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The use of aniline blue chromatin condensation test on prediction of pregnancy in mild male factor and unexplained male infertility

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Abstract

The aim of this study was to investigate the possibility of using sperm function tests (hypoosmotic swelling test [HOS], aniline blue [AB] staining test, and sperm chromatin dispersion [SCD]) to predict intrauterine insemination [IUI] success rate. A total of 243 couples with mild male factor or unexplained male infertility who underwent IUI were evaluated prospectively. The results of basic sperm analysis and sperm function tests were compared between pregnant or nonpregnant groups. The HOS (11.9 ± 9.6% vs. 10.1 ± 8.5%, $p = 0.35$) and SCD tests (32.9 ± 21.0% vs. 29.9 ± 19.0%, $p = 0.48$) were not significantly different between pregnant ($n = 22$) and nonpregnant ($n = 221$) groups. However, the AB staining negativity rate was significantly higher in the pregnant group compared to the nonpregnant group (35.2 ± 20.8% and 24.4 ± 18.0%, $p = 0.008$). On ROC analysis, a cut-off value of 24% for AB negativity showed a sensitivity and a specificity value of 82.35% and 51.38% (AUC) = 0.653; 95% confidence interval: 0.571–0.72 P (Area = 0.5) = 0.0267, respectively, for prediction of pregnancy. Our study showed that the sperm chromatin maturity, assessed by AB stain, may predict the pregnancy in couples with unexplained female infertility plus mild male factor or unexplained male infertility. The HOS and SCD failed to predict the pregnancy in this group of couples.

KEYWORDS

aniline blue test (chromatin condensation), HOS, IUI, sperm chromatin dispersion test

1 | INTRODUCTION

Total motile sperm count has been shown to be directly proportional to high rate of pregnancy in intrauterine insemination (IUI) technique (Branigan, Estes, & Muller, 1999; Brasch, Rawlins, Tarchala, & Radwanska, 1994; Dimarzo et al., 1992), whereas sperm morphology was indicated to be an important factor affecting the success rate of IUI (Kruger et al., 1988). Moreover, factors such as ovarian stimulation treatment, number of applications and sperm morphology and motility are influencing factors for IUI outcomes (Berker et al., 2012; Boomsma, Heineman, Cohlen, & Farquhar, 2007; Branigan et al., 1999; Campana et al., 1996; Cantineau, Cohlen, & Heineman, 2007). However, the basic semen analysis performed in couples with mild male factor and unexplained infertility may be insufficient in predicting the success of IUI. At this point, the sperm DNA damage, sperm membrane permeability and sperm chromatin condensation may be suggested as further parameters to evaluate the sperm quality. The sperm membrane permeability can be assessed by the hypoosmotic swelling test (HOS), the sperm chromatin maturity can be assessed by the aniline blue (AB) staining test and the sperm DNA fragmentation or chromatin decondensation may be assessed by the sperm chromatin dispersion test (SCD).

During spermiogenesis, mammalian nuclear histones are replaced by transition proteins followed by replacement with the

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protamines immediately after oxidisation of disulphide bonds, which are enhanced by the cysteine-thiol groups of protamine of sperm DNA molecule in the epididymis (Hou, Chen, & Jeyendran, 1995; Talebi et al., 2013). AB positivity shows the presence of histones, indicating immaturity; whereas AB stain negativity shows the normal chromatin maturity.

The stabilisation of sperm cells' chromatin provides a resistance against external factors. Evenson and Wixon (2006) have observed that any damage to the sperm chromatin has a large impact on fertilisation problems. A number of studies have investigated the role of the sperm damage evaluation tests in IUI cases (Agarwal & Said, 2003; Castilla et al., 2010; Evenson & Wixon, 2006). A close relationship between sperm chromatin condensation defects and sperm DNA damage has been shown in many studies (Agarwal & Said, 2003; Chapman & Michael, 2003; Rodriguez, Ohanian, & Bustos-Obregon, 1985; Steger et al., 2008).

