BIOLOGY OF REPRODUCTION **68,** 812–821 (2003)
Published online before print 13 November 2002.
DOI 10.1095/biolreprod.102.008656

Assessment of Oocyte Quality Following Repeated Gonadotropin Stimulation in the Mouse[1]

Catherine M.H. Combelles and David F. Albertini[2]

Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111

ABSTRACT

The present study assessed the effects of repeated ovarian stimulation on oocyte quality. Female mice were stimulated with eCG and hCG at 1-wk intervals for 4 wk. Germinal vesicle (GV)-stage oocytes were evaluated in relation to size, somatic cell association, and chromatin organization after each week of stimulation. In addition, ATP content and expression of meiotic competence were monitored in GV and in vivo (IVO) or in vitro (IVM)-matured oocytes. The developmental competence of ovulated oocytes was determined after in vitro fertilization and embryo culture, and reproductive outcome was evaluated after mating following repeated cycles of stimulation. In GV oocytes, the degree of somatic cell association, size, and timing of transcriptional repression were altered when comparing repeated with single cycle(s) of stimulation. Meiotic competence expression was unaffected for IVO oocytes while IVM oocytes exhibited a progressive decrease in meiotic competence with repeated stimulation. The ATP content of immature and IVO oocytes decreased with repeated stimulation. Although after one cycle of stimulation ATP content was lower in IVM than IVO oocytes, IVM oocytes exhibited stable levels of ATP across cycles of stimulation. Last, the in vitro developmental competence of IVO oocytes retrieved after repeated stimulation was not significantly different, and in vivo, similar implantation and resorption rates were observed following mating of animals subjected to repeated stimulation. Therefore, despite measurable consequences of repeated stimulation on specific parameters of follicular oocyte quality, compensatory mechanisms may exist in vivo to optimize the developmental competence of ovulated oocytes in the mouse.

follicle-stimulating hormone, human chorionic gonadotropin, meiosis, oocyte development, ovulation

INTRODUCTION

Gonadotropin injections have been recognized for many years as a method to induce development of supernumerary follicles and multiple ovulations in animal and human subjects [1]. This procedure, known as ovarian stimulation (OS), yields high numbers of oocytes for embryo production and/or in vitro reproductive studies. In rodent models, the classical OS regimen involves an injection of eCG with predominantly FSH-like activity, followed by hCG, with LH activity; eCG induces the development of multiple follicles while hCG results in oocyte maturation and ovulation [2]. Animal studies comparing natural and stimulated ovarian cycles have revealed detrimental effects of gonadotropin stimulation, possibly due to a loss in the coordination of folliculogenesis and oogenesis. While adult female rodents induced to ovulate mate and carry embryos to full term, pregnancy and fetal abnormalities were observed, which include increased embryonic mortality and fetal retardation [1, 3, 4]. These treatments induce teratogenicity in mice [5–7]. And in bovine and hamster models, in vitro developmental competence of embryos is reduced for oocytes from superovulated animals when compared with untreated controls [8, 9].

Given widespread concerns and potential abnormalities in embryo and offspring elicited by OS, it has become imperative to determine underlying defects associated with this procedure. Using mouse models, there is mounting evidence that embryo quality is a direct reflection of oocyte quality. More recently, nuclear transfer procedures have demonstrated experimentally the importance of oocyte cytoplasm and environment within which the oocyte develops on the ability to sustain later embryonic development [10, 11]. Moor et al. [12] showed alterations in protein synthesis profiles following OS, the significance of which is not understood. Adverse effects of OS on implantation and fetal development likely also derive from hormonal imbalance and/or modifications in the uterine environment [13, 14]. Gonadotropin stimulation results in modified steroid profiles, altering the microenvironment of the developing follicles and their enclosed oocytes [12, 15]. The nature of the defects caused by exogenous OS on oocyte quality remains to be established, in part because good markers of oocyte developmental competence are still ill defined.

In conclusion, OS enhances oocyte availability but compromises oocyte developmental competence. These observations have encouraged alternative strategies in the treatment of human infertility to optimize oocyte developmental potential and improve pregnancy rates in assisted reproductive technologies (ARTs) [16, 17]. Furthermore, the influence of repeated cycles of OS is relevant to ART because infertile couples often undergo several sequential in vitro fertilization-embryo transfer attempts whether or not pregnancy is achieved. Infertile women undergoing successive stimulation cycles in fact show decreased rates of pregnancy [18]. However, two recent retrospective studies by Caligara et al. [19] and Opsahl et al. [20] strongly suggested that, in egg donors, neither the number of repeated cycles nor the interval between cycles adversely influenced oocyte quality, based on fertilization, implantation, and pregnancy rates. Studies in mice, rabbits, rhesus monkeys, cows, and domestic cats reported a reduced response to repeated injections of gonadotropins, possibly attributed to an immunologically mediated ovarian refractoriness [21–24]. More

[1]This work was supported by research grant 1-FY01-248 from the March of Dimes Birth Defects Foundation. C.M.H.C. was supported in part by the NIH Training Program in Developmental Biology HD07403.
[2]Correspondence: David F. Albertini, Department of Anatomy and Cellular Biology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. FAX: 617 636 6536; e-mail: david.albertini@tufts.edu

Received: 24 June 2002.
First decision: 21 July 2002.
Accepted: 15 September 2002.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Pregnancy outcome following repeated ovarian stimulation.*

		Week 1	Week 2	Week 3	Week 4
E11	Implantation rate	0.78 ± 0.05[a] (16)	0.70 ± 0.17[a] (13)	0.59 ± 0.08[a] (12)	0.55 ± 0.06[a] (11)
	Resorption rate	0.15 ± 0.04[a] (16)	0.19 ± 0.09[a] (13)	0.12 ± 0.04[a] (12)	0.22 ± 0.05[a] (11)
	Number of fetuses/female	13.25 ± 1.44[a] (16)	9.40 ± 1.94[a] (13)	12.33 ± 2.72[a] (12)	9.83 ± 1.25[a] (11)
	Fetal weight (mg)	33.21 ± 0.77[a] (217)	24.05 ± 1.90[b] (117)	26.30 ± 0.77[b] (120)	27.22 ± 1.29[b] (106)
E18	Number of fetuses/female	11.09 ± 1.45[a] (13)	9.70 ± 1.30[a] (14)	10.00 ± 1.00[a] (12)	10.33 ± 0.95[a] (11)
	Fetal weight (g)	1.182 ± 0.011[a] (153)	1.311 ± 0.013[b] (127)	1.295 ± 0.016[b] (112)	1.315 ± 0.016[b] (120)

* Numbers of pregnant females (for implantation/resorption rates and number of fetuses/female) or fetuses (for fetal weight) are shown in parentheses. Different superscripts denote statistical significance (implantation/resorption rates, *Z*-test; number of fetuses/female and fetal weights, one-way ANOVA; $P < 0.0005$).

recently, Van Blerkom and Davis [25] reported an increased incidence of spindle defects with repeated rounds of ovarian stimulation in ovulated mouse eggs. Furthermore, given the possible influence of intra- and extraovarian changes on aneuploidy in female gametes [26], it is surprising that more specific oocyte function parameters have not been targeted for study in the context of repeated OS.

Experiments designed here address the effects of repeated cycles of stimulation on ovarian response and oocyte/embryo quality in the mouse. Oocyte quality is assessed using markers of oocyte differentiation, meiotic competence acquisition/expression, metabolism, developmental competence, and fetal development, covering a range of intraovarian and postfertilization parameters. Collectively, our work shows that, following repeated OS, 1) germinal vesicle (GV) size, transcription status, and association of somatic cells with the oocyte differ, 2) meiotic competence expression is impaired during in vitro and but not in vivo maturation, 3) metabolism, as indicated by ATP content, is decreased, and 4) in vitro preimplantation development as well as in vivo postimplantation development are only mildly affected.

MATERIALS AND METHODS

Animals

CF-1 outbred female mice (Harlan-Sprague Dawley, Indianapolis, IN) between 6 and 10 wk of age were used for all oocyte collection and mating experiments. Investigations were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* (National Academy of Science, 1996). Animals were maintained on a 14L:10D photoperiod under constant temperature and relative humidity conditions. Food and water were provided ad libitum. Typically, five females were kept per cage, and a total of 390 females were used to complete the present studies. In a first set of experiments (experiment 1), two replicates (experiments 1.1 and 1.2; 115 females each) were performed, with females stimulated 1–4 times. Results from experiment 1 are represented in Figures 1–7, with values shown in Figures 1–6 combined from experiments 1.1 and 1.2 because there was no evidence for statistical differences between the two replicates. In a second set of experiments (experiment 2; 160 females), only matings were carried out, also with females stimulated 1–4 times (see below). Results from experiment 2 are shown in Table 1. B6D2F1 hybrid male mice (Harlan-Sprague Dawley) between 8 and 12 wk of age were used for in vitro fertilization (IVF) and mating. Sexually mature males of proven fertility were used following successful mating and after abstaining for a minimum of 7 days and a maximum of 14 days.

