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Reproductive toxicity of ritonavir in male: Insight into mouse sperm capacitation

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ABSTRACT

Since COVID-19 began in 2019, therapeutic agents are being developed for its treatment. Among the numerous potential therapeutic agents, ritonavir (RTV), an anti-viral agent, has recently been identified as an important element of the COVID-19 treatment. Moreover, RTV has also been applied in the drug repurposing of cancer cells. However, previous studies have shown that RTV has toxic effects on various cell types. In addition, RTV regulates AKT phosphorylation within cancer cells, and AKT is known to control sperm functions (motility, capacitation, and so on). Although deleterious effects of RTV have been reported, it is not known whether RTV has male reproduction toxicity. Therefore, in this study, we aimed to investigate the effects of RTV on sperm function and male fertility. In the present study, sperm collected from the cauda epididymis of mice were incubated with various concentrations of RTV (0, 0.1, 1, 10, and 100 μM). The expression levels of AKT, phospho-AKT (Thr308 and Ser473), and phospho-tyrosine proteins, sperm motility, motion kinematics, capacitation status, and cell viability were assessed after capacitation. The results revealed that AKT phosphorylation at Thr308 and Ser473 was significantly increased, and the levels of tyrosine-phosphorylated proteins (at approximately 25 and 100 kDa) were significantly increased in a dose-dependent manner. In addition, RTV adversely affected sperm motility, motion kinematics, and cell viability. Taken together, RTV may have negative effects on sperm function through an abnormal increase in tyrosine phosphorylation and phospho-AKT levels. Therefore, individuals taking or prescribing RTV should be aware of its reproductive toxicity.

1. Introduction

Since the COVID-19 pandemic in 2019, researchers have been developing a treatment [1–3]. Among the various candidate chemical compounds, ritonavir (RTV), a protease inhibitor (PI), has been effective in treating COVID-19. RTV is a well-known therapeutic chemical for acquired immune deficiency syndrome (AIDS) [4] and is widely used to treat various viral infections. Moreover, several studies have recently reported that RTV can be used to treat cancer [5–7]. Several studies have shown that RTV affects various types of cancer cells by reducing the phosphorylation of protein kinase B (AKT) [6,8,9]. RTV inhibits the AKT pathway in a dose-dependent manner in pancreatic ductal adenocarcinoma cell lines [6], with a stable total AKT level; however, in another study, phospho-AKT (Ser473) level decreased and apoptosis was induced in breast cancer cells [8]. Moreover, RTV exhibits synergistic effect when treated with AKT siRNA, leading to apoptosis in ovarian cancer cells [9].

Although RTV can be used in anti-viral and anti-cancer treatment regimens, several studies have reported that RTV can affect abnormal functions in normal cells [10,11]. RTV exerts cytotoxicity and ER stress in Hepa RG cell lines in a concentration-dependent manner [10]. In addition, RTV affects cell viability, LDH release, mitochondrial DNA damage, and apoptosis in human dermal microvascular endothelial cells [11]. However, reproductive toxicity of RTV has not been reported.

Ejaculated sperm cells undergo maturation, called ‘capacitation,’ to facilitate sperm-egg fusion [12,13]. During capacitation, tyrosine phosphorylation occurs, and only capacitated spermatozoa undergo the acrosome reaction [14]. AKT plays an important role in spermatozoa and is related to sperm function during capacitation. It has been reported that motility, hyperactivation, acrosome reaction, and tyrosine phosphorylation are associated with AKT during capacitation [15,16]. Although RTV can be used for cancer treatment, the toxicity of RTV has also been emphasized in studies on various cell types [10,11]. RTV could also exert reproductive toxicity because AKT plays an important role in sperm function and male fertility. However, there is no research on the toxicity of AKT-related RTV on sperm function and male fertility.
Therefore, the present study was designed to investigate the effects of RTV on sperm function following AKT regulation in spermatozoa during capacitation.