Sperm chromatin dispersion may assess the decondensation ability of the spermatozoa after fertilisation (Gopalkrishnan, Hinduja, & Anandkumar, 1991; Rodriguez et al., 1985). The decondensation ability is also important to form the male pronuclei (WHO, 2010). The most commonly used sperm nucleus decondensation test is the one conducted by preparing the mixture of 1% SDS and 6 M EDTA solution (Shete, Hulke, Thakare, & Patil, 2012).

Van der Ven et al. (1986) have found a correlation between swelling ability of the spermatozoa in a hypoosmotic environment and the outcomes of in vitro fertilisation. Ramu and Jeyendran (2013) have shown that the sperm membrane integrity can be assessed using the HOS test. The sperm membrane plays a very important role during acrosome reaction, adherence of the spermatozoa and oocyte, as well as penetration. Irez et al. (2013) suggested that HOS test scores may play a role in development of the good embryos in normospermic patients' IVF fertilisation.

The aim of this prospective study was to investigate the possibility of using the AB sperm chromatin test, the HOS test, and the SCD test to predict IUI success rates and report our 3 years of IUI practice (243 cases) at the Universal German Hospital IVF Center.

2 | MATERIALS AND METHODS

A total of 243 couples with mild male factor or unexplained male infertility who underwent IUI between 2012 and 2015 in the Universal German Hospital, Istanbul, Turkey, were evaluated prospectively. The study was approved by the Yeni Yuzyil University Ethics Committee (2012/3-21), and informed consent was taken from the couples.

The couples with women <18 and >38 years old were excluded from the study. The basic semen analysis, sperm function tests and pregnancy outcomes were evaluated.

Couples with unexplained infertility had semen analyses with a sperm concentration >20 × 10^6/ml, motility (grade I + II) >39%, and normal morphological forms >4%. Couples with mild male factor infertility had semen analyses with one only of the above-mentioned parameters below the normal range.

Semen samples were obtained by masturbation following a 3–5-day sexual abstinence. A total of 262 semen samples were collected from men attending our andrology and IVF laboratory for mild male infertility disorders (n = 122) and unexplained cases as control (n = 121). Male infertility was classified according to WHO criteria (WHO, 2010, 21): oligozoospermia, <15 × 10^6 sperm/ml; asthenozoospermia, <39% of spermatozoa with grades a/b motility; teratozoospermia, <4% normal morphology. We also scored sperm movement as: immotile spermatozoa with 3 points, moving without progression with 2 points, slow nonlinear progression and forward linear progression with 1 point. In our series, 132 men had mild male factor, and 130 men had normospermia with unexplained infertility cases. The samples were evaluated for chromatin condensation with the percentage of unstained sperm head, sperm morphology, concentration and linear progressive motility.

2.1 | Sperm chromatin tests and membrane permeability

Two smears were prepared after semen processing by the gradient technique, air-dried and fixed for 30 min in 2.5% glutaraldehyde in phosphate-buffered saline for the assessment of morphology and nuclear maturity, which was carried out by the AB staining method. The smear was stained for 5 min in 5% aqueous AB solution (pH 3.5). The percentage of spermatozoa stained with AB was determined by counting 200 spermatozoa per slide under a bright-field illumination at a magnification of 100×. Sperm heads containing immature nuclear chromatin stain blue (chromatin not condensed) and those with mature nuclei do not take up the stain (chromatin condensed), and later were washed with a phosphate buffer. Semen analysis was performed according to WHO 2010 criteria (2010). In the next step, the following fixed sample was stained with the Diff-Quick staining method for sperm morphology, and the results were evaluated according to WHO 2010 criteria (2010).

HOS and SCD tests were conducted using 20 µl sample for each test. The hypoosmotic swelling test was performed in diluted HTF media (1/1) with sterile distilled water and 20 µl of prepared sperm solution mixed with HOS solution. In total, 200 spermatozoa were used. After 30 min, completely swollen sperm tails were counted (Irez et al., 2013).

The SCD test was carried out using EDTA, which provides chromatin dispersion. The results included the percentage of completely swollen spermatozoa as suggested by Fernández et al. (2003).

Figure 1 shows the image of the AB of the sperm chromatin condensation test. Chromatin structure of nonstained sperm head is considered normal. Figure 2 shows sperm chromatin decondensation with SDS-EDTA solution with the percentage of decondensed sperm heads taken into account.