Gonadotropin Stimulation

Throughout the present studies (experiments 1 and 2), cycles of OS were initiated in 6-wk-old females that received 5 IU i.p. injections of eCG followed 48 h later by 5 IU i.p. of hCG. Hormones were prepared in 0.9% (w/v) sterile NaCl (vehicle) and originated from the same lot number for the entire study (eCG, lot B41809; hCG, lot B39559; Calbiochem-Novabiochem Corp., La Jolla, CA). Cycles of ovarian stimulation were performed using a 1-wk interval between each cycle for a total duration of 4 wk. After each weekly cycle of stimulation, groups of females were used for collection of oocytes and mating experiments.

Collection and Culture of Oocytes

For experiments 1.1 and 1.2 and after each week of stimulation, two types of oocyte collection were carried out, with either ovarian immature oocytes obtained after a last single injection of eCG or ovulated mature eggs retrieved after both eCG and hCG injections.

Ovarian immature oocytes were obtained 44–48 h post-eCG injection by follicular puncture, and it is likely that oocytes were released from both preantral and antral follicles. All culture reagents were purchased from Life Technologies Gibco/BRL (Gaithersburg, MD) unless stated otherwise. Oocytes were expressed in collection medium, and cumulus-enclosed oocytes were matured in vitro in a humidified atmosphere of 5% CO_2 at 37°C as previously described [27]. In vitro maturation was terminated after 14 h of culture. In vivo-matured eggs were flushed from oviducts at 13.5–14 h post-hCG injection. If necessary, cumulus cells were removed with 0.01% hyaluronidase (Sigma Biosciences, St. Louis, MO) for 3–5 min.

After removal of cumulus cells using either hyaluronidase treatment for ovulated eggs or gentle mechanical stripping for ovarian oocytes and in vitro-matured eggs (after 14 h of culture), mean diameter measurements (not including the zona pellucida or perivitelline space) were determined from the maximum and minimum diameters of each oocyte.

Processing of Oocytes for Fluorescence Microscopy

Oocytes were fixed and processed for microtubules and chromatin organization as previously described [28]. Microtubules were labeled using a monoclonal anti-α-tubulin and anti-β-tubulin mixture (1:500; Sigma Biosciences) and an affinity-purified fluoresceinated donkey anti-mouse IgGs (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA). Chromatin was detected with Hoechst 33258 (Polysciences Inc., Warrington, PA). Processed oocytes were mounted and analyzed by conventional fluorescence as previously described [28].

Measurements of Oocyte ATP Content

The ATP content of oocytes, free of surrounding cumulus cells, was measured using a commercial assay kit based on the luciferin-luciferase reaction (Bioluminescent Somatic Cell Assay Kit, FL-ASC; Sigma Biosciences). A slightly modified protocol was used [29–32]. Briefly, oocytes were placed individually in 200 μl of sterile purified water, rapidly frozen, and kept at −80°C. Prior to analysis, 100 μl of ice-cold somatic cell ATP-releasing agent was added to 50 μl of thawed samples. The tubes were kept for 5 min on ice prior to adding 100 μl of ice-cold ATP assay mix (diluted 1:25 in assay mix buffer). Tubes were incubated for 5 min in the dark at room temperature to allow for the initial chemiluminescence flash period. Bioluminescence of individual samples was measured using a high-sensitivity luminometer (TD-20/20; Turner Designs, Sunnyvale, CA). An 11-point standard curve (0, 0.01, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0. 4.0, and 5.0 pmole of ATP) was included in each assay. Preliminary experi-

ments revealed minimal inter- and intraassay variability in ATP content when measuring a maximum of 52 samples at a time (including the standard curve). Furthermore, in order to measure samples at the same time, tubes were counted once (C_1) and then again in reverse order (C_2), and the geometrical mean of the two counts was obtained with the square root of $C_1 \times C_2$ [30]. The ATP content of individual oocytes was determined from a formula derived for the standard curve using linear regression analysis (SPSS 10.0 statistical package; SPSS, Chicago, IL). The values shown represent adjusted ATP content in 10^{-3} fmol/μm^3 following normalization against any variations in oocyte volume. Oocyte volumes were obtained using oocyte diameter measurements and the $V = 4/3\pi r^3$ formula. For in vitro-matured (IVM) and in vivo (IVO) samples, only metaphase II eggs, with a polar body apparent under the dissecting microscope, were included in the ATP analysis.

In Vitro Fertilization and Embryo Culture

In vitro fertilization followed by embryo culture was performed as previously described [33, 34] using in vivo-ovulated eggs from females stimulated once (control) or repeatedly (treatment) with eCG and hCG (see *Gonadotropin Stimulation* section above). Treatment and control groups were paired and cultured under identical conditions to limit interassay variability and possible differences on a given day.

All culture media were prepared fresh using frozen reagents (Sigma Biosciences) kept at −80°C for up to 3 mo. Drops of media overlaid with embryo-tested washed mineral oil (Sigma Biosciences) were set up the day before and allowed to equilibrate overnight in a humidified atmosphere of 5% CO_2 at 37°C.

For sperm preparation, caudae epididymes were obtained from B6D2F1 males and placed in a 200-μl drop of modified Tyrode medium under oil. The epididymal contents were gently released using watchmaker's forceps and allowed to swim out for 10 min prior to discarding the epididymes. Subsequently, a 100-μl volume of this preparation was diluted 1:4 in modified Tyrode medium under oil for capacitation. Sperm were capacitated for a total of 90 min at 37°C in 5% CO_2. Sperm concentrations were determined using a hemocytometer.

Oviducts were collected 13.5 h post-hCG injection into Hepes-free PBS supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. A single oviduct was placed directly through the oil surrounding the 200-μl fertilization drop of modified potassium simplex optimization medium (KSOM) [34]; intact cumulus masses were then released from the oviduct and gently transferred into the fertilization drop with a minimum amount of PBS and debris transferred. Capacitated sperm were gently added to the fertilization drop at a final motile concentration of 800 000 sperm/ml.

Four hours after insemination, fertilized zygotes were washed several times through 50-μl drops of KSOM [33]. Between 8 and 12 zygotes were placed in 50-μl drops of KSOM under oil and cultured in a humidified atmosphere of 5% CO_2 at 37°C. Fertilization was assessed by the presence of two pronuclei 6 h after fertilization and confirmed by development to the two-cell stage 40 h post-hCG. Evaluation of embryonic development (cleavage, compacted morulae, blastocysts, hatching/hatched blastocysts) was continued at 72, 96, 120, and 144 h post-hCG, at which time cultures were terminated.

Differential Staining of Blastocyst Nuclei

At Day 6 of culture (or 144 h post-hCG), differential counts of inner cell mass and trophectoderm cells were determined on in in vitro-produced blastocysts using a modification of Handyside and Hunter [35] and Papaioannou and Ebert [36] and as previously described [33, 34]. After removal of the zona pellucida using acid Tyrode, blastocysts were washed in Hepes-buffered KSOM (KSOM-H) supplemented with 10% fetal calf serum (FCS) for 15 min at room temperature. Blastocysts were placed then into KSOM-H containing 10% rabbit anti-mouse red blood cell antibody (Research Diagnostics Inc., Flanders, NJ) for 30 min on a shaker. Following three washes in KSOM-H supplemented with 10% FCS, blastocysts were placed into a complement-stain solution containing 10% guinea pig complement serum (Sigma Biosciences), 1 μg/ml propidium iodide (PI; Sigma Biosciences), and 1 μg/ml Hoechst 33258 (Polysciences Inc.). Samples were incubated in the dark for 30 min on a shaker. Labeled embryos were then fixed in 2% formalin in KSOM-H with PI and Hoechst 33258 for 30 min in the dark. Blastocysts were stored in KSOM-H with PI and Hoechst 33258 at 4°C for 48 h until mounting. Inner cell mass nuclei, labeled with Hoechst 33258, appeared blue, and trophectoderm nuclei, labeled with PI and Hoechst 33258, fluoresced pink. For each blastocyst, nuclei were counted differentially twice and a mean value for the two counts was used in the final analysis.

