal plasma to inhibit the oxidation of the 2,20-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTSt. Under the reaction conditions used, the antioxidants in the seminal plasma cause suppression of the absorbance at 750 nm to a degree that is proportional to their concentration. The capacity of the antioxidants present in the sample to prevent ABTS oxidation was compared with that of standard Trolox, a water soluble tocopherol analogue. Results are reported as micromoles of Trolox equivalent. This assay measures the combined antioxidant activities of all its constituents including vitamins, proteins, lipids, glutathione, uric acid, and so forth.

The technique for TAC assay used in our study has been described before (Miller and Rice-Evans, 1997). All seminal plasma samples were diluted 1:10 with the assay buffer before assaying to avoid variability because of interference by the plasma proteins or sample dilution. All reagents and samples were equilibrated to room temperature before beginning the assay. Samples as well as Trolox standards were assayed in duplicate. Trolox standards and reagent were prepared as per the manufacturer's instructions at the time of the assay. After the plate configuration, 10 µL of Trolox standard and samples were loaded on to the corresponding wells of a 96-well plate. Then 10 µL of metmyoglobin and 150 µL of chromogen were added to all standard/sample wells. The reaction was initiated by adding 40 µL of hydrogen peroxide as quickly as possible. The plate was covered and incubated for 5 minutes on a shaker at room temperature. Absorbance was monitored at 750 nm using ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT). Determination of the reaction rate was done by calculating the average absorbance of each standard and sample.

Evaluation of acrosome reaction

Peanut agglutinin (PNA) from Arachis hypogea is specific for b-D-galactose residues, and hence binds to, and labels, the outer acrosomal membrane. Subsequent to incubation of sperm samples for 2 h, sperm acrosome status was assessed by FITC-PNA staining (Sigma) similar to our previous report (Lee *et al.,* 2010). Briefly, 20 ml of sperm suspension was spread over a clean microscope slide and allowed to air-dry. The smear was then fixed in 95% ethanol for 5 min and again allowed to air-dry. Fixed slides were stained in FITC-PNA (600 ml aliquot of FITC-PNA in 15.4 ml of reagent water in a foil-covered Coplin jar) for 15 min at ambient temperature. Slides were rinsed by dipping in PBS twice before fixing for 15 min in paraformaldehyde at ambient temperature. Slides were air-dried, mounted and stored in the dark until scoring.

Between 100 and 250 spermatozoa were counted per slide and scored into three classes for PNA labeling: 1) acrosome intact, where whole acrosome labeling denotes an intact outer acrosomal membrane; 2) partially acrosome reacted, where patchy acrosome labeling is suggestive of a transition stage in which the outer acrosomal membrane is fenestrated; and 3) acrosome reacted, where the equatorial segment only is labeled, thus denoting a normally acrosome-reacted spermatozoa that has lost the outer acrosomal membrane over the anterior cap of the acrosome, but has retained the equatorial segment of the acrosome intact. Only

Class 3 spermatozoa were considered to have undergone an induced acrosome reaction.

Western blot analysis of tyrosine phosphorylation

After the incubation in capacitating condition, proteins from spermatozoa were analyzed by sodium dodecyl sulphate (SDS)±polyacrylamide gel electrophoresis and western immunoblot analysis. Briefly, samples were resuspended in Laemmli's sample buffer (final concentrations: 2% SDS, 10% glycerol, 5% b-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8) and heated at 100^{o}C for 5 min. Proteins were then separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Towbin *et al.*, 1979). Nonspecific binding sites on the membrane were blocked with 5% (w:v) non-fat milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6). The nitrocellulose membrane (0.22-mm pore size; Micron Separations Inc., Westboro, MA) was incubated for overnight at 4^{o}C with the Monoclonal anti-phosphotyrosine antibody (1/1000) (clone 4G10; Upstate Technology Inc., Lake Placid, NY). Blots were then incubated with a horseradish peroxidase goat anti-mouse IgG (Kirkegaard and Perry Lab., Gaithersburg, MD) for 1 h. Afterwards, signal was detected by using enhanced chemiluminescence (ECL) commercial kit (Amersham Biosciences) and relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corporation, San Leandro, CA, USA).

Part II. The association of redox status of male partners with fertilization and embryo development in ART cycles

A total of 30 UI couples undergoing ART cycles were recruited for this study; however, 5 male partners refused skin carotenoid status measurement by resonance Raman spectroscopy. As a result, 25 male partners of UI couples completed the evaluation process and provided the data for analysis. All the patients received long protocol of GnRH agonist administration for IVF treatment, which was similar to that described previously (Lee *et al.*, 2008).

