Article

Administration of increasing amounts of gonadotrophin compromises preimplantation development of parthenogenetic mouse embryos

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Abstract

The aim of the present study was to examine the effect of ovarian stimulation with increasing amounts of pregnant mare's serum gonadotrophin (PMSG) on preimplantation development of diploid parthenogenetic embryos *in vitro*. Administration of 5, 10 and 20 IU PMSG significantly increased the number of oocytes obtained per mouse in a dose-dependent manner. The amount of PMSG administered did not alter the proportion of degenerate oocytes. However, there was a significant decrease in the proportion of 8-cell/compacted embryos after 53 h of culture with the administration of increasing amounts of PMSG. Proportion of embryos reaching at the blastocyst stage after 79 h of culture was reduced significantly in both the 10 and 20 IU PMSG groups. Reduced blastocyst development after 96 h of culture, however, was significant only in the 20 IU PMSG group. Total blastocyst, trophectoderm and inner cell mass numbers were also reduced significantly with the administration of 20 IU PMSG. It is concluded on the basis of these observations that preimplantation development of diploid parthenogenetic oocytes, which depends virtually entirely on maternal molecules accumulated during oogenesis along with gene products derived from the maternal genome, is compromised with the administration of increasing amounts of PMSG.

Keywords: *blastocyst*, *culture*, *embryo*, *mouse*, *parthenogenesis*

Introduction

Administration of gonadotrophins aimed to increase the number of oocytes recruited in a single cycle is an important component of the current assisted reproductive treatment. However, there is a substantial amount of data indicating that both preimplantation development and post-implantation viability of mouse (Edgar *et al*., 1987; Elmazar *et al*., 1989; Ertzeid and Storeng, 1992, 2001; Ertzeid *et al*., 1993; Van der Auwera *et al*., 1999; Van der Auwera and D'Hooghe, 2001), rat (Vanderhyden and Armstrong, 1988) and hamster (McKiernan and Bavister, 1998) embryos are compromised with exogenous administration of gonadotrophins. There is also evidence demonstrating that ovarian stimulation causes congenital anomalies in the offspring (Sakai and Endo, 1987). Furthermore, clinical evidence obtained from human IVF suggests that high serum oestradiol concentrations, as a consequence of ovarian stimulation, are associated with decreased pregnancy and implantation rates (Simon *et al*., 1995; Pellicer *et al*., 1996; Ng *et al*., 2000).

It has been demonstrated that exogenous administration of gonadotrophins may have a direct effect on oocyte quality such as the ability to be fertilized (Evans and Armstrong, 1984), chromosomal anomalies (Luckett and Mukherjee, 1986; Vogel and Spielman, 1992; Ma *et al*., 1997) and spindle defects (Van Blerkom and Davis, 2001). Therefore, it appears that adverse effects of high dose gonadotrophins are exerted on

the oocyte prior to ovulation and that the underlying cause of compromised embryo development is reduced quality of the oocyte. Nevertheless, it is not clear to what extent oocyte quality contributes to impaired development of embryos obtained from women who have undergone ovarian stimulation. It also remains to be determined whether deleterious effects of ovarian stimulation on oocyte/embryo quality would occur in the absence of a paternal genome. One way to address this question would be to assess the development of experimentally produced parthenogenetic embryos, which has been demonstrated to be a useful tool in examining how maternally derived factors contribute to early development (Latham *et al*., 1999). Parthenogenesis is defined as the production of an embryo from an oocyte in the absence of any contribution from a spermatozoon (Rougier and Werb, 2001). Although it has been reported that parthenogenetic embryos cannot progress to term (Rougier and Werb, 2001), successful development as live fetuses for 17.5 days gestation can be obtained (Kono *et al*., 2002). Development of diploid parthenogenetic embryos has been reported to be normal up to the blastocyst stage (Latham *et al*., 2002; Liu *et al*., 2002). This clearly indicates that maternal molecules accumulated during oogenesis along with gene products derived exclusively from the maternal genome can support preimplantation development of the mouse embryo. However, the ability of these maternal molecules to support development of parthenogenetic embryos appears to be determined, at least in part, by the genetic background of the oocyte (Latham *et al*., 1999). It is proposed in the present study that exogenous administration of supra-physiological dosages of gonadotrophins may also alter the ability of maternally encoded messages to support pre-implantation development of parthenogenetic mouse embryos. With this in mind, experiments were performed to examine the effect of ovarian stimulation with increasing amounts of pregnant mare's serum gonadotrophin (PMSG) on pre-implantation development of diploid parthenogenetic oocytes *in vitro*.