Assessment of Implantation and Postimplantation Development

For examination of fetal development (experiment 2), mice were subjected to 1–4 cycles of gonadotropin stimulation (see *Gonadotropin Stimulation* above). Injections were scheduled in reverse order so that all mating and pregnancies occurred on age-matched females at 1-day intervals in order to keep conditions constant. Control mice that received a single cycle of eCG and hCG were also mated alongside mice that were repeatedly stimulated. After hCG injection, two females from a given stimulation or control group were placed overnight with one male of proven fertility (see above). The presence of a vaginal plug the following morning was considered a successful mating; this was designated as Day 0 of gestation (E0).

Pregnant mice were sacrificed at E11 (midgestation) or E18 (full term = E19/20) and the uterine contents examined. The number of live fetuses, stillborns, and early and late resorptions in each uterine horn was recorded. Each fetus was examined for gross morphological abnormalities and weighed following drying and removal of chorion and amnion. Ovaries were removed and the number of corpora lutea was counted under the dissecting microscope.

To address the possible adverse effects of handling and multiple injections on the stress level and endocrine profile of mice, an additional group was included. This control group was comprised of females that received three cycles of weekly i.p. injections of saline alone followed by a single cycle of eCG and hCG on the fourth week prior to mating. Reproductive outcome was analyzed as described above, and results were compared with simultaneous groups of mice that received either four cycles of weekly eCG and hCG or a single cycle of eCG and hCG.

Statistical Analysis

Data are presented as mean values, and variation is indicated with the SEM (errors bars on graphs). The data were analyzed using SPSS 10.0 Statistics Package for Social Sciences (SPSS). Comparison of proportions for independent samples was done with a two-tailed *Z*-test. Effects of oocyte category and weeks of stimulation on the diameters and ATP content of oocytes were evaluated using a nonparametric Kruskal-Wallis test followed by Mann-Whitney tests for two independent samples. Numbers of oocytes and blastocyst cell counts were analyzed using one-way ANOVA followed by least significant difference (LSD) and Tukey post hoc tests. When in disagreement, results using the more stringent Tukey post hoc were reported unless noted otherwise. Assumptions that the populations were normal (Shapiro-Wilk and Kolmogorov-Smirnov tests for normality) and population variances were all equal (Levene test) were checked prior to performing ANOVA. Furthermore, if homogeneity of variances could not be assumed, a Tamhane post hoc was used. Differences were considered significant at $P < 0.05$.

Distributions of total, inner cell mass, and trophectoderm cell numbers are compared using notched box and whisker plots (Slide Write Plus for Windows, Version 5.01; Advanced Graphics Software Inc., Encinitas, CA). Notched box plots display order statistics (5th, 25th, 50th or median, 75th, and 95th percentiles), and the notches on the box plots are the median confidence limits. Two medians are considered significantly different at the 0.05 level if their confidence limits do not overlap.

RESULTS

Female Mice Continue to Respond to OS Following Repeated Cycles

In the present study, the ovulatory responsivity of females to eCG and hCG, as evidenced by the presence of cumulus masses in the oviducts 14 h post-hCG, remained constant for Weeks 1, 2, and 3 of stimulation (87.5%–93.1%); in contrast, by Week 4 of stimulation, a significant decline in the number of females responding to eCG and hCG was observed (62.5%; *Z*-test, $P < 0.05$). Among females that responded to superovulation treatment with eCG and hCG, the mean number of ovulated eggs remained unchanged after 1–3 weekly repeated cycles of stimulation (from 13.5 to 17.5 eggs/female); however, following the fourth cycle of eCG and hCG, fewer eggs were retrieved per female (11.7 ± 1.6 eggs/female; ANOVA, LSD post-hoc, $P = 0.039$). Numbers reported here included all eggs

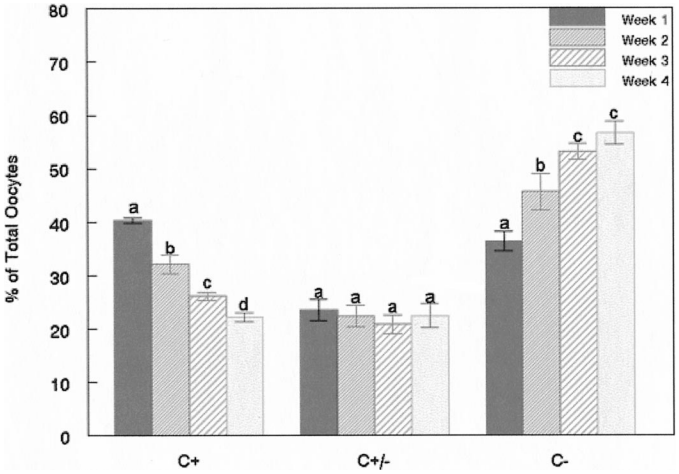


FIG. 1. Proportions of C^+, $C^{+/-}$, and C^- ovarian oocytes out of the total number of oocytes retrieved per animal by follicle puncture are shown after each week of stimulation (Weeks 1–4). Statistical differences between mean ± SEM percentages, as indicated with different letters, were obtained within each oocyte category (C^+, $C^{+/-}$, or C^-) across weeks of stimulation (*Z*-test, $P < 0.005$).

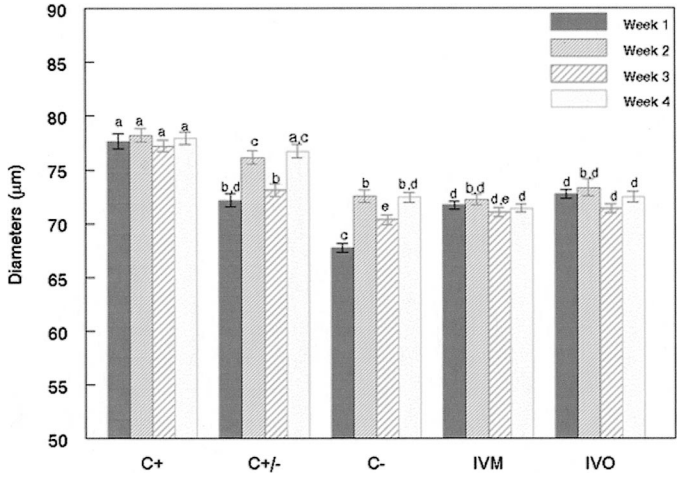


FIG. 2. Diameters of C^+, $C^{+/-}$, and C^- oocytes and IVM and IVO eggs after repeated stimulation. Mean ± SEM measurements (μm) are shown. Different letters represent statistical differences that were obtained across oocyte categories and weeks of stimulation using nonparametric Kruskal-Wallis and Mann-Whitney tests ($P < 0.05$).

except morphologically abnormal ones showing signs of degeneration, lysis, or fragmentation; no significant difference was observed in the proportion of abnormal eggs across weeks of stimulation (0.0%–5.6%, Weeks 1–4). After exogenous OS with eCG for follicular priming, the mean number of ovarian oocytes did not appear to change after each cycle of gonadotropin stimulation (42.7–50.7 oocytes/female; ANOVA, Tukey post hoc, $P > 0.05$). Therefore, under the present conditions, the majority of females continue to respond to repeated OS.

Ovarian Oocytes Vary in Degree of Somatic Cell Association and Size Following Repeated OS

The influence of gonadotropins on somatic cell adhesion to the oocyte was suggested by work in our laboratory [37]. To test the hypothesis that repeated OS modifies adhesion between somatic cells and the oocyte, ovarian GV-stage oocytes were categorized and quantified according to their degree of association with surrounding somatic cells. Oocytes with several layers of intact cumulus cells were referred to as C^+ oocytes, while oocytes with few or no attached cumulus cells were classified as $C^{+/-}$ and C^- oocytes, respectively. The mean percentage of oocytes in each category (C^+, $C^{+/-}$, C^-) out of the total number of oocytes retrieved per female is shown in Figure 1 following each cycle of stimulation (1346 oocytes, Week 1; 828 oocytes, Week 2; 1108 oocytes, Week 3; 884 oocytes, Week 4). At Week 1, the majority of oocytes belonged to the C^+ or C^- categories with similar proportions of each; fewer $C^{+/-}$ oocytes were observed. Following a second cycle of ovarian stimulation, a significantly lower percentage of C^+ oocytes were obtained and a significantly greater percentage of C^- oocytes. This trend with a greater proportion of C^- at the expense of the C^+ category of oocytes was consistent for Weeks 3 and 4 of stimulation. Regardless of the stimulation paradigm, the proportion of $C^{+/-}$ oocytes remained unchanged. By comparison, in unprimed adult cycling females, C^+, $C^{+/-}$, and C^- oocytes occurred in the following frequencies: 21.4 ± 1.9, 24.3 ± 2.3, and 54.3 ± 1.8 % (639 oocytes; data not shown). Therefore, repeated OS causes a shift in the proportions of oocytes from having more adherent somatic cells (C^+) to no somatic cells (C^-).

