



Being on the side of old findings: progesterone elevation on the day of oocyte maturation induction does not affect embryological parameters throughout the blastocyst culture period

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Abstract

Purpose To investigate whether there is any detrimental effect of progesterone elevation (PE) on the day of oocyte maturation induction on embryological development potentials.

Methods This retrospective single-center cohort study included a total of 1485 individual intracytoplasmic sperm injection (ICSI) cycles between January 2014 and December 2018. Serum progesterone (P) levels were measured on the day of oocyte maturation induction following the GnRH antagonist suppression protocol. Embryological parameters such as maturation, fertilization rate (FR), top-quality embryo (TQE) formation rate per 2PN on day 3, and excellent-quality blastocyst (EQB) formation rate per 2PN on day 5/6 were recorded. The inclusion criteria for women were an age ≤ 37 years, a BMI ≤ 30 kg/m², and access to a total sperm concentration ≥ 2 million. Groups were stratified according to the serum P levels using the cut-off levels of < 0.8 ng/ml; $0.8\text{--}1.49$ ng/ml; and ≥ 1.5 ng/ml.

Results Peak E2 level and total number of oocytes retrieved were significantly related to PE ($p < 0.001$). FR did not display a significance difference between groups ($p = 0.108$). The TQE and the blastulation rates were not affected by PE ($p = 0.82$ and $p = 0.68$, respectively). Chi square analysis revealed a significant relationship between PE and the EQB formation rate ($p = 0.01$). GEE analysis failed to present any statistical significance regarding the effect of PE on neither the TQE nor the EQB formation rates per 2PN [OR 1.07; 95% (0.98–1.16) $p = 0.113$ and OR 0.93; 95% (0.80–1.07) $p = 0.32$, respectively].

Conclusions In accordance with previously published papers, our study could not find any detrimental effect of PE on embryological outcomes throughout the blastocyst culture period.

Keywords Progesterone elevation · Blastocyst culture · Blastulation · Excellent-quality blastocyst formation rate

Purpose

The effect of late follicular phase progesterone elevation (PE) in controlled ovarian hyperstimulation (COH) cycles has been a major controversy that is of ongoing importance. Although many studies and meta-analysis have been carried out so far, and increased serum progesterone (P) levels have been shown to have a negative impact on pregnancy

outcomes, discussions about the precise P cut-off value and the use of different assays are still ongoing. Most authors conducted the discussion through embryo-endometrium asynchrony. Recent publications have verified the significant negative impacts of PE on pregnancy outcomes, especially when fresh embryo transfer is performed [1–3]. Moreover, gene expression studies have related this negative effect to premature development of the endometrium [4, 5].

Today, the most commonly used threshold for serum progesterone (P) level is 1.5 ng/ml, and it ranges between 0.8 and 2 ng/ml among published studies [6]. The aforementioned gene expression studies have found significant differences in gene expression profiles between two groups with P levels above and below 1.5 ng/ml on the day of triggering ovulation [4].

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There are only a few published reports regarding the effect of P levels on oocyte or embryo quality, and there is still no consensus on this issue. Initial studies were not able to find detrimental effects of increased P on oocyte/embryo quality [3, 7]; however, recent evidence suggests a strong association between rising levels of P and decreasing quality of embryos [8–10]. Two published studies focused on the embryo development process at the cleavage and blastocyst stages and found PE to be responsible for decreased embryo development potential [8, 9]. In another study, embryo development in the cleavage and blastocyst stage and pregnancy outcomes as well as fresh and cumulative live birth rates (CLBR) were evaluated, and PE was associated with worse embryo development and decreased LBRs [10].

Due to the paucity of literature, we aimed to determine whether there was a negative relationship between PE and gamete and/or embryo quality throughout the blastocyst culture period. In addition, we tried to answer the following question: where is the effect first occurring?

