Astaxanthin Ameliorates the Lipopolysaccharides-Induced Subfertility in Mouse via Nrf2/HO-1 Antioxidant Pathway

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Abstract
The endotoxin lipopolysaccharide (LPS) exists in human semen, which is associated with reduced sperm quality. Studying the LPS-impaired spermatozoa motility and viability, and discovering effective therapeutic treatments have crucial importance. The time-course and dose-response experiments were performed to optimize the treatment dose and time of astaxanthin and LPS on mouse spermatozoa motility and viability. Sperm kinetics and morphology, reactive oxygen species production, in vitro fertilization, and developmental competence were examined to evaluate the protective effects of astaxanthin on spermatozoa after LPS exposure. The activity of nuclear factor erythroid 2-related factor-2/heme oxygenase 1 (Nrf2/HO-1) pathway was detected by quantitative reverse transcription polymerase chain reaction and Western blot. Astaxanthin improves LPS-impaired spermatozoa motility, viability, morphology, and activity; reduces LPS-induced spermatozoa oxidative stress; and alleviates LPS-impaired fertilization and embryo development through activating Nrf2/HO-1 antioxidant signaling pathway. Astaxanthin might be a potential treatment for LPS-induced subfertility.

Keywords
astaxanthin, LPS, subfertility, Nrf2/HO-1 antioxidant pathway, spermatozoa

Introduction
Subfertility affects 10% to 15% of couples worldwide, and the male factor accounts for about half of the subfertility cases.¹⁴ Hence, determining and studying the pathogenic factors of male subfertility and discovering effective therapeutic treatments have crucial importance.

Lipopolysaccharide (LPS), the endotoxins produced by gram-negative bacteria, is responsible for the development of inflammatory response, and in extreme cases, it leads to endotoxic shock.⁵⁻⁷ Clinical studies reported the presence of LPS in human semen.⁷ Lipopolysaccharide is constantly released into the genital tract and results in the infection of genitourinary tract, which are the commonest cause of subfertility.⁵⁻⁸ Cumulative evidences showed that LPS decreases sperm motility, viability, and morphology in mouse and human and then causes infertility.⁵⁻¹¹ As an endotoxin, LPS could destroy the integrity of spermatozoa membrane, cause chromosome and DNA damage, and result in cell apoptosis. Fujita et al found that LPS directly reduced the sperm motility and increased the apoptotic rate of both mouse and human sperm and significantly impaired the potential for fertilization.⁵ Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system’s ability to readily detoxify the reactive intermediates or to repair the resulting damage.³⁻⁸ Urata et al demonstrated that LPS exposure induced excessive production of ROS and resulted in reduced sperm motility and viability in human.¹⁰ After a short-time LPS exposure, the function of oviduct was changed and the spermatozoa motility was dramatically reduced through impairing the tubal flow and the frequency of cilia movement.¹²

Antioxidants are substances that can prevent or slow damage to cells caused by free radicals, unstable molecules that the
body produces as a reaction to oxidative stress. Astaxanthin is a xanthophyll carotenoid of predominantly marine origin, which possesses potent antioxidant and anti-inflammatory effects. The antioxidant activity of astaxanthin was 10 times more than other family members, such as lutein, β-carotene, and canthaxanthin. Astaxanthin offered the best protection from oxidative stress relative to lutein and β-carotene. Astaxanthin has been reported to decrease the DNA oxidative damage and inflammation and then improve the immune response in human. It can prevent skin thickening and reduce collagen reduction against the ultrasound-induced skin damage. However, the effect of astaxanthin on mouse semen has not been investigated.

We, therefore, hypothesized that astaxanthin might inhibit the LPS-impaired spermatozoa motility and viability and restore the fertilization and developmental competence of resultant embryos. In this study, we demonstrated that astaxanthin improves LPS-impaired spermatozoa motility, viability, morphology, and activity; reduces spermatozoa oxidative stress; and alleviates LPS-impaired fertilization and embryo development through activating nuclear factor erythroid 2-related factor-2/heme oxygenase 1 (Nrf2/HO-1) antioxidant signaling pathway. These results suggest a potential therapeutic role of astaxanthin in the treatment of LPS-induced subfertility.

Materials and Methods

Animals

Twelve-week-old C57BL/6 male mice (Shanghai Laboratory Animal Center, Shanghai, China) were used in this study. The mice were bred and maintained following the standard rearing conditions of 12 hours light and 12 hours dark. All mice studies were performed following the guideline established by the Affiliated Yantai Yuhuangding Hospital of Qingdao University Institutional Animal Care and Use Committee.