Materials and methods

Animals

Oocytes were obtained from 7–10 week old female F1 hybrid (C57BL/6 × BALB/c) mice (Tubitak, GMBAE, Gebze, Turkey). The animals were maintained on a 14 h light: 10 h dark photoperiod (light on at 5 a.m.), with free access to food and water. Multiple ovulations were induced by administration of 5, 10 and 20 IU PMSG (Folligon, Intervet, Istanbul, Turkey) in 0.9% NaCl solution. All animals received 5 IU human chorionic gonadotrophin (Pregnyl; Organon, Istanbul, Turkey) 48 h following the administration of PMSG. The experiment was repeated three times and all three PMSG doses were tested simultaneously in the same experiment. Numbers of mice used for groups of females receiving 5, 10 and 20 IU PMSG were ten, eight and eight respectively.

Oocyte activation and production of diploid parthenogenons

Cumulus-enclosed eggs were collected from superovulated, but unmated, females at 22 h after human chorionic gonadotrophin (HCG) in medium G-MOPS (Vitrolife AB, Gothenberg, Sweden) supplemented with 5 mg/ml human serum albumin (G-MOPS/HSA; Vitrolife). Cumulus cells were removed by incubating the eggs in medium G-MOPS/HSA containing hyaluronidase (0.5 mg/ml; Sigma Chemical Co., St Louis, MO, USA) for a short period of time. Eggs were washed well first in three large drops of G-MOPS/HSA and then in medium G1.3 (Vitrolife) supplemented with 5 mg/ml HSA (Vitrolife). The number of degenerate oocytes was determined at oocyte recovery and only morphologically normal oocytes were processed for parthenogenic activation. Parthenogenic activation was performed by incubating oocytes in medium G1.3/HSA supplemented with 10 μmol/l ionophore A23187 (Sigma) for 7 min in 6% CO_2:5% O_2:89% N_2 at 37°C. Activated oocytes were washed through several changes of G1.3/HSA. Diploid parthenogenetic oocytes were produced by suppressing formation of the second polar body as described by Barton *et al*. (1987), with the exception of using medium G1.3/HSA instead of medium M16. Briefly, eggs were incubated in medium G1.3/HSA containing the cytoskeletal inhibitor cytochalasin B (CCB; Sigma) at a concentration of 5 μg/ml for 3–4 h in 6% CO_2:5% O_2:89% N_2 at 37°C. Eggs were washed through several changes of G1.3/HSA to remove CCB. Oocytes were classified as degenerate, non-activated, haploid and diploid under a differential interference contrast microscope. Oocytes showing no pronuclei following activation and CCB treatment were defined as non-activated oocytes; whereas oocytes with only one pronucleus and a second polar body were defined as haploid oocytes. Only diploid parthenogenetic oocytes (**Figure 1a**), defined as oocytes with two clearly visible pronuclei, were used in the current study. All embryos were cultured in 20 μl droplets of pre-equilibrated G1.3/HSA under embryo-tested mineral oil (Sigma) in 6% CO_2:5% O_2:89% N_2 at 37°C for 53 h to around 8-cell/compaction stage. After 53 h of culture, embryos were washed well in medium G2.3 (Vitrolife) supplemented with 5 mg/ml of HSA and then cultured in 20 μl droplets of pre-equilibrated G2.3/HSA for a further 43 h to the blastocyst stage.

Assessment of preimplantation development

Development of diploid parthenogenetic oocytes was assessed after 53 h of culture to determine the number of the 8-cell/compacted embryos (**Figure 1b**). Embryos were re-examined after 79 and 96 h of culture for blastocyst development (**Figure 1c**). Blastocyst cell numbers were determined immediately following the assessment of embryo development after 96 h of culture.

Differential labelling of ICM and TE nuclei

The number of inner cell mass (ICM) and trophectoderm (TE) cells of blastocysts were determined using the differential nuclear staining method of Hardy *et al*. (1989).

Statistical analysis

The chi-squared test was used in analysing differences in the number of morphologically normal and degenerate oocytes at the time of oocyte recovery; the number of degenerate, activated, haploid and diploid oocytes following activation and CCB treatment as well as differences in embryo development.

