Toona Sinensis Affects Reproductive Physiology of Male

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ABSTRACT

One of the major causes of the decline in semen quality appears to be the elevated levels of oxidative stress. Leaves of Toona Sinensis (TSL) have been used as a traditional medicine for treatment of certain male reproduction-related disorders in Chinese ancient medical books; however, the scientific data are limited. In the present study, human spermatozoa treated with hydrogen peroxide (H_2O_2) were used to investigate the protective function of TSL-6 under oxidative stress. The intracellular reactive oxygen species (ROS) levels of human spermatozoa were increased after 0.2 mmol/L H_2O_2 treatment and the elevated levels were diminished by TSL-6 dose dependently. The protection of TSL-6 against oxidative stress in human spermatozoa was further substantiated by increasing sperm motility assay including computer assisted sperm analysis (CASA), percentage of high MMP cells, ATP production, percentage of double strand DNA cells, and decreasing cell death. Our results suggested that TSL-6 improved functions of spermatozoa under oxidative stress *via* decreasing ROS level and cell death. In addition, TSL-6 increased sperm motility, MMP, ATP levels, and maintained the integrity of chromatin structure. TSL-6 is beneficial for human sperm and is potentially developed into functional foods for infertile men under oxidative stress.

Key words: *Toona sinensis* Roem, male infertility, human spermatozoa, oxidative stress

INTRODUCTION

Male infertility accounts for 40 - 50% of infertility and is commonly due to deficiencies in the semen quality. One of the major causes of the decline in semen quality appears to be the elevated levels of oxidative stress. *Toona sinensis* Roem (TS), a widely distributed arbor in Asia, is a nutritious food in Chinese society and a popular vegetarian cuisine in Taiwan. Leaves of TS (TSL) have been used as an oriental medicine for treatment of certain male reproduction-related disorders[(1)]. However, the scientific evidences are limited. Recently, TSL provides novel functions including strong DPPH radical scavenging activities and inhibitory effects on lipid peroxidation[(2)], protection against hydrogen-peroxide- induced oxidative stress and DNA damage in MDCK cells[(3)]. Our previous findings suggested that TSL-6, one of TSL extracts, exhibited antioxidant effects at both low and high concentrations. In this study, we elucidated that TSL-6 protected human spermatozoon from oxidative stress via the decreasing the intracellular ROS levels, increasing sperm motility, elevating the percentage of high MMP cells and production of ATP, and maintaining chromatin integrity and repressing the cell death.

MATERIALS AND METHODS

I. *Materials*

Acridine orange (AO), 2'-7'-Dichlorofluorescin diacetate (DCFH2-DA), Ethidium bromide (EtBr), Ethylene Glycol-bis-N,N,N',N'-tetraacetic acid (EGTA), Propidium iodide (PI), and Triton X-100 were purchased from Sigma (MO, USA). 3-3'-Dihexyloxacarbocyanine (DiOC6) was purchased from Fluka (Steinheim, Switzerland). Citric acid and Dimethyl sulfoxide (DMSO) were obtained from J. T Baker (NJ, USA). Ethylenediaminetetraacetic acid disodium salt (EDTA) was obtained from Boehringer (Mannheim GmbH, Germany). Human tubal fluid (HTF) and synthetic serum substitute (SSS) were obtained from Irvine Scientific (CA, USA). Hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), and sodium dihydrogen phosphate (Na_2HPO_4) were purchased from Katayama Chemical Co., Ltd. (Osaka, Japan). Potassium chloride (KCl) and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Showa (Tokyo, Japan). PureSperm was from Nidacon International AB (Gothenburg, Sweden). Quantitative ATP monitoring kit was from Thermo Lybsystems (Helsinki, Finland). Sodium chloride (NaCl) was purchased from Riedel-de Haën (Seelze, Germany).

II. *Preparation of TSL Extracts*

Crude TSL extracted powder (TSL-1) was purchased from Taiwan Toona Biotech Corporation (Kaohsiung, Taiwan). The TSL-1 powder was then dissolved in 99.5%

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# Equal Contribution

ethanol and centrifuged at 3000 rpm at 4°C for 12 min. The pellet (TSL-2P) was lyophilized and further dissolved in serial ethanol for the serial extractions to obtain TSL-6.

