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Addition of peroxiredoxin 6 (PRDX6) to IVF fertilization medium maintains motility and longevity of human spermatozoa

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Abstract: This study aims to investigate the protective effects of peroxiredoxin 6 on the total motility and progressive motility of human spermatozoa. Semen samples with normal parameters were collected from 23 males and supplemented with different concentrations of peroxiredoxin 6. All the semen samples were measured according to the WHO 5th manual, and the motile spermatozoa were extracted using IVF fertilization medium supplemented with different peroxiredoxin 6 concentrations. Total motility and progressive motility were observed at different time-points of culture at room temperature. After peroxiredoxin 6 supplementation, all groups had a significant increase in total motility and progressive motility compared to the control group. The difference in total motility and progressive motility between the 0 and 10^{-7} mM groups was observed at 24 and 48 h of culture at room temperature. At 24 h, the total motility increased by 30% in the control group (16.03 ± 11.91 *vs.* 11.51 ± 7.84), and progressive motility increased by 21% (10.53 ± 9.4 *vs.* 8.31 ± 6.04). A similar trend was observed in the 48 h group. In addition, we also found that peroxiredoxin 6 had a well protective effect on sperm kinetic parameters at 10^{-7} mM. The findings of this study suggest that peroxiredoxin 6 can enhance sperm total motility and progressive motility in IVF fertilization medium. Peroxiredoxin 6 may have potential benefits for sperm preparation in assisted reproductive technology.

Introduction

Sperm parameters, including semen concentration, general volume, color, consistency, motility, vitality, and morphology, are used to determine the quality of an ejaculate and for *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (Alessandro *et al.*, 2018; Shu *et al.*, 2013; Ohlweiler *et al.*, 2019). Human sperm motility is of utmost importance for improving the efficacy of IVF and intracytoplasmic sperm injection (Stanic *et al.*, 2002; Sun *et al.*, 2018). In particular, maximizing the clinical pregnancy rate following artificial insemination necessitates the development of strategies to manage sperm metabolism so that human sperm cells retain full structural and functional characteristics during their collection, *in vitro* culture, and subsequent insemination (Lee *et al.*, 2018). Therefore, hyperactivity is one of the necessary processes for IVF efficiency and increasing embryonic development in assisted reproductive technologies (Stauss *et al.*, 1995; Suarez and Ho, 2010).

PRDX6 (one of six members of the peroxiredoxins (PRDXs) family) plays an important role in protecting spermatozoa against oxidative stress (Gong *et al.*, 2012; Liu and O'Flaherty, 2017). O'Flaherty and his colleagues observed that PRDX6 could affect the viability of spermatozoa and promote oxidative stress, thus increasing the levels of lipid peroxidation, and hence increasing sperm motility *in vivo* (O'Flaherty and Souza, 2011). Studies have also found that PRDX6 has peroxidase and calcium-independent phospholipase A2 activities and is a major factor in the protection of sperm motility, fertilization, and blastocyst development (Fisher, 2017; Moawad *et al.*, 2017).

Studies have demonstrated that the peroxidase and phospholipase A2 activities of PRDX6 are important for sperm quality *in vivo* (Moawad *et al.*, 2017; Ozkosem *et al.*, 2016; O'Flaherty, 2018). In our previous study, we found that PRDX6 promoted total and progressive motility of human spermatozoa after cryopreservation (Sun *et al.*, 2020). However, its effect on sperm motility *in vitro* is not

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known. Therefore, in the present investigation, we examined the hypothesis that PRDX6 can exert beneficial effects on total sperm motility and progressive motility under *in vitro* conditions.

Materials and Methods

Patients

Twenty-three semen samples were collected from 23 healthy donors at the clinical laboratory of the infertility center between December 2019 and January 2020. The mean age of healthy donors was 32.88 ± 4.64 years old. All men were asked to maintain abstinence for 2–7 days before sample collection and to release semen into sterile containers by masturbation. The study was approved by the Institutional Ethical Committee of Peking University International Hospital.

