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Effect of ovarian stimulation on embryo aneuploidy and mosaicism rate

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ABSTRACT

There is a high incidence of chromosome abnormalities in human embryos that leads to a failed IVF cycle. Different studies have shown that maternal age is the determining factor in the appearance of chromosomal alterations in the embryo. However, the possible influence of ovarian stimulation on oocyte and embryo aneuploidies and mosaicism is controversial. A retrospective study was carried out in which 835 embryos from 280 couples undergoing reproductive treatment using their oocytes were chromosomally analyzed. A binary logistic regression analysis was performed to evaluate the relationship between different parameters characterizing controlled ovarian stimulation (COS) and the rate of aneuploidy and embryonic mosaicism. The embryo aneuploidy rate showed no association with the use of oral contraceptives, type, total and daily doses of gonadotropins, stimulation protocol type, and drugs used for ovulation trigger ($p > 0.05$). In contrast, the duration of the ovarian stimulation treatment was correlated with the aneuploidy rate: patients requiring more days of stimulation presented a lower rate of aneuploid embryos ($p = 0.015$). None of the variables studied showed any association with the rate of embryo mosaicism. However, the duration of COS showed association with the appearance of aneuploidy, suggesting that faster recruitment could be deleterious for those reassuming meiosis, yielding more abnormal karyotype.

Abbreviations: IVF: in vitro fertilization; COS: controlled ovarian stimulation; PGT-A: preimplantation genetic test for aneuploidy; hCG: human chorionic gonadotropin; GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; NGS: next-generation sequencing; a-CGH: comparative genomic hybridization; TUNEL: Terminal transferase dUTP Nick End Labeling; FISH: fluorescent in situ hybridization

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Ovarian stimulation; embryo aneuploidy; embryo mosaicism; preimplantation genetic testing

Introduction

One of the main objectives of controlled ovarian stimulation (COS) for IVF is to obtain an ideal number of mature oocytes to optimize the success rate of the reproductive treatment. It is therefore essential to adequately carry out ovarian stimulation by administering exogenous gonadotropins to induce the development of multiple dominant follicles and thus increase the number of oocytes available for laboratory fertilization (de Ziegler et al. 1998). However, a high number of obtained oocytes is not a guarantee of successful treatment. Several studies affirm that the chromosomal status of the embryo is crucial (Hodes-Wertz et al. 2012; Capalbo et al. 2014; Morin et al. 2014).

Chromosomal abnormalities in embryos are a major cause of reproductive failure since they are responsible for stopping embryonic development as well as for implantation failures and repeated miscarriages (Alfarawati et al. 2011; Hodes-Wertz et al. 2012). These alterations are normally caused by failures in the meiosis during gametogenesis generating nullisomic or disomic gametes, which later give rise to chromosomally altered embryos (Pacchierotti et al. 2007; MacLennan et al. 2015). These chromosomal abnormalities can also occur after fertilization, when meiosis resumes, or in subsequent mitoses. These may result in aneuploid embryos or ones with chromosomal mosaicism (Munné et al. 2007).

The progress in genetic diagnosis techniques has enabled pre-implantation genetic testing for aneuploidy (PGT-A) on embryos generated in the laboratory before being transferred to the maternal uterus, thus improving the rates of implantation and ongoing pregnancies (Alfarawati et al. 2011; Neal and Werner 2018).

Different studies have shown that advanced maternal age is the determining factor in the appearance of chromosomal abnormalities in the embryo (Barash et al. 2017; Babariya et al. 2017). Nevertheless, a considerably high

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incidence of embryo aneuploidy has been also observed in young patients and oocyte donors (Munné et al. 2017). This leads us to question whether other factors involved in assisted reproduction treatments could interfere with the normal physiology of the oocyte, increasing aneuploidy rates?

In recent years, there has been speculation that ovarian stimulation treatments applied in IVF cycles interfere with the physiological process of dominant follicle selection, increasing errors in chromosome division during meiosis in the oocytes (Macklon et al. 2006; Patrizio and Sakkas 2009) and in the process of chromosomal imprinting (Sato et al. 2007).