Sample Collection and Treatment

Mouse spermatozoa were collected from the cauda epididymides of 12-week-old C57BL/6 male mice following the previously described methods. The cauda epididymides were separated from surrounding fat and connective tissue and placed in a sterile 3.5-cm culture dish with 2 mL preincubated culture medium (1.5 mmol/L KCl, 98 mmol/L NaCl, 0.4 mmol/L NaH2PO4, 0.5 mmol/L MgCl2, 1.8 mmol/L CaCl2, 25 mmol/L NaHCO3, 50 μg/mL gentamycin, 25 mmol/L Na-lactate, 5.6 mmol/L n-glucose, and 0.5% bovine serum albumin). The epididymides were cut into small pieces and spermatozoa suspensions were collected and incubated at 37°C with 5% CO2 for 1 hour to allow dispersing spermatozoa into medium. The spermatozoa suspensions were transferred to a 10-cm culture dish and adjusted to 1 x 107 cells/mL.

In order to identify the effective dose and optimize the treatment time of astaxanthin, 5 x 106 spermatozoa cells/mL was incubated for 0, 1, 2, 3, 4, and 5 hours with 0.5, 1, 5, 10, and 25 μmol/L astaxanthin (#SML0982, Sigma-Aldrich, St Louis, Missouri) dissolved in dimethyl sulfoxide. The LPS was directly dissolved in medium. The doses and incubation time were determined as 0, 1, 2, 3, 4, and 5 hours with 0.01, 0.1, and 1 μg/mL LPS (#L4391, Sigma-Aldrich).

Spermatozoa Motility, Kinetics, Viability, and Morphology Assessment

The spermatozoa motility, kinetics, viability, and morphology assessment were performed according to the previously described methods. For the motility and kinetics, spermatozoa solution was placed on the Neobar slide and covered with a cover glass and then assessed using BX41 phase-contrast microscope (Olympus, Shinjuku, Japan). For the viability assessment, 20 μL spermatozoa sample was stained by the same volume eosin and nigrosin solution. The colorless alive spermatozoa and colored dead spermatozoa were counted using a light microscope. Spermatozoa morphology was evaluated using aniline blue (abnormal appearance spermatozoa) and eosin–nigrosin staining, imaged by BX41 phase-contrast microscope. The videos were analyzed using Spermatozoa Class Analyzer CASA System (Microptic, Barcelona, Spain).

Reactive Oxygen Species Production Assay

The ROS production of treated spermatozoa groups was determined by using the 2',7'-dichlorofluorescin diacetate (DCFDA)/H2DCFDA – cellular ROS assay kit (ab113851, Abcam, Cambridge, the United Kingdom). Briefly, DCFDA was loaded to spermatozoa solution and incubated at room temperature for 30 minutes. The samples were analyzed using the FACSCalibur flow cytometer (BD Biosciences, San Jose, California) according the previous studies.

DNA Damage Assay

The DNA damage of treated spermatozoa groups was assessed using the acridine orange test (AOT, A3568, Thermo Fisher Scientific, Waltham, Massachusetts). The spermatozoa smears were fixed by methanol and acetic acid solution before the acridine orange staining. After 15 minutes staining, the slides were assessed using Nikon Eclipse E600 Eclipse Fluorescence Motorized Stage Microscope (Nikon, Minato, Japan). The DNA damage was visualized as red or yellow fluorescence. The AOT-positive spermatozoa were counted and analyzed with the ImageJ software (http://rsb.info.nih.gov/ij/).

Glutathione Assay and γ-Glutamylcysteine Synthetase Activity Determination

The spermatozoa glutathione (GSH) was measured by using glutathione colorimetric detection kit (#EIAGSHC, Thermo Fisher Scientific) following the manufacturer’s description. A colorimetric substrate reacts with the free thiol group on GSH to yield a highly colored product. The oxidized...
glutathione (glutathione disulfide) can be determined by using 2-vinylpyridine to block any free GSH in the sample. The concentration of GSH can be determined at 405 nm and analyzed using the Molecular Devices SPECTRAmax microplate reader (Molecular Devices, San Jose, California).