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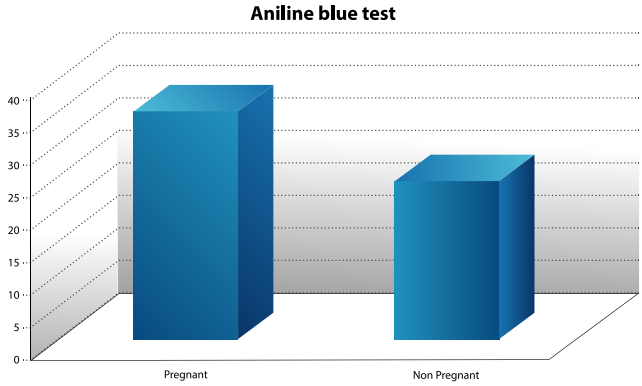

FIGURE 1 Percentage of chromatin condensation (aniline blue staining test negativity) in pregnant and nonpregnant patients

2.2 | IUI protocol

Ovulation induction was performed with clomiphene citrate between days 5 and 10 of menstruation. HCG (5,000 IU) was injected when at least three follicles had reached a diameter of ≥16 mm. IUI was scheduled for 36 hr after HCG injection (day 0).

Semen specimens were collected by masturbation on site, evaluated according to standardised methods, and prepared for IUI within an hour after collection (Berker et al., 2012; Boomsma et al., 2007). Semen was diluted 1:2 (vol/vol) with HEPES-buffered HTF medium (SAGE) containing 1.5% serum albumin (Merck, Germany). Two layers of gradient protocols were used for motile sperm separation.

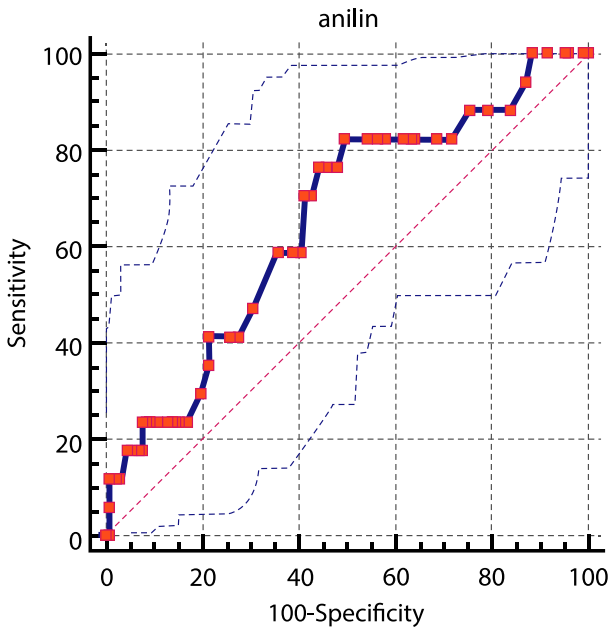

FIGURE 2 Receiver operating characteristic (ROC) curve to test the sensitivity and the specificity of the sperm chromatin condensation to predict the clinical pregnancy rate per cycle. The Area under the ROC curve (AUC) = 0.653, 95% confidence interval:0.571–0.72 Significance level P (Area = 0.5) = 0.0267 Associated criterion: >24, and 82.35 sensitivity with 51.38 specificity

After centrifugation at 2,000 rpm for 10 min, the pellets were resuspended and combined in 3 ml of the medium and washed again two times at 300g centrifugation. The sperm pellet was resuspended in 0.45 ml of the medium. Approximately 0.05 ml was used to determine the concentration and motility of the spermatozoa. The remaining sample was drawn into a Cooper Surgical IUI catheter (Cooper Surgical, NYSE, Co., USA) attached to a 1-ml syringe for IUI, which was performed within 2 1/2 hr after semen collection.

2.3 | Definition of pregnancy

Serum β-hCG was measured 15 days after IUI (luteal day 15). If the value exceeded 10 mIU per millilitre, the measurement was repeated on luteal day 17. Pregnancy was indicated by an increase in β-hCG concentration of the serum between luteal days 15 and 17. Subsequently, the outcome of pregnancy, sperm morphology and concentration, chromatin condensation by AB staining test, HOS scores, and percentage of SCD were recorded.