Recent studies have failed to establish a clear relationship between the ovarian stimulation process and the appearance of aneuploidies in embryos, obtaining contradictory results (Verpoest et al. 2008; Barash et al. 2017; Labarta et al. 2017; Sekhon et al. 2017; Hong et al. 2019). As it is a subject of great controversy and given that experimental evidence and clinical data suggest that, in addition to advanced maternal age, the appearance of embryo aneuploidies and mosaicism could be influenced by the ovarian stimulation process, the main objective of this study was to evaluate this incidence and its relationship with the different variables of the ovarian stimulation currently in use for IVF.

Results

A total of 280 IVF cycles paired with oocytes were included in the study, in which 835 embryos were genetically analyzed. Table 1 shows the mean values studied per cycle.

Each patient who took part in the study followed a personalized ovarian COS protocol, custom-designed by a gynecologist according to their medical history, BMI, and ovarian reserve markers. All the information about types of ovarian stimulation protocols, types of gonadotropins, and oocyte maturation triggers used in IVF cycles is presented in Table 2.

Embryo aneuploidy and mosaicism

Of the 835 embryos, 453 (54.3%) were included in the study were analyzed with array-CGH and the remaining 382 (45.7%) were analyzed with NGS. We observed that 484 embryos (58.0%) were euploid, while 332 (39.8%) presented as aneuploidy. The incidence of mosaicism was 15.6%. Only 2.2% of all the embryos were non-informative.

The association between the appearance of embryo aneuploidies and mosaicism with the total number of oocytes and MII oocytes recovered as well as with the number of embryos biopsied per cycle is shown in Table 1. No statistically significant association was observed for any of the variables. As expected, mean maternal age was higher in the group of aneuploid embryos, showing a direct relationship with the increase of these chromosomopathies ($p < 0.001$), although not with the incidence of mosaicism. Paternal age was not related neither was aneuploidy nor to embryo mosaicism (Table 1). The embryo aneuploidy rate showed no association with the use of oral contraceptives, type of protocol, type of gonadotropin nor ovulation trigger ($p > 0.05$). The daily and total doses of gonadotropins administered could not be related to aneuploidy ($p > 0.05$) either (Table 3). The only variable that showed association with the embryo aneuploidy rate was the duration of the ovarian stimulation process: patients requiring more days of stimulation had a lower rate of aneuploid embryos ($p = 0.015$).

Due to the significant relationship obtained for the number of days of stimulation, a second statistical analysis was performed to corroborate the impact of

Table 1. Summary statistics of study population and IVF cycles outcomes.

	Global	Euploidy	Aneuploidy	*p*value	No mosaicism	Mosaicisim	*p*value
Maternal age (mean±SD)	35.14 ± 2.16	34.79 ± 2.20	35.46 ± 2.035	<0.001 [a]	35.02 ± 2.17	35.35 ± 2.05	0.111 [a]
Paternal age (mean±SD)	37.34 ± 4.91	37.00 ± 4.64	37.42 ± 5.16	0.223 [a]	37.08 ± 4.70	37.66 ± 5.61	0.208 [a]
# Biopsied embryos per patient (mean±SD)	2.88 ± 1.78	3.88 ± 2.14	4.04 ± 2.25	0.317 [a]	3.98 ± 2.22	3.77 ± 2.02	0.322 [a]
# Aspirated oocytes per patient (mean±SD)	13.67 ± 6.77	15.62 ± 7.61	14.97 ± 6.95	0.214 [a]	15.36 ± 7.19	15.25 ± 8.20	0.878 [a]
# Aspirated oocytes MII per patient (mean±SD)	10.73 ± 6.05	12.24 ± 6.41	11.65 ± 5.37	0.169 [a]	11.97 ± 5.69	12.18 ± 7.49	0.707 [a]
RIF (%)	20.2	22.5	16.9	0.048 [b]	19.9	21.5	0.661 [b]
RPL (%)	21.8	22.5	20.8	0.555 [b]	22.2	20.0	0.580 [b]
Biopsy day							
D + 5	63.1	65.9	59.0	0.039 [b]	62.3	66.2	0.606 [b]
D + 6	36.9	34.1	41.0		37.7	33.8	
Embryo quality							
A	45.2	53.4	33.1	<0.001 [b]	46.6	38.3	0.009 [b]
B	50.1	43.9	59.3		49.8	51.6	
C	3.3	1.9	5.5		2.6	7.0	
D	1.4	0.8	2.1		1.0	3.1	

Test performed for statistical analysis: a:Student t-test; b: Pearson's Chi-Square test.
RIF: Repeated implantation failure; RPL: Recurrent pregnancy loss

Table 2. Descriptive analysis of the cycles.

