A Quantitative and Morphological Analysis of Oocytes Collected During 438 IVF Cycles

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Purpose: Our purpose was to determine if the number of retrieved oocytes, oocyte maturity, morphology, and other embryological parameters are related to the outcome of treatment.

Methods: This retrospective study on 438 IVF cycles analyzes the numbers of retrieved oocytes, fractured zona oocytes, germinal vesicle-stage oocytes, normally and abnormally fertilized oocytes, pregnancy rate, age of female partner, ovarian stimulation protocol, day of hCG injection, and serum estradiol concentration.

Results: (1) Pregnancy rate increases with an increase in the number of retrieved oocytes, (2) a high incidence of fractured zona pellucida oocytes has a negative effect on fertilization rate but none on pregnancy rate, (3) a high incidence of immature oocytes is associated with improved fertilization and pregnancy rates, and (4) an inverse relationship between the presence of immature oocytes and oocytes with fractured zona pellucida is suggested.

Conclusions: Precise oocyte assessment in IVF cycles provides informations useful for the analysis and improvement of ovarian stimulation protocols.

KEY WORDS: fractured zona pellucida oocyte; germinal vesicle-stage oocyte; IVF; oocyte maturity; polyspermy.

INTRODUCTION

In assisted conception treatments, the combined use of GnRH analogues and gonadotropins for multiple follicular development has become widely used

because it allows the recovery of numerous preovulatory oocytes. The influence of oocyte number on outcome of in vitro fertilization (IVF) treatment has already been studied with controversial results. The retrieval of a large number of oocytes seems to be correlated with oocytes of lower quality and lower implantation rate (1). However, for other authors, the retrieval of large number of preovulatory oocytes has a small impact on oocyte quality (2) and the pregnancy rate even increases with the number of retrieved oocytes (3). Concerning intracytoplasmic sperm injection (ICSI) technique, oocyte morphology seems to be not correlate with fertilization rate (4) but with outcome of ICSI (5). Assisted conception units are usually interested in the number of embryos available for transfer, the cryopreservation, and the pregnancy rates. However, as shown in the literature, the number and quality of retrieved oocytes appear to be important factors of predicting the outcome of treatment. Ovarian stimulation protocols for IVF indeed produce cohorts of oocytes presenting a great heterogeneity in number and quality (6).

The aim of this study was to determine if in IVF treatments only (ICSI attempts were excluded), the information provided by an analysis of the total number of retrieved oocytes and of different oocyte classes, based on their maturity and intactness of the zona pellucida, would allow a better evaluation of the ovarian stimulation protocol and lead to an improvement of the IVF treatments.

MATERIALS AND METHODS

This analysis is based on 438 consecutive IVF treatment cycles in 410 couples who were treated at our center from January 1 to September 30, 1997. Ovarian stimulation was carried out using human menopausal gonadotropin (hMG; Humegon; 75 IU/ml; Organon

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Laboratories, Saint-Denis, France) and/or follicle stimulating hormone (FSH) injections (MetrodinHP; Serono Laboratories, Boulogne, France). Prior to this, pituitary downregulation and continued desensitization was achieved using daily subcutaneous injections of Buserelin (Suprefact; 1 mg/ml; Hoechst, Paris La Défense, France) administered at a dose of 0.6 mg once daily. The GnRH analogue was used either in a long protocol, with ovarian desensitization starting at the end of the luteal phase of the previous cycle, or in a short protocol begun in early follicular phase to take advantage of the "flare-up" effect. The FSH/hMG dose range administered to each patient was determined according to basal hormonal profile and, if possible, the results of previous stimulation treatments. Follicular development was monitored by ultrasound scanning and serial assay of serum estradiol concentration. The dose of 5000 IU of human chorionic gonadotropin (hCG; Gonadotrophine Chorionique "endo"; Organon Laboratories, Saint-Denis, France) was routinely administered as ovulation trigger. Thirty-four to thirty-seven hours later, oocyte retrieval was performed by transvaginal oocyte aspiration under ultrasound guidance using a 20-ml syringe directly connected to the aspiration needle (Luer Lock needle ovum recovery, gauge 17; CCD Laboratories, Paris, France) without flushing the follicles. The aspirates were examined immediately in the IVF laboratory using a stereoscopic microscope (SMZ-2T; Nikon Corporation, Tokyo) and the oocyte-cumulus complexes isolated and rinsed in culture medium (IVF MEDIUM, MediCult, Denmark). The cumulus spreading technique described by Veeck (7) was immediately carried out to observed oocytes. This was performed using an inverted phase-contrast microscope (Nikon, Diaphot 200, Nikon Corporation, Tokyo) at a magnification of $\times 200$. The microscope was equipped with the Nomarski system to allow color photography using the microscope. This technique allows classification of oocytes as fractured zona pellucida oocytes (FZO), immature prophase I oocytes or germinal vesicle-stage oocytes (GV), mature oocytes (metaphase II), and nearly mature oocytes (metaphase I).

