Research Article

Clinical Pregnancy Is Not Associated with Sperm Quality in POSEIDON Group 1 and 2 Patients

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This study is aimed at establishing the effects of varying sperm quality on IVF-ICSI/IMSI outcomes in unexpected poor ovarian responder subjects of POSEIDON groups 1 and 2. In the present study, 1,263 couples with female partners who fulfilled the POSEIDON group 1 and 2 criteria were recruited. All couples underwent ICSI or IMSI at Morula IVF Jakarta Clinic, Indonesia. Patients were subsequently classified into six groups, according to semen conditions of the respective male partners: (1) normozoospermic, (2) teratozoospermia, (3) oligoasthenoteratozoospermia (OAT), (4) asthenozoospermia, (5) severe OAT, and (6) cryptozoospermia. Laboratory and clinical outcomes of the IVF-ICSI/IMSI program were then evaluated. Early and late embryonic development parameters including the number of fertilization, cleavage and blastocyst stages, and blastocyst quality differed significantly among the different sperm quality groups ($p < 0.05$). No difference was observed in the number of embryo transfers and clinical pregnancy among the studied groups ($p > 0.05$). Our study has demonstrated the effect of sperm quality on embryo development at the early and later stages; however, the clinical pregnancy was not impaired in the unexpected poor responders of POSEIDON groups 1 and 2.

1. Introduction

Poor ovarian responders (PORs), indicated as women who fail to respond sufficiently to standard ovarian stimulation, continue to impede the success of in vitro fertilization (IVF) practice [1]. With a high prevalence of more than 20%, such condition is therefore challenging for both the patients and clinicians [2]. Consequently, the identification

of POR is vital to individualize the patient's IVF treatment for an optimized outcome [3]. The Bologna criterion, refined by the European Society of Human Reproduction and Embryology (ESHRE) (2011), was the principal consent to homogenize the characterization of a poor response which varied considerably at that time [4]. However, critical appraisal of Bologna criteria has unveiled heterogeneity in the POR population [4, 5]. Therefore, a group of scientists introduce new criteria known as POSEIDON (Patient-Oriented Strategies Encompassing Individualized Oocyte Number) to define a poor ovarian response, reinstating the previous consensus [6].

In contrast to the previously established criteria, the POSEIDON concept (2016) stratifies poor ovarian responders into four groups according to (i) age, (ii) ovarian reserve measures (level of AFC and/or AMH), and (iii) previous response to ovarian stimulation, i.e., "unexpected" (groups 1 and 2) and "expected" PORs (groups 3 and 4) [5, 6]. Implementation of POSEIDON criteria has evidently heightened the understanding of POR including its therapeutic management and research design [5]. Several studies have demonstrated that POR subjects consistently exhibit a suboptimal IVF pregnancy rate compared to normal responder subjects [7–9] in addition to a reduced retrieval of mature oocytes and a higher cancellation rate [10, 11]. Suboptimal quality and quantity of oocytes obtained through the controlled ovarian stimulation were then considered as one of the underlying factors contributing to the unsatisfactory IVF outcomes [3].

While the maternal factor has been well-analyzed, the role of sperm quality on IVF outcomes in poor responders interestingly remains inconclusive [12]; despite a staggering, 37.1% of poor responders were reported by Garcia-Velasco et al. [3] to have infertile male partners [3]. Correspondingly, the impact of sperm quality on IVF outcomes regardless of the female partner's prognosis remains obscure [13]. Lee et al. [14] revealed that rather than embryo development or quality, sperm quality influenced implantation and pregnancy [14]. Meanwhile, Loutradi et al. described a remarkable decline in fertilization rate, cleavage-stage formation, and blastocyst development in women whose partners had poor semen quality [15], confirming previous existing evidence [16, 17]. On the contrary, no significant impact of severe male factor infertility on implantation and subsequent pregnancy outcome has been reported [15].

This study intends to fulfill the essential need to elucidate the link between semen conditions and IVF outcomes by evaluating the effects of varying sperm quality in IVF treatment of poor ovarian responders who fulfilled groups 1 and 2 of the POSEIDON criteria.