	IVF cycles n (%)
OCP	
Yes	54 (19.3%)
No	226 (80.7%)
Stimulation protocol	
Long	65 (23.2%)
Short	5 (1.8%)
Antagonist	201 (71.8%)
Mild	1 (0.4%)
Agonist-Antagonist	3 (1.1%)
Free	5 (1.8%)
Gonadotropin	
uFSH	37 (13.2%)
rFSH	30 (10.7%)
uFSH+rFSH	186 (66.4%)
rLH	25 (8.9%)
rFSH+rLH	1 (0.4%)
uFSH+rLH	1 (0.4%)
Trigger	
hCG	140 (50.0%)
GnRH antagonist	94 (36.9%)
Dual	45 (16.1%)

OCP: oral contraceptive pills; FSH: follicle-stimulating hormone; LH: luteinizing hormone; rFSH: recombinant FSH; uFSH: urinary FSH; rLH: recombinant LH; hCG: human chorionic gonadotropin; GnRH: gonadotropin-releasing hormone; Dual: hCG+GnRH antagonist

Table 3. Association between aneuploidy rate and IVF cycles parameters.

	Aneuploid embryos (n/%)	*p* value	OR (95% CI)
OCP			
No	266 (40.5%)		Reference
Yes	66 (41.2%)	0.222	0.793 (0.546–1.151)
Stimulation protocol			Reference
Long	55 (36.4%)		
Short	9 (45.0%)	0.139	2.143 (0.781–5.880)
Antagonist	262 (42.3%)	0.077	1.423 (0.962–2.105)
Mild	0 (0.0%)	0.999	0.000 (0.000–0.000)
Agonist-Antagonist	3 (30.0%)	0.550	0.640 (0.148–2.949)
Free	3 (23.1%)	0.667	0.737 (0.184–2.949)
Gonadotropin			
uFSH	51 (46.8%)		Reference
rFSH	41 (38.0%)	0.255	0.719 (0.408–1.269)
uFSH+rFSH	204 (40.1%)	0.438	0.840 (0.540–1.306)
rLH	33 (39.8%)	0.401	0.771 (0.420–1.415)
rFSH+rLH	1 (25.0%)	0.922	0.890 (0.086–9.266)
uFSH+rLH	2 (66.7%)	0.648	1.174 (0.149–21.345)
Trigger			
hCG	142 (39.6%)		Reference
GnRH antagonist	128(41.0%)	0.516	1.119 (0.797–1.571)
Dual	58 (42.6%)	0.101	1.423 (0.933–2.172)
# Aspirated oocytes per patient	-	0.857	0.998 (0.978–1.019)
# Aspirated oocytes MII per patient	-	0.589	0.993 (0.969–1.018)
Duration of stimulation	-	0.015*	0.897 (0.822–0.979)
Total dosage of gonadotropins	-	0.151	1.000 (1.000–1.000)
Daily dosage of gonadotropins	-	0.822	1.000 (0.998–1.002)

P values are calculated with a binary logistic regression statistical test using as confounding factors maternal age, embryo quality and biopsy day and genetic analysis technique (NGS or a-CGH) ($p < 0.05$ is considered significantly different).
OCP: oral contraceptive pills; FSH: follicle-stimulating hormone; LH: luteinizing hormone; rFSH: recombinant FSH; uFSH: urinary FSH; rLH: recombinant LH; hCG: human chorionic gonadotropin; GnRH: gonadotropin-releasing hormone; Dual: hCG+GnRH antagonist

treatment duration on the aneuploidy rate. We analyzed only the results obtained in the cycles that followed a long agonist protocol and the antagonist one, which differ in duration (11–12 vs. 8–9 days, approximately). The effect of the COS duration was observed again, obtaining a higher percentage of aneuploid embryos in cycles with antagonists (shorter duration). Although this difference did not become significant, it did show a certain tendency (36.4% vs 42.3%; $p = 0.084$) (Supplemental data Table 1). In comparison, no statistically significant relationship was observed between the incidence of mosaicism in embryos and any of the variables of stimulation treatment studied ($p > 0.05$) (Table 4).