2. Materials and methods

All procedures were conducted according to the guidelines for the ethical treatment of animals and approved by the Institutional Animal Care and Use Committee of Kyungpook National University. All the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Modified Tyrode’s medium was used as the basic medium (BM) (97.84 mM NaCl; 1.42 mM KCl; 0.47 mM MgCl$_2$·6H$_2$O; 0.36 mM Na$_2$HPO$_4$·H$_2$O; 5.56 mM D-glucose; 25 mM NaHCO$_3$; 1.78 mM CaCl$_2$·0.2 H$_2$O; 24.9 mM Na-lactate; 0.47 mM Na-pyruvate; 50 μg/mL gentamycin; and 0.005 mM phenol red). Bovine serum albumin (0.4 %) was added to BM to induce capacitation [17,18].

2.1. Media and chemicals

ICR mice were bred at 22 ± 2 °C with proper humidity under 12 h light/dark cycle. All mice were supplied with food (Cargill Agripurina Inc., Korea) and water ad libitum. 3 mice were utilized per experiment as previously described [19]. Mature male mice aged 8–12 weeks were sacrificed by cervical dislocation, and the cauda epididymis was extracted. Finally, the spermatozoa were collected by puncturing the epididymis using a sterile needle. The spermatozoa were incubated with different concentrations of RTV (0, 0.1, 1, 10, and 100 μM) under 5 % CO$_2$ and 37 °C for 90 min to induce capacitation [17]. The concentration range of RTV was based on previous studies [9,20,21].

2.2. Preparation and treatment of spermatozoa

Levels of AKT, phospho-AKT (Thr$^{308}$ and Ser$^{473}$), and tyrosine phosphorylation were measured using western blotting. Each sample was washed twice with Dulbecco’s phosphate-buffered saline (DPBS). The samples were resuspended in modified Laemmli sample buffer (315 mM Tris, 10 % glycerol, 10 % SDS, 5 % 2-mercaptoethanol, and 5 % bromophenol blue) and incubated at room temperature (RT) for 10 min. Then, the samples were centrifuged at 10,000 g for 5 min, and the supernatants were boiled at 95 °C for 3 min. The protein samples were separated via 12 % SDS-PAGE (MiniPROTEIN Tetra Cell, Bio-Rad, Hercules, CA, USA) and then transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with 3 % ECL blocking agent (GE Healthcare, Chicago, IL, USA) for 2 h at RT. After washing the membranes with DPBS containing 0.1 % Tween 20 (PBS-T), the membranes were incubated with primary antibodies. The primary antibodies were diluted with 3 % ECL blocking agent [anti-alpha Tubulin antibody [DM1A] (Abcam, Cambridge, UK), 1:5000; anti-AKT antibody (Cell Signaling Technology, Danvers, Massachusetts, USA), 1:1000; anti-phospho-AKT (Thr$^{308}$) antibody (Cell Signaling Technology, Danvers, Massachusetts, USA), 1:1000; anti-phospho-AKT (Ser$^{473}$) antibody (Genetex, Inc, Irvine, CA, USA), 1:1000; anti-phosphotyrosine antibody [PY20] (HRP) (Abcam, Cambridge, UK), 1:50000]. After the membranes were incubated with each antibody, they were washed with PBS-T. Goat anti-mouse IgG H&L (HRP) (Abcam, Cambridge, UK) and goat anti-rabbit IgG H&L (HRP) (Cell Signaling Technology, Danvers, Massachusetts, USA) were diluted at 1:2000 with 3 % ECL blocking agent and used as secondary antibodies. Proteins were detected using the iBright™ CL1500 Imaging System (ThermoFisher Scientific, Waltham, MA, USA) by the enhanced chemiluminescence method. The Image Studio Lite (Version 5.2, LI-COR Corporate, Lincoln, NE, USA) system was used to analyze the protein expression levels. The density ratio of each treatment band was normalized to that of α-tubulin.