Oocytes with fractured zona pellucida have two main morphological characteristics: an obvious break in the zona pellucida and a darkened ooplasm which is often bilobular.

GV are characterized by their distinct germinal vesicle and refractile nucleolus. An irregular shape, a darkened center, and granular ooplasm are almost always seen. Attached cumulus cells are usually compact.

The metaphase I oocyte is distinguished by the absence of both germinal vesicle and polar body usually associated with luteinized cumulus cells. The metaphase II oocyte is characterized by the presence of the first polar body, the rounded shape, and homogeneous granularity of the ooplasm and is usually associated with an expanded luteinized cumulus and by a "sun-burst" corona radiata.

All oocytes limited by an obvious zona pellucida were cultured at 37°C in tissue cultured dishes (Falcon 35 3001; Becton Dickinson France SA, Le Pont de Claix, France) in an atmosphere of 5% CO₂ and were inseminated 4 to 5 hr after retrieval.

Spermatozoa were prepared using a three-layer Percoll gradient (45, 70, and 90%) and incubated in IVF-medium for 2 to 5 hr before inseminating the oocytes. Oocytes were inseminated by adding 100,000 to 200,000 progressive sperm per milliliter of culture medium.

The removal of corona cells for evidence of fertilization was done the day after insemination using smooth micropipettes carefully avoiding doing any damage to the egg.

Embryo transfer took place 44–48 hr after insemination.

Clinical pregnancy was diagnosed by a positive β -hCG pregnancy test performed 20 days after embryo transfer and the visualization of an intrauterine sac by ultrasound 6 weeks after embryo transfer. Only clinical pregnancies were considered in this analysis. The later evolution of these pregnancies has not been included, as it was not the subject of this study.

Data collected for analysis included the total number of retrieved oocytes, number of FZO and GV, number of normally and abnormally fertilized oocytes, pregnancy rate, age of the female partner, ovarian stimulation protocol, day of hCG injection, and serum estradiol concentration on the day of hCG injection.

Results were expressed as means ($\pm$ SD) and percentages. Analysis of variance was used to compare group means, while the χ^2 test was used to compare proportions. The level of significance was set at $P < 0.05$.

RESULTS

General Characteristics

A total of 410 couples was eligible for the study. The woman's mean age was 33.8 years (SD, 4.56 years). In 368 IVF cycles (84% of all the cycles)

patients were treated with the long protocol of GnRH analogue and the mean age of the women in this group was 33.2 years (SD, 4.33 years). In 70 cycles (16%) ovarian stimulation was carried out using the short protocol and the mean age of the women was 37.4 years (SD, 4.36 years). The difference between woman's age in these two groups of patients was highly significant ($P < 0.00001$).

The total number of retrieved oocytes was 4066, giving a mean of 9.28 oocytes per retrieval (SD, 5.49). The number of FZO was 352 (8.66% of the collected oocytes). The number of GV was 268 (6.6% of the collected oocytes). The total number of fertilized oocytes was 2160 and the fertilization rate was 53.1%. The number of trippronuclear oocytes was 223, giving a 10.3% polyspermic rate. Eighty clinical pregnancies were initiated giving a 18.26% pregnancy rate per oocyte retrieval. Seventy-three women delivered a total of 84 healthy babies.

The hCG injection was performed at day 11.6 (mean) of stimulation (SD, day 1.58) by hMG and/or FSH and the mean serum estradiol concentration on the day of hCG was 2233.7 (SD, 924.2) pg/ml.