Semen analysis and separation

The semen characteristics were assessed according to World Health Organization (WHO) criteria (volume ≥1.5 mL, total motility ≥40%, sperm concentration ≥15 × 10^6 sperm/mL, and ≥4% normal). If all semen parameters meet the WHO criteria, the untreated semen was washed twice using IVF fertilization medium (G-IVF™ PLUS, Vitrolife, Sweden). Then, the semen samples were mixed with 2 mL of IVF fertilization medium and divided into five groups.

PRDX6 supplementation

PRDX6 (Sigma-Aldrich, Saint Louis, MO, USA) at the concentration of control, 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9} mM was added into the IVF fertilization medium of the five groups of semen samples, respectively. The influence of PRDX6 supplementation on motility was assessed at 1 h, 12 h, 24 h and 48 h at room temperature. At least 200 spermatozoa were scored for motility evaluation under 200× magnification using a computer-assisted sperm analysis program (CASA, WeiLi, Beijing, China) and graded as rapid progressive (PR), non-progressive (NP), and immotility (IM) spermatozoa. All kinetic parameters including straight-line velocity (VSL) (μm/s), curvilinear velocity (VCL) (μm/s), average path velocity (VAP) (μm/s), linearity (LIN) (%), straightness (STR) (%) and wobble (WOB) (%) were recorded.

Statistical analyses

Data were analyzed by two-way repeated-measures ANOVA using SPSS 3.0 software (USA). The Student's paired *t*-test was performed to compare data between groups. Multiple comparisons were made using the Bonferroni procedure. $P < 0.05$ was considered statistically significant.

Results

Initial seminal analysis after ejaculation

All semen characteristics (volume, concentration, progressive rate, non-progressive rate, immotility, and morphology) were found to be normal, according to the WHO 5th edition (Tab. 1). The mean sperm concentration was 120.03 ± 19.07 × 10^6/mL; volume was 3.1 ± 0.56 (mL); total motility was 75.66 ± 8.96%; progressive motility was 60.91 ± 7.95%; non-progressive motility was 14.75 ± 3.01%; and the percentage of spermatozoa with normal morphology was 5.13 ± 1.13%.

TABLE 1

The characteristics of the patients who underwent semen analyses (N = 23)

Parameter	Mean	SD	Median	25%–75% range
Age (years old)	32.88	4.64	32.5	30.25–37
Volume (mL)	3.1	0.56	3.3	2.9–3.45
Concentration (10^6/mL)	120.03	19.07	112.92	110.91–124.25
Total motility (PR + NP) (%)	75.66	8.96	77.28	74.49–80.53
PR (%)	60.91	7.95	63	60.01–65
NP (%)	14.75	3.01	14.48	12.51–15.55
Normal morphology (%)	5.13	1.13	5	4–6

PR, progressive motility; NP, nonprogressive motility; SD, standard deviation

Effects of different concentrations of PRDX6 on sperm total and progressive motility

The classic negative effects of long-term *in vitro* culture on sperm motility were clearly observed (Tab. 2). In particular, we observed a significant decrease in total motility and progressive motility in all sperm samples (Fig. 1). *In vitro* culture of spermatozoa resulted in approximately 25%–75% reduction in total and progressive motility compared to the 12 h group after the incubation. The difference in total and progressive motility was also observed at 24 h and 48 h after the incubation. At 1 h, the total motility (PR+NP) was the highest, but with the prolongation of culture time, sperm motility decreased significantly (Fig. 1A). A similar trend was observed in progressive (PR) motility (Fig. 1B). As we observed, it seems that the decline speed of total motility is more obvious than that of progressive (PR) motility. At 12 h, the total motility decreased by 60% in the 1 h group (20.69 ± 1.96 *vs.* 56.99 ± 2.3); in the 24 and 48 h groups, total motility decreased by 75% (13.78 ± 2.03 *vs.* 56.99 ± 2.3) and 90% (5.56 ± 1.45 *vs.* 56.99 ± 2.3), respectively ($P < 0.001$ compared to the 1 h).

Optimum concentration of PRDX6 on total and progressive motility

The total and progressive motility in all samples decreased gradually with culture time (Fig. 2). Although there was a significant decrease in sperm motility with the prolongation of culture time, we found that regardless of total motility or progressive motility, 10^{-7} mM and 10^{-9} mM PRDX6 were the best concentrations to protect sperm motility at 24 h and 48 h. Even though the other PRDX6 groups had a marginally higher percentage of total or progressive motility, statistically, it was not found to be significant compared to the 10^{-7} mM group at any time point.