2.3. Western blotting of AKT, phospho-AKT (Thr$^{308}$ and Ser$^{473}$), and tyrosine phosphorylation

Sperm motility and motion kinematics

Sperm motility and motion kinematics were analyzed using a computer-assisted sperm analysis program (CASA) (FSA 2016, Medical Supply, Seoul, Korea). Sperm motility (total sperm motility (MOT, %), rapid sperm motility (RPP, %), medium sperm motility (MED, %), slow sperm motility (SLW, %), and progressive sperm motility (PRG, %)), motion kinematics (curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), beat-cross frequency (BCF, Hz), mean angular displacement (MAD, degree), wobble (WOB, %), dance (DNC, μm$^2$/s), mean dance (DNM, μm), and amplitude of lateral head displacement (ALH, μm) were measured. Briefly, 10 μL of the treated sample was laid on a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel), which was placed on a 37 °C heating plate. More than five random fields were recorded. Finally, the images were analyzed using the FSA 2016 program.

2.4. Sperm motility and motion kinematics

In the present study, we used the dual staining method (combined Hoechst 33258/chlortetracycline fluorescence assessment) to evaluate the capacitation status [22]. The samples were centrifuged at 100 × g for 2.5 min. The supernatant (135 μL) was discarded, Hoechst 33258 (H33258) solution was added, and the samples were incubated for 2 min at RT. Then, 250 μL of 2 % (w/v) polyvinylpyrrolidone in DPBS was added, and the samples were centrifuged at 100 × g for 2.5 min. All supernatants were removed, and 100 μL of DPBS and chlortetracycline fluorescence (CTC) solution (750 mM CTC, 20 mM Tris, 130 mM NaCl, and 5 mM cysteine, pH 7.4) were added. Finally, capacitation status was observed using an OLYMPUS BX43 (Olympus, Tokyo, Japan) with fluorescence illumination, using ultraviolet BP340–380/LP425 and BP450–490/LP515 excitation/emission filters for H33258 and chlortetracycline fluorescence, respectively. At least 400 spermatozoa per slide were sorted into four patterns according to the capacitation status: dead (D pattern, blue fluorescence spread evenly on the sperm head), live non-capacitated (F pattern, bright green fluorescence spread evenly on the sperm head), live capacitated (B pattern, bright green fluorescence spread over the acrosomal region and a dark post-acrosomal region), and live acrosome reacted (AR pattern, no fluorescence over the sperm head or bright green fluorescence only in the post acrosomal region) [22].

2.5. Sperm capacitation status

Cell viability was evaluated using a Cell Cytotoxicity Assay Kit (Abcam, Cambridge, UK). The assay solution was added to a 96-well plate containing each sample. The samples were incubated at 37 °C in the dark for 3 h. After incubation, absorbance was measured at 570 and 650 nm (Gemini Em; Molecular Devices Corporation, Sunnyvale, CA, USA). Cell viability was assessed as the ratio of OD 570 nm to OD 650 nm (SoftMax Pro 7; Molecular Devices Corporation, Sunnyvale, CA, USA).

2.6. Assessment of cell viability

Data were analyzed using one-way ANOVA with the SPSS software (version 26.0, IBM, Armonk, NY, USA). Tukey’s multiple comparison test was used to compare the four RTV-treated groups with the control group. Each experiment was conducted at least three times. Differences were considered significant when the p-value was less than 0.05 (P < 0.05). The data are expressed as the mean ± SEM.
3. Results

3.1. Expression levels of AKT, phospho-AKT (Thr\(^{308}\) and Ser\(^{473}\)), and tyrosine-phosphorylated proteins

The expression levels of AKT, phospho-AKT (Thr\(^{308}\) and Ser\(^{473}\)), and tyrosine-phosphorylated proteins were analyzed using western blotting. AKT and Thr\(^{308}\) were detected at approximately 60 kDa, and Ser\(^{473}\) was detected at approximately 56 kDa (Fig. 1D). Phospho-AKT (Thr\(^{308}\) and Ser\(^{473}\)) showed increasing patterns in dose-dependent manners. The level of Thr\(^{308}\) was significantly increased at 1 µM RTV concentration, and the level of Ser\(^{473}\) was significantly increased at highest RTV concentration (100 µM) (P < 0.05, Fig. 1A). These results indicate that the treatment of RTV on sperm increased the expression levels of phospho-AKT but not AKT. Phosphotyrosine substrates were detected at approximately 25 kDa, 60 kDa, and 100 kDa (Fig. 2B). The levels of phosphotyrosine substrates at approximately 25 and 100 kDa were significantly increased, whereas those at approximately 60 kDa were not significantly different (P < 0.05, Fig. 2). This indicates that the RTV significantly altered the tyrosine phosphorylation during the capacitation.