We measured ROS and TAC levels for neat semen and ROS levels for washed spermatozoa with HTF+P for preparation media as described in the part I study. To evaluate the whole body antioxidant status of the male partner, the skin carotenoid levels was evaluated by resonance Raman spectroscopy (RRS). Such parameters were utilized to represent the redox status of male partners of UI couples. The relationship between those redox markers and sperm motion characteristics, fertilization rates, and good embryo rates on day 3 in ART cycles were explored.

Assessment of skin carotenoid status

Details of RRS instrumentation have been reported elsewhere (Ermakov *et al.*, 2001) with minor modifications in this study. Briefly, the instrument uses a compact, portable, continuous wave solid-state laser operating at a wavelength of 488 nm to shine blue visible light onto a tissue of interest (that is, the palm of patients). This laser is based on a frequency double near-infrared semiconductor diode laser. The spectrograph is equipped with a linear charge coupled device array operating at room temperature. The array was interfaced to a laptop computer for data acquisition, processing and display. At a power of 0.2 W/cm2 and an elliptical laser spot size of 2.5 × 1.5 mm, it is safe to expose skin to the laser light for 30h. The light exposure is ~1000 times less than the exposure limit set by the ANSI z136.1-2007 standard, which assures that the instrument was safe for use in human. The palm of each patient was scanned two times for reliability at an exposure time of 30 s per scan, and scans were performed immediately before the semen collection on the day of ovum pick up for female partners of UI couples. Mean RRS values for total carotenoid status, measured as skin Raman intensity, were obtained to provide an assessment of total skin carotenoid status for each patient.

Statistical analysis

The sperm motion characteristics, intensity of western immunoblot for tyrosine phosphorylation and acrosome reaction rate were subjected to Wilcoxon singed ranks test to evaluate the significance of the results compared to the HTF+P group. Spearman correlation coefficient was utilized to evaluate the relationship between the redox index to sperm motion characteristics, fertilization rates, and good embryo rates in ART cycles. A confidence level of $p < 0.05$ was considered to constitute the limit of statistical significance.

Part I. The effects of antioxidants in preparation media on sperm capacitation

Thirty semen samples were collected from male partners of couples with UI for semen analysis. The results of basic semen analysis, sperm motion characteristics, and ROS levels of neat semen and washed spermatozoa of the 30 patients are demonstrated in Table 1.

Subsequent to the basic semen analysis and CASA, the 30 semen samples were divided into 3 groups and treated with HTF+P, GSH, and TAU. Table 2 shows that the motility [median (interquartile range): 76.1% (58.9~89.2) vs. 68.7% (38.2~88.4), p=0.014 by Wilcoxon signed-rank test] and ALH [5.0 μM (3.6~6.4) vs. 3.8 μM (0~5.4), p=0.018 by Wilcoxon signed-rank test] decreased with the addition of GSH under capacitating conditions (the addition of pentoxifylline in the current study).

The ROS levels of washed spermatozoa with various sperm preparation media were observed for 180 min (Figure 1). The ROS levels of spermatozoa were lower in GSH media than HTF+P media throughout the observation period. The GSH treatment was associated with reduced ROS levels of spermatozoa under capacitating conditions.

The results of tyrosine phosphorylation by Western immunoblot and acrosome reaction by immnunostaining revealed similar findings to the semen motion characteristics. The addition of GSH under capacitating conditions decreased the levels of tyrosine phosphorylation [median (interquartiles): 0.87 (0.72~0.97) vs. 1, p=0.002; Figure 2B] and acrosome reaction [39.0% (27.0~69.2) vs. 52.1% (34.0~74.2), p=0.024; Figure 2D] compared to the HTF+P group.

Part II. The association of the redox index of male partners in couples with UI and fertilization and embryo development in IVF cycles

Thirty couples with UI undergoing IVF treatment completed the investigation measurements in this part of study. The skin carotenoid status, TAC in seminal plasma, neat semen ROS levels, and washed spermatozoa ROS levels of the male partners in couples with UI were measured as the redox status of the male partners. Table 3 shows the demographic and redox status data of the male partners. Washed spermatozoa had higher levels of ALH [4.3 μM (2.9~5.5) vs. 1.9 μM (1.4~3.3), p=0.001 by Wilcoxon signed-rank test] and ROS [17520 RLU (12873~26561) vs. 4110 RLU (2270~7180), p<0.001 by Wilcoxon signed-rank test] than neat semen.