Methods

This is a retrospective, single-center cohort analysis of fresh/autologous intracytoplasmic sperm injection (ICSI) cycles performed in Bahceci IVF center, Istanbul, between January 2014 and December 2018. Study protocol was approved by the institutional review board. In total, 20,001 oocytes from 1485 treatment cycles were included in the study. Repetitive treatment cycles of the same patient were not included. Based on our experience and reports in the literature, we decided to switch to freeze-all and subsequent frozen-thawed embryo transfer (FET) strategy in all treatment cycles since 2013, due to its superior reproductive outcomes compared to fresh transfer [11, 12]. Inclusion criteria were the use of a GnRH antagonist protocol, available data on the serum P levels on the day of maturation induction, and blastocyst culture up to the 5th or 6th day. To prevent bias, intrinsic prognostic factors, such as advanced maternal age (> 37 years old), BMI ≥ 30 kg/m² and severe male factor (sperm concentration ≤ 2 million/ml), were considered as exclusion criteria. Furthermore, patients who had two or more failed IVF/ICSI attempts were excluded from the analyses.

COH was initiated on the second day of the menstrual cycle with either recombinant FSH (150–300 IU, Gonal-F; Serono) and/or highly purified hMG (75–150 IU, Merional; IBSA). Dosages were adjusted according to the patient's characteristics and ovarian response to the stimulation. Pituitary down regulation was achieved by using daily 0.25 mg of cetrorelix (Cetrotide, Merck Serono Pharmaceuticals) injections when the leading follicle was 14 mm in diameter. When at least two or more follicles reached ≥ 18 mm in mean diameter, final oocyte maturation was induced either

with the use of 250 μ g of human chorionic gonadotropin (hCG; Ovitrelle, Serono) or 0.2 mg triptorelin (Gonapeptyl, Ferring), depending on the physician's preference. Cycles were monitored by means of serial vaginal sonography and serum estradiol (E2) and P measurements. Final blood analyses were performed on the same day with oocyte maturation induction, and egg retrieval was scheduled 34–36 h later.

Hormonal measurement

Samples were tested by an electrochemiluminescence immunoassay (Cobas[®] Elecsys Progesterone III, Roche diagnostics GmbH, Germany) with a measured sensitivity and total imprecision of 0.03 μ g/l and < 7%, respectively.

Laboratory process, embryo grading

ICSI was performed almost 1 h after denudation, and micro-injected oocytes were cultured individually in a special pre-equilibrated culture dish. Fertilization was confirmed 16–18 h after insemination. Cleavage-stage controls of embryos were initiated on day 3 of development. The number of blastomeres, any observed variation in blastomeric symmetry, the percentage of fragmented blastomeres, and the presence of vacuolization, multinucleation and granulation were evaluated. Embryo grading throughout the entire process was performed by two senior embryologists. An embryo with 6–10 even blastomeres and < %20 fragmentation, without vacuolization, granulation and multinucleation, was regarded as a top-quality embryo (TQE) [13]. Second and third assessments were performed on the 5th and 6th days, respectively. Blastocyst assessments were performed according to a morphology-based three-part scoring system as described previously [14, 15]. Thereafter, every blastocyst reached an expansion score of at least 3 and was frozen either on day 5 or day 6. Categorization of blastocysts was as follows: excellent (≥ 3 AA), good (3, 4, 5, or 6 and AB, BA, BB, or BC), poor (3, 4, 5, or 6 and CB, CC, or CA).

Outcome measures

The main outcome measures in the study were the TQE and excellent-quality blastocyst (EQB) formation rate per 2PN [8, 9]. As secondary outcomes, the fertilization rate (FR) and the blastulation rate (total blastocyst/2PN) were also evaluated.

Statistics

Continuous variables in the study did not follow a normal distribution. Therefore, such parameters were reported as median (minimum–maximum) values. Categorical variables are presented as a proportion and equivalent percentage

figures. Chi square tests were used to test whether there was a statistically significant difference regarding the proportion of categorical variables between the groups.

Patients were divided into three groups according to their P levels on the day of maturation induction: P levels less than 0.8 ng/ml (n : 806), P levels between 0.8 and 1.49 ng/ml (n : 475) and P levels greater than and equal to 1.5 ng/ml (n : 204). These cut-off levels were selected from previously published studies [1, 6, 9].

The association between serum P levels on the day of maturation induction and the patient's basic and COH characteristics were calculated using Spearman's correlation coefficient. In addition, multicollinearity analysis was performed to prevent bias formation and to determine whether there was a correlation between independent variables. Existing correlation was re-scaled by grand mean centering that helps the interpretation of the terms associated with the intercept.