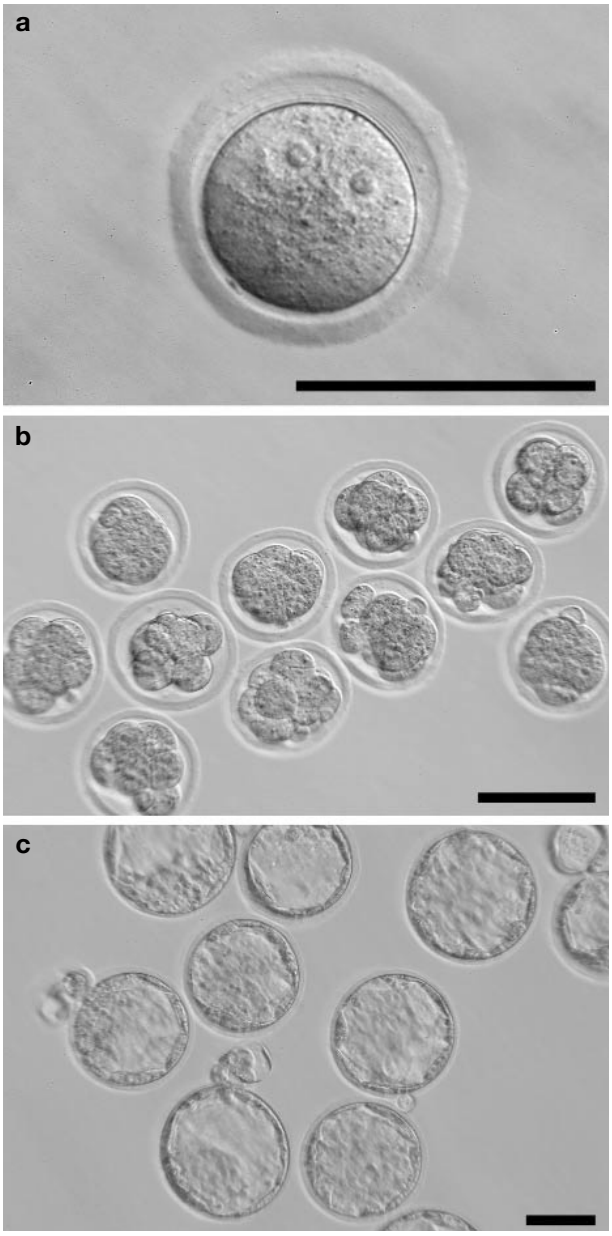

Figure 1. Morphological appearance of parthenogenetic oocytes and embryos. (**a**) A diploid parthenogenetic oocyte showing two pronuclei. (**b**) Parthenogenetic embryos at the 8-cell/compaction stage after 53 h of culture. (**c**) Parthenogenetic embryos at the blastocyst stage after 96 h of culture. Scale bar = 100 μm.

Cell numbers were analysed using analysis of variance (ANOVA) followed by Bonferroni procedure for multiple comparisons. A value of $P < 0.05$ was considered significantly different. The statistical package SPSS (version 10.0; SPSS Inc., Chicago, IL, USA) was used for all analyses.

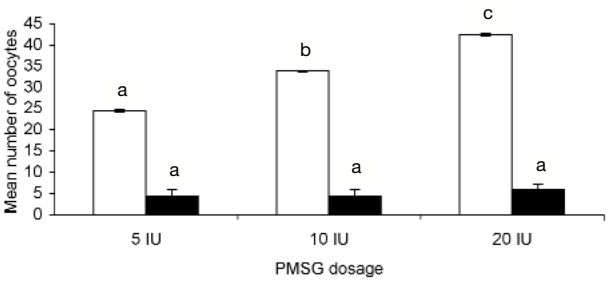

Figure 2. Effect of PMSG dosage on the number of morphologically normal and degenerate oocytes. Open bars represent the number of oocytes obtained per mouse (mean ± SE), whereas solid bars represent the number of degenerate oocytes (mean ± SE). [a,b,c]Bars labelled with different letters were significantly different from each other ($P < 0.05$).

Results

Effect of PMSG dosage on the number of oocytes

Administration of PMSG significantly ($P = 0.027$) increased the number of oocytes obtained per mouse in a dose-dependent manner (**Figure 2**). The mean numbers of oocytes obtained after the administration of 5, 10 and 20 IU PMSG were 24.4 ± 0.26, 33.8 ± 0.18 and 42.4 ± 0.35 (mean ± SEM) respectively. There was no significant difference in the number of degenerate oocytes with increasing amounts of PMSG (**Figure 2**).