When we reviewed the Spearman correlation coefficient among the four redox indices and sperm motion characteristics of neat semen and washed spermatozoa, the only significant association was between the ROS levels and ALH of washed spermatozoa (rho= 0.496, p=0.012; Table 4). Furthermore, none of the four redox indices were significantly correlated with the motion characteristics in pre-washed semen (data not shown).

The relationship between the four redox indices of the male partners with the fertilization rates and embryo development in ART cycles showed that the skin Raman intensity rather than the ROS levels in neat semen or washed spermatozoa was significantly correlated with normal fertilization rates (rho= 0.46, 95% CI = 0.07~0.72, p=0.02; Figure3) and good embryo rates (rho= 0.61, 95% CI = 0.27~0.81, p=0.002; Figure 3).

討論

The results of this study indicate that sperm capacitation is related to sperm ROS levels. The addition of antioxidants to preparation media decreases the amount of ROS during capacitation, but also decrease the motile proportion, the tyrosine phosphorylation levels and the percentage of acrosome reaction in washed spermatozoa. In the setting of ART cycles, the washed spermatozoa ROS levels are correlated to ALH (a surrogate marker for hyperactivation); however, the sperm ROS or seminal TAC levels are not relevant to fertilization rates nor good embryo rates in those cycles. By contrast, the skin Raman intensity in male partners are correlated to the fertilization rates and the good embryo rates in ART cycles for UI couples.

During sperm capacitation, ROS plays an important role for signal transduction (de Lamirande (de and Lamothe, 2009). It has been demonstrated that ROS production last for hours in spermatozoa incubated in capacitating condition (de Lamirande (de and Lamothe, 2009). The addition of antioxidant to sperm preparation media may interfere the production of ROS during the capacitating condition. The ROS levels of spermatozoa incubated in GSH media are persistently lower than those in HTF+P media in the present study. The ROS patterns of spermatozoa in HTF+P and GSH media in this study are similar to those patterns of spermatozoa in PSW (PureSperm washed) and AA (ascorbic acid) media, respectively, in a previous report (Dona *et al.*, 2011). The previous report indicates that if the threshold of ROS production is not achieved, spermatozoa may not ensure correct cell function (Dona *et al.*, 2011).

An abundance of antioxidants were discovered in reproductive tract fluid and seminal plasma, including glutathione and taurine (Lewis *et al.*, 1997; Donnelly *et al.*, 2000). Although high levels of glutathione are found in the testis of rats, much lower levels are found in the ejaculate (Donnelly *et al.*, 2000). Other antioxidants, such as ascorbate, are much more abundant in seminal plasma (Lewis *et al.*, 1997). According to the results of this study, the taurine did not affect the capacitation process. By contrast, the addition of glutathione into sperm preparation media did reduce the levels of ROS and capacitation of spermatozoa. This suggests that some kind of antioxidants may interfere the sperm capacitation if they significantly reduce the ROS levels of spermatozoa in capacitating condition. The levels of antioxidants have to fit the needs of spermatozoa, otherwise, some unexpected adverse effect may be resulted (Menezo *et al.*, 2007; Chi *et al.*, 2008).

In this study, the parameters regarding to sperm motion characteristics, including motility and ALH (as a surrogate marker of hyperactivation), decreased after incubation in capacitating condition with antioxidant addition (glutathione but not taurine). This is different from the report that hyperactivation is not affected by adding ROS inducing agent (NADPH) or antioxidants (ascorbic acid) into sperm preparation media (Aitken *et al.*, 1998b; Dona *et al.*, 2011). Nonetheless, the effect of glutathione on spermatozoa incubating in capacitation condition is consistent with the effect of ascorbic acid as a reduction of tyrosine phosphorylation (Dona *et al.*, 2011). The difference may result from the different formula of antioxidants (glutathione vs. taurine vs. ascorbic acid). Another possible explanation is that we used ALH in a counting chamber with 20μM in depth and did not utilize the formal definition of hyperactivation (ALH>7,) in a counting chamber with 30μM in depth. However, the 25% percentile of ALH in GSH group decreased to 0 means that the lateral head movement spermatozoa did affected by glutathione supplementation. These varieties further suggest that sperm capacitation is a redox - regulated and complex events.