Generalized estimating equation (GEE) models were performed to further investigate which factors affected the

cycles in which a TQE on day 3 or an EQB on day 5/6 were generated. Since the dependent variable was dichotomous (in reference to whether or not the patient had TQE or EQB), the GEE models were evaluated by logistic regression using GEE logit estimates. Serum P levels were evaluated as a continuous variable rather than a categorical variable in the models.

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 25.0 (SPSS Incl. USA), and statistical significance was considered if the p value < 0.05 .

Results

The baseline characteristics and all of the embryological parameters obtained from 1485 cycles, 20,001 oocytes and 5279 blastocysts according to P groups are detailed in Table 1. The average age in this study was 32 years (ranging between 18 and 37). There was no significant difference

Table 1 Descriptive characteristics of patients separated by serum P levels (ng/ml) on the day of maturation induction

	Serum P levels (ng/ml) on the day of maturation induction				p values
	All ($n = 1485$)	< 0.8 ng/ml $n = 806$ (54.3%)	0.8–1.49 ng/ml $n = 475$ (32%)	≥ 1.5 ng/ml $n = 204$ (13.7%)	
Age (years)	32 (18–37)	32 (18–37)	32 (21–37)	32 (19–37)	0.852
BMI (kg/m ²)	24.4 (18.2–29.9)	24.4 (19.2–29.7)	24.3 (18.2–28.9)	24.2 (18.4–29.9)	0.69
Sperm concentration ($\times 10^6$ /ml)	40 (2–103.4)	40 (2–97)	40 (2–103.4)	44 (3–97)	0.516
Total dose of gonadotrophins	2175 (300–21,150)	2100 (300–21,150)	2287.5 (450–10,800)	2250 (375–7000)	0.026*
Peak E2 level (pg/ml)	1795 (224–15,000)	1570 (224–10,386)	2058 (326–15,000)	2211.5 (390–11,992)	$< 0.001^*$
Total no of oocytes retrieved	12 (1–62)	10.5 (2–58)	13 (1–56)	15 (3–62)	$< 0.001^*$
No. of mature oocytes	9 (1–55)	8 (1–54)	10 (1–55)	13 (2–55)	$< 0.001^*$
Fertilization rate (%)	80 (0–100)	81.82 (0–100)	80 (0–100)	80 (0–100)	0.108
Day 3 TQE rate per 2PN	7142/12,328 (57.9%)	3425/5884 (58.2%)	2500/4341 (57.6%)	1217/2103 (57.9%)	0.82
Blastulation rate	40 (0–100)	42.1 (0–100)	40 (0–100)	40 (0–100)	0.68
Blastocyst quality/D5					
Excellent	1121/4116 (27.2%)	561/1967 (28.5%)	405/1439 (28.1%)	155/710 (21.8%)	$< 0.001^*$
Good	2798/4116 (68%)	1337/1967 (68%)	962/1439 (66.9%)	499/710 (70.3%)	
Low	197/4116 (4.8%)	69/1967 (3.5%)	72/1439 (5%)	56/710 (7.9%)	
Blastocyst quality/D6					
Excellent	132/1163 (11.3%)	63/537 (11.7%)	49/433 (11.3%)	20/193 (10.4%)	0.869
Good	784/1163 (67.4%)	358/537 (66.7%)	298/433 (68.8%)	128/193 (66.3%)	
Low	247/1163 (21.2%)	116/537 (21.6%)	86/433 (19.9%)	45/193 (23.3%)	
EQB rate per 2PN	1253/12,328 (10.2%)	624/5884 (10.6%)	454/4341 (10.4%)	175/2103 (8.3%)	0.01*

Continuous variables were tested using an independent samples median test to test the median values between the three groups of serum P levels. Categorical variables were assessed using chi-square tests to determine the proportion between the three groups of serum P levels.

Binary comparison revealed statistical significance between only Group 1 and 2 with respect to total dose of gonadotrophins ($p = 0.008$).