PRDX6 on sperm kinetic parameters

With the powerful analysis capability of CASA, we recorded almost all dynamic parameters, including VCL (μm/s), VSL (μm/s), VAP (μm /s), LIN (%), STR (%) and WOB (%). In our study, most individual sperm velocity parameters (VSL, VCL, VAP, LIN, WOB and STR) were significantly decreased along with the extension of culture time (Tab. 3). Surprisingly, our

TABLE 2

Effects of different concentrations of PRDX6 supplemented to IVF fertilization medium on total and progressive motility

Parameter	Time points (h)	PRDX6 (mM)				
		control	10^{-3}	10^{-5}	10^{-7}	10^{-9}
Concentration (M/mL)	0	28.27 ± 13.56	29.2 ± 15.82	30.98 ± 17.04	26.28 ± 14.21	29.81 ± 18.08
	12	23.15 ± 12.53	20.42 ± 13.18	20.98 ± 14.05	20.0 ± 12.0	23.82 ± 15.75
	24	20.63 ± 10.91	23.83 ± 12.98	23.43 ± 12.75	22.31 ± 12.32	21.73 ± 12.45
	48	26.27 ± 17.29	23.88 ± 14.94	23.84 ± 14.83	22.28 ± 11.83	24.19 ± 15.83
Total motility (%)	0	57.34 ± 16.31	57.88 ± 19.53	56.74 ± 16.51	56.36 ± 16.76	59.65 ± 19.52
	12	22.56 ± 23.61	19.4 ± 26.02	22.61 ± 23.26	20.13 ± 22.44	20.73 ± 24.47
	24	11.51 ± 7.84	12.74 ± 12.26	12.79 ± 10.03	**16.03 ± 11.91****	**15.83 ± 10.95***
	48	5.23 ± 6.6	4.03 ± 4.26	6.94 ± 7.82	6.44 ± 6.58	5.18 ± 3.89
PR (%)	0	51.29 ± 15.2	51.33 ± 18.62	48.35 ± 16.0	50.24 ± 17.33	53.75 ± 15.27
	12	17.59 ± 21.92	13.77 ± 24.1	12.27 ± 21.86	15.78 ± 22.26	13.99 ± 23.44
	24	8.31 ± 6.04	8.27 ± 8.64	9.42 ± 8.14	**10.53 ± 9.4***	**11.63 ± 8.15****
	48	3.77 ± 5.21	3.0 ± 3.68	4.93 ± 6.12	3.65 ± 3.75	3.46 ± 3.18
NP (%)	0	6.09 ± 2.49	6.56 ± 2.79	6.5 ± 2.9	5.0 ± 2.7	5.91 ± 3.27
	12	4.97 ± 2.68	5.62 ± 3.66	6.83 ± 3.81	5.86 ± 2.84	6.74 ± 4.03
	24	3.2 ± 2.1	4.46 ± 4.35	3.37 ± 3.03	5.5 ± 4.03	4.2 ± 3.2
	48	1.46 ± 1.57	1.03 ± 1.07	2.01 ± 2.37	2.79 ± 2.95	1.71 ± 1.61

PR, progressive motility; NP, non-progressive motility; SD, standard deviation; *Statistically significant. (*) indicates *P*-values: significant ($P < 0.05$); (**) highly significant ($P < 0.01$) *vs* 0 concentration