The addition of glutathione in sperm preparation media is able to decrease the tyrosine phosphorylation and acrosome reaction levels in the present study. In some ART settings, the cumulus cells

and spermatozoa were removed 2-3 hrs after insemination. The effects of reducing ROS levels and sperm capacitation by antioxidants in sperm preparation media might bring significant impact on fertilization and subsequent embryo development. Therefore, we executed the second part of investigation to define the relevance of the redox status of male partners of UI couples to fertilization and embryo development in ART cycles.

The second part of investigation is consistent with the first part that the ROS levels of washed spermatozoa, but not those of neat semen, are significantly relevant to ALH. However, the sperm ROS levels did not correlate with the fertilization rates or good embryo rates in ART cycles for UI couples. In the first part of study, although the amount of capacitated spermatozoa decreased at a level of 10%, the amount of progressive motile spermatozoa is still substantially enough for fertilization. We speculated that the decreased amount of ROS levels by physiological levels of antioxidants (5mM glutathione) in sperm preparation media might not interfere fertilization or embryo development process in ART cycles for UI couples. Nevertheless, the sample size of the present study is small, further studies in large cohorts are needed to confirm the findings.

Carotenoids are plant pigments resulting in red, orange and yellow coloring. They are classified as antioxidants and their contents in the body/tissue are the best current biomarkers for fruit and vegetable intake. Biochemical analysis, like high performance liquid chromatography, can be used to measure carotenoids in plasma or tissues. However, resonance Raman spectroscopy (RRS) is a less expensive, non-invasive and rapid method to assess carotenoid status for estimating fruit and vegetable intake (Mayne *et al.*, 2010; Scarmo *et al.*, 2012). It has been proved that RRS is a valid way to measure carotenoid status in health adult population (Scarmo *et al.*, 2010).

The antioxidant status within the human body (skin carotenoid status), but not TAC in seminal plasma, in this study is related to fertilization rate and good embryo rates in ART cycles. It has been reported that the most ROS-mediated damage of spermatozoa occurred during epididymal storage (Greco *et al.*, 2005b). During spermatogenesis and epididymal storage, the spermatozoa are not in contact with the antioxidants in seminal plasma. This may explain that the seminal TAC is not directly correlated with fertilization rates or good embryo rates in ART cycles for UI couples. Furthermore, nuclear remodeling process during late spermatogenesis rather than apoptosis is a cause of ROS-induced DNA damage (Moustafa *et al.*, 2004). The deficient protamination for sperm DNA package in infertile men lead to vulnerability of sperm DNA to ROS attack (Oliva, 2006). Taking together, we suggest that the insufficient amount of antioxidants during spermatogenesis, instead of those during in vitro sperm preparation, may be the major cause of oxidative stress related spermatozoa damage, especially for UI couples. Such results further supported the idea that oral supplementation of antioxidants is beneficial for sperm function, which may reduce the ROS-related damage of spermatozoa prior to ejaculation (Tremellen, 2008). Consequently, oral supplement of antioxidants may improve the fertilization rate and probably the pregnancy outcome in ART cycles.

In conclusion, the addition of antioxidants, like glutathione, into sperm preparation media may reduce the level of ROS and, in the mean time, the level of sperm capacitation, such as tyrosine phosphorylation and acrosome reaction. Nonetheless, the ROS levels in washed spermatozoa did not demonstrate significant connection with fertilization rate or embryo development in ART cycles for UI couples. By contrast, the skin carotenoid status, instead TAC in seminal plasma, of male partners of UI couples is closely related to fertilization and embryo development in ART cycles. The protecting action of antioxidants from ROS-related damage of spermatozoa may be more prominent in male reproductive tract than in ejaculated semen.

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Table I

Sperm motion characteristics and levels of reactive oxygen species (ROS) in the semen samples from 30 male partners of couples with unexplained infertility. The data are presented as the median (interquartile range). The TAC and RLU denote total antioxidant capacity and relative light units, respectively. The units for TAC are micromoles of Trolox equivalents.