* Statistically significant difference was indicated by $p < 0.05$ between the three groups of serum P levels.

between groups with respect to female age, total sperm concentration and BMI. Although the total dose of gonadotrophins was different between groups ($p=0.026$), binary group comparisons revealed statistical significance only between groups 1 and 2 ($p=0.008$). Peak E2 level, total number of oocytes retrieved and MII oocytes displayed a significant upward trend with PE. FR was not affected by elevated P levels ($p=0.108$). Regarding embryo development during the first 3 days, the TQE formation rate did not show any significant difference ($p=0.82$). Similarly, the blastulation rate (total number of blastocysts/2PN) was comparable between groups ($p=0.68$). PE seemed to have a negative influence on the number of excellent-quality day 5 blastocysts among the groups ($p<0.001$), but no difference was seen in any category of day 6 blastocysts ($p=0.869$). The EQB formation rate per 2PN showed a significant inverse relationship with PE ($p=0.01$).

Table 2 shows the correlation analysis of factors related to PE, and it was found that there was a statistically significant negative association between serum P level on the day of maturation induction and age, meaning that a year increase in age decreased the serum P level by 0.0039. Conversely, statistically significant positive relationships were found between the serum P level and the total dose of gonadotrophins, type of gonadotrophins (r-FSH and hMG), peak E2 level and total number of oocytes retrieved. No statistically significant correlation was found between BMI and serum P levels.

Before proceeding to regression analysis, we first tried to find out whether multicollinearity exist among the independent variables when considering both the TQE and EQB as the dependent outcomes. For all the parameters except

total dose of gonadotrophins, variance inflation factor (VIF) values were less than 3 which meant that there was no multicollinearity generated due to age of the patient, type of gonadotrophins, peak E2 level, serum P level, sperm concentration, total number of retrieved and mature oocytes and fertilization rate. Therefore, to overcome bias formation, grand mean centered levels of the total dose of gonadotrophins were re-scaled and used in the further GEE models.

As shown in Tables 3, 4, when considering all data from the same patient using the GEE model with logistic regression and independent covariance structure, p values of the Wald Chi square test indicated that the only parameter affecting whether the patient would have a TQE or an EQB was the FR [OR 2.96; 95% (1.99–4.43) $p=0.000$ and OR 2.65; 95% (1.46–4.81) $p=0.001$, respectively]. Serum P levels on the day of maturation induction were not related to neither the TQE nor the EQB formation rate [OR 1.07; 95% (0.98–1.16) $p=0.113$ and OR 0.93; 95% (0.80–1.07) $p=0.32$, respectively].

Discussion

Today, blastocyst culture is the major tool used to evaluate embryo survival. PE is a factor that has been criticized as to whether it may harm embryo development at any stage during the process. In our large retrospective analysis, we observed the embryo characteristics throughout the blastocyst culture period. Even though the number of EQB within day 5 blastocysts seemed negatively affected by PE, we could not find any detrimental effect of PE on the TQE formation rate at day 3 or on the EQB formation rate.

It is well known that premature PE on the day of triggering oocyte maturation is linked to increasing number of antral follicles, peak E2 level and oocyte yield [1, 3, 4, 16]. In accordance with the literature, this phenomenon was also observed in our study. However, the exact mechanism of PE is still unresolved. One translational research study using in vitro tools has shed light on this mechanism and has shown a direct stimulating effect of FSH on 3β -hydroxysteroid dehydrogenase (3β -HSD) enzyme activity in human granulosa cell sections. The same effect has not been shown for 17α -hydroxylase (17α -OH) enzyme activity, which mainly acts through the conversion of progesterone (P) into androgens within the theca layer by means of LH activity. As a consequence, because of the suppressed levels of LH up to the preovulatory follicular stage during IVF stimulation cycles, the enhanced enzymatic activity of 3β -HSD exceeds the conversion capacity of the theca layer; hence, accumulated P may leak into systemic circulation [17].

Until now, only a small number of studies have been published about PE and its effect on embryological parameters,

Table 2 Relationship between serum P level on the day of maturation induction and the patient's basic and COH characteristics

	Serum P levels (ng/ml) on the day of maturation induction	
	CC	p value
Age	−0.0039	<0.001*
Total dose of gonadotrophins (IU)	0.025	<0.001*
Type of gonadotrophins		
r-FSH (IU)	0.050	<0.001*
hMG (IU)	−0.065	<0.001*
Peak E2 level (pg/ml)	0.229	<0.001*
No. of oocytes retrieved	0.315	<0.001*
BMI (kg/m ²)	−0.013	0.867

The correlation between the serum P level on the day of maturation induction and the patient's basic and COH characteristics given in the table were calculated by Spearman's Correlation Coefficient