2. Materials and Methods

2.1. Study Population.
This retrospective cross-sectional study was performed in Morula IVF Jakarta Clinic, Jakarta, Indonesia. Data of 1,263 female subjects who fulfilled the POSEIDON group 1 (aged < 35 years) and 2 (aged ≥ 35 years) criteria with normal ovarian reserve features including AMH ≥ 1.2 ng/mL and/or AFC ≥ 5 and <9 retrieved oocytes in the previous stimulation were enrolled and classified according to the respective male partner's semen analysis as follows: (1) normozoospermia, (2) teratozoospermia, (3) OAT, (4) asthenozoospermia, (5) severe OAT, and (6) cryptozoospermia. Semen analysis was conducted based on WHO criteria [18]. All data were extracted from the medical records between January 2015 and November 2021.

2.2. Ovarian Stimulation.
Recruited patients underwent mild, agonist, or antagonist ovarian stimulation protocol. In the mild stimulation, subjects were administered 150 mg of clomiphene citrate per day continuously from the second until the sixth day of the menstrual period plus a daily injection of 150 IU hMG (Menopur, Ferring) or 150 IU/75 IU rFSH/rLH (Pergoveris, Merck Serono). 0.25 mg (Cetrotide, Merck Serono) antagonist injections were performed on the seventh day. Treatment was prolonged until 18 mm follicles developed, and a trigger injection with 250 mcg rhCG (Ovidrel, Merck Serono; equivalent to 6500 IU) was performed. In antagonist protocol, 300 IU/150 IU of rFSH/rLH (Pergoveris, Merck Serono) or 150-450 IU rFSH (Gonal F, Merck Serono) or 150-450 IU hMG (Menopur, Ferring) was given daily, beginning on the second or third day of the menstrual period. GnRH antagonist administration has begun on the fifth day of the stimulation and afterward a 250 mcg of rhCG trigger injection when the growing follicles reached adequate sizes. In agonist protocol, 250 pg agonist administration commenced on the twenty-first day of the menstrual cycle which continued daily for 14 days. Through monitoring with blood tests and transvaginal ultrasound, stimulation with daily 150-450 IU rFSH or hMG was performed if follicles of size < 5 mm and E2 level of <40 pg/ mL were confirmed. GnRH agonist administration was prolonged until hCG injection. In all protocols, rhCG was injected after the diameter of at least two leading follicles reached ≥18 mm.

2.3. Ovum Pickup and Nuclear Maturation Assessment.
Under transvaginal ultrasound guidance, ovum pickup was performed 36 hours after hCG trigger shot to retrieve oocytes in G-MOPS Plus medium (Vitrolife, Sweden). Cumulus-oocyte complexes (COCs) were rinsed and incubated for 3 hours in an incubator under 6% CO_2 at 37°C covered with paraffin oil (OVOIL; Vitrolife). Upon incubation, cumulus cells were stripped off using hyaluronidase (Vitrolife) and the oocytes were washed in a culture medium prior to maturation assessment. Only mature oocytes with a distinct first polar body observed under an inverted microscope were viable for ICSI or IMSI.

2.4. Sperm Preparation.
After abstinence for 3-5 days, fresh ejaculate semen was obtained by masturbation on the day of oocyte retrieval. Semen was collected into a sterile sample container and allowed to liquefy for 30 minutes at room temperature. Sperm volume, concentration, and motility were evaluated according to the "WHO laboratory manual for the examination and processing of human semen" (2010). Semen samples were subsequently processed by an albumin gradient, density gradient, swim-up,

or simple wash technique according to our clinic's standard operating procedure as described previously [19]. The final sperm postwash count and motility were then examined.