III. *Semen Samples*

The study was approved by the institutional review board of the National Science Council. Semen samples (n = 178) were collected from TUBE Fertility Clinic (Tainan, Taiwan) and the semen quality (concentration, motility, and morphology) was measured. The semen samples confirmed to normozoospermia criteria of WHO were used as experimental materials in the following studies[4]. In addition, all semen samples were allowed to liquefy at least 1 h before each experiment.

IV. *Sperm Preparation*

Human spermatozoa were isolated by discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient separation (80 and 40% layers) using HTF. After an initial 30-min period of centrifugation at 2000 rpm, the population of 80% layer (live sperm) was collected and the population of 40% layer (immature or dead sperm) was discarded. The collected live sperms were centrifuged at 2000 rpm for 10 min (to remove PureSperm) and resuspended in 1 mL HTF containing 3% SSS[5]. Aliquots of live sperms (2×10^6 sperms/mL) were treated H_2O_2 for 3 h as a negative control and then treated with different concentration of TSL-6 (0.01 - 1 mg/mL) for 3 h. These sperm sample were used for the assessment of following indicators.

V. *Flow Cytometry*

Human spermatozoa (2×10^6 sperms/mL) were incubated with different fluorescent dye at optimal concentration and were analyzed on a COULTER flow cytometer (Epics XL-MCL, Coulter, Miami, FL, USA). The intracellular ROS was measured by detecting intracellular $DCFH_2$-DA oxidation in spermatozoa adapted from van Reyk[6]. $DiOC_6$ can bind to mitochondrial inner membrane and was used for quantitative measurement of MMP[7]. Sperm samples were labeled with propidium iodide stain which binds to DNA in dead cells and can therefore commonly be used for recording membrane integrity as well as labeling dead cells[8].

VI. *Sperm Motility*

The motility characteristics of spermatozoa were assessed on the Computer Assisted Sperm Analysis (CASA) using Hamilton-Thorn analyzer (Version 10, HTM-IVOS, Hamilton-Thorn Research, Beverly, MA, USA). For each measurement, a 10-μL aliquot was loaded on a Makler chamber and analyzed the average path velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL). Instrument settings for the CASA analysis were as follows: Apply Sort: 0; Frames Acquired: 30; Frame Rate: 60 Hz; Minimum Contrast: 80; Minimum Cell size: 3 Pixels; Minimum Static Contrast: 30; Straightness (STR) Threshold: 80%; Low VAP cutoff: 5 μm/s; Medium VAP cutoff: 25 μm/s; Low VSL cutoff: 11 μm/s.

VII. *ATP Production*

The ATP assay is based upon the quantitative measurement of a stable level of light produced as a result of an enzyme reaction catalyzed by firefly luciferase. The amount of light generated by this enzymatic reaction is then measured in a luminometer and is directly related to the amount of ATP in the sample. Briefly, ATP was extracted from spermatozoa (2×10^6 sperm/ mL) using Triton X-100 (0.2%) containing EDTA (4 mmol/L). The samples (100 μL) were diluted with Tris-acetate buffer (0.1 mol/L; pH 7.75) (700 μL) and ATP monitoring reagent (200 μL) and assayed using the firefly luciferase bioluminescent assay kit (Labsystems, Helsinki, Finland).

VIII. *Sperm Chromatin Structure Assay*

The method of sperm chromatin structure assay (SCSA) was adopted from Evenson and colleagues[9]. In brief, every 0.20-mL aliquot of sperm (2×10^6 sperm/mL) samples was mixed with 0.40-mL of acid-detergent solution (0.08 mol/L HCl, 0.15 mol/L NaCl, 0.1% Triton X-100, pH 1.2). After 30 s, the cells were stained by 1.2 mL acridine orange (AO) stain solution containing 6 mg AO (chromatographically purified; Polysciences Inc., Warrington, PA, USA) per mL buffer [0.037 mmol/L citric acid, 0.126 mmol/L Na_2HPO_4, 0.0011 mmol/L EDTA (di-sodium), 0.15 mmol/L NaCl, pH 6.0]. Three minutes after the staining, fluorescence intensity was collected on a flow cytometer with an excitation wavelength of 488 nm and emission wavelength of 525/630 nm, respectively. The red fluorescence represents the binding of AO with single strand DNA[10] while the green fluorescence depicts the binding of AO with double strand DNA.