Whether C^+, $C^{+/-}$, and C^- categories reflect growth states of oocytes was next evaluated by determining oocyte diameters (Fig. 2). Following a single injection of eCG (Week 1), C^+ oocytes were significantly larger in size (n = 45) than $C^{+/-}$ (n = 55) and C^- (n = 55) oocytes. Furthermore, $C^{+/-}$ oocytes were themselves significantly greater in diameter than C^- oocytes (Mann-Whitney, $P < 0.0005$). Similar relationships in diameters between C^+, $C^{+/-}$, and C^- oocytes were observed in Weeks 2–4 of stimulation, with one exception at Week 4, for which C^+ oocytes were equivalent in size to $C^{+/-}$ oocytes. When oocyte diameters were compared within each oocyte category across weeks of stimulation, we found that 1) diameters of C^+ oocytes were unchanged regardless of the weeks of stimulation, 2) $C^{+/-}$ oocyte diameters were significantly increased at Weeks 2 and 4 when compared with oocytes from Week 1, and 3) C^- oocyte diameters were significantly greater at Weeks 2, 3, and 4 when compared with Week 1 (Mann-Whitney, $P < 0.05$). Therefore, C^-, $C^{+/-}$, and C^+ categories reflect sequential developmental transitions during oocyte growth. Furthermore, while oocytes with no adherent cumulus cells arise in greater proportion following repeated OS, these oocytes are larger in diameter, suggesting their advanced state of oocyte development and prompting analysis of GV chromatin patterns to monitor transcriptional status.

Following Repeated OS, Oocytes Prematurely Undergo Transcriptional Repression

The transition from NSN (nonsurrounded nucleolus, transcriptionally active) to SN (suurounded nucleolus, transcriptionally inactive) GV chromatin patterns has been related to progressive stages of oocyte and follicular growth and coincides with the time of antrum formation [38–41]. To analyze whether repeated OS affects oocyte transcriptional profiles, the proportion of NSN and SN oocytes were reported for each category of oocytes (C^+, $C^{+/-}$, and C^-) across weeks of stimulation (Fig. 3). At each cycle of stimulation, the majority of C^+ oocytes exhibited the SN type of chromatin organization; furthermore, a significant increase in the proportion of SN chromatin oocytes was observed in C^+ oocytes by the fourth cycle of stimulation (*Z*-

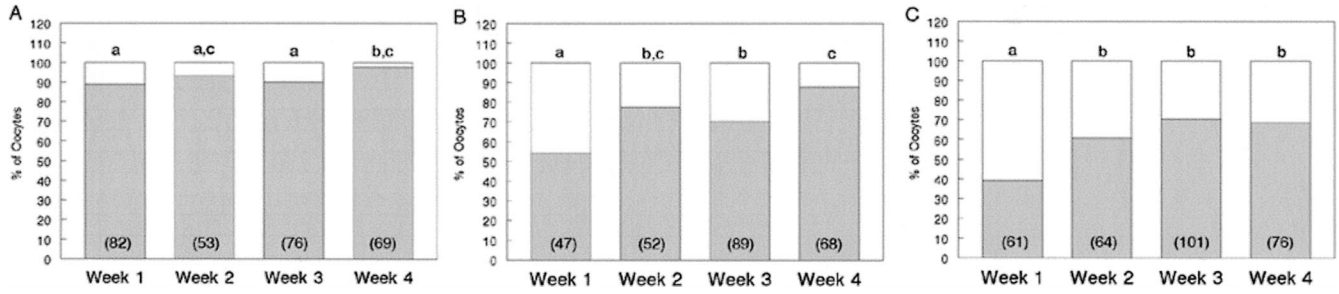


FIG. 3. Stacked bar graphs showing germinal vesicle chromatin patterns following repeated ovarian stimulation for C^+ (**A**), $C^{+/-}$ (**B**), and C^- (**C**) oocytes. Gray and white coloration represent oocytes with SN and NSN types of chromatin, respectively. Values represent mean percent of oocytes, and number of oocytes analyzed are shown in parentheses at the bottom of each bar. Within **A**, **B**, and **C**, bars that do not share a superscript are different (*Z*-test, $P < 0.05$).

test, $P < 0.05$). At Week 1, relatively equal proportions of NSN and SN chromatin patterns were observed for $C^{+/-}$ oocytes; however, following repeated ovarian stimulation, the proportion of SN oocytes significantly increased. Last, while C^- oocytes mainly possessed NSN chromatin at Week 1, SN chromatin became the prominent pattern at Weeks 2, 3, and 4 of stimulation. In summary, C^+ oocytes mostly possess SN chromatin regardless of the stimulation regimen, while $C^{+/-}$ and C^- oocytes show a shift toward SN chromatin following repeated OS, indicative of precocious transcriptional repression.

Repeated OS Compromises In Vitro but Not In Vivo Meiotic Competence

Because the onset of transcriptional repression coincides with meiotic competence acquisition [42], we tested the ability of oocytes to initiate and complete maturation to metaphase II under both in vitro and in vivo conditions. No difference in oocyte diameters was apparent between IVM (C^+ at the start of culture) and IVO oocytes across weeks of stimulation (Fig. 2; Mann-Whitney, $P < 0.05$). Maturation to metaphase II was evaluated from analysis of chromatin patterns and spindle organization by immunofluorescence. Control mice that received a single cycle of gonadotropin stimulation were analyzed at the same time as repeatedly stimulated mice (data not shown). Comparison of several independent control groups, equivalent to Week 1 of stimulation, failed to reveal any significant differences in the incidence of maturation. Therefore, statistical tests were performed against Week 1 of stimulation for the remainder of the analysis. Figure 4 shows the percentage of oocytes that were arrested at metaphase II after 14 h of IVM or 14 h post-hCG injection IVO after each cycle of stimulation. For IVM, the majority of oocytes from Weeks 1 and 2 were arrested at metaphase II (n = 157, Week 1; n = 132, Week 2); at Weeks 3 and 4, a significant decrease in the proportion of oocytes arrested at metaphase II was observed (n = 130, Week 3; n = 111, Week 4; *Z*-test, $P < 0.05$). In the case of IVO, the percentage of oocytes in metaphase II was only reduced at Week 3 of stimulation when compared with all other groups except at Week 2 (n = 102, Week 1; n = 96, Week 2; n = 95, Week 3; n = 87, Week 4). Nonetheless, the maturation incidence remained elevated for IVO oocytes at Week 3 of stimulation (83.0%). Thus, following repeated OS, a progressive decrease in meiotic competence expression was apparent after IVM and not IVO.

ATP Content of Oocytes Varies with Developmental Stages of the Oocyte and Repeated OS

The progressive impairment in in vitro meiotic competence expression together with the shift to C^- oocytes suggests that repeated OS may alter metabolism of the oocyte. Therefore, ATP content of individual oocytes was measured across weeks of stimulation (Weeks 1–4) and for each oocyte category (C^+, $C^{+/-}$, C^-, IVM, IVO). Figure 5 shows

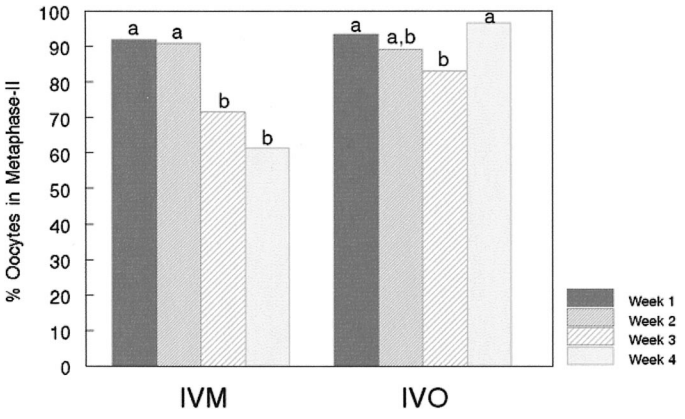


FIG. 4. Meiotic competence expression in vitro (IVM) and in vivo (IVO) following repeated stimulation. Mean percent of oocytes reaching metaphase II, as evidenced by immunostaining for chromatin and microtubules, are shown after each week of stimulation. For IVM and IVO groups, statistical differences are shown with different letters across weeks of stimulation (*Z*-test, $P < 0.05$).