Body mass index was calculated as body weight/height^2 and was measured before the beginning of the ovulation induction. Serum follicle stimulating hormone (FSH), luteinising hormone (LH), thyroid stimulating hormone (TSH) and E2 levels were measured at the third day of menstruation. Endometrial thickness was measured between days 5 and 14 with vaginal ultrasonography as part of the routine follow-up.

2.4 | Statistics

All parameters were equally distributed as shown by Levene's test and Kolmogorov–Smirnov's test. The noncategorical data were presented as mean and standard deviation and compared between the groups with an independent *t* test. Categorical data were presented as proportions and were compared with chi-square test between the groups. The receiver operating characteristic (ROC) curves were used to determine the sensitivity and specificity values and area under curve. A *p*-value <0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, software version 20 SPSS, Chicago, USA).

3 | RESULTS

The demographic characteristics of mild male factor and unexplained infertility cases were presented in Table 1. Although there were significant differences in basic sperm parameters (concentration, motility and morphology), there was no significant difference in the age of the men, sperm function tests (HOS, SCD and AB tests) and pregnancy rates (10.6% vs. 9.9%; $p = 0.850$) between the mild male factor ($n = 122$) and unexplained infertility ($n = 121$) groups. Moreover, there was no significant difference in male or female BMI, endometrium thickness or women's hormone levels between the two groups (Table 1).

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TABLE 1 Demographic characteristics of mild male factor and unexplained infertility cases

	Mild male factor (132)	Unexplained infertility (130)	*p*
Women age (years)	31.5 ± 4.67	32.31 ± 4.2	0.109
Male age (years)	35.7 ± 3.56	34.4 ± 4.36	0.499
BMI	25.30 ± 4.54	24.95 ± 4.35	0.515
Endometrium thickness (mm)	9.23 ± 2.38	8.85 ± 2.36	0.732
FSH (mIU/ml)	6.41 ± 2.14	6.61 ± 2.34	0.475
LH (mIU/ml)	4.23 ± 3.52	4.25 ± 2.07	0.970
E2 (IU/ml)	41.63 ± 21.47	44.82 ± 22.09	0.274
Sperm concentrations	32.56 ± 16.1	55.34 ± 18.8	0.098
Sperm motility	36.65 ± 17.98	41.67 ± 5.86	0.109
Sperm morphology	3.8 ± 3.98	5.1 ± 3.1	0.051
Aniline (–)	27.23 ± 21.14	31.17 ± 19.34	0.480
HOS (+)	9.90 ± 9.12	11.23 ± 9.87	0.430
SCD (+)	29.25 ± 21.98	30.23 ± 12.8	0.890
Pregnancy rate (%)	9.9	9.1	0.867

A total of 25 pregnancies were observed during the study period. The comparison of the pregnant and nonpregnant groups is presented in Table 2. There was no significant difference in male or female age or basic sperm parameters between the pregnant (n = 22) and nonpregnant (n = 221) groups. The HOS (11.9 ± 9.6% vs. 10.1 ± 8.5%, p = 0.35) and SCD tests (32.9 ± 21.0% vs. 29.9 ± 19.0%, p = 0.48) were not significantly different between pregnant (n = 22) and nonpregnant (n = 221) groups (Table 2, Figures 3 and 4). However, the AB staining test negativity rate was significantly higher in the pregnant group compared to the nonpregnant group (35.2 ± 20.8% and 24.4 ± 18.0%, p = 0.008; Table 2, Figures 1 and 3). On ROC analysis, a cut-off value of 24% for AB staining test negativity showed a sensitivity and a specificity value of 82.35% and 51.38% (AUC) = 0.653; 95% confidence interval: 0.571–0.72 P (Area = 0.5) = 0.0267, respectively, for prediction of pregnancy (Figure 2).

4 | DISCUSSION

The diagnosis of male infertility is made by semen analysis. In semen analysis, sperm morphology and basal values such as motility and concentration can be used to evaluate the production process.