2.5. Insemination and Assessment of Fertilization, Embryo Grading, and Transfer. All denuded mature oocytes were treated with ICSI under 200x magnification (Olympus IX71) or IMSI under 6000x (Eclipse Ti) magnification on a heated stage (Tokai-Hit, Olympus). Briefly, oocytes were each placed in a $5\,\mu$L G-MOPS Plus medium (Vitrolife, Sweden) droplet covered with mineral oil on a Petri dish (Nunc IVF Dish for ICSI, Thermo Scientific and glass-bottomed dish for IMSI (Fluorodish, WPI)). A selected spermatozoon was immobilized by nicking its tail and aspirated into an injection pipette (TPC, CooperSurgical Fertility). The spermatozoon was subsequently injected into a mature oocyte through the zona pellucida at the 3 o'clock position with the polar body at the 12 o'clock orientation. 17 ± 2 hours after the insemination, an assessment of fertilization, characterized by the appearance of two distinct pronuclei, was conducted. Embryos were then cultured in G1 (Vitrolife, Sweden) for three days and transferred into G2 (Vitrolife, Sweden) medium covered with mineral oil until day 5/6 (37°C, 6% CO_2, and 5% O_2). Cleavage-stage embryos were morphologically graded according to blastomere regularity, degree of fragmentation, and cell number. Meanwhile, trophectoderm density, inner cell mass, and blastocyst cavity expansion were evaluated at the blastocyst stage [20]. The day of embryo transfer was decided according to the standard operating procedure in our clinic. Specifically, when less than three good-quality embryos were available on day 2 or 3 observation, the patients were suggested to perform embryo transfer at the cleavage stage. Conversely, patients were suggested to prolong embryo culture up to the blastocyst stage when at least three good-quality cleavages were available to allow natural selection. To yield a comprehensive review, all couples who underwent fresh embryo transfer on either cleavage or blastocyst stage were included in the analyses. A viable embryo was transferred, and after 14 days, a biochemical pregnancy test was carried out through serum bHCG level measurement. A clinical pregnancy ultrasound was performed approximately 2 weeks after confirming positive for the biochemical pregnancy. Clinical pregnancy measurement was described previously [21].

2.6. Statistical Analysis. Descriptive statistics outline the characteristics of the enrolled participants. Categorical variables and numerical variables were shown as the number of subjects and percentage (%) and as mean ± standard deviation or median and interquartile range (Q1 and Q3), if applicable. Categorical and numerical data were, respectively, analyzed by chi-square and *t*-test or Mann–Whitney. To adjust for potential confounders which might interfere with the outcomes of interest, a multiple analysis was performed. Data analyses were done using SPSS software (release 20.0, Chicago, USA) at a 95% confidence level.

3. Results

Data of 1,263 IVF-ICSI/IMSI treatment cycles were examined. Overall basal and clinical parameters of the recruited subjects are summarized in Table 1. As shown in the table, the median female and male ages were 35 and 37 years old, respectively. Median AMH level and AFC indicated normal ovarian reserve markers in addition to normal median BMI ($<25\,$kg/m^2). Asthenoteratozoospermia ($n = 520$ (41.2%)) was the most prevalent abnormal semen analysis in this study followed by teratozoospermia ($n = 340$ (26.9%)), OAT ($n = 204$ (16.2%)), severe OAT ($n = 90$ (7.1%)), cryptozoospermia ($n = 76$ (6%)), and normozoospermia ($n = 33$ (2.6%)). The median female age in each group was relatively young with BMI in the normal reference ($<25\,$kg/m^2). The median of both AMH levels and AFC in each group indicated normal ovarian parameters (Table 1).

According to the analysis, inconclusive result in clinical pregnancy was observed with varying sperm quality ($p = 0.658$) (Table 2) notwithstanding adjustment for female age and progesterone level on the trigger day (adjusted OR 1.027, 95% CI 0.93–1.134, adjusted *p* value = 0.594). Subgroup analysis of clinical pregnancy according to the day of embryo transfer and sperm selection methods among studied groups also did not differ ($p > 0.05$). Embryo outcomes are presented in Table 2. Statistically significant differences were observed in the number: fertilized oocytes ($p = 0.003$), cleavage-stage embryos ($p = 0.001$), blastocyst ($p = 0.003$), good-quality blastocyst ($p = 0.036$), and the proportion of cleavage-stage embryo (day 2/3) and blastocyst (D5/6) transfer ($p = 0.003$). No differences were observed in the proportion of abnormal fertilization, number of good cleavage-stage embryo quality, and number of embryos transferred (Table 2). In addition, both sperm selection strategies were shown to have equal number of fertilized oocytes across sperm quality groups ($p > 0.05$).

4. Discussion

The current study demonstrated that varying semen quality had no impact on the clinical outcomes of IVF-ICSI or -IMSI cycles in POSEIDON groups 1 and 2, alternately referred to as unexpected poor ovarian responders. While the effect of sperm quality has long been investigated in the general infertile population undergoing IVF programs [15, 22], we endeavor to scrutinize the impacts of semen quality in IVF cycles of poor ovarian responders. Age-associated decline in oocyte quality and quantity has been well-established in poor ovarian responders whereas the mechanism underlying a poor response in women with normal or adequate ovarian reserve remains obscure. Several studies have suggested particular genetic polymorphisms, inadequate starting gonadotropin dose during stimulation, and asynchronous follicular development as contributing factors to the unexpected poor ovarian response [23].