Effect of PMSG dosage on the number of degenerate and activated oocytes following activation and treatment with CCB

Twelve per cent of oocytes did not survive following activation and CCB treatment (**Table 1**). However, the proportion of degenerate oocytes was not affected by the PMSG dosage used ($P > 0.05$). Significantly higher ($P < 0.01$) proportion of oocytes obtained from females induced by 20 IU PMSG formed haploid parthenogenetic embryos upon activation and CCB treatment compared with those obtained from animals receiving 5 and 10 IU PMSG (**Table 1**). There was also a significant decrease ($P \leq 0.01$) in the proportion of oocytes forming diploid parthenogenetic oocytes with the administration of 20 IU PMSG (**Table 1**).

Effect of PMSG dosage on embryonic development *in vitro and* blastocyst cell numbers

Development of diploid parthenogenetic oocytes was assessed after 53 h of culture to determine the number of the 8-cell/compacted embryos. There was a significant ($P \leq 0.01$) decrease in the proportion of 8-cell/compacted embryos with the administration of increasing amounts of PMSG (**Figure 3**). There was a dramatic and significant ($P \leq 0.01$) decrease in the proportion of embryos reaching at the blastocyst stage after 79 h of culture in groups of females receiving 10 and 20 IU

Table 1. Effect of PMSG dosage on the number of degenerate and activated oocytes following activation and CCB treatment.

PMSG dosage (IU)	*n*	*No. of degenerate oocytes after CCB treatment (% ± SE)[a]*	*No. of non-activated oocytes (% ± SE)[a]*	*No. of activated oocytes (% ± SE)[a]*	*No. of haploid oocytes (% ± SE)[a]*	*No. of diploid oocytes (% ± SE)[a]*
5	218	29 (13.3 ± 2.3)	18 (8.3 ± 2.5)[a]	171 (78.4 ± 2.8)	44 (20.2 ± 2.7)a	127 (58.3 ± 3.3)[a]
10	253	28 (11.1± 2.0)	38 (15.0 ± 2.2)[b]	187 (73.4 ± 2.8)	56 (22.1 ± 2.6)[a]	131 (51.8 ± 3.1)[a]
20	316	37 (11.7 ± 1.8)	55 (17.4 ± 2.1)[b]	224 (70.9 ± 2.6)	109(34.5± 2.7)[b]	115 (36.4 ± 2.7)[b]
P-value		>0.05	0.007	>0.05	<0.001	<0.001

[a]Data are expressed as mean percentages (± SE). [a,b]Within a column, values with different superscripts were significantly different ($P < 0.05$).

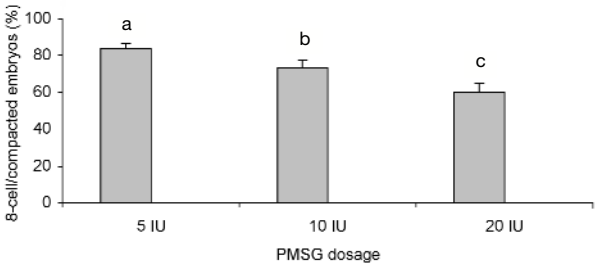

Figure 3. Effect of PMSG dosage on the development of diploid parthenogenetic oocytes cultured for 53 h. [a,b,c]Bars labelled with different letters were significantly different from each other ($P < 0.05$).

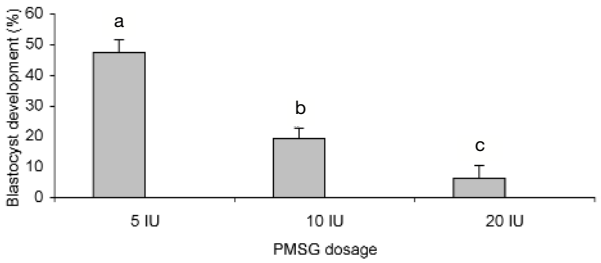

Figure 4. Effect of PMSG dosage on blastocyst development of diploid parthenogenetic oocytes cultured for 79 h. [a,b,c]Bars labelled with different letters were significantly different from each other ($P < 0.05$).