*Statistically significant correlation was indicated by $p<0.05$ when comparing the two variables

Table 3 Generalized Estimating Equations (GEE) Model using logit link function on the outcome of Day 3 TQE

Parameter	<i>B</i>	95% Wald confidence interval		Wald Chi square	<i>p</i> value	OR	95% Wald confidence interval for OR	
		Lower	Upper				Lower	Upper
(Intercept)	0.464	−1.333	2.261	0.256	0.613	1.590	0.264	9.589
Age (years)	−0.007	−0.023	0.009	0.680	0.409	0.993	0.978	1.009
Total dose of gonadotrophins (IU)	1.382E-6	0.000	0.000	0.001	0.979	1.000	1.000	1.000
Type of gonadotrophins								
r-FSH (IU)	0.000	0.000	1.839E-5	5.182	0.063	1.000	1.000	1.000
hMG (IU)	0					1		
Peak E2 level (pg/ml)	−1.798E-5	−5.148E-5	1.553E-5	1.106	0.293	1.000	1.000	1.000
P levels (ng/ml) on the day of maturation induction	0.069	−0.016	0.155	2.515	0.113	1.072	0.984	1.167
Sperm concentration ($\times 10^6$ /ml)	−0.001	−0.001	0.000	6.206	0.093	0.999	0.999	1.000
No. of oocytes retrieved	0.000	−0.009	0.008	0.011	0.916	1.000	0.991	1.009
No. of mature oocytes	−0.562	−2.225	1.101	0.439	0.508	0.570	0.108	3.008
Fertilization rate (%)	1.088	0.688	1.488	28.372	0.000*	2.968	1.989	4.430

The GEE model was evaluated by logistic regression using GEE logit estimates

independent covariance structure was assumed for the dependence of each observation with other observations in the same cluster

*Statistically significant effect on the patient to have an outcome of Day 3 Top Quality Embryo was indicated by $p < 0.05$

Table 4 Generalized Estimating Equations (GEE) Model using logit link function on the outcome of EQB

Parameter	<i>B</i>	95% Wald confidence interval		Wald Chi square	<i>P</i> value	OR	95% Wald confidence interval for OR	
		Lower	Upper				Lower	Upper
Intercept	−1.506	−3.416	0.404	2.388	0.122	0.222	0.033	1.498
Age (years)	−0.016	−0.041	0.009	1.627	0.202	0.984	0.960	1.009
Total dose of gonadotrophins (IU)	1.141E-5	0.000	0.000	0.022	0.882	1.000	1.000	1.000
Type of gonadotrophins								
r-FSH (IU)	0.000	0.000	4.164E-5	2.143	0.143	1.000	1.000	1.000
hMG (IU)	0					1		
Peak E2 level (pg/ml)	4812E-6	−5.299E-5	6.261E-5	0.027	0.870	1.000	1.000	1.000
P levels (ng/ml) on the day of maturation induction	−0.073	−0.218	0.071	0.987	0.320	0.929	0.804	1.074
Sperm concentration ($\times 10^6$ /ml)	−4.094E-5	−0.001	0.001	0.005	0.943	1.000	0.999	1.001
No. of oocytes retrieved	−0.009	−0.026	0.008	1.185	0.276	0.991	0.974	1.008
No. of mature oocytes	−0.643	−2.219	0.933	0.639	0.424	0.526	0.109	2.542
Fertilization rate (%)	0.977	0.382	1.571	10.374	0.001*	2.656	1.466	4.813

The GEE model was evaluated by logistic regression using GEE logit estimates

independent covariance structure was assumed for the dependence of each observation with other observations in the same cluster

*Statistically significant effect on the patient to have an outcome of EQB embryo was indicated by $p < 0.05$

and they have had conflicting results. In the last decade, most IVF practitioners thought there were detrimental effects of PE on endometrial histology rather than embryo quality, but debate is ongoing with respect to a precise cut-off level for PE due to different progesterone assays and study designs.