The γ-glutamylcysteine synthetase (γ-GCS) activity was measured using the γ-GCS assay kit (#CA257, Signalway Antibody, College Park, Maryland) following the manufacturer’s description. The total protein was detected using the Pierce Rapid Gold BCA protein assay kit (#A53225, Thermo Fisher Scientific). The final result of γ-GCS assay activity is displayed as units per mg protein.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

The total RNA was isolated and extracted from treated spermatozoa groups using the Invitrogen TRIzol (Thermo Fisher Scientific). One microgram of total RNA was converted into complementary DNA using random hexamers and M-MLV (Thermo Fisher Scientific). The quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in iCycler Multicolor RT-PCR system using SYBR Green (Bio-Rad, Hercules, California). The expression of tested gene was normalized to internal control GAPDH, and fold change was calculated by the $2^{-\Delta\Delta CT}$ method. Primers for the qRT-PCR were listed below: Nrf2, upstream 5′-ACAGT-GCTCCTATGCGTGAA-3′, downstream 5′-GAGCCTC-TAACGCGCTTGAA-3′; HO-1, upstream 5′-CAGAAGAG-GCTAAGACCGCC-3′, downstream 5′-CTCTGACGAAGT-GACGCCAT-3′; and GAPDH (internal reference), upstream 5′-ACCACAGTCCATGCCCCATC-3′ and downstream 5′-TCCACCACCCCTGTTGGCTGA-3′. In all of these qRT-PCR measurements, assays of samples were performed in duplicate. Three or more independent experiments were carried out and assayed.

Western Blot

The control and treated spermatozoa cells were lysed using the radioimmunoprecipitation assay buffer. Samples were subjected to immunoblotting analysis as described previously. Nuclear factor erythroid 2-related factor-2 (#ab76026, 1:1000

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**Figure 1.** Effect of astaxanthin on mouse sperm motility and viability. Sperm cells were incubated with various doses of astaxanthin (0.5, 1, 5, 10, and 25 μmol/L) for 0, 1, 2, 3, 4, and 5 hours. Total motility (A) and viability (B) were measured at different time points. Three independent experiments were carried out. Data presented as mean (standard deviation). *P <.05 compared to control.
dilution) and HO-1 (#ab13248, 1:1000 dilution) primary antibodies were purchased from Abcam (Shanghai, China); β-actin antibody (#4967, 1:3000 dilution) was purchased from Cell Signaling Technology, Inc (Danvers, Massachusetts).

In Vitro Fertilization and Embryo Culture

Both spermatozoa cells and cumulus oocyte complexes (1 day after human chorionic gonadotropin injection) were collected and cultured in KSOM fertilization medium, respectively. The spermatozoa were treated with 0.5 μmol/L astaxanthin, 0.01 μg/mL LPS, or 0.5 μmol/L astaxanthin plus 0.01 μg/mL LPS for 1 hour, then separated by gradient centrifugation (40%-80% grades). The spermatozoa pellets were collected and diluted into 1000 cells/μL, then inseminated with oocytes. The fertilization was confirmed by checking the pronuclear formation using the BX41 phase-contrast microscope. The embryos were placed in KSOM medium with mineral oil at 37°C and 5% CO₂. Three days after the fertilization, the blastocyst rates, morula, and cleavage were checked following the previous study.²²

Statistical Analysis

Statistical analyses were carried out using SPSS version 11.0 package. The differences between groups were analyzed using 1- or 2-way analysis of variance followed a Tukey post hoc test. All data represented mean (standard deviation). *P < .05, **P < .01, and ***P < .001 compared to control group, #P < .05 and ##P < .01 compared to the LPS-treated group.

Results

Effect of Astaxanthin and LPS on Mouse Spermatozoa Motility and Viability

In order to identify the effective dose and optimize the treatment time of astaxanthin on mouse spermatozoa motility and viability, we performed time-course or dose–response experiments. As shown in Figure 1A, the spermatozoa motility was decreased with prolonged incubation in control group. However, the astaxanthin treatment significantly alleviates the downtrend of spermatozoa motility relative to control (Figure 1A).

Similarly, in comparison with control, the spermatozoa viability was increased after astaxanthin treatment (Figure 1B).
Figure 3. Effect of astaxanthin on LPS-impaired mouse sperm motility and viability. Sperm cells were incubated with a combination of various doses of astaxanthin (0.5, 5, and 25 μmol/L) and 0.01 μg/mL LPS for 5 hours. Total motility (A) and viability (B) were measured at different time points. Three independent experiments were carried out. Data presented as mean (standard deviation). **p < .01 compared to control, #p < .05 compared to LPS group. LPS indicates lipopolysaccharide.