Total Number of Retrieved Oocytes

The mean number of retrieved oocytes per IVF attempt was 10.0 (SD, 5.4) for long protocol cycles and 5.5 (SD, 4.1) for short protocol cycles. The difference between mean number of retrieved oocytes in these two groups of attempts was highly significant ($P < 0.00001$). According to the type of protocol, oocyte retrievals were classified according to the number of collected oocytes. For long protocol cycles, three groups were defined: Group 1, where 5 or fewer oocytes were retrieved (92 attempts); Group 2, where 6 to 10 oocytes were collected (122 attempts); and Group 3, where more than 10 oocytes were retrieved (154 attempts). For short protocol cycles, two groups were defined: Group 1, where from one to five oocytes collected (41 attempts); and Group 2, where at least six oocytes were retrieved (29 attempts).

The main results obtained when oocyte retrievals are classified according to the total number of oocytes are presented in Table IA for long protocol cycles and in Table IB for short protocol cycles.

Concerning long-protocol cycles, there was a significant change in the median age within the patient groups. Patients in Group 1 were older than patients of Groups 2 and 3 (37.5, 32.9, and 32.4 years, respectively; $P < 0.01$). The day for hCG administration was significantly different among the three groups.

The duration of stimulation decreased from Group 1 to Group 2 and Group 3 (14.0, 11.8, and 11.4 days, respectively; $P < 0.025$). Estradiol values increased significantly from Group 1 to Group 2 and Group 3 (1096, 2100.9, and 2877.2 pg/ml, respectively; $P < 0.001$). There was a significant difference in the fertilization rate within the three groups. The best result was obtained in Group 2: 57.8%. The pregnancy rate per oocyte retrieval increased with an increase in the number of oocytes collected (7.6, 20.5, and 24.0%, respectively; $P < 0.01$); there were significant differences within the three groups.

Concerning short protocol cycles, there was a highly significant difference between patient's age in Group 1 and Group 2 (38.5 and 35.7 years; $P < 0.0001$). Estradiol values increased significantly from Group 1 to Group 2 (1465.4 and 2263.2 pg/ml; $P < 0.0001$). The day of hCG administration, the fertilization rate, and the pregnancy rate were not significantly different among the two groups of short protocol cycles.

Fractured Zona Oocytes (FZO)

In 184 of the 438 studied cycles (35.1%), FZO were retrieved, while in 254 of them (57.9% of the cycles) FZO were not observed. FZO represented 8.6% of the total number of oocytes recovered and 17.6% of the oocytes recovered in fractured zona cycles (FZC). In 10 FZC, more than 60% of the oocytes retrieved were FZO. Nine patients in this group had 48% FZO during their previous IVF cycles. Two patients had 100% FZO during all their IVF attempts (two and four previous attempts, respectively). Concerning long protocol, the number of FZC and intact zona cycles (IZC) were 207 and 161, respectively. In short protocol, there were 23 FZC and 47 IZC. There was no significant difference in the proportion of FZC and IZC between long and short protocol.

Table II shows the comparison between these two groups of IVF cycles: FZC and IZC.

There were no significant differences in the maternal age (33.9 and 33.8 years), the rate of GV oocytes (7.02 and 6.15%), the polyspermic rate (9.41 and 11.53%), the day of hCG administration (11.54 and 11.71 days), and the pregnancy rate per cycle (17.3 and 19.6%) between IZC and FZC groups. The mean number of collected oocytes (10.87 and 8.13; $P < 0.0001$), the fertilization rate per mature oocyte (64.13 and 60.85%; $P < 0.05$), and the estradiol concentration on the day of hCG administration (2120.8 and 2389.7 pg/ml; $P < 0.01$) were significantly different between the

Table I. Classification of the 438 IVF Attempts According to the Type of Protocol and Number of Collected Oocytes