Data (n=30)	Median	(25% ~ 75% range)
Concentration (M/ml)	86.8	49.2~172.9
Motility (%)	77.3	66.1 ~83.5
Progressive motility (%)	37.8	28.4~58.7
VAP (μm/s)	28.4	25.5~35.5
VSL (μm/s)	19.5	16.1~22.4
ALH (μm)	1.7	1.0~3.0
Neat semen ROS (RLU)	5482	3950~10106
Washed spermatozoa ROS (RLU)	19870	11570~29573
Seminal plasma ROS (RLU)	3254	2642~5246
Seminal plasma TAC	113	79~175

Table II

The sperm motion characteristics of the washed spermatozoa incubated with capacitating condition. The data are presented as the median (interquartile range). HTF+P denotes human tubular fluid media with the addition of pentoxyphylline as a capacitating condition for spermatozoa. GSH and TAU denote the further addition of 5 mM glutathione and 5mM taurine into HTF+P media, respectively.

n=30	HTF+P	GSH	TAU
Motility (%)	76.1 (58.9~89.2)[a]	68.7 (38.2~88.4)[a]	71.7 (57.1~88.6)
Progressive motility (%)	44.0 (23.7~54.9)	35.5 (25.0~56.7)	47.4 (27.9~58.3)
VAP (μm/s)	38.1 (30.8~45.1)	37.1 (32.6~43.7)	33.2 (42.9 ~50.3)
VSL (μm/s)	17.9 (15.4~25.5)	17.4 (15.1~23.1)	18.7 (15.9~25.7)
ALH (μm)	5.0 (3.6~6.4)[b]	3.8 (0~5.4)[b]	4.9 (0.7~6.5)

a P=0.014, b P= 0.018 by Wilcoxon signed-rank test

Table III

Demographic data for unexplained infertility couples (n=30) undergoing ART cycles, including sperm motion characteristics and levels of reactive oxygen species (ROS). The parameters for neat semen and washed spermatozoa are presented as the median (interquartile range). The TAC and RLU denote total antioxidant capacity and relative light units, respectively. The units for TAC are micromoles of Trolox equivalents.

N=30	Pre-washed	Post-washed
Age (Years)	34 (30-38)	----
Concentration (M/ml)	114 (61-148)	----
Morphology (%)	18 (15.5~24.5)	----
Motility (%)	78 (68.5-87)	80 (45.5-87.5)
VAP (μm/s)	35 (25.5-42)	43 (34.5-48.5)
VSL (μm/s)	27 (20.5-33.5)	27 (19.5-32)
ALH (μm)	1.9 (1.4-3.3)[a]	4.3 (2.9-5.5)[a]
Semen ROS (RLU)	4110 (2270-7180)[b]	17520 (12873-26561)[b]
Seminal plasma TAC	109 (86-153)	---
Skin Raman intensity [c]	21000 (16250-26000)	---

a P= 0.001 , b: P<0.001 by Wilcoxon signed-rank test

c. The value detected by resonance Raman spectroscopy

Table IV

The relevance of the redox index of the male partner of couples with unexplained infertility (n=30) to sperm motion characteristics of post-washed spermatozoa. The TAC and ROS denote total antioxidant capacity and reactive oxygen species, respectively.

Spearman correlation coefficient	Neat semen (Pre-washed) ROS	Post-washed spermatozoa ROS	Seminal plasma TAC	Skin carotenoid status
Motility	-0.382	0.041	0.281	-0.080
VAP	-0.003	0.032	-0.089	-0.218
VSL	-0.244	-0.232	-0.200	-0.188
ALH	0.047	0.496[a]	0.155	-0.199
STR	-0.336	-0.221	-0.109	-0.104