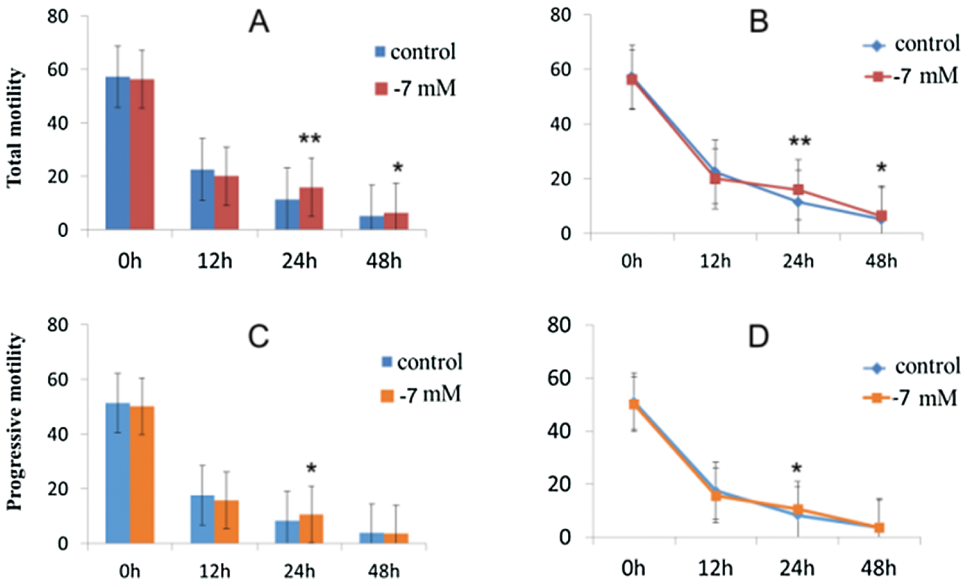

FIGURE 1. Effects of PRDX6 on total and progressive motility at different hours after culture *in vitro*.
(A and B) Total motility and all time-points. (C and D) Progressive motility and all time-points. *significant difference *vs.* control ($P < 0.05$); **significant difference *vs.* control ($P < 0.01$).

results showed that the sperm showed a high VCL and VSL at concentrations of 10^{-7} mM and 10^{-9} mM (Tab. 3). In addition, we also found that sperm velocity parameters of STR and LIN also had the same trend at concentrations of 10^{-7} mM and 10^{-9} mM (Tab. 3). The above results suggest that PRDX6 has a better protective effect on sperm kinetic parameters at 10^{-7} mM and 10^{-9} mM concentrations than at other concentrations.

Discussion

Our study was carried out to investigate the effects of different concentrations of PRDX6 on human sperm motility using a CASA system. The results showed that PRDX6 increased human sperm total and progressive motility. The positive effects of PRDX6 on sperm motility have also been demonstrated previously (Shi *et al.*, 2018). The beneficial effect of PRDX6 was observed at 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9} mM, respectively.

It has been reported that PRDX6 can promote sperm movement, improve sperm fertilization rate, and increase the penetration rate of boar spermatozoa and zona pellucida (Fisher, 2017; Moawad *et al.*, 2017). The total and progressive motility of human sperms were increased by using PRDX6 at 10^{-7} mM and 10^{-9} mM at different

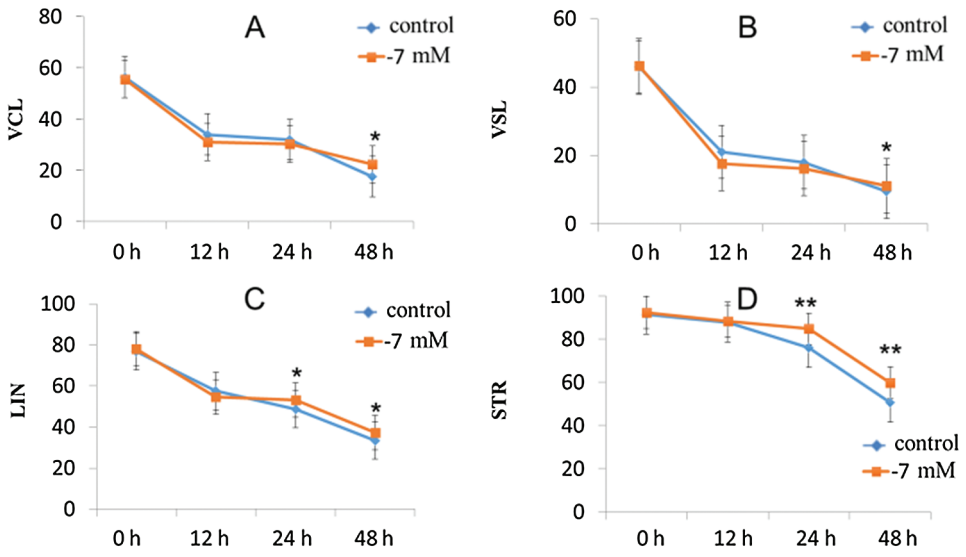

FIGURE 2. Effects of PRDX6 on sperm kinetic parameters (VCL, VSL, LIN and STR).
(A) Curvilinear velocity (VCL) (μm/s) and all time-points. (B) Straight-line velocity (VSL) (μm/s) and all time-points. (C) Linearity (LIN) (%) and all time-points. (D) Straightness (STR) (%) and all time-points. *significant difference *vs.* control ($P < 0.05$); **significant difference *vs.* control ($P < 0.01$).