Variable	(A) Long protocol			<i>P</i>
	IVF attempt group (range of retrieved oocytes)			
	Group 1, from 1 to 5	Group 2, from 6 to 10	Group 3, more than 10	
Number of treatment cycles	92	122	154	
Patient's age (mean ± SD years)	37.5 (2.12)	32.9 (4.43)	32.4 (4.13)	<0.01
Day of hCG (mean ± SD)	14.0 (1.41)	11.8 (1.55)	11.43 (1.37)	<0.025
Estradiol value at hCG (mean ± SD pg/ml)	1096 (247.5)	2100.9 (724.4)	2877.2 (818.2)	<0.001
Total number of collected oocytes	360	970	2351	
Fertilized oocytes (fertilization rate)	167 (46.4)	561 (57.8)	1232 (52.4)	<0.001
Pregnancy (rate per attempt)	7 (7.6)	25 (20.5)	37 (24.0)	<0.01
Variable	(B) Short protocol			<i>P</i>
	IVF attempt group (range of retrieved oocytes)			
	Group 1, from 1 to 5	Group 2, 6 and more		
Number of treatment cycles	41	29		
Patient's age (mean ± SD years)	38.5 (3.68)	35.7 (4.76)	<0.0001	
Day of hCG (mean ± SD)	11.27 (2.34)	11.17 (1.77)	>0.05	
Estradiol value at hCG (mean ± SD)	1465.4 (769.9)	2263.2 (826.7)	<0.0001	
Total number of collected oocytes	113	272		
Fertilized oocytes (fertilization rate)	58 (51.3)	142 (52.2)	>0.05	
Pregnancy (rate per attempt)	6 (14.6)	5 (17.2)	>0.05	

group of intact zona cycles and the group of fractured zona cycles.

Prophase I Oocytes

Of 438 stimulated cycles, 337 (76.9%) had 10% or less of their oocytes as germinal vesicle-stage oocytes (GV), while in 101 cycles (23.1%) GV represented more than 10% of all oocytes retrieved. Concerning long-protocol cycles, 242 of 3681 oocytes were at the germinal vesicle stage (6.57%), while in short-protocol cycles, 26 of 385 oocytes were GV (6.75%). The difference between these two groups was not significant.

Table III presents the comparison between cycles with 10% or less GV and cycles with more than 10% GV.

The number of collected oocytes was significantly different between the two groups (8.9 and 10.4, respectively; $P < 0.03$), as well as the incidence of fractured zona oocytes (9.65 and 5.80%; $P < 0.001$). The fertilization rate per mature oocyte (i.e., without FZO and GV) was significantly increased when more than 10% of the oocytes were immature (58.94 versus 40.16%; $P < 0.0001$). Polyspermic fertilization occurred more frequently in the group with 10% or less GV (17.16 versus 8.51%; $P < 0.0001$). The pregnancy rate per

Table II. Comparison Between Results Obtained in Intact Zona Cycles (IZC) and Fractured Zona Cycles (FZC)

Variable	IZC	FZC	<i>P</i>
Number of treatment cycles	254	184	
Patient's age (mean $\pm$ SD years)	33.9 (4.56)	33.8 (4.65)	>0.05
Oocytes collected per cycle (mean $\pm$ SD)	8.13 (5.21)	10.87 (5.48)	<0.0001
Germinal vesicle oocytes [number (rate)]	145 (7.02)	123 (6.15)	>0.05
Fertilization rate per mature oocyte	64.13	60.85	<0.05
Polyspermic rate	9.41	11.53	>0.05
Day of hCG (mean $\pm$ SD)	11.54 (1.57)	11.71 (1.61)	>0.05
Estradiol value at hCG (mean $\pm$ SD)	2120.8 (928.6)	2389.7 (897.5)	<0.01
Pregnancy (rate per attempt)	44 (17.3)	36 (19.6)	>0.05

Table III. Comparison Between Cycles with 10% or Less GV and Cycles with More Than 10% GV

Variable	IVF cycles with		P
	≤10% GV	>10% GV	
Number of treatment cycles	337	101	
Patient's age (mean ± SD years)	34.0 (4.7)	33.4 (4.2)	>0.05
Oocytes collected per cycle (mean ± SD)	8.9 (5.35)	10.4 (5.8)	<0.03
Fractured zona oocytes (rate per collected oocytes)	291 (9.65)	61 (5.80)	>0.01
Fertilization rate per mature oocyte	40.16	58.94	<0.0001
Polyspermic rate	17.16	8.51	<0.0001
Day of hCG (mean ± SD)	11.67 (1.57)	11.44 (1.62)	>0.05
Estradiol value at hCG (mean ± SD)	2220.7 (922.0)	2277.3 (934.7)	>0.05
Pregnancy (rate per attempt)	55 (16.3)	25 (24.7)	<0.05

cycle was significantly decreased in Group 1 (≤10% GV) compared to Group 2 (>10% GV) (16.3 and 24.7%, respectively; $P < 0.05$).