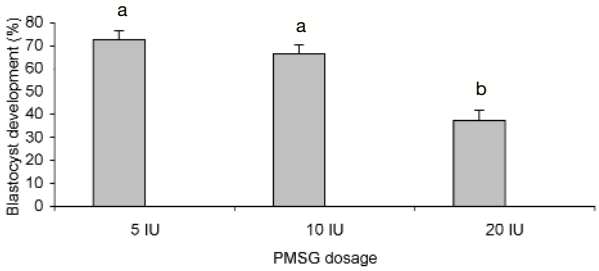

Figure 5. Effect of PMSG dosage on blastocyst development of diploid parthenogenetic oocytes cultured for 96 h. [a,b]Bars labelled with different letters were significantly different from each other ($P < 0.05$).

Table 2. Effect of PMSG dosage on blastocyst cell number and differentiation.

PMSG dosage (IU)	*TE cell number (mean ± SE)*	*ICM cell number (mean ± SE)*	*Total cell number (mean ± SE)*
5	48.3[a] ± 1.29	13.3[a] ± 0.38	61.6[a] ± 1.38
10	44.4[a] ± 2.13	12.1[ab] ± 0.61	56.5[a] ± 2.22
20	36.8[b] ± 1.45	10.8[b] ± 0.58	47.5[b] ± 1.44
P-value	<0.001	0.032	<0.001

Data are expressed as mean ± SE.
[a,b]Within a column, values with different superscripts were significantly different ($P < 0.05$).

PMSG compared with those induced by 5 IU PMSG (**Figure 4**). Blastocyst development after 96 h of culture was also reduced significantly ($P \leq 0.01$) in embryos derived from females receiving 20 IU PMSG compared with blastocyst development of embryos derived from females that received 5 and 10 IU PMSG (**Figure 5**). In addition, total blastocyst as well as TE and ICM cell numbers were significantly ($P < 0.05$) reduced with the administration of 20 IU PMSG compared with cell numbers of blastocysts derived from females that received 5 and 10 IU PMSG (**Table 2**).

Discussion

The effect of ovarian stimulation on embryo development has been a relatively intense area of research. It has been demonstrated that ovarian stimulation results in a significant increase in the proportion of mouse embryos that are delayed in their development (Ertzeid and Storeng, 1992, 2001). Implantation rate and post-implantation development of both mouse and hamster embryos are also reduced with exogenous administration of gonadotrophins (McKiernan and Bavister, 1998; Van der Auwera *et al*., 1999; Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001). However, it is not clear from these studies to what extent oocyte quality is compromised with the administration of exogenous gonadotrophins and as such contributes to relatively poor development of embryos obtained from stimulated females. The extent to which oocyte competence declines as a consequence of repeated cycles of ovarian stimulation has been examined recently by Van Blerkom and Davis (2001). It was demonstrated that repeated rounds of ovarian stimulation are associated with a progressive increase in the frequency of oocytes with spindle defects (Van Blerkom and Davis, 2001). A different approach was taken in the present study, which used parthenogenetic rather than fertilized embryos to examine developmental consequences of ovarian stimulation. The specific premise was that administration of increasing amounts of gonadotrophin would proportionally and adversely affect oocyte quality and thus preimplantation development of parthenogenetic embryos. Compromised development of diploid parthenogenetic oocytes with the administration of increasing amounts of PMSG, as shown in the current study, supports this hypothesis and suggests that ovarian stimulation does indeed diminish the quality and the developmental potency of the oocyte. These results support and corroborate previous observations demonstrating adverse effects of PMSG on preimplantation development of normally fertilized embryos of various species (Vanderhyden *et al*., 1988; Edgar *et al*., 1987; Elmazar *et al*., 1989; Ertzeid and Storeng, 1992, 2001; Ertzeid *et al*., 1993; McKiernan and Bavister, 1998; Van der Auwera *et al*., 1999; Van der Auwera and D'Hooghe, 2001).