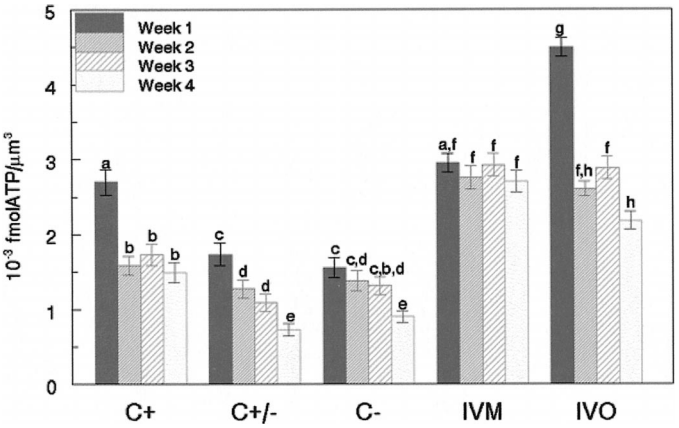
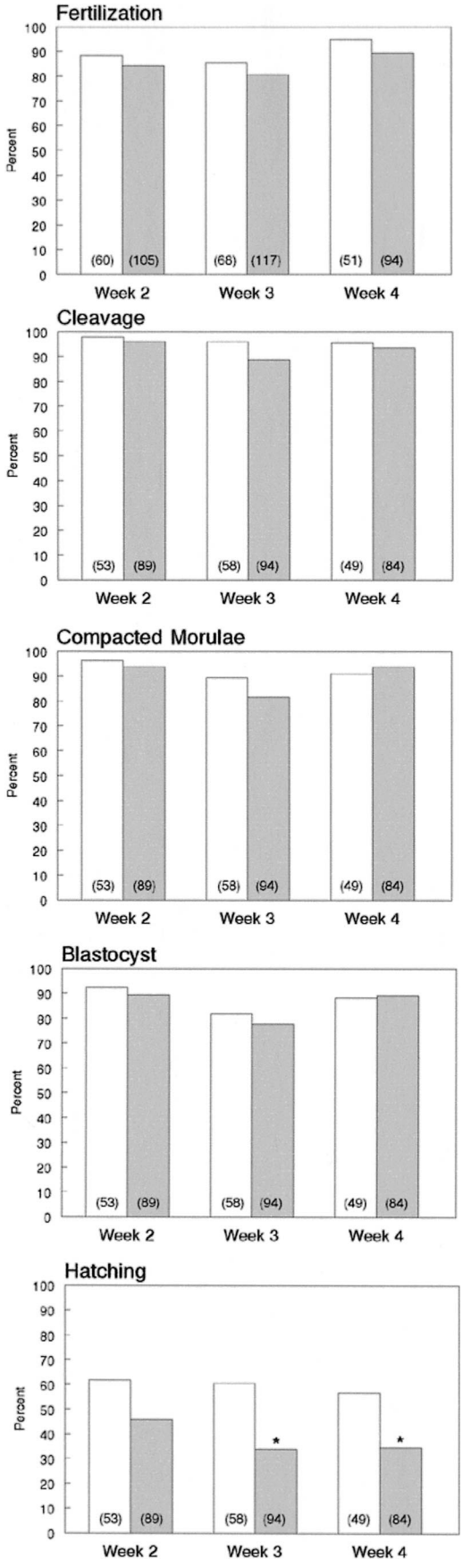


FIG. 5. ATP content of individual C^+, $C^{+/-}$, and C^- oocytes and IVM and IVO eggs. Mean ± SEM values (10^{-3} fmol ATP/μm^3) are shown. Statistical analysis was performed for both oocyte category and week of stimulation (Mann-Whitney, $P < 0.05$), and different letters indicate statistical significance.



mean ± SEM values for two independent experimental replicates, and a total of 1040 oocytes with 60 individual C^+ oocytes, IVM, and IVO eggs and 40 $C^{+/-}$ and C^- oocytes were analyzed at each week of stimulation. Mann-Whitney tests on the raw data were done to analyze differences in the two factors analyzed here, i.e., oocyte category and week of stimulation. However, following a logarithmic transformation of the data to reach normality, a two-way ANOVA revealed an interaction between the two factors ($P < 0.0005$). Therefore, relationships between ATP content and oocyte category appeared to be different with weeks of stimulation, and conversely, oocyte category ought to be considered when analyzing ATP content and week of stimulation.

When comparing ATP content across categories of oocytes at Week 1 of stimulation, changes in ATP values were apparent (Mann-Whitney, $P < 0.05$). C^+ oocytes exhibited significantly higher ATP content than $C^{+/-}$ and C^- oocytes. No difference was observed between $C^{+/-}$ and C^- oocytes. C^+ oocytes that were IVM possessed similar ATP levels to C^+ oocytes at the start of culture, and it is noted that C^+ and IVM oocytes originated from the same cohort. In contrast, IVO eggs exhibited the highest ATP content when compared with other oocyte categories. Similarly, for Weeks 2–4 of stimulation, C^+ oocytes showed consistently higher ATP levels than $C^{+/-}$ and C^- oocytes, with one exception at Week 3, when C^- oocytes had similar ATP levels to C^+ oocytes. Thus, oocytes in different developmental states exhibit variations in ATP content, with C^+ oocytes possessing the greatest ATP content among immature oocytes and ATP content significantly increasing after IVO.

In addition, ATP levels were compared within each oocyte category across weeks of stimulation. C^+ oocytes showed reduced ATP content with repeated cycles of stimulation (Weeks 2–4) when compared with C^+ oocytes collected following a single cycle (Week 1; Mann-Whitney, $P < 0.05$). Likewise, ATP levels decreased in $C^{+/-}$ oocytes with repeated ovarian stimulation. In C^- oocytes, a decrease in ATP levels was apparent only at Week 4 of stimulation. All IVM eggs exhibited similar levels of ATP regardless of the stimulation paradigm. Last, ATP content was significantly reduced in IVO eggs from Weeks 2, 3, and 4 when compared with IVO eggs from Week 1. Therefore, repeated OS resulted in reduced ATP content in C^+, $C^{+/-}$, and C^- oocytes and in IVO eggs.

Repeated OS Results in Slight Impairment in Preimplantation Development In Vitro

Given the reduction in ATP content of IVO eggs following repeated OS, we next determined the developmental competence of IVO eggs using IVF and embryo culture. The mean frequencies of fertilization and development to cleavage, compacted morula, blastocyst, or hatching stages are reported in Figure 6 for two independent experimental replicates (experiments 1.1 and 1.2). Comparisons were always made between paired control (single eCG + hCG)

FIG. 6. In vitro fertilization and development of in vivo-ovulated eggs following repeated ovarian stimulation. Bar graphs show mean percent of eggs or embryos that fertilized, developed to cleavage, compacted morulae, blastocyst, and hatching stages after each week of stimulation. White and gray bars represent embryos derived after a single or repeated cycle(s) of stimulation, respectively. Numbers of cells analyzed for each group are shown in parentheses, and * denotes statistical significance (*Z*-test, $P < 0.05$).