TABLE 3

Effects of PRDX6 on sperm kinetic parameters (mean ± SD)

Parameter	Time points (h)	PRDX6 (mM)				
		control	10^{-3}	10^{-5}	10^{-7}	10^{-9}
VCL (μm/s)	0	56.27 ± 10.45	57.77 ± 9.74	58.85 ± 9.46	55.53 ± 9.16	55.86 ± 10.52
	12	34 ± 7.01	33.49 ± 8.75	33.18 ± 7.93	31.04 ± 6.69	32.42 ± 10.09
	24	31.96 ± 14.41	28.33 ± 12.3	27.97 ± 8.27	**30.26 ± 5.54**	**32.16 ± 7.24***
	48	17.57 ± 15.28	23.03 ± 12.16	28.09 ± 14.98	22.31 ± 16.54	**29.33 ± 16.92***
VSL (μm/s)	0	45.91 ± 11.18	47.9 ± 10.39	48.22 ± 9.55	46.34 ± 10.74	46.54 ± 10.66
	12	21.11 ± 7.65	19.49 ± 6.7	19.83 ± 6.1	17.69 ± 5.01	17.62 ± 8.56
	24	18.14 ± 8.51	15.08 ± 7.01	16.11 ± 5.38	16.25 ± 5.94	**19.17 ± 5.73***
	48	9.55 ± 8.59	14.93 ± 13.2	14.51 ± 12.49	11.24 ± 9.54	**15.7 ± 9.71***
VAP (μm/s)	0	48.25 ± 10.91	50.37 ± 10.5	50.49 ± 9.5	48.44 ± 10.82	48.48 ± 10.87
	12	22.74 ± 7.15	21.59 ± 6.72	21.55 ± 6.79	19.41 ± 5.54	20.4 ± 7.53
	24	20.1 ± 9.34	16.54 ± 7.37	17.51 ± 5.9	16.16 ± 7.46	**21.56 ± 5.59***
	48	10.65 ± 9.52	16.33 ± 13.51	16.91 ± 11.86	13.33 ± 10.0	**17.95 ± 10.96***
LIN (%)	0	76.82 ± 6.11	77.02 ± 6.11	76.93 ± 5.29	78.15 ± 6.42	78.23 ± 5.24
	12	57.55 ± 14.26	56.08 ± 8.45	57.34 ± 5.47	54.57 ± 5.45	50.25 ± 18.69
	24	48.78 ± 18.93	46.57 ± 20.42	56.97 ± 5.08	53.16 ± 10.01	**58.05 ± 9.28***
	48	33.46 ± 28.07	43.39 ± 40.43	41.68 ± 27.71	37.32 ± 23.66	**46.78 ± 21.22***
WOB (%)	0	82.81 ± 4.69	83.69 ± 4.82	82.56 ± 4.35	83.87 ± 5.29	83.47 ± 4.49
	12	64.29 ± 9.32	63.86 ± 6.14	63.61 ± 7.11	61.72 ± 6.74	61.36 ± 10.7
	24	55.11 ± 22.7	52.33 ± 21.92	62.5 ± 5.24	61.26 ± 6.52	**66.95 ± 9.48***
	48	38.61 ± 32.12	50.31 ± 32.94	51.13 ± 25.09	45.61 ± 28.27	53.19 ± 22.37
STR (%)	0	91.35 ± 2.71	90.67 ± 2.86	91.99 ± 2.54	92.2 ± 2.24	92.59 ± 1.93
	12	87.82 ± 12.49	85.73 ± 8.26	88.13 ± 2.49	88.3 ± 7.2	77.4 ± 24.61
	24	76.05 ± 31.67	76.32 ± 31.52	**90.84 ± 5.17***	84.74 ± 11.66	84.71 ± 9.39
	48	50.67 ± 42.35	63.03 ± 40.41	65.27 ± 40.8	59.78 ± 39.16	**75.76 ± 31.69***

