Original Paper

UCP2 Mitigates the Loss of Human Spermatozoa Motility by Promoting mROS Elimination

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Key Words

Uncoupling protein 2 • human spermatozoa motility • mROS

Abstract

Background/Aims: To demonstrate the function of uncoupling protein 2 (UCP2) in the regulation of human spermatozoa motility. **Methods:** Semen samples were collected from donors with either normal spermatozoa motility (normospermia [NS]) or poor spermatozoa motility (asthenospermia [AS]). UCP2 protein in spermatozoa was quantified by Western blotting. The level of mitochondrial reactive oxygen species (mROS) was evaluated by MitoSOX Red. The activity of mitochondrial membrane potential (MMP) in spermatozoa was evaluated by a JC-1 assay and the ATP level was monitored by a luciferin-luciferase assay. **Results:** UCP2 was expressed in both NS and AS groups, with the former exhibiting a higher level than the latter. Immunofluorescence analysis shows that UCP2 is mainly located at the mid-region of human spermatozoa. The inhibition of UCP2 by a highly selective inhibitor, Genipin, results in not only impaired spermatozoa mobility ($P < .05$) but also an elevated level of mROS ($P < .05$), suggesting that UCP2 is involved in the maintenance of the spermatozoa mobility, which probably is achieved by promoting mROS elimination. Furthermore, H_2O_2 treatment of spermatozoa increases the mROS level coupled with the loss of spermatozoa mobility. Unexpectedly, this treatment also has a positive impact on the expression of UCP2 within a certain range of supplemental H_2O_2 , indicating the moderate mROS level possibly serves as a feedback signal to stimulate the expression of UCP2. Finally, the treatment of spermatozoa by an ROS scavenger, *N*-acetyl-L-cysteine (NAC), decreases the level of mROS and increases the curvilinear velocity (VCL) of spermatozoa, but the UCP2 level is not affected. **Conclusion:** These results suggest an UCP2–mROS–motility regulatory system exists for maintaining

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spermatozoa mobility in humans. In such a system, UCP2 fulfills its function by promoting mROS elimination, and slightly over-produced mROS in turn serves as a signal to stimulates the expression of UCP2. This regulatory system represents a new potential target for the discovery of novel pharmaceuticals for the treatment of patients with low spermatozoa motility.

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Introduction

Approximate 15% to 20% of couples worldwide have infertility, with males accounting for about 40% to 50% of cases [1]. Male infertility is associated with abnormalities in various aspects, such as semen volume, spermatozoa concentration, spermatozoa motility, and morphology [2-5]. Poor spermatozoa motility (also called asthenozoospermia) is a major cause of male infertility [4-6]. Spermatozoa motility, a key indicator of semen quality, is driven by flagellae, which is dependent on an adequate and continuous supply of ATP. Lack of ATP supply [7], overproduction of reactive oxygen species (ROS) [8], calcium dyshomeostasis [6], aging [9], and certain forms of environmental pollution [10-12] can lead to impaired spermatozoa motility.

Mitochondria plays critical roles in intracellular ATP production and maintenance of calcium homeostasis. It is also the major source of ROS. Previous studies have revealed the close relationship between morphology and functions of mitochondria and spermatozoa motility [13-15]. These studies demonstrated that the maintenance of mitochondrial homeostasis is critical for spermatozoa motility and semen quality. When mitochondria are damaged, a major outcome is the overproduction of mitochondrial reactive oxygen species (mROS), which then elevates the level of total cellular ROS. Excessive ROS can cause severe oxidative damages to spermatozoa membranes and DNA [14, 16], leading to lowered integrity of these subcellular components, impaired spermatozoa motility, and subsequent infertility [8, 16, 17]. Spermatozoa motility is considered the earliest and most sensitive indicator of spermatozoa oxidative damage [17].

Uncoupling protein 2 (UCP2), a mitochondrial membrane protein, is widely expressed in mammalian tissues. UCP2 plays important roles in a variety of physiological processes, such as food intake [18], insulin secretion, and immunology [19, 20]. UCP2 is also vital to many pathological conditions [21]. However, the function of UCP2 in male reproduction remains unclear. Recently, a research group found expression of UCP2 in the spermatozoa of zebrafish and yellow catfish and revealed the close relevance between the expression of UCP2 and spermatozoa ROS level, degree of oxidative damage, and post-thaw viability [22, 23]. In human spermatozoa, the transcription of UCP2-encoding gene has been observed by quantifying mRNA, however, the UCP2 protein level was not further measured in the same study [24]. The aims of the present study are to determine the correlation between the expression of UCP2 and human spermatozoa mobility and to elucidate the underlying mechanism for the regulation of spermatozoa motility by UCP2.