Discussion

Advances in preimplantation genetic diagnosis techniques in recent years allowed us to increase the success rate of in vitro fertilization cycles, especially for elderly patients who are at greater risk of having aneuploid embryos (Barash et al. 2017). For these patients, it is of great importance to obtain a higher number of oocytes to increase the chances of having at least one euploid embryo to transfer.

Although the majority of embryo chromosomopathies originate in the meiosis of the gametes, the effect of the COS process on the induction of this genetic alteration is questioned. Studies, such as McCulloh et al (McCulloh et al. 2019), reinforce this idea showing in their analysis that euploidy rates vary among physicians and speculating that this variation is due to physician-specific ovarian stimulation protocols used for oocyte donors. In contrast with our results, they found that euploidy rates were associated with the ratio of hMG to total gonadotropins. This difference may be due to the type of women included in each study (donors vs patients) and the mean maternal age.

To our knowledge, this is the first study evaluating the use of previous oral contraceptives and its relationship with the rate of aneuploidy and mosaicism in embryos. The effect of the hormones administered

Table 4. Association between mosaicism rate and IVF cycles parameters.

	Mosaic embryos (n/%)	*p* value	OR (95% CI)
OCP			
No	111 (16.9%)		Reference
Yes	19 (12.0%)	0.172	1.455 (0.850–2.490)
Stimulation protocol			Reference
Long	25 (16.4%)		
Short	3 (15.0%)	0.687	0.759 (0.199–2.902)
Antagonist	96 (15.5%)	0.445	0.820 (0.494–1.363)
Mild	1 (50.0%)	0.152	7.974 (0.465–136.843)
Agonist-Antagonist	2 (20.0%)	0.782	1.260 (0.246–6.466)
Free	3 (23.1%)	0.597	1.467 (0.354–6.078)
Gonadotropin			
uFSH	19 (17.3%)		Reference
rFSH	19 (17.8%)	0.737	1.134 (0.545–2.359)
uFSH+rFSH	78 (15.4%)	0.789	1.083 (0.603–1.948)
rLH	13 (15.7%)	0.889	0.944 (0.421–2.116)
rFSH+rLH	0 (0.0%)	0.999	0.000 (0.000-)
uFSH+rLH	1 (33.3%)	0.608	1.911 (0.161–22.662)
Trigger			
hCG	59 (16.5%)		Reference
GnRH antagonist	54(17.3%)	0.626	0.897 (0.579–1.389)
Dual	16 (11.8%)	0.351	0.748 (0.406–1.377)
# Aspirated oocytes per patient	-	0.819	1.041 (0.949–1.141)
# Aspirated oocytes MII per patient	-	0.790	1.004 (0.973–1.037)
Duration of stimulation	-	0.754	1.018 (0.912–1.136)
Total dosage of gonadotropins	-	0.348	1.000 (1.000–1.000)
Daily dosage of gonadotropins	-	0.431	1.001 (0.998–1.004)

P values are calculated with a binary logistic regression statistical test using as confounding factors maternal age, embryo quality and biopsy day and genetic analysis technique (NGS or a-CGH) ($p < 0.05$ is considered significantly different).
OCP: oral contraceptive pills; FSH: follicle-stimulating hormone; LH: luteinizing hormone; rFSH: recombinant FSH; uFSH: urinary FSH; rLH: recombinant LH; hCG: human chorionic gonadotropin; GnRH: gonadotropin-releasing hormone; Dual: hCG+GnRH antagonist

during stimulation at the cellular level is still unknown. Sekhon et al. (Sekhon et al. 2017) observed a dose-dependent relationship between embryo aneuploidies and the amount of gonadotropins administered, but only in patients who required COS beyond cycle day 12. This may be attributed to induction during a prolonged time of a high level of intracellular stress in the antral follicles which may be associated with an increase in the number of meiotic and mitotic oocyte errors (Munne et al. 1997; Baart et al. 2007). Nevertheless, our results, as others carried out previously (Barash et al. 2017; Labarta et al. 2017; Wu et al. 2018; Hong et al. 2019), do not show a relationship between the total and daily dose of gonadotropins administered and the rate of embryo aneuploidies. Our data show that embryo aneuploidy rates are independent of the number of embryos biopsied per cycle, as well as the total number of oocytes and MII oocytes obtained, supporting the results previously reported by other groups (Ata et al. 2012; Barash et al. 2017; Labarta et al. 2017).