3.2. Effects of RTV on sperm motility and motion kinematics

MOT, RPD, PRG, VCL, BCF, DNC, and ALH were significantly decreased in all RTV treatment groups compared to those of the control group, whereas STR was significantly increased at highest concentration (100 µM) (P < 0.05, Table 1). In addition, WOB was significantly increased, whereas DNM was significantly decreased at high concentrations (10 and 100 µM) (P < 0.05, Fig. 2). This indicates that treatment of RTV increased acrosome reaction but decreased the capacitation abnormally.

3.3. Effects of RTV on capacitation status and cell viability

The ratio of the AR pattern was significantly increased at high concentrations (10 and 100 µM), whereas the ratio of the B pattern was significantly decreased at highest RTV concentration (100 µM) compared to that of the control group (P < 0.05, Fig. 3A and B). There was no significant change in the proportion of the F pattern (P < 0.05, Fig. 3C). The ratio of cell viability significantly decreased at high concentrations (10 and 100 µM) (P < 0.05, Fig. 3D). These results mean that treatment of RTV increased acrosome reaction but decreased the capacitation abnormally.

4. Discussion

RTV is a PI that prevents viral replication by binding to the viral protease and was originally used for managing AIDS [4]. RTV is mainly used as a boosting agent because it can disrupt the metabolism of another PI. Thus, RTV has been used to assist various anti-viral agents [23]. Using this mechanism for COVID-19 treatments, RTV has been used for related research and has recently been adopted as a component of medicine [24]. In addition, RTV is listed in the WHO Model List of Essential Medicines, which lists drugs that are considered safe and effective to meet the most important requirements of the health system [25]. However, previous studies have shown that RTV is toxic to several cells [10,11]. Nevertheless, the toxicity of RTV to spermatozoa remains unknown.

AKT signaling is involved in cell survival, growth, and proliferation in cancer and normal cells [26]. In spermatozoa, AKT also regulates sperm function during capacitation [15,16]. AKT should be phosphorylated for its activation [27]. RTV regulates cancer cells by suppressing AKT phosphorylation [6,8,9]. However, there have been no studies on how RTV affects AKT phosphorylation in spermatozoa and its effects on sperm function. Therefore, this study was designed to investigate the changes in AKT phosphorylation caused by RTV and its effects on sperm function and male fertility.

In the present study, AKT and phosphorylated AKT (Thr\(^{308}\) and Ser\(^{473}\)) levels were measured to investigate how RTV affects AKT phosphorylation in spermatozoa. Interestingly, contrary to previous studies [6,8,9], our result showed that RTV treatment did not decrease the phosphorylation of Thr\(^{308}\) and Ser\(^{473}\), however, the phosphorylation increased significantly compared to that of the control group in mouse spermatids.