The women in Group 1 were older than those in Group 2 but the difference was not significant. There were no significant differences in the day of hCG administration and the estradiol value concentration on the day of hCG administration between the two groups.

DISCUSSION

Ovarian stimulation protocols in IVF aim to produce a large number of oocytes so as to increase the number of embryos. The first aim of this study was to evaluate the relationship between the number of collected oocytes and the patients' age, ovarian stimulation protocol, and fertilization and pregnancy rates. The results of this study indeed show that the higher the number of retrieved oocytes, the higher the resulting pregnancy rate following IVF treatment. We also showed clearly the effect of age and stimulation protocol on oocyte recruitment.

With the increasingly more sophisticated laboratory techniques, biologists now have the tools to attempt an assessment of oocyte quality. The morphology of the oocyte can be defined and a great deal of effort is made to correlate the morphological evaluation of the oocytes with the chance of fertilization and successful outcome of treatment. The other aim of this study was to show that a precise evaluation of oocytes collected during IVF treatment provides information which may be useful to members of the team. Oocytes are usually not immediately classified because the cumulus cells are considered to interfere with a correct evaluation of oocyte maturity. We have demonstrated here that, by using a cumulus spreading technique (7) performed immediately after retrieval, a good evaluation of the

oocytes can be made. Intracytoplasmic sperm injection (ICSI) treatment cycles were not included in this study. When ICSI is performed, the cumulus spreading technique is not used because only metaphase II oocytes are injected and the assessment of oocyte maturity before denudation from surrounding cells by hyaluronidase solution is useless.

In this analysis of 438 IVF cycles we considered only the female parameters. In our practice, standard and more specific semen criteria (computer-aided sperm analysis and acrosomal reaction) are used to target the most effective assisted reproduction technology between IVF and ICSI (8). This strategy allows us to minimize the role of the male factor in fertilization and pregnancy processes in IVF treatments.

Total Number of Retrieved Oocytes

As the mean number of retrieved oocytes per IVF attempt was significantly different between long and short protocol, cycles were grouped according to the stimulation protocol.

Among the long-protocol group, the treatment cycles were grouped into three classes for two reasons. First, these classes reflect the main groups of responders to ovarian stimulation within IVF patients: a poor retrieval group, a middle retrieval group, and a high retrieval group. Second, these classes have been selected by other authors (2,3) and allow comparisons between the results of different teams. Group 1 (from one to five oocytes collected) fits into a defined group of IVF patients who are clearly older than those in the two other groups. The age factor is probably responsible for the other results observed in this group: an abnormal duration of stimulation with a lower estradiol value on the day of hCG administration, which directly reflects the number of collected oocytes, according to the results of Toner *et al.* (2). The fertilization and

pregnancy rates were low and underline once more the adverse impact of age on the chance of successful IVF treatment (2,3,9,10). In Group 2 and Group 3, the stimulation regimen appeared to be adapted considering duration and estradiol values. The fertilization rate per retrieved oocyte was better for Group 2 than for Group 3. This may represent the recruitment of more divergent cohorts of follicles rather than a decreased oocyte quality. The better pregnancy rate in Group 3 was in agreement with Meniru and Craft's results (3).

The short protocol was used to stimulate ovulation in patients who had decreasing ovarian responsiveness to gonadotropin (10). Among these patients, the treatment cycles were grouped into two classes because of the low number of cases. Two significant differences were noted between Group 1 and Group 2 concerning age factor and estradiol values. Clearly as in long-protocol cycles, the older the patients, the lower was the response to ovarian stimulation.