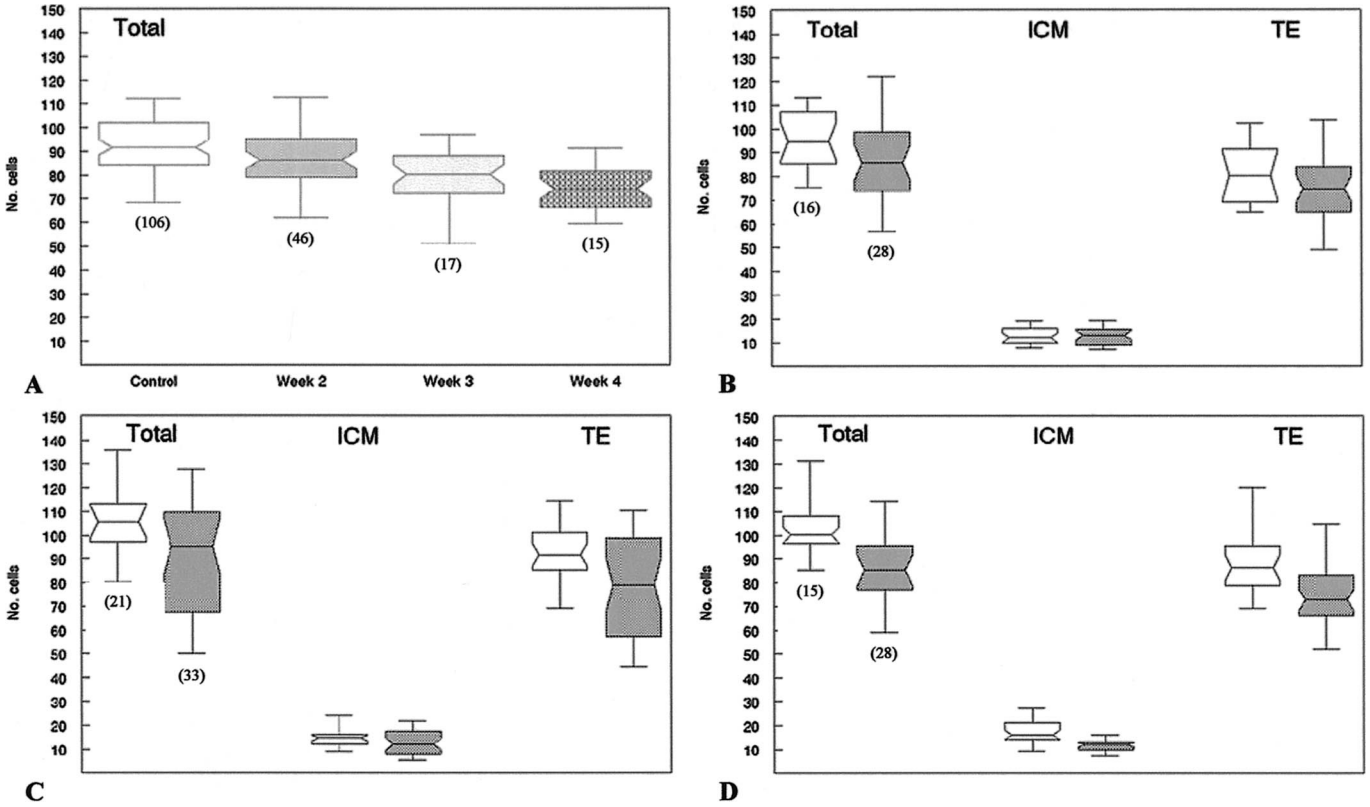


FIG. 7. Notched box and whisker plots showing the distribution of total cell (Total), inner cell mass (ICM), and trophectoderm cell (TE) numbers in blastocysts produced in vitro following repeated cycles of stimulation. **A**) Total cell counts for experiment 1.1. **B**–**D**) Total, ICM, and TE counts after each cycle of stimulation (**B**, Week 2; **C**, Week 3; **D**, Week 4) for experiment 1.2. White and gray boxes represent blastocysts derived after a single or repeated cycle(s) of stimulation, respectively. Numbers of blastocysts analyzed for each group are shown in parentheses.

and treatment (repeated eCG + hCG) groups. No differences were apparent in the proportions of embryos that developed to the blastocyst stage by Day 6 (*Z*-test, $P < 0.05$). The rate of hatching, as evidenced by blastocysts that at least started to hatch, was reduced significantly at Weeks 3 and 4 when compared with respective control groups.

To evaluate in vitro-produced blastocysts, total inner cell mass (ICM) and trophectoderm (TE) cells were counted using differential nuclei staining after each week of stimulation (Fig. 7; experiments 1.1 and 1.2). In experiment 1.1, data from all control groups were pooled because total cell counts were similar (one-way ANOVA, $P = 0.377$). Total cell counts were decreased at Weeks 3 and 4 when compared with control values (Fig. 7A). At Week 3, ICM counts were comparable with control groups, while the number of TE cells was decreased (one-way ANOVA, $P < 0.05$). At Week 4, both ICM and TE cell counts were reduced, while the ICM/TE ratio remained unchanged (ICM/TE ratio: Week 4, 0.21 ± 0.02; control, 0.24 ± 0.02). Because of significant interassay variation in cell counts for experiment 1.2 (one-way ANOVA, $P < 0.0005$), blastocysts derived from a single cycle of stimulation (control) were compared with blastocysts derived from repeated cycles of stimulation (Weeks 2–4; Fig. 7, B–D). Cell counts remained similar for Weeks 2 and 3 when compared with control values, while lower total cell counts were observed at Week 4 of stimulation. At Week 4, the number of both ICM and TE cells was reduced, a decrease not observed for any other paired control/repeated stimulation groups. There were no changes in ICM/TE ratios regardless of stimulation paradigm (Weeks 2–4, 0.15–0.17; control, 0.16–0.19). Thus, IVO eggs derived from repeated OS display similar in vitro developmental competence to IVO eggs from a single cycle of OS, with the exception of hatching and total/ICM/trophectoderm cell counts, which were all reduced in in vitro-produced blastocysts after the fourth cycle of OS.

Comparable Implantation Rates, Resorption Rates, and Fetal Counts Are Observed but Fetal Weights Vary Following Repeated OS

Given detectable alterations in oocyte differentiation state, oocyte ATP content, and in vitro developmental competence (notably reduced number of blastocyst cells) following repeated OS, we next asked whether repeated OS alters postimplantation development in vivo (experiment 2). Table 1 summarizes reproductive outcome at E11 and E18 after mating at each week of stimulation. Pregnancy rates, as assessed by the number of females that became pregnant following mating, did not change with repeated OS (Week 1, 72.5%; Week 2, 67.5%; Week 3, 60.0%; Week 4, 55.0%; 40 mice per week). Similarly, the mean number of corpora lutea (CL) per female was comparable across weeks of stimulation. Furthermore, a similar proportion of CL resulted in implantation at E11 (implantation rate). Resorption rates, defined as number of resorptions (early and late) over total number of implantation sites, were similar across weeks of stimulation (E11, Table 1; E18, data not shown). At E11, fetal weights (excluding the placenta) were lower following repeated OS, with weight differences of 6–9 mg on average, corresponding to a 18.0–27.6% decrease between repeated and single OS. It is noted that, at E11 and E18, similar numbers of viable fetuses were observed at

Weeks 1–4, with a mean of 9.3–13.3 fetuses per female. At E18 and by Week 2 of OS, fetal weights were elevated significantly, an increase that persisted through Week 4 (10.6% increase in weight relative to Week 1 of stimulation). In comparing fetal weights, the effect of litter size on any weight difference was also considered; however, no significant difference in the overall decrease (at E11) and increase (at E18) in fetal weight between single and repeated OS was observed for any of the litter size subgroups. Sex ratios of male to female at E18 remained constant regardless of the stimulation regimen (0.57:0.43; n = 502). There were no gross morphological aberrations observed across groups of offspring. Last, no difference in any of the parameters measured above was apparent between control females that received a single cycle of eCG + hCG, preceded or not by three cycles of saline + saline injections. In conclusion, fetal weights were reduced at midgestation (E11) and increased near term (E18) when comparing repeated with single OS. However, repeated OS did not result in changes in either implantation/resorption rates or fetal counts when compared with a single cycle of OS.

DISCUSSION

This study, to our knowledge, is the first to assess the overall effects of repeated OS on oocyte quality using intraovarian, embryonic, and pregnancy criteria. Our main findings are that repeated OS leads to 1) modifications in associated somatic cells, size, and transcriptional status of GV-stage oocytes; 2) reductions in in vitro meiotic competence, with no changes in in vivo maturation; 3) significant variations in oocyte ATP content according to oocyte types and stimulation cycles; 4) in vitro preimplantation embryonic development only compromised at the level of hatching at Weeks 3 and 4 of stimulation and total/inner cell mass/trophectoderm cell counts at Week 4 of stimulation; and 5) comparable implantation/resorption rates and fetal counts, although E11 and E18 fetal weights were modified by Week 2 of OS. Collectively, these observations suggest that, in a mouse model, while repeated OS has specific effects on follicular oocyte quality, following ovulation in vivo, oocytes can be rescued from consequences of repeated OS.