VCL, Curvilinear velocity (μm/s); VSL, Straight-line velocity (μm/s); VAP, Average path velocity (μm/s); LIN, Linearity (%); WOB, Wobble (%); STR, Straightness (%); SD, standard deviation; *Statistically significant. (*) indicates *P*-values: significant ($P < 0.05$); (**) highly significant ($P < 0.01$) *vs.* control

time-points (1 h, 12 h, 24 h, and 48 h) after culture at room temperature, as revealed in our study. However, other concentrations of PRDX6 did not dramatically affect sperm movement. Therefore, the effect of PRDX6 on sperm characteristics may be specific; sperm motility may be adversely affected by other concentrations of PRDX6.

The optimum value of sperm motility is a key factor for successful fertilization. According to the WHO 5th manual, less than 40% of the proportion of motile spermatozoa is an essential prognostic fertility factor (Shu *et al.*, 2013). Sperm motility parameters, including ALH, VCL, VAP, and LIN, have been shown to be important markers of sperm motility and fertilizing ability (Lee *et al.*, 2018; Shih *et al.*, 2016; Rondanino *et al.*, 2015). To our knowledge, no study has found a significant improvement in motility after PRDX6 treatment *in vitro*. Some have observed either no improvement in sperm total motility or VCL and VSL. Our results for the first time indicate that PRDX6 can not only improve the total sperm motility but also improve progressive motility.

Oxidative stress has a negative effect on sperm motility through reactive oxygen species (ROS) and ROS-dependent proteins. Before assisted reproductive technology, human spermatozoa were processed through *in vitro* preparation and cultured for a long time, which could induce certain levels of ROS (Shih *et al., 2016*). PRDXs have been confirmed to have antioxidant enzyme activities to control the levels of ROS production and avoid oxidative damage in the spermatozoa (O'Flaherty, 2018). Previous studies have shown that PRDX6 is the primary antioxidant defense in human spermatozoa and maintains viability by regulating the phosphoinositide 3-kinase/AKT pathway (Fernandez and O'Flaherty, 2018; Fernandez *et al.*, 2019). Our results showed that PRDX6 significantly enhanced the sperm total motility of sperm under oxidative stress. As for the protective effect of PRDX6 on sperm motility, our results were consistent with those reported by O'Flaherty C *et al.* (Moawad *et al.*, 2017). The enhanced total and progressive motility induced by PRDX6 is due to its inhibitory effect on ROS. It is difficult to elucidate the exact mechanism of action of PRDX6 from this preliminary data. However, through indirect methods, we attempted to know whether this effect is mediated by the inhibition of ROS and ROS-related pathways. Surprisingly, we observed that the addition of different concentrations of PRDX6 to the IVF fertilization medium not only significantly improved sperm motility but also maintained 48 h of motility. Moreover, the 10^{-7} mM or 10^{-9} mM PRDX6 supplemented group maintained a significantly higher percentage of total and progressive motility than the other groups at all time- points. Although our research shows that PRDX6 may have potential application value as human sperm motility "vigor" *in vitro*, further research is needed to clarify the exact molecular mechanism of PRDX6-induced sperm motility enhancement.

Conclusion

Collectively, our novel finding has demonstrated that supplementing PRDX6 to IVF fertilization medium can increase sperm total and progressive motility *in vitro*. Additionally, lower concentrations of PRDX6 have better protective effects than higher concentrations. Therefore, we recommend the addition of PRDX6 at 10^{-7} mM concentration to the IVF fertilization medium.

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Author Contribution: The authors confirm contribution to the paper as follows: Study conception and design: (X. Chen and L. Tian); data collection: (Xin Ping Sun, Hong Yu and Ling Li Song); analysis and interpretation of results: (Tie Cheng Sun, Yan Dong Zhang and Jian Hua Li); draft manuscript preparation: (Tie Cheng Sun, Yan Dong Zhang and Jian Hua Li). All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval: The study was approved by the Institutional Ethical Committee of Peking University International Hospital, ethical approval code: S2020-04.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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