Figure 4. Effect of astaxanthin on LPS-impaired mouse sperm motility, kinetics, and morphology. Sperm cells were incubated with a combination of 0.5 μmol/L astaxanthin and 0.01 μg/mL LPS for 1 hour. Percentage of progressive spermatozoa (A), rapid spermatozoa, rapid >45 μm/s (B), normal morphology (C), and straight line velocity (D), average path velocity (E) curvilinear velocity (F) were measured. Three independent experiments were carried out. Data presented as mean (standard deviation). *p < .05 compared to control, #p < .05 compared to LPS group. LPS indicates lipopolysaccharide.
There was no dose-dependent effect among various doses of astaxanthin (0.5, 1, 5, 10, and 25 μmol/L) in different time points.

We further evaluated the effect of LPS on mouse spermatozoa motility and viability. After 1 to 5 hours LPS treatment, both motility and viability of mouse spermatozoa were significantly decreased compared with control group (Figure 2A and B). However, there was no dose-dependent effect among various doses of LPS (0.01, 0.1, and 1 μg/mL) in different time points (Figure 2).

Astaxanthin Improves LPS-Impaired Spermatozoa Motility and Viability

Next we investigated whether astaxanthin treatment can inhibit the impaired spermatozoa motility and viability induced by LPS exposure. The sperm cells were incubated with a combination of various doses of astaxanthin (0.5, 5, and 25 μmol/L) and 0.01 μg/mL LPS for 5 hours. As shown in Figure 3, after astaxanthin coadministration, both motility and viability of the sperm cells were significantly improved compared with the LPS group. In agreement with the above result, there was no dose-dependent effect of various doses of astaxanthin (Figure 3A and B).

Astaxanthin Rescues LPS-Impaired Spermatozoa Morphology and Activity

In order to further assess the protective effective effects of astaxanthin on the morphology and activity of spermatozoa after LPS exposure, we evaluated the spermatozoa motility, kinetics, and morphology 1 hour after astaxanthin treatment (0.5 μmol/L), LPS exposure (0.01 μg/mL), and coadministration. In comparison with control group, the 1-hour LPS exposure could significantly reduce the proportion of progressive spermatozoa (Figure 4A), rapid spermatozoa (Figure 4B), and normal morphology (Figure 4C). Moreover, the straight line velocity (Figure 4D), average path velocity (Figure 4E), and curvilinear velocity (Figure 4F) of the LPS-treated spermatozoa were decreased. Notably, the coadministration of astaxanthin with LPS dramatically improved the spermatozoa morphology and activity, all the above important parameters that reflect the quality spermatozoa were significantly increased relative to the LPS-
treated group (Figure 4A-F). These results suggested that astaxanthin (0.5 μmol/L) treatment could rescue LPS-impaired spermatozoa morphology and activity.

Astaxanthin Reduces LPS-Induced Spermatozoa Oxidative Stress

Since LPS exposure can increase the oxidative level, we asked whether astaxanthin treatment suppress the detrimental effects of LPS. To this end, we measured the spermatozoa ROS production and DNA damage after LPS exposure. In comparison with control group, astaxanthin treatment decreased the levels of ROS production and DNA damage; in contrast, LPS exposure increased both (Figure 5A and B). Notably, coadministration of astaxanthin with LPS for 5 hours could significantly inhibit the LPS-induced ROS production and DNA damage (Figure 5A and B). Furthermore, we detected the content of GSH, an antioxidant in semen, and the activity of γ-GCS, the synthetase of GSH. As shown in Figure 5C and D, astaxanthin treatment could dramatically suppress the inhibitory effect of LPS on GSH content and γ-GCS activity. The above results indicated that astaxanthin treatment can reduce LPS-induced spermatozoa oxidative stress.

Astaxanthin Blocks LPS-Induced Oxidative Stress Through Nrf2/HO-1 Antioxidant Pathway

The Nrf2/HO-1 signaling pathway controls numbers of cytoprotective genes that can combat the detrimental effects of oxidative stress. The transcriptional level of Nrf2 and HO-1 in the treated sperm cells was detected using qRT-PCR, 5 hours astaxanthin treatment could significantly increase the expression of both genes (Figure 6A and B). The LPS exposure reduced the messenger RNA (mRNA) levels of Nrf2 and HO-1, which could be rescued by the astaxanthin coadministration. Consistent with the transcriptional result, astaxanthin treatment increased the protein levels of Nrf2 and HO-1; more importantly, astaxanthin could partially restore the 2 proteins after LPS exposure (Figure 6C and D). These results suggested that astaxanthin blocked LPS-induced oxidative stress through Nrf2/HO-1 antioxidant pathway.