Several hypotheses can be put forward to explain reduced developmental competence of the oocyte in response to administration of increasing amounts of PMSG. First, administration of high doses of PMSG may perturb normal signal transduction pathways (Williams, 2002) that function at the earliest stages of development, whether a genomic contribution is exclusively maternal or has a paternal element as well. Second, administration of increasing amounts of PMSG might alter the quantity and distribution of various regulatory proteins within the oocyte such as STAT3, leptin, transforming growth factor β-2 (TGFβ-2), Bcl-x, Bax, epidermal growth factor R (EGF-R), vascular endothelial growth factor (VEGF) and c-kit (Antczak and Van Blerkom, 1997, 1999). Third, administration of increasing concentrations of PMSG might cause meiotic spindle abnormalities. Spindle alterations as a consequence of repeated PMSG use have been demonstrated previously (Van Blerkom and Davis, 2001). While PMSG-related spindle alterations may not prevent pronuclear formation and cleavage after activation, embryos may not develop consistently through the preimplantation stages. This may be seen as a progressive reduction in the capacity of the maternally derived spindle to continue to function appropriately with each cell division. In this case, embryo developmental abnormalities would have a maternal origin regardless of whether activation was parthenogenetic or fertilization induced.

Paternal factors may affect preimplantation development of embryos (Tesarik *et al*., 2002). Conceivably, the use of parthenogenetic embryos would eliminate any paternal contribution. In the present study, only diploid parthenogenetic oocytes were used, as haploid parthenogenetic embryos are known to be developmentally compromised compared with their diploid counterparts (Latham *et al*., 2002; Liu *et al*., 2002). It was determined that higher number of oocytes can be obtained by increasing the amount of PMSG administered, which is in accordance with previous observations (Ziebe *et al*., 1993; Ozgunen *et al*., 2001). However, the dose-dependent increase in oocyte number was not accompanied by an increase in the proportion of degenerate oocytes and majority of oocytes appeared morphologically normal irrespective of the amount of PMSG administered. However, it should be noted that proportionally fewer parthenogenetic oocytes reached at the 8-cell/compaction and blastocyst stages with the administration of increasing amounts PMSG. This would suggest that the number of developmentally less competent, albeit morphologically normal, oocytes increases in proportion with the administration of increasing amounts of PMSG.

The parthenogenetic activation protocol used in the current study resulted in the activation of majority of oocytes irrespective of the amount of PMSG administered. Nevertheless, the proportion of oocytes forming diploid parthenogenetic oocytes following activation and CCB treatment differed between groups of females induced by 5, 10 and 20 IU PMSG. Administration of PMSG at a dosage of 20 IU per mouse significantly reduced the proportion oocytes yielding diploid parthenogenetic oocytes. As the dosage of PMSG administered was the only difference between groups of donor females, the amount of PMSG administered appears to have altered the response of oocytes to activation and CCB treatment.

The most significant finding of the current study was the negative effect of increasing dosages of PMSG on preimplantation development of diploid parthenogenetic oocytes. There was a significant decrease in the proportion of eight-cell/compacted embryos with the administration of increasing amounts of PMSG. However, administration of 10 and 20 IU PMSG had its most dramatic effect on blastocyst development after 79 h of culture. The proportion of diploid parthenogenetic oocytes reaching at the blastocyst stage after 96 h of culture as well as blastocyst cell numbers were reduced

significantly with the administration of 20 IU PMSG compared with experimental groups receiving 5 and 10 IU PMSG. Relatively low cell numbers and blastocyst development reported in this study for diploid parthenogenetic oocytes derived from females induced by 5 IU PMSG are in accordance with previous observations demonstrating delayed development and reduced total and ICM cell numbers of parthenogenetic embryos compared with fertilized embryos. (O'Neill *et al*., 1991; Hardy and Handyside, 1996; Uranga and Arechaga, 1997). It may also be important to note that blastocyst development and cell numbers obtained using zygotes from the same population of mice induced by 5 IU PMSG are in fact significantly higher than those reported for parthenogenic embryos in the present study (data not shown). It is well established that compromised development of parthenogenetic embryos is mainly the result of genomic imprinting (Tilgham, 1999). Insufficient parthenogenetic activation and suboptimal culture conditions are also indicated as possible causes of reduced developmental potential of parthenogenetic embryos (Liu *et al*., 2002). The data presented in the current study demonstrate that the amount of PMSG administered for ovarian stimulation is another contributing factor that might lead to compromised development of diploid parthenogenetic oocytes .

Taken together, compromised development of diploid parthenogenetic oocytes with the administration of increasing amounts of PMSG as shown in the current study provides further support for oocyte cytoplasmic influences related to ovarian stimulation whose adverse consequences are not expressed until the embryonic stages.

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Received 8 December 2003; refereed 17 December 2003; accepted 27 February 2004.