Materials and Methods

Spermatozoa preparation

Human semen samples were collected from 200 male fertility evaluation donors between 25 to 35 years old according to World Health Organization (WHO) criteria. All participants signed a written informed consent. The study was approved by the Ethics Committee of the hospital and the protocols were carried out in accordance with the approved guidelines. Semen samples were collected by masturbation after a stipulated period of 2-7 days of abstinence. The collected semen samples were analyzed by computer-aided spermatozoa analysis (CASA, IVOS, Hamilton, USA) and identified as normospermic (NS, n = 120) and asthenospermic (AS, n = 80) according to WHO Guidelines. Spermatozoa were isolated by density gradient centrifugation after liquefaction for 15-30 min at 37 °C. In brief, semen samples (2 mL) were added on top of a colloidal silica suspension (Percoll, Sigma Aldrich, UK) made of 80% and 40% layers (1 mL each), and

centrifuged at $1,300 \times g$ for 15 min. Then the pellets were collected and washed twice with HTF medium (Quinn's, SAGE, USA). Finally, the spermatozoa were resuspended in HTF medium.

Immunofluorescence staining

Spermatozoa were fixed using 4% paraformaldehyde for 15 min. Spermatozoa membrane was permeated with cold 95% ethyl alcohol for 15 min. After washing, spermatozoa were blocked for 1 h with 1% fetal bovine serum (FBS) in phosphate-buffered saline (PBS). The spermatozoa were incubated with mouse polyclonal anti-UCP2 (1:50, Abcam) for 4 h at room temperature, and then with FITC-conjugated anti-mouse (1:5000, Bioword) for 2 h at room temperature. Cells were then rinsed with PBS three times for 5 min each. The negative control was only incubated with secondary antibody. Images were captured by an Olympus FluoView FV500 microscope.

Western blotting

Semen samples were incubated with 100 μ l SDS lysis buffer (Beyotime, China) for 30 min and then centrifuged at $12,000 \times g$ for 10 min at 4 °C, and the supernatant was analyzed for protein concentration. Equal amount of protein (40 μ g) was separated by 12% SDS-PAGE and transferred to PVDF membrane (Millipore, USA). The membranes were blocked with 5% non-fat milk for 1 hour at room temperature. After that the membranes were then incubated overnight at 4 °C with primary antibodies of both polyclonal anti-UCP2 (1:500, Abcam, USA) and polyclonal anti- β -actin (1:200, Boster, China) followed by the incubation with HRP-conjugated anti-rabbit IgG secondary antibodies (1:500, Beyotime, China) for 2 h at room temperature, and then washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBST). The chemiluminescence signal was captured using Super Signal West Pico (Pierce, USA), and the results were analyzed using Quantity One software (AlphaEaseFc).

Measurement of ATP content

The ATP content of spermatozoa samples was measured using the ATP Detection Kit (Beyotime, China). Spermatozoa samples were lysed by ATP lysis buffer for 30 min, and then centrifuged at $12,000 \times g$ for 10 min at 4 °C. The supernatant (40 μ l) after being mixed with 100 μ l ATP detection buffer was added into 96-well black plates. The ATP content (nmol/mg) was analyzed using an automatic microplate reader (Biotek, USA).

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was analyzed with a JC-1 kit (Sigma). The spermatozoa samples ($1 \sim 2 \times 10^6$ /mL) were washed with PBS, and incubated with 1.5 μ l JC-1 working solution (2.5 mg/mL) at 37 °C for 30 min. After washing with PBS, the spermatozoa samples were suspended and analyzed by Flow Cytometry (BD, USA). Ten thousand spermatozoa were recorded in each group, and the MMP level was calculated using the ratio of green fluorescence intensity to red fluorescence intensity.

Measurement of ROS content in mitochondria

The ROS content in mitochondria (mROS) was measured using MitoSOX™ Red (Invitrogen). Spermatozoa were incubated with 10 μ M Mitosox at 37 °C for 10 min. Then samples were washed with PBS and analyzed for mROS content using Flow Cytometry. Ten thousand spermatozoa were recorded in each group, and the mean fluorescent intensity (MFI) was measured in a PE channel.