Despite widespread utility, OS has been associated with a number of detrimental reproductive effects, including decreased fertilization and rates of embryonic development, increased chromosomal abnormalities, delayed and impaired implantation, and retarded fetal growth [1, 3, 5–7, 13, 43]. Why OS compromises gametic and embryonic development remains poorly understood. Blondin et al. [8] proposed that, induced by OS, accelerated follicular growth would yield oocytes with decreased developmental competence, possibly because of incomplete oogenesis. Few studies have rigorously assessed oocyte quality following OS. The present studies materially extend previous efforts to determine the effects of repeated OS on oocyte quality. First, we defined three types of oocytes (C^+, $C^{+/-}$, and C^-) that differed in their association with somatic cells within the total population of ovarian oocytes. When comparing unprimed and primed adult females, the proportion of C^+ oocytes increased following eCG stimulation, while fewer C^- oocytes were observed. In addition, C^+ oocytes were larger in diameter than $C^{+/-}$ and C^- oocytes (Fig. 2). Given that eCG is known to promote follicle development, we conclude that C^-, $C^{+/-}$, and C^+ oocytes represent progressive stages of oocyte growth during folliculogenesis, with the latter category achieving maximum size expected. Oocytes with a fully formed cumulus (C^+) represent full-grown oocytes derived from Graafian follicles, while $C^{+/-}$ and C^- oocytes with fewer and no associated cells are growing oocytes at transition stages around the time of antrum formation and from preantral follicles, respectively, consistent with previous work on GV configuration [38] and studies on prepubertal mice (Combelles and Albertini, unpublished observations). Furthermore, we show that somatic cell-oocyte interactions are influenced by repeated OS because a greater proportion of C^- oocytes result from repeated OS (Fig. 1). Interestingly, the fact that C^- oocytes achieve larger diameters with repeated OS (Fig. 2) suggests that, with repeated cycles of OS, either folliculogenesis and oogenesis are discoordinated and/or cumulus cells exhibit diminished affinity for the oocyte. These observations resemble the oocyte phenotype described in the GDF-9 (growth differentiation factor-9) knockout mouse. In the absence of GDF-9, oocyte growth is misregulated, giving rise to large-diameter oocytes and precocious transcriptional repression within follicles arrested at the primary stage [44, 45]. It seems plausible then that decreased adhesion alters somatic cell-oocyte interactions, enhancing the rate of oocyte growth following repeated OS. Whether such loss of coordination results in other alterations in oocyte quality was also evaluated.

We analyzed the NSN to SN transition in nuclear morphology that coincides with meiotic competence acquisition and transcriptional repression during oocyte growth [40, 42]. This transition occurs around the time of antrum formation and is promoted by exogenous gonadotropin stimulation [38, 39]. Work by De La Fuente and Eppig [41] and Carabatsos et al. [46] further suggested the involvement of gap junctions and somatic cells in controlling oocyte transcriptional status. The observation that repeated OS leads to an increased incidence of oocytes devoid of somatic cells (C^-), in conjunction with transcriptional repression (NSN to SN shift; Fig. 3), is again consistent with the idea that compromised somatic cell input influences key aspects of oogenesis. Two additional criteria we evaluated were consistently influenced by repeated OS.

Meiotic competence is the ability of an oocyte to resume meiosis, and upon repeated OS, deficiencies were apparent for IVM because a progressive reduction in the incidence of oocytes progressing to metaphase II was observed (Fig. 4). This was accompanied by an increase in oocytes that failed to form normal spindles during meiosis 1 (data not shown), again illustrating a loss in meiotic competence in vitro with repeated OS. This contrasts with our data on IVM because no differences in either maturation frequency or spindle/centrosome organization were apparent across weeks of stimulation following hCG-induced ovulation (data not shown) and differs from previous observations of spindle defects and detached chromosomes in ovulated oocytes using a similar stimulation paradigm [25]. Whether repeated OS influences ploidy in IVO and/or IVM eggs remains an interesting area of study, but our findings do reinforce the idea that differences exist between IVO and IVM conditions and encourage caution in using IVM, which may augment potential risk factors or deficiencies associated with oocytes of lower quality following repeated OS. Whether the follicular environment for IVM corrects intrafollicular defects remains an interesting question.

Finally, to support energy-consuming cellular processes during oocyte maturation and early cleavage divisions, oocytes rely predominantly on ATP reserves gained during oogenesis, and our work demonstrates that mouse oocytes

vary significantly in ATP content depending on their meiotic stage and on stimulation protocol used. After normalizing for variations in oocyte volume, ATP content was lowest in C^- oocytes, highest in C^+ oocytes, and intermediate in $C^{+/-}$ oocytes (Fig. 5). Variations in ATP content were reported previously in bovine oocytes by Stojkovic et al. [32], in which different grades of cumulus morphology correlated with ATP content. However, no previous reports have examined oocyte ATP content in relation to somatic cell association in the mouse. The exact source of oocyte ATP remains poorly understood but may derive from metabolism of pyruvate or other substrates by the oocyte itself or from oxidative phosphorylation in surrounding cumulus cells [47, 48]. Our data show that increased ATP is present in oocytes during progressive stages of oocyte growth (C^+ versus $C^{+/-}$ and C^-). When C^+ oocytes were matured to metaphase II in vitro, ATP content did not change and IVO eggs displayed significantly higher ATP when compared with all other oocyte types (Fig. 5). To our knowledge, this is the first comparison of ATP content in IVM, IVO, and GV-stage mammalian oocytes, and the differences reported here between IVM and IVO eggs after a single cycle of OS may be due to lower metabolic activity of cumulus cells and/or oocytes after removal from the follicular milieu and/or the specific IVM conditions utilized here. Whether exogenous gonadotropin stimulation influences oocyte metabolism and ATP content was also addressed. A reduction in ATP content was observed for all oocyte categories with the exception of IVM eggs after repeated OS. This suggests that metabolic cooperativity between cumulus cells and oocyte may be compromised with repeated cycles of OS and contribute to the diminished levels of ATP measured in the oocyte presently. Thus, again these data are consistent with the proposed effect of repeated OS on somatic cell adhesion to the oocyte (see above).

Metabolic impairment in the embryo has been shown to lead to preimplantation failure in several mammalian species, and oocyte ATP content may determine the developmental potential of embryos [29, 31, 32, 49–51]. Our experimental design allowed us to test the developmental competence of oocytes derived from repeated OS, working on the assumption that deficits in oocyte ATP might impair later development. To this end, it was somewhat surprising that, regardless of the stimulation cycles, IVO eggs gave rise to blastocysts at high rates, and only modest effects were noted in resulting blastocysts (Fig. 6). These effects included decreased blastocyst hatching by Weeks 3 and 4 of OS at Day 6 of culture. The physiological significance of hatching deficiencies in vitro remains unknown, but in addition, it was found that blastocyst cell numbers (both ICM and trophectoderm) were reduced by Week 4 of stimulation (Fig. 7). Papaioannou and Ebert [52] and Tsunoda and McLaren [53] showed that blastocysts with different cell numbers may implant at similar rates but develop into normal fetuses at divergent rates. To better discern whether postimplantation development was compromised, we next tested the developmental competence of oocytes in vivo following repeated OS. While repeated OS did not result in altered pre- and postimplantation embryonic development, it seems to contribute to variations in fetal weights (Table 1). Thus, the physiological significance of possible relationships between ATP, hatching, and weight differences remains to be established and prompts further experiments to better discern the origins (ovarian, embryonic, or uterine) of detrimental consequences of repeated OS.

In conclusion, these studies show significant effects of repeated OS on oocyte quality parameters that, based on in vitro preimplantation and in vivo postimplantation development outcome, may not be of particularly useful prognostic value. Our work suggests that either mechanisms compensating for variations in oocyte quality exist in mice or that better markers are required to evaluate oocyte quality. Equally germane is the question of whether comparable findings apply to human and other monovular species with single dominant follicle selection. These studies reinforce directly the idea that repeated OS does affect the coordination of oogenesis with folliculogenesis and in particular suggests that direct contact interactions may be compromised.

ACKNOWLEDGMENTS

We wish to thank Lynda McGinnis and Dr. John Biggers for sharing protocols and precious advice with in vitro fertilization and embryo culture conditions and Dr. Gao for use of the luminometer.