[a] p=0.012

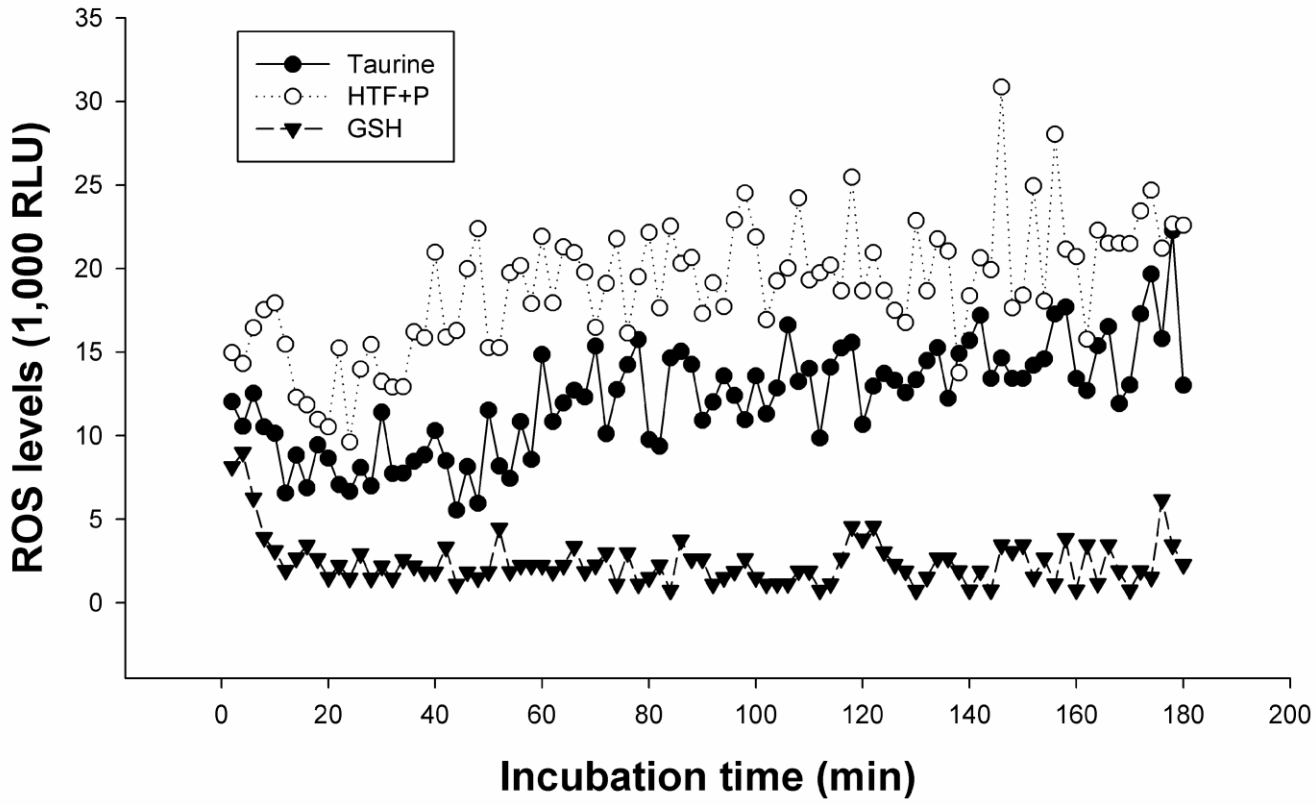

Figure 1.

The dynamic pattern of reactive oxygen species (ROS) levels for washed spermatozoa in capacitating conditions (HTF with pentoxyphylline [HTF+P]). The units for ROS are relative light units (RLU). The GSH and taurine group denote addition of 5 mM glutathione (GSH) and 5 mM taurine to HTF+P media, respectively.