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**Fig. 1.** Effects of ritonavir (RTV) on AKT and phosphorylated AKT (Thr\(^{308}\) and Ser\(^{473}\)) levels. (A) AKT level was measured at approximately 60 kDa. (B) Phosphorylated AKT (Thr\(^{308}\)) level was measured at approximately 60 kDa. (C) Phosphorylated AKT (Ser\(^{473}\)) level was measured at approximately 56 kDa. (D) AKT and phosphorylated AKT (Thr\(^{308}\) and Ser\(^{473}\)) were probed. Lane 1: Control; Lane 2: 0.1 µM RTV; Lane 3: 1 µM RTV; Lane 4: 10 µM RTV; Lane 5: 100 µM RTV. Data were evaluated by one-way ANOVA and expressed as mean ± SEM; n = 3. Values with different superscripts (a, b, and c) indicate significant differences between the control and treatment groups (P < 0.05).
Addition, AKT phosphorylation at Thr308 in spermatozoa (Fig. 1). Based on this result, we expect that RTV may affect AKT signaling methods compared to that of other cells. Meanwhile, AKT regulates sperm motility and kinematics. When AKT phosphorylation was inhibited by the AKT inhibitor SH-5, the AKT signaling pathway is also involved in capacitation and the acrosome reaction [15,32]. Akti-2 inhibits the acrosome reaction in spermatozoa [12,13]. In this study, the levels of tyrosine-phosphorylated proteins were measured. The results showed that the treatment of RTV significantly increased tyrosine phosphorylation (Fig. 2). In addition to tyrosine phosphorylation, AKT regulates sperm motility and kinematics. When AKT phosphorylation was inhibited by the AKT inhibitor SH-5, sperm motility, VCL, VSL, and VAP were significantly decreased in the CASA system. RTV treatment significantly reduced MOT, RPD, and SLW, and suppressed VCL, BCF, DNC, DNM, and ALH (Table 1), despite motility (%); RPD, rapid sperm motility (%); MED, medium sperm motility (%); SLW, slow sperm motility (%); PRG, progressive sperm motility (%); VCL, curvilinear velocity (μm/s); VSL, straight-line velocity (μm/s); VAP, average path velocity (μm/s); LIN, linearity (%); STR, straightness (%); BCF, beat cross frequency (Hz); MAD, mean angular displacement (degree); WOB, wobble (μm); DNC, sperm dance (μm²/s); DNLM, sperm mean dance (μm); ALH, mean amplitude of lateral head displacement (μm). Values with different superscript characters (a, b, and c) indicate significant differences within the same row indicate significant differences (P < 0.05), as determined by one-way ANOVA.

Spermatozoa undergo a special process called capacitation, which occurs in the female genital tract and enables spermatozoa to acquire the ability to fertilize. Tyrosine phosphorylation is caused by enzymes that are activated during capacitation. Finally, the sperm motility and motion kinematics are altered by the tyrosine phosphorylation [12,13]. In this study, the levels of tyrosine-phosphorylated proteins was measured at approximately 100, 60, and 25 μM. (B) Tyrosine phosphorylated proteins were probed. Lane 1: Control; Lane 2: 0.1 μM RTV; Lane 3: 1 μM RTV; Lane 4: 10 μM RTV; Lane 5: 100 μM RTV. Data were evaluated by one-way ANOVA and expressed as mean ± SEM; n = 3. Values with different superscripts (a, b, and c) indicate significant differences between the control and treatment groups (P < 0.05).

Table 1
Effects of Ritonavir (RTV) on sperm motility and motion kinematics.