Astaxanthin Alleviates LPS-Impaired Fertilization and Developmental Competence

Finally, we examined the effect of preincubation of mouse spermatozoa with a combination of astaxanthin and LPS on the resultant fertilization and developmental competence. As shown in Figure 7, oocytes inseminated by LPS significantly
reduced the fertilization (Figure 7A), cleavage (Figure 7C), compaction (Figure 7D), and blastocyst rates (E) and increased the degeneration (Figure 7B) compared to the control group. Notably, in comparison with LPS group, astaxanthin coadministration dramatically alleviated the detrimental effects of LPS on fertilization and embryo developmental competence (Figure 7).

Discussion

More than 80 million people are affected by the inability to have children worldwide, and the male factor contributes to about 51.2% of these cases. Clinical studies have reported the presence of LPS or peptidoglycans in human semen, and these components are associated with reduced sperm quality. Antioxidants are thought to improve sperm quality by reducing oxidative damage. Astaxanthin is a natural xanthophyll carotenoid with potent antioxidant and anti-inflammatory effects, which is thought to improve sperm quality. Here, we demonstrated the protective roles and mechanism of astaxanthin on abrogating the detrimental effects of LPS on sperm motility, viability, and fertility.

The LPS exposure significantly increased spermatozoa ROS production, reduced sperm motility and viability, and finally impaired the fertilization and embryo development (Figures 3–5 and 7). Consistent with our results, LPS can be detected in human semen, and the addition of LPS in the absence of leukocytes dramatically decreased the motility and increased apoptosis of both human and mouse sperm. The LPS exposure damaged the integrity of spermatozoa membrane and increased the apoptotic rate of both mouse and human sperm and significantly impaired the potential for fertilization. After 20 minutes of LPS exposure, the sperm ROS production was induced excessively, and both sperm motility and viability were reduced dramatically in human. O’Doherty et al investigated the imparted effects of short-time LPS exposure (2 hours) on particle transport, cilia function, and sperm motility using an ex vivo oviduct model, which revealed that the LPS-induced oxidative response had implications for both male and female fertility.
The natural antioxidants are widely available and inexpensive compared to other fertility treatments. As a xanthophyll carotenoid, astaxanthin possesses potent antioxidant ability than other family members, which is about 10 times more than lutein, β-carotene, and canthaxanthin. Astaxanthin has been reported to reduce DNA oxidative damage and inflammation and improve the immune response in human. More and more evidence indicate that oxidative stress-induced sperm damage is an important contributing pathology in 30% to 80% subfertility. Exploring the therapeutic effects of antioxidant, such as astaxanthin, become a hot topic in subfertility research. In present study, we found that astaxanthin treatment improved the sperm morphology and kinetics and positively affected fertilization and embryo developmental competence. More importantly, coadministration of astaxanthin with LPS could significantly alleviate LPS-impaired spermatozoa motility and viability and then improve fertilization and embryo development after the LPS exposure. Basioura et al demonstrated the beneficial and protective effect of astaxanthin on boar semen quality under 17°C storage, 37°C and 38.5°C in vitro incubation. The nuclear transcription factor Nrf2 is one of the important antioxidant regulators involved in the maintenance of the redox state for the defense of intracellular oxidative stress. Heme oxygenase 1, a phase II detoxifying enzyme regulated by Nrf2, has been reported to be a critical beneficial regulator in oxidative stress-induced diseases. In this study, we found that astaxanthin treatment could reactivate Nrf2/HO-1 signaling pathway on mRNA and protein levels in sperm cells after the LPS exposure (Figure 6), which are consistent with a previous report that astaxanthin is able to prevent oxidative challenge. Xue et al found that astaxanthin suppressed coronary microembolization-induced oxidative stress through activating Nrf2/HO-1 signaling pathway. Niu et al reported that astaxanthin treatment induced the Nrf2/HO-1 antioxidant pathway and decreased ROS production in human umbilical vein endothelial cells. Our results and previous studies indicate that astaxanthin has the potential therapeutic effects on LPS-induced subfertility. However, how astaxanthin regulates the Nrf2/HO-1 pathway still remain unclear, which require further investigations.

**Conclusion**

Taken together, the findings of this study showed that LPS inhibits motility and viability in mouse spermatozoa and impairs fertilization and embryo developmental competence. The astaxanthin coadministration suppresses ROS production and improves LPS-impaired sperm motility, viability, and quality through reactivating Nrf2/HO-1 signaling pathway. This study implies that astaxanthin, a natural antioxidant, might be a potential treatment for LPS-induced subfertility.

**Declaration of Conflicting Interests**

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