Some studies, such as those by Weghofer et al. (2008, 2009), observed an incremental increase in the success rates of IVF cycles when the combination of FSH and LH gonadotropins were used. This observation suggests the existence of a synergistic effect between the two gonadotropins that, subsequently, considerably improves ovarian steroidogenesis, especially in patients with low endogenous LH levels and low responders (Balasch et al. 2001; Mochtar et al. 2007). However, despite observing an increase in implantation and ongoing pregnancy rates, these studies found a significant relationship between the use of gonadotropins with LH activity and embryonic euploidy rates only in long agonist cycles. In the present study, we have not found this association in any of the cases, nor in the function of FSH gonadotropins of urinary or recombinant origin.

The use of GnRH agonists as ovulation triggers has gained popularity in recent years as an alternative to the use of hCG in the cycles with antagonist protocols, partly because they significantly decrease the risk of ovarian hyperstimulation (Engmann et al. 2008; Thorne et al. 2019). Moreover, the GnRH agonist trigger has been suggested to improve oocyte maturity rates (Griffin et al. 2014). We can speculate that since the use of GnRH agonists evoke an LH peak and an additional FSH peak similar to that which occurs physiologically compared to hCG, there may exist a difference in the final maturation process of the oocyte that affects meiotic segregation and therefore the aneuploidy rate. However, like the results reported by Thorne et al. (Thorne et al. 2019), the data obtained in our study show that the use of one or the other drug, as well as the combination of both, ovulation triggers is independent of the appearance of embryonic aneuploidies.

Some authors, such as Baart et al. and Nargund et al. (Nargund et al. 2001; Baart et al. 2007), suggest that chromosomal imbalance in embryos is affected by the type of stimulation protocol followed. The proportion of aneuploid embryos analyzed in these studies is lower when patients undergo mild stimulation or when the stimulation takes place after a natural cycle. In comparison, in the study by Verpoest et al. (Verpoest et al. 2008), astonishingly high aneuploidy rates were observed in embryos coming from cycles without prior stimulation. As per our data, we found no statistically significant relationship between the aneuploidy rate and any type of stimulation protocol, thus we cannot associate the appearance of these chromosomal alterations with the protocol used.

Our results show the existence of an inverse relationship between the number of days of ovarian stimulation and the appearance of aneuploidies in the embryos. In this way, a greater number of days of treatment are associated with a lower incidence of these chromosomal alterations. This longer duration may indicate that the process of oocyte maturation occurs more gradually, similar to that which occurs physiologically, giving time to the processes of intracellular self-repair to act (Kuliev and Verlinsky 2004; Vanneste et al. 2009). This association is also observed when we compare the protocols that differ most in duration (long agonist vs. antagonist), confirming that the rate of embryonic aneuploidy is lower in long agonist protocols due to their longer duration.

Since mosaicism originates in the mitotic division of the embryo (McCulloh et al. 2019), it would be expected that the appearance of this alteration is not related to the ovarian stimulation process. Our results reinforce this model by not having found an association between embryonic mosaicism and any of the stimulation protocol variables.

This study presents some limitations, mainly due to its retrospective nature. Although the patients included in the study were of similar age, other factors, such as ovarian reserve, were not taken into consideration for the statistical analysis. Additionally, the results of the present study are limited with the number of analyzed cases. For that reason, it would be interesting to extend it by increasing the number of included patients.

Based on our results, we found a significant relationship between the duration of stimulation and the incidence of aneuploidy, detecting a higher rate of affected embryos in cycles of shorter duration. Thus, an adequate ovarian stimulation treatment adapted to the clinical history of each patient remains a fundamental rule to successfully perform an IVF cycle, trying to reduce as much as possible the cell damage in the oocytes both in cycles with or without PGT-A.