Fractured Zona Oocytes (FZO)

When oocyte morphology evaluation is performed by the cumulus spreading technique, FZO are the most easily identifiable. Although these oocytes were not subsequently inseminated with sperm, their detection during oocyte retrieval was very interesting. The recovery of oocytes with an abnormal zona pellucida from patients undergoing in vitro fertilization and embryo transfer in stimulated cycles has been reported when either gonadotropins or a combination of clomiphene citrate and human menopausal gonadotropin was employed for stimulation. However, little information is available about the nature of this phenomenon as well as its consequence. Some authors have suggested that the technique used for follicle puncture may damage the integrity of the zona pellucida (11) and, especially, that the use of a manual syringe suction system, instead of an electric pump, to aspirate follicles was associated with a significant increase in the proportion of oocytes with fractured zona pellucida (12). The inherent zona fragility suggested by Testart and Mendoza-Oltras (13) seems to be confirmed by our results concerning nine patients with more than 60% FZO. All these patients were affected by the same phenomenon during their previous IVF cycles, whatever the ovarian stimulation protocol. Exceptionally patients present 100% FZO during different ART attempts (2 of 410 women in our analysis). Oocyte donation could be proposed for such cases. The available literature shows a wide range in the incidence of FZO (from 5 to 40%). Our incidence of FZO is 8.6%

among all retrieved oocytes; this result is significantly higher ($P < 0.0001$) than that obtained by Cordeiro *et al.* (14), who studied 267 IVF cycles with 5.8% FZO. Cycles where only oocytes with an intact zona were recovered (intact zona cycles; IZC) were compared with cycles where one or more fractured zona oocytes were recovered (fractured zona cycles; FZC). In our study, as described elsewhere (15), the number of oocytes recovered was higher in the FZC group than in the IZC group. We confirm in our study the results obtained by Mendoza and Testart (11), who demonstrated that there was a higher fertilization rate for mature oocytes coming from patients who did not yield any fractured zona oocytes. However, as noted by Cordeiro *et al.* (14), FZOs have no clear influence on the overall pregnancy rate. The polyspermic rate seems to be increased in FZC. This suggests that this abnormal fertilization event is likely to be linked with overmature oocytes. However, there is no clear consensus regarding the role of immature or postmature oocytes in the literature (7,16,17).

Prophase I Oocytes

The other group of oocytes of interest is the germinal vesicle-stage oocytes (VG). A cutoff value of 10% VG was chosen, as it corresponds to the rate of immature oocytes usually found during classical ovarian stimulation (18). Previous works have stated that oocyte immaturity has a negative effect on the fertilization rate (19). But this phenomenon was not confirmed in our study, where cycles with more than 10% GV presented a significantly higher fertilization rate compared to cycles with less than 10% GV. The multipronuclear fertilization rate was significantly lower in cycles with more than 10% GV. Different factors have been implicated in the etiology of multipronuclear fertilization (12), for example, increased pronuclear formation may indicate retention of the second polar body in poorer-quality oocytes. We hypothesize that there is a relationship among the lower polyspermic rate, the lower incidence of fractured zona oocytes, and the higher number of immature oocytes. This observation indicates that oocytes collected during cycles with more than 10% GV are closer to the optimal stage of maturation than oocytes collected during cycles with 10% or less GV. It is known that embryos developing from GV are capable of implantation but their overall potential for live birth is lower than those developed from mature oocytes (7). However, the total number of GV in an IVF attempt is rarely high. The majority of embryos are developed from mature oocytes and

so the rate of GV has no clear influence on the overall pregnancy rate, as demonstrated in our study, where the pregnancy rate was significantly higher in cycles with more than 10% GV. These observations suggest that to optimize the stimulation protocol, the number of days of stimulation should not exceed 12 days with a limited estradiol level.

CONCLUSIONS

The total number of retrieved oocytes and the number of FZO and GV, which can easily be determined by every trained biologist, allow evaluation of the IVF attempt.

The findings of this analysis were that with an increase in the number of oocytes recovered, the pregnancy rate increased, that the presence of fractured zona oocytes had a negative effect on the fertilization rate but no direct influence on the pregnancy rate, and that, paradoxically, a high incidence of immature oocytes ultimately was associated with an improved fertilization and pregnancy rate. In addition, there seems to be an inverse relationship between the presence of immature oocytes and fractured zona oocytes. We propose to consider FZO and GV as criteria to appreciate the stimulation protocol; the incidence of GV being a good predictive factor of success contrary to the incidence of FZO.

The cumulus spreading technique is easy to perform in an IVF laboratory; it has no negative effect on the fertilization rate and pregnancy rate and could be considered as an internal quality control method. For clinicians, this technique analyzes the efficiency of the ovarian stimulation protocols and for biologists, this technique could constitute a good control of the culture conditions month after month.

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