IX. *Statistics*

Data were analyzed using the statistics software package SAS (version 8e; SAS Inst. Inc., Cary, NC, USA). Duncan's test was used to perform the analysis of variance on the various experimental groups. The Pearson correlation analysis was performed (CORR procedure) to investigate the relationship between different experimental groups. Unless otherwise stated, data are presented as means ± S.E. Differences were considered to be significant if the calculated probability of their occurring by chance was less than 5% ($p < 0.05$).

Table 1. The Characteristics of human seminal parameters

	n	Range	Mean ± SE
Concentration ($\times 10^6$ sperms/mL)	178	28 - 147	81.4 ± 13.3
Motility (%)	178	50 - 85	65.3 ± 2.7
Normal morphology (%)	178	30 - 41	34.1 ± 1.3

RESULTS AND DISCUSSION

I. *TSL-6 Decreased the Oxidative Stress in Human Spermatozoa*

The semen quality showed that all semen samples were confirmed to the seminal parameters of normozoospermia[4] (Table 1). Here, we used H_2O_2 as the oxidative stress to investigate the protective function of TSL-6 for human spermatozoa. The intracellular ROS levels of human spermatozoa were increased after 0.2 mmol/L H_2O_2 treatment for 3 h; however, the elevated levels of intracellular ROS were diminished dose dependently by TSL-6 treatment (Figure 1).

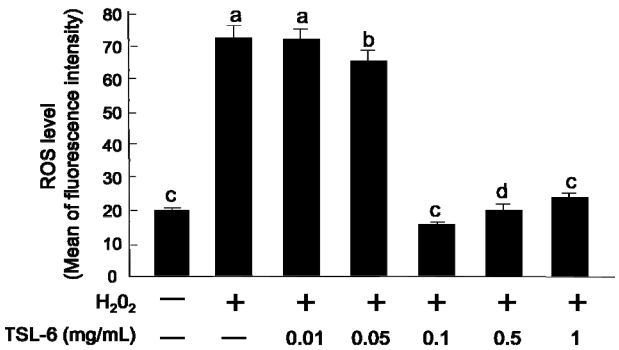

Figure 1. Effects of TSL-6 on intracellular ROS levels of human spermatozoa treated with H_2O_2. The intracellular ROS levels of human spermatozoa were measured in human spermatozoa treated with or without 0.2 mmol/L H_2O_2, followed by incubation with various concentrations of TSL-6 for 3h. Each bar represents Mean ± S.E. (n = 7). Means with different superscript letters are significantly different from each other ($p < 0.01$) as determined by Duncan's test.

II. *TSL-6 Restores the Motility of Human Spermatozoa Under Oxidative Stress*

The CASA revealed that VAP, VSL, and VCL values of human spermatozoa treated with H_2O_2 were significantly decreased and were reversed dose-dependently by TSL-6 treatment (Figure 2A-C).

III. *TSL-6 Increases the Percentage of High MMP Cells and Production of ATP in the Human Spermatozoa Under Oxidative Stress*

Studies showed that the levels of ATP and MMP are responsible for sperm motility[11]. Treating human spermatozoa with H_2O_2 resulted in the decreased percentage of the high MMP cells was also reversed by TSL-6 treatment (Figure 3A), indicating that TSL-6 alleviated H_2O_2-induced damage on mitochondria. Moreover, we found that decrease of ATP levels in human spermatozoa with H_2O_2 treatment was reversed by TSL-6 treatment (Figure 3B).