However, the conventional methods are not efficient to evaluate the cases with unexplained infertility (Imade, Towobola, Sagay, & Otubu, 1993; Kruger et al., 1993; Morshedi, Duran, Taylor, & Oehninger, 2003; Ombelet et al., 1994; Overstreet, Gould, Katz, & Hanson, 1980). Therefore, in the case of unexplained infertility, the spermatozoa should be evaluated by new tests in addition to conventional semen analysis, such as the sperm DNA integrity assessment tests and chromatin condensation tests, which examine the protein structure of the chromatin, as well as the HOS test, which shows the permeability of the sperm membrane (Irez et al., 2013; Muriel et al., 2005).

In our study, we observed that the sperm morphology, motility and concentration were not significantly different in pregnant and nonpregnant groups in normospermic and mild male factor cases (Table 2). However, the chromatin condensation was significantly higher in the pregnant group compared to the nonpregnant group. These results show that the chromatin condensation test is important in predicting IUI pregnancies in normospermic or mild male factor cases. A threshold of 24% had a sensitivity, and the specificity of the sperm chromatin condensation test with AB staining were

TABLE 2 Comparison of the sperm function parameters in patients with pregnancy positive and negative cases of IUI. Bold values are p < 0.05 in significant.

	Pregnancy	*n*	Mean ± *SD*	*t*	*p*
Women age	Positive	22	27.5 ± 5.6	2.342	**0.020**
	Negative	240	30.2 ± 5.1		
Male age	Positive	22	34.2 ± 5.6	0.304	0.761
	Negative	240	35.5 ± 5.0		
Sperm const	Positive	22	43.0 ± 38.7	0.122	0.903
	Negative	240	39.9 ± 37.6		
Total motility	Positive	22	38.9 ± 18.7	0.751	0.453
	Negative	240	26.4 ± 14.7		
Normal morphology	Positive	22	4.6 ± 3.2	1.335	0.183
	Negative	240	3.7 ± 3.3		
HOS (+)	Positive	22	11.9 ± 9.6	0.920	0.359
	Negative	240	10.0 ± 8.5		
SCD (+)	Positive	22	32.9 ± 21.0	0.644	0.520
	Negative	240	29.8 ± 18.9		
Aniline (–)	Positive	22	35.2 ± 20.8	2.208	**0.029**
	Negative	240	24.6 ± 18.2		

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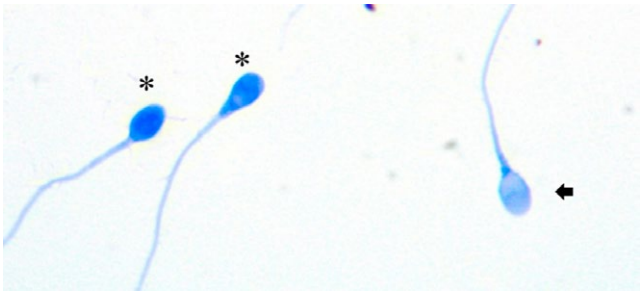

FIGURE 3 Aniline blue staining test positive (*) uncondensed, histone included and negative, condensed spermatozoa, completed protamination (←)

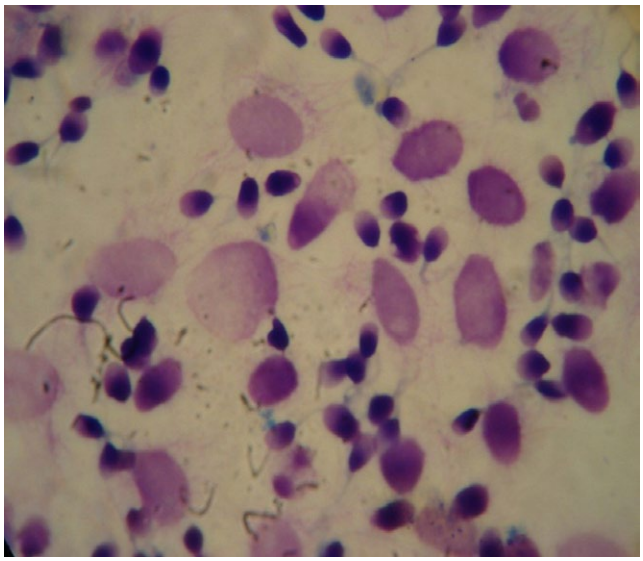

FIGURE 4 Sperm chromatin decondensation test with SDS-EDTA, shown decondensed sperm head, Haematoxylin–eosin staining ×100