Our results seem to indicate that the semen conditions which reflect both the microscopic sperm quantity and quality influenced the early and late preimplantation embryonic

Table 1: Baseline and clinical characteristics of studied participants according to semen analysis.

Parameters	Overall subjects (n = 1,263)	Normozoospermia (n = 33)	Teratozoospermia (n = 340)	OAT (n = 204)	Astenoteratozoospermia (n = 520)	Severe OAT (n = 90)	Cryptozoospermia (n = 76)
Female characteristics							
Age (year)	35 (32, 38)	33 (31, 35)	34 (32, 38)	35 (31, 38)	36 (33, 38)	34 (31, 38)	34 (30, 38)
BMI (kg/m²)	23.21 (21.19, 25.86)	22.81 (21.02, 26.94)	22.99 (20.84, 25.50)	23.52 (21.48, 25.82)	23.06 (21.12, 25.79)	23.61 (21.63, 26.90)	23.90 (21.59, 27.06)
Number of previous failed IVF cycles	1 (1, 1)	1 (1, 1)	1 (1, 1)	1 (1, 1)	1 (1, 1)	1 (1, 1)	1 (1, 1)
Infertility duration (year)	5 (3, 8)	5 (3, 8)	5 (3, 8)	5 (3, 8)	6 (3, 9)	6 (3, 8)	6 (4, 10)
Basal FSH (mIU/mL)	7.51 (6.44, 8.99)	6.69 (5.94, 7.64)	7.5 (6.62, 8.85)	7.12 (6.25, 8.76)	7.81 (6.39, 9.41)	7.57 (6.65, 8.96)	7.38 (6.68, 8.55)
Basal progesterone (ng/mL)	0.26 (0.16, 0.40)	0.23 (0.11, 0.31)	0.21 (0.11, 0.33)	0.28 (0.18, 0.41)	0.30 (0.20, 0.45)	0.24 (0.15, 0.36)	0.26 (0.12, 0.45)
Basal estradiol (pg/mL)	38 (28.93, 48)	34.15 (28, 42)	35.93 (28.70, 46.74)	38 (28, 46)	39.08 (30, 50)	38.27 (27.12, 47.15)	37.58 (28.88, 47)
Estradiol on trigger day (pg/mL)	1839 (1389, 2350)	1695.5 (1346, 2180.5)	1883.5 (1393.5, 2399)	1913 (1386, 2394)	1828.5 (1403.5, 2310)	1690 (1238, 2332)	1963 (1478, 2169)
Progesterone on trigger day (ng/mL)	0.65 (0.43, 0.89)	0.55 (0.38, 0.83)	0.6 (0.37, 0.84)	0.66 (0.43, 0.89)	0.71 (0.47, 0.92)	0.64 (0.35, 0.85)	0.57 (0.41, 0.86)
AFC	9 (7, 11)	9 (8, 11)	9 (7, 12)	9 (7, 12)	8 (6, 11)	10 (7, 12)	8 (7, 11)
AMH (ng/mL)	2.36 (1.73, 3.53)	2.04 (1.66, 3.18)	2.15 (1.63, 3.09)	2.55 (1.76, 3.88)	2.42 (1.74, 3.64)	2.41 (1.92, 3.46)	2.49 (1.87, 4.18)
Endometrial thickness (mm)	10.45 (9.30, 11.90)	10.9 (9.9, 12)	10.11 (9, 11.8)	10.90 (9.7, 12)	10.20 (9.1, 11.5)	10.6 (9.65, 12)	10.95 (9.95, 11.70)
History of miscarriage	229 (18.1%)	5 (15.2%)	71 (20.9%)	27 (13.2%)	107 (20.6%)	11 (12.2%)	8 (10.5%)
Male characteristics							
Age	37 (33, 41)	35 (33, 38)	36 (33, 40)	37 (33, 41)	38 (34, 42)	37 (33, 41)	36 (33, 42)
Semen volume (mL)	3 (2, 4)	2.5 (2, 3)	3 (2, 3.5)	3 (2, 4.3)	2.5 (2, 3.5)	2.5 (2, 4)	3 (2, 3.8)
Concentration (×10⁶)	33 (12, 64)	63 (41, 102)	53.5 (36, 86)	9 (7, 11)	41 (25, 73)	3 (2, 4)	1 (1, 1)
Motility (%)	20 (10, 37)	45 (37, 51)	44 (39, 51)	10 (6, 17)	18 (12, 24)	5 (2, 8)	1 (1, 1)
Morphology (%)	1 (1, 2)	4 (4, 5)	2 (1, 2)	1 (1, 2)	2 (1, 2)	1 (1, 1)	1 (1, 1)