In recent years, three publications have rekindled the discussion again. Huang et al. [8] stratified groups according to five distinct P levels and investigated the effect of PE on day 2 and 3 TQEs. Contrary to our findings, the TQE formation rate was negatively influenced by increased levels of P. The

cut-off level of 2 ng/ml was found to be the most significant predictor. Vanni et al. [9] went one step further and followed-up the embryos up to the blastocyst stage. They included only cycles in which at least one blastocyst was formed and tried to determine whether there was any inverse relationship between PE and top-quality blastocyst (TQB) formation rate. Four distinct groups were evaluated, and PE was indeed found to be a significant factor. Binary comparisons of each P group that had levels increased above 1 ng/ml with a group in which the P level was below 1 ng/ml revealed statistical significance in terms of TQB formation rate. According to the ROC curve analysis, a P level > 1.49 ng/ml was the best cut-off value for identifying patients at risk for the absence of TQB. Similar to our findings, the blastulation rate was not different between groups. In our study, we tried to clarify the real effect of PE in all stages of embryo development, even for cycles in which no blastocyst was developed; thus, we thought that the results would be more representative of a general population. In addition, the average age of patients in our study was lower, which might have a positive effect on embryo quality. Finally, Racca et al. [10] investigated the deleterious impact of PE on embryo quality and CLBR in their retrospective analysis. They categorized patients according to three distinct P cut-off levels. The embryo utilization rate, LBR after fresh transfer and CLBR were lower in the high P group. In this study, embryos on day 3 were reported to be of good quality even if they had fragmentation up to 50% and were selected either to be transferred or to be frozen. Although they could not find a significant difference in terms of LBR between FET among P groups after a failed fresh embryo transfer, the number of FET cycles to reach a healthy baby was greater in the high P group, which was the opposite of our findings (unpublished data). In a retrospective analysis, the relationship between elevated P levels (cut-off level 1.5 ng/ml) on the day of oocyte maturation and ploidy status as well as pregnancy outcomes in the subsequent FET cycle was investigated [18]. Elevated P levels were not associated with the percentage of available blastocysts suitable for biopsy or pregnancy outcomes. In this study, the quality of the blastocysts was assessed through their genetic structure not through morphological criteria.

The same topic has been discussed in many studies of donation cycles. In a retrospective analysis, the effects of PE on embryological parameters as well as pregnancy outcomes were analyzed among repeated oocyte donation cycles using the cut-off level as 1.2 ng/ml [19]. The same donor, acting as its own control, had two stimulations in which the peak P level was less than or greater than the cut-off level. No detrimental effect of PE could be found on fertilization and cleavage rates, embryo quality parameters or pregnancy outcomes. Another study that was conducted on oocyte donors and in which the cut-off level was 1.0 ng/ml reported similar pregnancy rates (PR) between groups. They concluded that

PE had an adverse effect on the endometrium rather than on embryo quality [20]. Three other studies commenting on embryo quality and PR published the same results by using the cut-off P level as 1.1, 1.2 and 1 ng/ml, respectively [21–23]

Despite all the developments in the IVF field, 35–40% of patients still cannot achieve the desired goal, live birth, even after many attempts [24]. Analyzing the poorly understood factors affecting gamete and embryo qualities may help us to illuminate them along with the embryonic development process to improve the IVF outcomes. As far as we know, no previous research has been published focusing on both day 3 and day 5/6 embryo development potentials under the effect of PE. The uncertainty of whether there could be a negative impact of PE on embryo quality should be illuminated; hence, we built our study on a large sample size to make our findings more reliable. The main limitation of the present study is its retrospective design. To potentiate our findings, we analyzed various confounders and adjusted them via advanced methods. Although a homogenous population was constructed, using only the ICSI method and including only antagonist cycles might be potential confounders regarding the usage of these findings in global practice.

Conclusions

In conclusion, our study failed to demonstrate a negative effect of PE on embryo development characteristics, which is compatible with many previous studies. Embryo morphology as well as quality assessment may vary in various clinics. Thus, comparing PGT results or pregnancy outcomes as final end-points in well-designed studies would produce a more reliable result. Furthermore, homogenization of the stages of embryos that are destined for transfer and freezing may provide more accurate information than what has been previously found.

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Availability of data and materials The datasets analyzed during the current study are available from the corresponding author on reasonable request

Compliance with ethical standards

Conflict of interests The funder provided support in the form of salaries for authors [NET,SE,FKB,MG, ZY, NF, MB], but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Ethics approval This study was approved by Institutional Review Board with the reference number of 42.

Consent for publication Not applicable

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