REFERENCES

1. Fowler RE, Edwards RG. Induction of superovulation and pregnancy in mature mice by gonadotrophins. J Endocrinol 1957; 15:374–384.
2. Murphy BD, Martinuk SD. Equine chorionic gonadotropin. Endocr Rev 1991; 12:27–44.
3. Beaumont HM, Smith AF. Embryonic mortality during the pre- and post-implantation periods of pregnancy in mature mice after superovulation. J Reprod Fertil 1975; 45:437–448.
4. Ertzeid G, Storeng R. Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice. J Reprod Fertil 1992; 96:649–655.
5. Elbling L. Does gonadotrophin-induced ovulation in mice cause malformations in the of offspring? Nature 1973; 246:37–39.
6. Fujimoto S, Pahlavan N, Dukelow WR. Chromosome abnormalities in rabbit preimplantation blastocysts induced by superovulation. J Reprod Fertil 1974; 40:177–181.
7. Sakai N, Endo A. Potential teratogenicity of gonadotropin treatment for ovulation induction in the mouse offspring. Teratology 1987; 36: 229–233.
8. Blondin P, Coenen K, Guilbault LA, Sirard MA. Superovulation can reduce the developmental competence of bovine embryos. Theriogenology 1996; 46:1191–1203.
9. McKiernan SH, Bavister BD. Gonadotrophin stimulation of donor females decreases post-implantation viability of cultured one-cell hamster embryos. Hum Reprod 1998; 13:724–729.
10. Kono T, Obata Y, Yoshimzu T, Nakahara T, Carroll J. Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. Nat Genet 1996; 13:91–94.
11. Liu H, Krey LC, Zhang J, Grifo JA. Ooplasmic influence on nuclear function during the metaphase II-interphase transition in mouse oocytes. Biol Reprod 2001; 65:1794–1799.
12. Moor RM, Osborn JC, Crosby IM. Gonadotrophin-induced abnormalities in sheep oocytes after superovulation. J Reprod Fertil 1985; 74:167–172.
13. Ertzeid G, Storeng R. The impact of ovarian stimulation on implantation and fetal development in mice. Hum Reprod 2001; 16:221–225.
14. Van der Auwera I, D'Hooghe T. Superovulation of female mice delays embryonic and fetal development. Hum Reprod 2001; 16:1237–1243.
15. Szoltys M, Galas J, Jablonka A, Tabarowski Z. Some morphological and hormonal aspects of ovulation and superovulation in the rat. J Endocrinol 1994; 141:91–100.
16. Macklon NS, Fauser BC. Follicle-stimulating hormone and advanced follicle development in the human. Arch Med Res 2001; 32:595–600.
17. Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA. Manipulation of follicular development to produce developmentally competent bovine oocytes. Biol Reprod 2002; 66:38–43.
18. Meldrum DR, Silverberg KM, Bustillo M, Stokes L. Success rate with repeated cycles of in vitro fertilization-embryo transfer. Fertil Steril 1998; 69:1005–1009.
19. Caligara C, Navarro J, Vargas G, Simon C, Pellicer A, Remohi J. The effect of repeated controlled ovarian stimulation in donors. Hum Reprod 2001; 16:2320–2323.
20. Opsahl MS, Blauer KL, Black SH, Dorfmann A, Sherins RJ, Schul-

man JD. Pregnancy rates in sequential in vitro fertilization cycles by oocyte donors(1). Obstet Gynecol 2001; 97:201–204.
21. Lin TP, Bailey DW. Difference between two inbred strains of mice in ovulatory response to repeated administration of gonadotrophins. J Reprod Fertil 1965; 10:253–259.
22. Onuma H, Hahn J, Maurer RR, Foote RH. Repeated superovulation in calves. J Anim Sci 1969; 28:634–637.
23. Bavister BD, Dees C, Schultz RD. Refractoriness of rhesus monkeys to repeated ovarian stimulation by exogenous gonadotropins is caused by nonprecipitating antibodies. Am J Reprod Immunol Microbiol 1986; 11:11–16.
24. Swanson WF, Roth TL, Graham K, Horohov DW, Godke RA. Kinetics of the humoral immune response to multiple treatments with exogenous gonadotropins and relation to ovarian responsiveness in domestic cats. Am J Vet Res 1996; 57:302–307.
25. Van Blerkom J, Davis P. Differential effects of repeated ovarian stimulation on cytoplasmic and spindle organization in metaphase II mouse oocytes matured in vivo and in vitro. Hum Reprod 2001; 16: 757–764.
26. Hodges CA, Ilagan A, Jennings D, Keri R, Nilson J, Hunt PA. Experimental evidence that changes in oocyte growth influence meiotic chromosome segregation. Hum Reprod 2002; 17:1171–1180.
27. Combelles CM, Albertini DF. Microtubule patterning during meiotic maturation in mouse oocytes is determined by cell cycle-specific sorting and redistribution of gamma-tubulin. Dev Biol 2001; 239:281–294.
28. Messinger SM, Albertini DF. Centrosome and microtubule dynamics during meiotic progression in the mouse oocyte. J Cell Sci 1991; 100: 289–298.
29. Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. Hum Reprod 1995; 10:415–424.
30. Rieger D. Batch analysis of the ATP content of bovine sperm, oocytes, and early embryos using a scintillation counter to measure the chemiluminescence produced by the luciferin-luciferase reaction. Anal Biochem 1997; 246:67–70.
31. Van Blerkom J, Davis P, Alexander S. Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: relationship to microtubular organization, ATP content and competence. Hum Reprod 2000; 15:2621–2633.
32. Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalves PB, Wolf E. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. Biol Reprod 2001; 64: 904–909.
33. Erbach GT, Lawitts JA, Papaioannou VE, Biggers JD. Differential growth of the mouse preimplantation embryo in chemically defined media. Biol Reprod 1994; 50:1027–1033.
34. Summers MC, McGinnis LK, Lawitts JA, Raffin M, Biggers JD. IVF of mouse ova in a simplex optimized medium supplemented with amino acids. Hum Reprod 2000; 15:1791–1801.
35. Handyside AH, Hunter S. A rapid procedure for visualising the inner cell mass and trophectoderm nuclei of mouse blastocysts in situ using polynucleotide-specific fluorochromes. J Exp Zool 1984; 231:429–434.
36. Papaioannou VE, Ebert KM. The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass of the blastocyst in vivo and in vitro. Development 1988; 102:793–803.
37. Combelles CMH, Carabatsos MJ, Albertini DF. Developmental regulation of oocyte-granulosa connectivity during folliculogenesis in the mouse. Biol Reprod 2001; 64(suppl): 294 (abstract).
38. Mattson BA, Albertini DF. Oogenesis: chromatin and microtubule dynamics during meiotic prophase. Mol Reprod Dev 1990; 25:374–383.
39. Zuccotti M, Piccinelli A, Rossi PG, Garagna S, Redi CA. Chromatin organization during mouse oocyte growth. Mol Reprod Dev 1995; 41: 479–485.
40. Bouniol-Baly C, Hamraoui L, Guibert J, Beaujean N, Szollosi MS, Debey P. Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes. Biol Reprod 1999; 60:580–587.
41. De La Fuente R, Eppig JJ. Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. Dev Biol 2001; 229:224–236.
42. Wickramasinghe D, Ebert KM, Albertini DF. Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes. Dev Biol 1991; 143:162–172.
43. Fraser LR. Fertilization and preimplantation development in vitro of mouse eggs obtained following stimulation with different doses of pregnant mare serum: a comparison of the responses in two strains. Differentiation 1977; 9:29–35.
44. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 1996; 383:531–535.
45. Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev Biol 1998; 204:373–384.
46. Carabatsos MJ, Sellitto C, Goodenough DA, Albertini DF. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. Dev Biol 2000; 226:167–179.
47. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci U S A 1967; 58:560–567.
48. Downs SM. The influence of glucose, cumulus cells, and metabolic coupling on ATP levels and meiotic control in the isolated mouse oocyte. Dev Biol 1995; 167:502–512.
49. Quinn P, Wales RG. The relationships between the ATP content of preimplantation mouse embryos and their development in vitro during culture. J Reprod Fertil 1973; 35:301–309.
50. Slotte H, Gustafson O, Nylund L, Pousette A. ATP and ADP in human pre-embryos. Hum Reprod 1990; 5:319–322.
51. Spindler RE, Pukazhenthi BS, Wildt DE. Oocyte metabolism predicts the development of cat embryos to blastocyst in vitro. Mol Reprod Dev 2000; 56:163–171.
52. Papaioannou VE, Ebert KM. Development of fertilized embryos transferred to oviducts of immature mice. J Reprod Fertil 1986; 76:603–608.
53. Tsunoda Y, McLaren A. Effect of various procedures on the viability of mouse embryos containing half the normal number of blastomeres. J Reprod Fertil 1983; 69:315–322.