Statistical Analysis

All data was analyzed by one-way ANOVA followed by a post-hoc comparison test using either LSD (when equal variances were assumed) or Dunnett's T3 (when equal variances were not assumed) method when more than two groups were compared. The data was presented as the mean $\pm$ SEM, with a statistically significant difference defined as $P < 0.05$. All the statistical procedures were performed using SPSS 18.0.

Results

Expression of UCP2 in human spermatozoa and its correlation with spermatozoa motility

In human spermatozoa, the transcription of UCP2-encoding gene has been observed by quantifying mRNA level [24], but no study has been performed to track UCP2 expression at the protein level. In the present study, to determine whether the UCP2 protein is expressed in human spermatozoa, the prepared spermatozoa were subjected to immunofluorescence staining using antibodies against UCP2. The results shown in Fig. 1A strongly suggest that UCP2 is expressed in human spermatozoa and mainly located at the mid-region of spermatozoa. To our knowledge, this is the first time that the UCP2 protein is found to be expressed in human spermatozoa. Moreover, the UCP2 protein levels of both NS and AS groups were quantified and compared by western blotting to investigate the correlation of UCP2 expression and spermatozoa motility. The results show that the UCP2 expression is significantly higher in the NS group than in the AS group ($P < 0.05$, Fig. 1B), indicating that UCP2 is likely involved in the regulation of spermatozoa motility.

Effect of the UCP2-specific inhibitor on spermatozoa motility, mROS content, and mitochondrial function

To further understand how UCP2 regulates spermatozoa motility, Genipin (Sigma Chemical Co., St. Louis, MO, USA), a highly selective UCP2 inhibitor, was used to block UCP2 activity. The concentrations of Genipin used in this study are based on a published study [23]. Interestingly, as shown in Fig. 2B, the progressive motility (PR), the total motility (progressive plus nonprogressive motility), and the curvilinear velocity (VCL) of spermatozoa are all remarkably decreased in the presence of Genipin ($P < 0.05$). A significant increase in the mROS level is observed after Genipin treatment ($P < 0.05$, Fig. 2C). On the other hand, no significant difference is observed for mitochondrial membrane potential (MMP) activity and ATP content between Genipin-treated and nontreated spermatozoa ($P > 0.05$, Fig. 2D and 2E),

Fig. 1. UCP2 expression in human spermatozoa. (A) Immunofluorescence images show that UCP2 is located in the neck of sperm. Right images are the magnification of arrows indicated areas. Scale bars: 50 μ m. (B) UCP2 protein levels in both NS and AS groups were quantified and compared by western blotting. The optical density values were normalized to β -actin loading control, and means $\pm$ SD were graphed (relative expression) to semi-quantitatively compare the protein levels. Difference with $*P < 0.05$ (AS group versus NS group).

Fig. 2. Effects of the UCP2 inhibition on spermatozoa motility, mROS content, and mitochondrial function. (A) UCP2 protein levels were analyzed using western blot. The values were normalized to β-actin loading control, and means ± SD were calculated to compare the protein levels. (B) Spermatozoa motility was analyzed by spermatozoa analysis system (CASA). (C) Spermatozoa mROS level was determined using MitoSOX Red probes and analyzed using cytoFLEX software. The mean fluorescence intensity (MFI) with MitoSOX Red staining was determined by flow cytometry. (D) The MMP of spermatozoa was determined using JC-1 probes and analyzed by cytoFLEX software. (E) ATP content was analyzed by ATP detection kit. *P < 0.05.

although a slight decrease of ATP content occurs in spermatozoa treated with 100 $\mu\text{mol/L}$ Genipin (Fig. 2E). These results indicate that the specific inhibition of UCP2 by Genipin in spermatozoa can down-regulate the motility of spermatozoa, which is probably achieved by either promoting the mROS production or lowering the mROS elimination rather than by blocking the ATP production in human spermatozoa. In addition, the UCP2 protein level in Genipin-treated spermatozoa is slightly reduced compared with the level in nontreated spermatozoa ($P < 0.05$, Fig. 2A), which is likely due to the interference of Genipin with the UCP2 translation process.

Fig. 3. Effects of H_2O_2 treatment on UCP2 expression and spermatozoa motility. (A) Spermatozoa mROS was determined using MitoSOX Red probes and analyzed using cytoFLEX software. The mean fluorescence intensity (MFI) of sperm with MitoSOX Red staining was determined by flow cytometry. (B) Sperm motility was analyzed using sperm analysis system (CASA). (C) Western blot analysis of UCP2. The values were normalized to β -actin loading control, and means $\pm$ SD were calculated to compare the protein levels. * $P < 0.05$.