82.5% and 50.38% respectively (see Table 2 and Figure 2). We may conclude that IVF may be considered for a chromatin condensation value of <24%. These findings showed the importance of the chromatin condensation test with AB in mild male factor infertility cases, as demonstrated by Irez et al. (2015) in IUI and intracytoplasmic sperm injection (ICSI) cases in oligospermic and teratozoospermic cases.

In the chromatin condensation test with AB dye, the immature sperm bearing the histone is stained with AB (Morshedi et al., 2003). Sperm chromatin organisation takes place during spermiogenesis. An abnormal chromatin packaging has been shown to be more frequent in infertile men (Abu, Franken, Hoffman, & Henkel, 2012; Jayaraman, Upadhya, Narayan, & Adiga, 2012). The sperm head anomalies are associated with an increase in positive AB tests (Abu et al., 2012; Jayaraman et al., 2012). Kazerooni et al. (2009) have shown a positive correlation between the AB positivity and morphological anomalies of spermatozoa. Moreover, Zini Boman Belzile and Ciampi (2008) found higher rates of AB positivity in men with teratozoospermic sperm. Sellami et al. (2013) compared the sperm maturation defects over and under 20% and detected no significant difference in terms of sperm motility, concentration or morphological abnormalities.

Esteves (2016) showed that 23%–62% of the infertile normospermic men were found to have an impaired chromatin condensation level in normospermic fertile and infertile men, and infertile normospermic men had a statistically significant chromatin defect when compared to the two groups. In this context, our work supports the findings of Esteves. (2016).

In our study, the SCD tended to be higher in the pregnant group compared to the nonpregnant group; although this difference remains statistically not significant (Table 2). The relationship between sperm DNA damage and pregnancy have been evaluated in many studies (Jayaraman et al., 2012; Mohamed & Mohamed, 2012; Muriel et al., 2005; Ribas-Maynou, García-Peiró, & Fernández-Encinas, 2013; Tomlinson et al., 2001; Tomlinson, Kessopoulou, & Barratt, 1999). Ribas-Maynou García-Peiró and Fernández-Encinas (2013) have suggested that the SCD test may fail to show efficiency in sperm DNA damage. In our study, we observed that there were no differences in SCD values between pregnant and nonpregnant groups.

The HOS test measures the sperm membrane integrity according to the WHO WHO, 2010 criteria. The lower limit is 58% (Van der Ven et al., 1986). Irez et al. (2013) suggested a possible relationship between low HOS scores and fertilisation problems in IVF patients. The diagnostic threshold values of the HOS test reported by many investigators show a broad range (Bollendorf, Check, & Kramer, 2012; Carreras, Ramirez, & Mendoza, 1992; Check, Tubman, & Wilson, 2013). The common finding of all those studies was the correlation between the sperm viability and the HOS test scores (Hossain, Rizk, Barik, Huff, & Thorneycroft, 1998). In our study, we observed that the rate of HOS positive spermatozoa was not significantly different between pregnant and nonpregnant groups in mild male factor and unexplained infertility cases. The application duration and the threshold values of HOS test scores still need to be clarified.

5 | CONCLUSION

Our study showed that the sperm chromatin maturity, assessed by AB stain, may predict the pregnancy in couples with unexplained female infertility plus mild male factor or unexplained male infertility. The men with high positive AB staining percentage may be potential candidates for IVF treatment instead of IUI treatment. The other sperm function tests (HOS and SCD) failed to predict the pregnancy in this group of couples.

DISCLOSURES

Conflict of interest: The authors declare no conflict of interest. Human and Animal Rights: The protocol for the research was approved by Yeni Yuzyil University, Faculty of Medicine, Istanbul, Turkey. All the procedures were followed in accordance with the

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ethical standards of the responsible committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all the patients to be included in the study. This article does not contain any study with animal participants that has been performed by any of the authors.

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