Note: data are presented as median (Q1 and Q3); data are presented as the number of subjects and percentage (n (%)).

TABLE 2: IVF outcomes of studied participants according to semen analysis.

Parameters	Normozoospermia (n = 33)	Teratozoospermia (n = 340)	OAT (n = 204)	Astenoteratozoospermia (n = 520)	Severe OAT (n = 90)	Cryptozoospermia (n = 76)	p value
Number of retrieved oocytes	5 (4, 7)	6 (5, 7)	6 (5, 7)	6 (5, 7)	7 (5, 8)	6 (5, 8)	NS
Number of matured oocytes following injection	5 (3, 6)[c]	5 (4, 6)[e]	5 (4, 6)[e]	5 (4, 6)[e]	5 (4, 7)[f]	5 (3, 6)	0.042
Number of fertilized oocytes	3 (2, 4)	4 (3, 5)[c,f]	3 (2, 4)	3 (2, 5)[f]	3 (2, 5)	3 (2, 4)	0.003
ICSI method	3 (2, 5)	4 (3, 5)	4 (2, 4)	3 (2, 4)	3 (2, 5)	—	NS
IMSI method	4 (2, 4)	4 (3, 5)	3 (2, 5)	4 (3, 5)	4 (2, 5)	—	NS
Abnormal fertilization (1PN, 3PN, etc.) (n (%))	3 (9.1%)	44 (12.9%)	27 (13.2%)	66 (12.7%)	9 (10%)	15 (19.7%)	NS
Number of cleavage embryos	3 (2, 4)	4 (3, 5)[c]	3 (2, 4)	3 (2, 5)[f]	4 (2, 5)[f]	3 (2, 4)	0.001
Number of good cleavage quality	1 (1, 3)	2 (1, 3)	2 (1, 2)	2 (1, 3)	1.5 (1, 2)	1 (1, 2)	NS
Number of blastocyst embryos	2 (1, 4)[f]	2 (1, 4)[d,f]	2 (0, 4)	2 (0, 4)[f]	2 (0, 4)[f]	1 (0, 3)	0.003
Number of good blastocyst quality	1 (0, 2)[d,e,f]	0 (0, 1)[d,f]	0 (0, 1)	0 (0, 1)	0 (0, 1)	0 (0, 1)	0.036
Number of embryo transfer	1 (1, 2)	2 (1, 2)	2 (1, 2)	2 (1, 2)	2 (1, 2)	2 (1, 2)	NS
Embryo transfer day							
D2/3 embryo transfer (n (%))	14 (42.4%)	217 (63.8%)	136 (66.7%)	360 (69.2%)[a]	60 (66.7%)	58 (76.3%)[a]	0.003
D5/6 embryo transfer (n (%))	19 (57.6%)[d,f]	123 (36.2%)	68 (33.3%)	160 (30.8%)	30 (33.3%)	18 (23.7%)	
Overall clinical pregnancy (n (%))	13 (39.4%)	126 (37.1%)	82 (40.2%)	181 (34.8%)	32 (35.6%)	34 (44.7%)	NS
ICSI method (n (%))	5 (26.3%)	62 (35.4%)	36 (37.5%)	110 (37.3%)	19 (35.8%)	—	NS
IMSI method (n (%))	8 (57.1%)	64 (38.8%)	46 (42.6%)	71 (31.6%)	13 (35.1%)	—	NS
D2/3 clinical pregnancy (n (%))	6 (46.2%)	82 (65.1%)	51 (62.2%)	126 (69.6%)	22 (68.8%)	22 (64.7%)	NS
D5/6 clinical pregnancy (n (%))	7 (53.8%)	44 (34.9%)	31 (37.8%)	55 (30.4%)	10 (31.3%)	12 (35.3%)	NS