Effect of H_2O_2 treatment on UCP2 expression and spermatozoa motility

Many studies have demonstrated that mROS is a critical factor regulating spermatozoa motility. In addition, it has been confirmed that there is a close correlation between UCP2 expression and cellular oxidative stress. However, the role that UCP2 could play in regulating mROS content in spermatozoa remains unclear. To further understand the relevant mechanism, H_2O_2 was applied to the spermatozoa for 30 min with the concentrations ranging from 0 to 100 $\mu\text{mol/L}$ [25]. As expected, the mROS content is significantly increased after H_2O_2 -treatment ($P < 0.05$, Fig. 3A). Meanwhile, the total motility, the progressive motility, and VCL are significantly decreased after H_2O_2 -treatment compared with controls ($P < 0.05$, Fig. 3B). Interestingly, the UCP2 level is significantly elevated in spermatozoa treated with 25 $\mu\text{mol/L}$ and 50 $\mu\text{mol/L}$ H_2O_2 compared with controls ($P < 0.05$, Fig. 3C), indicating that, within a certain range, mildly excessive ROS can promote UCP2 expression. Combined with the data obtained in the Genipin-treatment experiments, it is clearly shown that the elevation of UCP2 expression is helpful for the maintenance of mROS content at a normal physiological level and thus mitigates the negative impact of ROS on spermatozoa motility.

Effect of ROS scavenger on UCP2 expression and spermatozoa motility

To illuminate whether ROS scavengers can affect UCP2 expression or spermatozoa motility, *N*-acetyl-L-cysteine (NAC), a widely used ROS scavenger, was used in this study. The concentration and duration of NAC treatment were determined in a spermatozoa viability experiment (data not shown). As expected, the level of spermatozoa mROS is reduced after NAC treatment (Fig. 4A). In addition, as shown in Fig. 4B, a significant increase is observed in the VCL mobility in the treatments with 50 $\mu\text{mol/L}$ and 100 $\mu\text{mol/L}$ NAC ($P < 0.05$). However, there is no significant difference of UCP2 levels between NAC-treated and nontreated

Fig. 4. Effects of NAC treatment on UCP2 expression and spermatozoa motility. (A) Sperm mROS was determined using MitoSOX Red. The mean fluorescence intensity (MFI) of sperm with MitoSOX Red staining was determined by flow cytometry. (B) UCP2 protein levels was detected by western blot. The optical density values were normalised to β -actin loading control, and means $\pm$ SD were graphed (relative expression) to semi-quantitatively compare the protein levels. (C). Sperm motility and VCL were analyzed using CASA after treatment with different concentration of NAC. * $P < 0.05$.

spermatozoa ($P > 0.05$, Fig. 4C), indicating that the decrease of mROS level caused by ROS scavenger has no impact on the expression of UCP2. Combined with the data obtained in the experiments involving H_2O_2 treatment, we conclude that only mildly excessive mROS can induce an elevated UCP2 expression level, while normal physiological level or a lower level of mROS has no impact on the expression of UCP2 in human spermatozoa.

Discussion

Poor spermatozoa motility has been found associated with various factors, such as the over-production of ROS [8], mitochondrial dysfunction in energy production (i.e. the abnormalities in levels of ATP and MMP) [7, 26], excessive DNA fragmentation [27], defective morphology [3], and so on. In the present study, UCP2 is found to have an intimate relationship with spermatozoa motility. The UCP2 expression levels are significantly higher in the NS group than in the AS group, while the specific inhibition of UCP2 by Genipin results in a significant decrease in spermatozoa total motility, progressive motility, and VCL. These observations raise a question: how does UCP2 regulate human spermatozoa motility?

It is known that UCP2 plays vital roles in maintaining cellular mitochondrial homeostasis via regulating energy balance [28], maintaining calcium homeostasis, controlling mROS levels [29, 30], and regulating cellular autophagy and apoptosis [31]. UCP2 can protect cells from oxidative damages via eliminating mROS [30]. Published studies have suggested that a UCP2 homologue, UCP1, regulates ATP production and maintains cellular energy balance in many cell types through uncoupling electron transport and ATP generation [32, 33]. Theoretically, protons pass through the inner mitochondrial membranes and form electrochemical gradients resulting in the production of ATPs coupled with the production of a large number of mROS [34]. Hence, the content of mROS is positively correlated with the levels of ATP and MMP. A positive correlation between mitochondrial energy function and spermatozoa motility has been demonstrated [14, 26, 35, 36]. In these studies, it was found that the levels of MMP and ATP are significantly higher in the NS group than in the AS group, but the mROS levels show a reverse trend, implying that one special mechanism exists in the NS group to either inhibit mROS production or promote mROS elimination. Interestingly, in the present study, upon the inhibition of UCP2, ATP and MMP levels are found unaffected while the mROS level is observed significantly elevated, indicating that UCP2 may fulfill its function by promoting mROS scavenging rather than by facilitating ATP production in human spermatozoa.