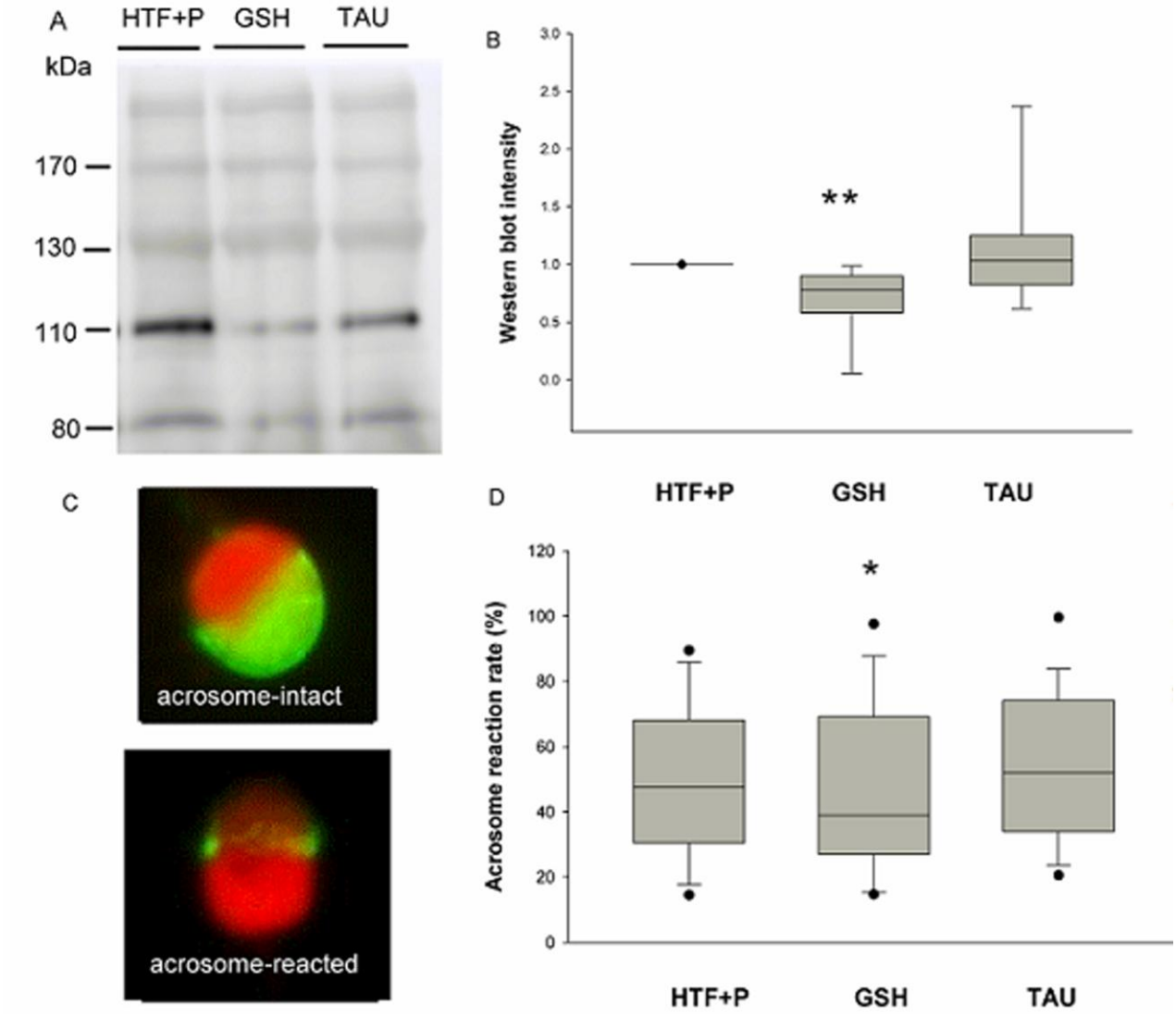

Figure 2.

Western blot analysis for tyrosine phosphorylation (A, B) and acrosome reaction rates (C, D) of spermatozoa incubated in various sperm preparation media. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, compared to spermatozoa in capacitating conditions (HTF plus pentoxyphylline [HTF+P]) by Wilcoxon signed-rank test. The GSH and TAU denote 5 mM glutathione and 5 mM taurine into HTF+P media, respectively. The horizontal line within the boxes, the box margins, and the error bar outside the boxes represent the median, 25th-75th percentile, and 5th-95th percentile of the acrosome reaction rates for the corresponding group, respectively.