Note: data are presented as median (Q1 and Q3), and the Kruskal-Wallis test is used. Data are presented as the number of subjects and percentage (n (%)), and chi-square tests are used for the categorical variable. [a]Compared by normozoospermia. [b]Compared by teratozoospermia. [c]Compared by OAT. [d]Compared by astenoteratozoospermia. [e]Compared by severe OAT. [f]Compared by cryptozoospermia.

development but did not adversely impact the clinical pregnancy of women in POSEIDON groups 1 and 2. We propose three prominent factors underpinning our findings. Firstly, natural selection of embryos occurred during culture period in the form of fertilization failure, arrested, or poor-quality embryos during development which lead to the derivation of only good-quality embryos from a healthy male and female gamete for subsequent embryo transfer. Secondly, ICSI and IMSI were sufficiently effective in overcoming the diminished reproductive potential of poor semen quality as suggested previously [24]. In our study, sperm selection methods through IMSI and ICSI were shown to have comparable outcomes among studied groups in terms of the number of fertilized oocytes as well as clinical pregnancy rate. Thirdly, intrinsic oocyte quality factors in women of POSEIDON groups 1 and 2 might render the clear-cut impacts of sperm quality obscure.

Our study observed an indisputable effect of poor sperm quality on fertilization rate as remarkedly discovered in other studies [15, 22]. Physiologically, the sperm delivers phospholipase C zeta (PLC-zeta) which triggers the smooth endoplasmic reticulum to release calcium to the ooplasm; consequently, activating signal transduction pathways would mediate the fertilization process [25]. Defective expression and localization of PLC-zeta have therefore been closely associated with the severity of male infertility [26] which could be manifested by a low fertilization rate as observed in this study. Sperm also passes on functional centrosomes, mRNA, microRNA (miRNA), and most importantly, a well-packaged haploid genome into the oocyte. Excluding the genome, those components purportedly affect early embryo development which earns the term early paternal effect. At the end of fertilization, the sperm centrosome mediates microtubule assembly into the first mitotic spindle [27]. Hence, a centrosome dysfunction could also potentially cause fertilization failure [28].

Late paternal effect commences later as sperm-derived genes are mostly activated after embryonic genome activation. Prior to the embryonic genome activation, maternally inherited mRNA predominantly controls the initial stage of embryo development [29]. Qualitative changes in protein expression during embryonic development have been well studied. Expressions of embryonic genes were only detected starting from the 4-cell stage in concurrence with the decrease in maternal transcript expression [30]. Moreover, microarray analysis showed a definitive maternal transcript degradation and overexpression of EGA-related genes in 5- to 8-cell embryos [31]. In both reports, paternal effect genes were expressed at the 4-cell cleavage stage. Our results seem to coincide with the notion that abnormal sperm quality could presumably influence the preimplantation embryonic development at a later stage as indicated by the significant differences in the number of cleavage-stage embryos, blastocyst quantity, and quality [28].

The comparable clinical pregnancy rates in this study supported a previous finding which suggested that oocyte quantity was solely affected in poor responders aged < 38 [32]. Although impaired preimplantation embryo development was observed, clinical pregnancy rates remained unaffected [24, 33]. Other investigations have likewise demonstrated that sperm aberrations did not affect clinical pregnancy rates [15, 34].

As increasing reports showed that both implantation and clinical pregnancy rates were significantly associated with sperm DNA integrity [35, 36], the use of the DNA fragmentation index might be a more robust indicator of sperm quality than that of routine semen parameters [37]. This study's limitations include its retrospective nature and the heterogeneity of IVF treatments among the recruited subjects such as the ovarian stimulation protocols, sperm preparation, and selection methods. Nonetheless, control for potential confounders was attempted through multiple analyses.

5. Conclusion

According to the present study, sperm quality according to semen analysis is significantly associated with fertilization and preimplantation embryonic development. However, clinical pregnancy was not affected.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

The study was approved by the Ethical Committee of the Faculty of Medicine of the University of Indonesia (KET-853/UN2.F1/ETIK/PPM.00.02/2021).

Conflicts of Interest

We declare that there are no conflicts of interest in the content of this study.

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