IV. *TSL-6 Maintains the Chromatin Integrity in Human Spermatozoa Under Oxidative Stress*

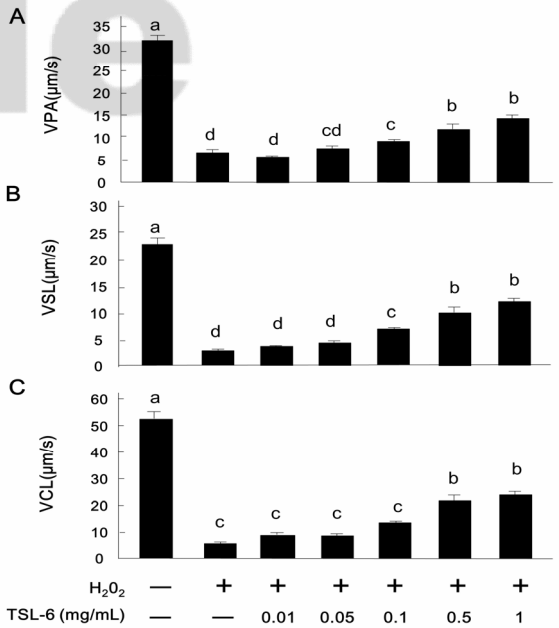

Figure 2. Effects of TSL-6 on the motility of human spermatozoa treated with H_2O_2 measured by CASA. The VAP (A), VSL (B), VCL (C) of human spermatozoa were measured in human spermatozoa treated with or without 0.2 mmol/L H_2O_2, followed by incubation with various concentrations of TSL-6 for 3 h. Each bar represents Mean ± S.E. (n = 7). Means with different superscript letters are significantly different from each other ($p < 0.01$) as determined by Duncan's test.

The results of SCSA suggested that reduced levels of double strand DNA by H_2O_2 was reversed by TSL-6 treatment in a dose dependent manner (Figure 4A). Meanwhile, the increased levels of single strand DNA in H_2O_2 treated human spermatozoa were reduced by treating TSL-6 (Figure 4B). In addition, percent of cells with single strand DNA (denatured cell) suggested that H_2O_2 contributed to the increased levels of denatured cells and that was reversed by TSL-6 treatment (Figure 4C).

V. *TSL-6 reverses H_2O_2-induced cytotoxicity of human spermatozoa*

To investigate the anti-cytotoxic effect of TSL-6, human spermatozoa treated with H_2O_2 were co-incubated with various concentrations of TSL-6 and revealed that H_2O_2-induced cell death was diminished by TSL-6 treatment at high concentration (0.5 or 1 mg/mL) by flow cytometry (Figure 5).

The most important finding of this study is that TSL-6 protects human spermatozoa against oxidative stress *via* the decreasing intracellular ROS levels, increasing sperm motility, MMP levels, and ATP production, reducing the percent of cell death, and maintaining chromatin structure. A majority of antioxidants and its co-activators such as vitamin C and vitamin E, were utilized for the prevention or treatment of male infertility[12]. The antioxidant functions of crude TSL extracts were demonstrated as

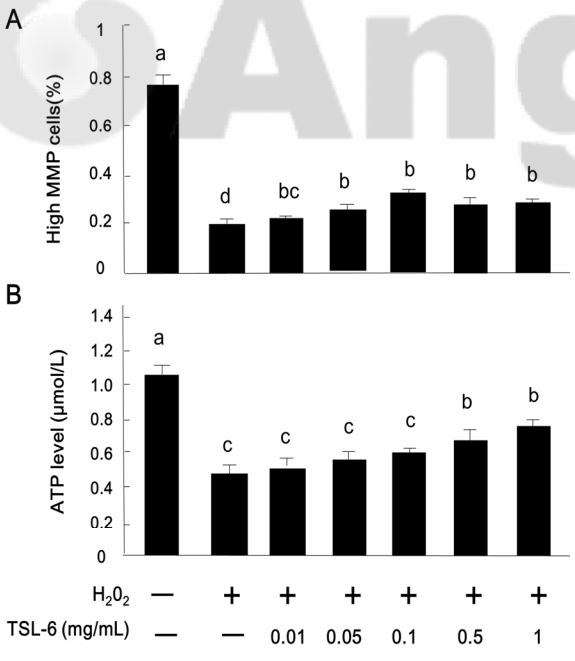

Figure 3. Effect of TSL-6 on the mitochondrial functions, MMP and ATP production, in human spermatozoa treated with 0.2 mmol/L H_2O_2. The high MMP cells (A) and ATP production (B) were measured in human spermatozoa treated with or without 0.2 mmol/L H_2O_2, followed by incubation with various concentrations of TSL-6 for 3 h. Each bar represents Mean ± SE (n = 7). Means with different superscript letters are significantly different from each other ($p < 0.01$) as determined by Duncan's test.