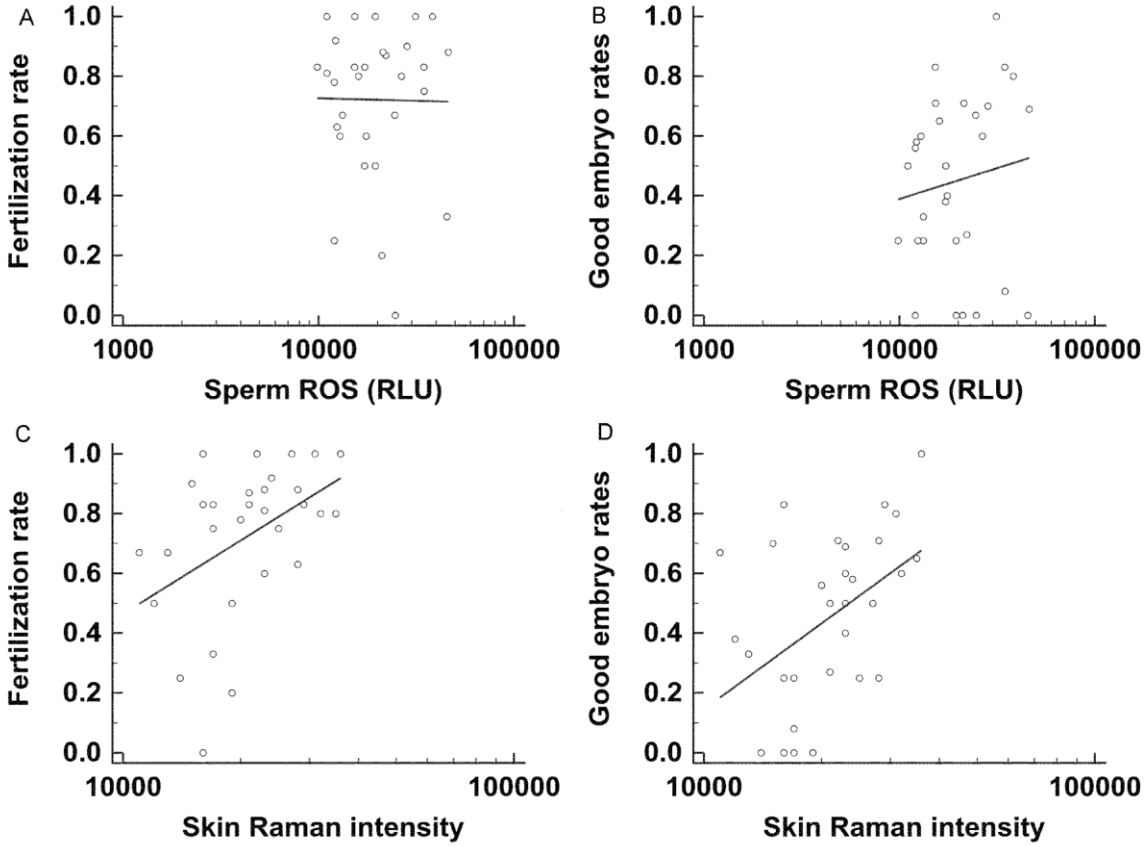

Figure 3.

The relevance of the redox index of the male partner in couples with unexplained infertility to the fertilization and day 3 good embryo rates. Skin Raman intensity is the value detected by resonance Raman spectroscopy and sperm ROS denotes reactive oxygen species levels of washed spermatozoa. The Spearman correlation coefficients are rho= 0.46 (p=0.02) and rho= 0.61 (p=0.002) in C and D, respectively.

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■ 達成目標

□ 未達成目標（請說明，以 100 字為限）

□ 實驗失敗

□ 因故實驗中斷

□ 其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 □無

技轉：□已技轉 □洽談中 □無

其他：（以 100 字為限）此三年計劃之內容較多，部份資料已經先行發表於 SCI 期刊，另外還有撰寫中的論文資料。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

研究結果指出精子活化過程中，需要氧化自由基的作用。此時給予過多的抗氧化物，可能對精子的活化有所影響，如果是期望在 2-3 小時內完成精卵受精的過程，則不適合加入過多的抗氧化物。而氧化自由基雖然對染色質有所影響，影響的結果在於受精後胚胎的發育。研究成果指出試管嬰兒療程中，氧化自由基是精子活化所必須，在精子活化階段加入過多的抗氧化物並沒有太多的益處，但是後續胚胎體外培養過程中，加入抗氧化物並降低氧化自由基的含量，則是有助於胚胎的發育。這些結果可用來增進不孕症實驗室的品質管制，也可改善體外受精及相關培養液的改良，以增進試管嬰兒療程的成功率。

國科會補助計畫衍生研發成果推廣資料表

日期:2013/10/27

國科會補助計畫	計畫名稱: 氧化自由基及抗氧化物質於人類精子活化與染色質損壞的角色
	計畫主持人: 李宗賢
	計畫編號: 99-2314-B-040-009-MY3　　學門領域: 婦產科

無研發成果推廣資料

99 年度專題研究計畫研究成果彙整表

計畫主持人：李宗賢	計畫編號：99-2314-B-040-009-MY3
計畫名稱：氧化自由基及抗氧化物質於人類精子活化與染色質損壞的角色	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	1	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	2	2	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	2	2	100%		
國外	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

此三年計劃之內容較多，部份資料已經先行發表於 SCI 期刊，另外還有撰寫中的論文資料。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

研究結果指出精子活化過程中，需要氧化自由基的作用。此時給予過多的抗氧化物，可能對精子的活化有所影響，如果是期望在 2-3 小時內完成精卵受精的過程，則不適合加入過多的抗氧化物。而氧化自由基雖然對染色質有所影響，影響的結果在於受精後胚胎的發育。研究成果指出試管嬰兒療程中，氧化自由基是精子活化所必須，在精子活化階段加入過多的抗氧化物並沒有太多的益處，但是後續胚胎體外培養過程中，加入抗氧化物並降低氧化自由基的含量，則是有助於胚胎的發育。這些結果可用來增進不孕症實驗室的品質管制，也可改善體外受精及相關培養液的改良，以增進試管嬰兒療程的成功率。