Materials and methods

Study population

A retrospective study was conducted. We included 835 embryos belonged to 280 couples undergoing reproductive treatment with PGT-A in our fertility clinic between March 2013 and February 2018. These embryos were biopsied on day 5 or 6 of their development. Only patients under 38 years of age who participated in IVF cycles with their own oocyte were included in the study. In addition, both members of the couple had a normal karyotype and, in the case of the male, FISH test showed that chromosomal composition of the sperm was normal and TUNEL test did not reveal sperm DNA fragmentation. The indication to perform PGT-A was recurrent implantation failure or repeated pregnancy loss. Only one IVF cycle per patient was included in the study.

Ovarian stimulation and oocyte retrieval

The COS protocol followed by the patients was customized by a specialized physician, adjusting the dose, type of exogenous gonadotropin and duration of treatment based on their clinical history, BMI and ovarian reserve markers (antral follicle count and anti-Mullerian hormone) and could be adjusted according to ovarian response. In addition, some of the patients took oral contraceptives pills during the months prior to the start of the stimulation treatment.

There is a great variety of ovarian stimulation protocols that are classified according to the type of exogenous hormones used and to the moment of the cycle when the gonadotropins are started. It is worth mentioning that mild stimulation protocol is characterized by the administration of a low dose of gonadotropins with or without oral compounds.

The final oocyte maturation can be triggered in two different ways. The first one via the administration of hCG, which molecular structure is very similar to LH and therefore mimics its effect in terms of inducing ovulation. Or in antagonist protocols, via administration of GnRH agonists which exert a flare-up effect and produce the release of endogenous LH and FSH.

During the stimulation cycle, the evolution of follicle growth was monitored ultrasonographically and the ovulation trigger was administered when the follicles reached the appropriate size (at least 2 follicles major than 18 mm). The oocytes were recovered 36 hours later by ultrasound-guided transvaginal aspiration under sedation.

Mature oocytes were fertilized in the laboratory by intracytoplasmic sperm injection (ICSI) following IVF laboratory guidelines. The generated embryos were

biopsied between day 5 and day 6 of embryonic development with the help of a 200 mW diode laser (Saturn, Research Instruments LtD, Cornwall,UK or Hamilton Thorne, Berverly, USA). The biopsied cells (5 to 10) were transferred to PCR tubes with 1 µl of PBS.

Genetic analysis

Firstly, a complete genome amplification was done (Picoplex kit, Rubicon Genomics ®, Ann Arbor, MI, USA).

The embryos were then analyzed by comparative genomic hybridization (array-CGH) using Agilent SurePrint G3 8x60K microarrays (Agilent Technologies ®, Palo Alto, CA, USA) or by massive sequencing (NGS) with a synthesis sequencer (Veriseq Illumina ®, San Diego, CA, USA). Embryos with ≤25% aneuploid cells were considered euploid, between 25% and 50% were classified as mosaic and aneuploid with >50%.

All the genetic analysis was performed at the center in the genetics and molecular biology laboratory.

Statistical analysis

Continuous variables were presented as mean value ± SD and categorical variables as percentages. The data were analyzed with Statistical Package for the Social Sciences (SPSS) software (version 20.0, SPSS, Inc., Chicago, IL, USA).

We evaluated the association between the incidence of aneuploidy and embryonic mosaicism with the different parameters of ovarian stimulation: previous oral contraceptives, days of stimulation, type, total and daily dose of gonadotropins, type of protocol, type of ovulation trigger, total number of oocytes and number of metaphase II oocytes recovered. A first approximation was made using Student's t-test for continuous data and Pearson's chi-square test for categorical variables. Finally, a multivariate analysis was performed through binary logistic regression. Maternal age, embryo quality and the day of biopsy were introduced as confounding variables. In order to avoid any bias that might be caused because the array-CGH platform is less sensitive than NGS technology, especially for detecting mosaicism, we also presented the type of technique used in the genetic analysis as a confounding factor. The results were considered significant for $p < 0.05$.

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Ethical approval

Ethical approval for this study was obtained from Institutional Review Board.

Disclosure statement

The authors declare that they have no conflict of interest.

Authors' contributions

Study design: AC, JAO, BL, RM; Laboratory experiments: BL, RM, JAO, JT; Data collection: AC, JAO; Statistical analysis and interpretation of data: JAO, AC, BL, RM; All authors contributed to drafting and revising the manuscript, and approved the final version to be published: AC, BL, JAO, RM, JT, JL, RB.

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