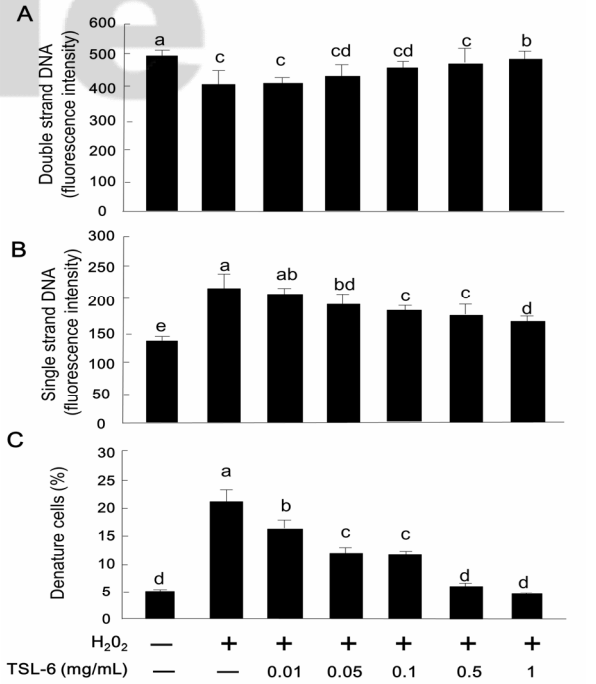

Figure 4. Effects of TSL-6 on the chromatin integrity of human spermatozoa treated with H_2O_2. The double strand DNA (A), single strand DNA (B), and percent of denatured cells (C) of human spermatozoa were measured by flow cytometry in human spermatozoa treated with or without 0.2 mmol/L H_2O_2, followed by incubation with various concentrations of TSL-6 for 3 h. Each bar represents Mean ± S.E. (n = 7). Means with different superscript letters are significantly different from each other ($p < 0.01$) as determined by Duncan's test.

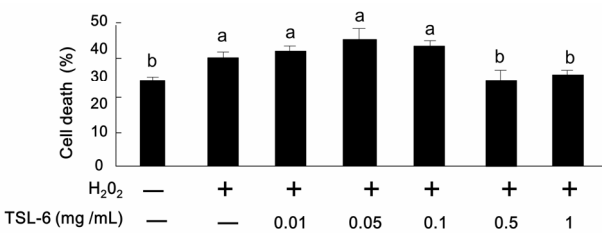

Figure 5. Effects of TSL-6 on the cell death of human spermatozoa treated with H_2O_2. The percent of cell death of human spermatozoa was measured by flow cytometry in human spermatozoa treated with or without 0.2 mmol/L H_2O_2, followed by incubation with various concentrations of TSL-6 for 3 h. Each bar represents Mean ± S.E. (n = 7). Means with different superscript letters are significantly different from each other ($p < 0.01$) as determined by Duncan's test.

effective antioxidants against various oxidative systems *in vitro*, prevention of oxidative modification of human LDL, and other antioxidant functions[2,3]. Here, TSL-6 was found to protect effectively human spermatozoa from oxidative stress. TSL-6 significantly diminished H_2O_2-induced ROS in a dose dependent manner (Figure 1) and rescued H_2O_2 impaired sperm motility (Figure 2A - C) as well as cell death (Figure 3). Treatment of H_2O_2-induced human spermatozoa with TSL-6 recovered impaired high MMP cells (Figure 2D) as well as ATP levels (Figure 2E). Recently, certain flavonoids in TS leaves have been isolated and identified, including quercetin, rutin and catechin, which possess the anti-oxidant properties[13,14]. In the present study, we found that TSL-6 possessed anti-oxidant properties in human spermatozoa. However, the identification of the main active compounds contributed to the protective effects of TSL-6 against oxidative stress in sperm is undergoing in our laboratory.

Taken together, these results suggested that TSL-6 improves functions of human spermatozoa under oxidative stress *via* the decreasing ROS levels, increasing sperm motility, elevating the MMP cells and ATP production, and maintaining the chromatin structure. Therefore, protective functions of TSL-6 in human spermatozoa potentially benefit the development of functional foods for infertile men under oxidative stress.

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