To further demonstrate the relationship between UCP2 and the mROS level, H_2O_2 and NAC were individually applied to increase and decrease the mROS levels, respectively. The H_2O_2 -treatment induces an elevated measured mROS level coupled with the impaired spermatozoa mobility as expected. Unexpectedly, the moderate level of H_2O_2 can slightly induce the UCP2 expression although highly excessive H_2O_2 shows a negative impact on the UCP2 expression. The NAC treatment facilitates the mROS scavenging, but does not have an impact on the UCP2 expression. Combined with the data obtained in the Genipin-treatment of UCP2, it is suggested that an UCP2-mROS-motility regulatory system exists in human spermatozoa. In this system, the elevation of UCP2 expression can enhance spermatozoa motility by promoting mROS scavenging, while slightly over-produced mROS in turn can serve as a feedback signal for the induction of UCP2 expression. The results obtained in the Genipin-, H_2O_2 -, and NAC-treatment experiments, combined with the observation of higher level of UCP2 expression in the NS group, suggest that the spermatozoa with high motility are more vigorous in mROS scavenging.

Normal physiological levels of ROS play roles in spermatogenesis and maturation [37], capacitation [38, 39], acrosome reaction [38], and the fusion programme of spermatozoa and oocyte [37, 40, 41]. Nevertheless, excessive ROS will induce severe damage to spermatozoa membrane lipids, DNA, and ultra-structures, leading to a decreased spermatozoa motility and the subsequent infertility [8, 37, 42]. Several antioxidant enzymes, such as catalase

(CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX), were detected in the seminal plasma [43, 44]. However, few intracellular antioxidant mechanisms were discerned in spermatozoa. In this study, the results demonstrate that UCP2 plays a pivotal role in ROS scavenging in human spermatozoa, and thus affects spermatozoa motility.

Recently, a research group observed similar effects of UCP2 expression in the spermatozoa of zebrafish and yellow catfish [22, 23]. It was found that a UCP2 promoter significantly improved the viability of post-thaw spermatozoa, accompanied by the decreased levels of ROS and MDA [23]. The UCP2 expression in the zebrafish spermatozoa can be down-regulated by Genipin [30] consistent with our observation in human spermatozoa, and furthermore, could be up-regulated by acute cold exposure [30]. It is noteworthy that the spermatozoa are terminally differentiated cells, with DNA tightly compacted in the head, most of organelles removed during spermatozoa maturation, and only a few organelles such as mitochondria retained. In addition, it is believed that the spermatozoa lack transcription and translation systems. Nevertheless, Gur *et al.* (2006) showed mitochondrial ribosomes are actively involved in protein translation during capacitation of spermatozoa. Moreover, they confirmed that the nuclear-encoded mRNA in spermatozoa could be translated on the spermatozoa mitochondrial ribosomes, and such an ability of translation is related to the spermatozoa viability, motility, capacitation, and acrosome reaction [45]. These studies proved that matured spermatozoa retain the ability of self-regulation, which can explain why the UCP2 expression in human spermatozoa can be regulated by Genipin and H₂O₂. These evidences further support the existence of UCP2 regulation system in human spermatozoa.

Conclusion

In summary, in this study, it is the first time to demonstrate the existence of an UCP2-mROS-motility regulatory system in human spermatozoa. UCP2 protein is expressed in human spermatozoa, and regulates spermatozoa motility. UCP2 controls mROS levels by promoting mROS elimination, and mildly over-produced mROS serving as a signal can induce the UCP2 expression. Therefore, the exploitation and regulation of UCP2-mROS system might pose a new strategy to treat patients with poor spermatozoa motility. However, for this purpose, more studies with the aim of revealing the in-depth mechanism of the UCP2-mROS-motility regulatory system and its roles in regulating other functions in spermatozoa are needed.

Disclosure Statement

The authors declared that no competing interests exist.

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