| Concentration of RTV (μM) | Control | 0.1 | 1 | 10 | 100 |
|--------------------------|---------|-----|---|----|-----|
| MOT (%)                  | 74.34 ± 0.76a | 67.31 ± 0.26b | 68.66 ± 0.51b | 67.91 ± 0.65b | 67.78 ± 1.70b |
| RPD (%)                  | 53.87 ± 0.05a | 48.81 ± 0.55b | 46.30 ± 0.26b | 47.03 ± 0.04b | 46.18 ± 1.32b |
| MED (%)                  | 14.54 ± 1.12 | 11.31 ± 0.32 | 15.44 ± 0.34 | 13.97 ± 1.23 | 12.91 ± 0.37 |
| SLW (%)                  | 4.94 ± 0.05  | 5.54 ± 0.17  | 6.94 ± 0.59  | 7.92 ± 0.07  | 6.91 ± 1.24  |
| PRG (%)                  | 68.40 ± 1.17a | 60.12 ± 0.87b | 61.72 ± 0.08b | 60.99 ± 1.19b | 60.69 ± 0.53b |
| VCL (μm/s)               | 85.40 ± 1.37a | 76.46 ± 0.05a | 74.72 ± 0.81b | 71.22 ± 2.05b | 74.14 ± 0.85b |
| VSL (μm/s)               | 28.78 ± 0.27 | 30.85 ± 1.04 | 31.10 ± 3.36 | 30.26 ± 0.46 | 33.56 ± 0.11 |
| VAP (μm/s)               | 41.45 ± 2.37 | 42.86 ± 0.95 | 41.34 ± 2.35 | 41.42 ± 0.07 | 42.06 ± 2.14 |
| LIN (%)                  | 25.62 ± 0.92 | 26.74 ± 1.24 | 26.77 ± 0.63 | 29.80 ± 1.99 | 30.29 ± 1.41 |
| STR (%)                  | 64.61 ± 0.24c | 71.42 ± 0.58bc | 69.69 ± 1.22b | 72.43 ± 0.98bc | 74.88 ± 0.05b |
| BCF (Hz)                 | 3.61 ± 0.10bc | 5.64 ± 0.02bc | 5.26 ± 0.03bc | 5.13 ± 0.00bc | 5.30 ± 0.05bc |
| MAD (degree)             | 40.64 ± 0.16 | 40.45 ± 0.24 | 35.73 ± 1.12 | 37.36 ± 0.04 | 38.18 ± 2.24 |
| WOB (%)                  | 52.12 ± 0.80a | 56.19 ± 1.12b | 55.06 ± 1.29b | 58.11 ± 1.77b | 60.49 ± 0.87b |
| DNC (μm²/s)              | 300.09 ± 9.37a | 233.44 ± 1.85b | 203.86 ± 14.57b | 199.45 ± 10.94b | 212.74 ± 5.84b |
| DNLM (μm)                | 13.92 ± 0.68a | 11.38 ± 0.49bc | 10.86 ± 0.89bc | 9.69 ± 0.89b | 9.48 ± 0.61b |
| ALH (μm)                 | 2.42 ± 0.05a | 2.93 ± 0.01b | 2.76 ± 0.10a | 2.75 ± 0.08b | 2.75 ± 0.04b |

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addition, in the ZP3/ZPse-induced acrosome reaction, ZP3/ZPse stimulation increased AKT activation; moreover, when the sperm were treated with an AKT antagonist, PI3 kinase-dependent acrosome reaction decreased significantly in mouse spermatozoa [32]. Our results showed that RTV caused an increase in the ratio of acrosome-reacted spermatozoa (Fig. 3A), while the ratio of live-capacitated spermatozoa decreased (Fig. 3B), indicating that an abnormal acrosome reaction occurred after RTV treatment. Based on these results, we hypothesized that an abnormal increase in AKT phosphorylation and tyrosine phosphorylation also detrimentally affects sperm capacitation status. However, it is still unknown whether the change in tyrosine phosphorylation levels was due to AKT phosphorylation by RTV or whether RTV directly influenced tyrosine phosphorylation. According to previous research, AKT regulates tyrosine phosphorylation in spermatozoa. When AKT phosphorylation is disturbed, tyrosine phosphorylation level decreases in mouse spermatozoa [15]. Moreover, the recent study conducted in boar sperm showed that inhibition of GRP78 increased the AKT level with increased phospho-tyrosine levels [30]. Our results also showed that RTV treatment increased both the AKT phosphorylation and tyrosine phosphorylation. Therefore, we assume that there may be an interaction between AKT phosphorylation and tyrosine phosphorylation. However, direct evaluation is needed to determine the exact signal transduction system between AKT and tyrosine phosphorylation in spermatozoa.

RTV is known to have adverse effects on the viability of various cell types [11,33]. Similar to these results, RTV treatment suppressed cell viability in mouse spermatozoa in the present study (Fig. 3D). It has also been reported that phosphorylated AKT is important for stallion spermatozoa [31]. Thus, the decrease in cell viability observed in this study might be related to abnormally increased AKT phosphorylation.

5. Conclusion

RTV is one of the anti-viral agents currently used for COVID-19 treatment; it is also used for anti-cancer-related research involving inhibition of AKT phosphorylation. Our results showed that RTV abnormally increased AKT and tyrosine phosphorylation and suppressed sperm functions during capacitation. Therefore, we suggest that special attention is required when prescribing this drug and the patients must be warned about its reproductive toxicity. In addition, the present study is the first to elucidate the reproductive toxicity of RTV in association with AKT. Thus, we anticipate that the present study will provide useful data for further reproductive toxicity studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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