pattern of estradiol variation similar to women [37]). In particular, in Fischer 344 rats, serum levels of estradiol display one single cycle of increasing and decreasing levels during the 4- or 5-day estrous cycles. Serum concentration of estradiol shows a single peak of ≈ 257 pmol/L on the morning of proestrus, preceding the zenith of the LH surge (≈ 0.076 IU/L) that arises during the transition from the light to the dark phase. The peak of estradiol is followed by a nadir of ≈ 55 pmol/L on the night of proestrus, with this baseline level being kept during estrus and diestrus-1. Finally, on the morning of diestrus-2, estradiol levels start to rise, reaching a level of ≈ 165 pmol/L on the night of diestrus-2, and a level of ≈ 239 pmol/L on the night of diestrus-3 in 5-day cyclers [26].

On the other hand, in women, serum concentration of estradiol shows 2 cycles of increasing and decreasing levels. Serum baseline levels of 147-165 pmol/L estradiol start to increase ≈ 5 or 6 days before the preovulatory peak of LH in women with 3 or 2 follicular waves, respectively. It reaches a maximum level of ≈ 808 pmol/L 1 day before the preovulatory peak of LH (≈ 26 IU/L) in women with 2 follicular waves, or coinciding with the peak of LH (≈ 35 IU/L) in women with 3 follicular waves. After reaching this maximum, serum levels of estradiol drop to the baseline level of 147-165 pmol/L on day 3 after ovulation but elevate thereafter to ≈ 367 pmol/L for a week in women with 2 or 3 follicular waves, although women with 3 follicular waves exhibit more fluctuating values. At the end of the luteal phase, serum concentration of estradiol drops to the baseline level of 147-165 pmol/L, with this level being kept steady during the early follicular phase [13].

HPA axis response to acute stress

Effect of ovarian cycle

Table 1 shows the effect of ovarian cycle on HPA axis response to acute stress in rats, rhesus monkeys and women.

Rats

In rats with 5-day estrous cycles, the surgical stress of laparotomy on the morning of proestrus leads to release of adrenal estradiol and progesterone preceding an early surge of LH from the pituitary [38]. Moreover, intact 4- and 5-day cycling rats exhibit increased HPA sensitivity, releasing higher amounts of ACTH and corticosterone into the blood stream, immediately after a 20-min restraint on the morning of proestrus than animals at either estrus or diestrus (combined diestrus-1, 2 and 3) [39]. Notwithstanding, Carey et al. [40] found higher plasma levels of ACTH and corticosterone immediately after a 20-min restraint in 4-day cycling rats but did not evidence a significant effect of the estrous cycle on plasma concentrations of these hormones

Non-human primates

In cycling female rhesus monkeys, a 30-min intracerebroventricular administration of interleukin-1α, which simulates an inflammatory/immune-like challenge, induces a higher increase in plasma levels of cortisol and progesterone if administered during the mid-follicular phase compared to the early-follicular phase [41].

Women

Women with normal cycles undergoing bilateral ovariectomy plus total hysterectomy under general anesthesia during the mid- to late-follicular phase of the menstrual cycle respond with a small increase in plasma levels of progesterone 12 h after surgery preceding a small rise of LH. Likewise, after the surgical stress of cholecystectomy for benign conditions of the gallbladder under general anesthesia during the early- to mid-follicular phase, women exhibit a small surge-like increase in plasma levels of progesterone 12 h after the operation [42]. These increases, however, are not observed in women undergoing bilateral ovariectomy plus total hysterectomy during the early- to mid-luteal phase [42,43].

Other studies show that women in the mid-luteal phase of the menstrual cycle display enhanced plasma levels of ACTH in response to a 20-min progressive treadmill exercise compared to women in the early follicular phase [44]. Mid-luteal phase women also display reduced sensitivity to glucocorticoid feedback (lower suppression of plasma cortisol in response to a low dose of synthetic glucocorticoid dexamethasone) and decreased glucocorticoid receptor (type II) mRNA expression in lymphocytes compared to women in the early follicular phase [45]. In addition, the cortisol response to a 90-min submaximal bicycle [46] or treadmill [47] exercise is higher in the mid-luteal phase than in the early-follicular [47], mid-follicular [46] and late-follicular phases [47]. Likewise, a lower adrenocortical reactivity (decreased plasma cortisol levels) to the psychological stress of self-evaluation has been evidenced during the ovulatory period (between 12-16 days from the onset of the previous menstruation) than during the premenstrual phase (between 1-3 days prior to the onset of the next menstruation) [48]. There are, however, other studies that either do not find menstrual cycle-related differences in the HPA axis responsivity to intense and moderate physical exercise [49-51] or to the psychological stress of remembering stressful situations in their lives and self-evaluation [52]. Interestingly, although Galliven et al. [51] did not find a significant effect of the menstrual cycle on plasma cortisol levels after a 20-min progressive submaximal treadmill, the analysis of the net integrated area under the curve revealed a marginal ($P = 0.056$) lower response of cortisol in the periovulatory (between 10-16 days after the start of menses) phase compared with the early-mid-follicular (between 3-9 days after the start of menses) and mid-late-

Table 1: Effect of ovarian cycle on HPA axis response to acute stress.

Species	Stressor	Phase of the cycle	HPA axis response	References
Rats	Surgery	On the morning of proestrus	Positive	[38]
	20-min restraint	On the morning of proestrus vs either estrus or diestrus	Increased	[39]
		Proestrus, estrus and diestrus II	No cycle effect	[40]
Rhesus monkeys	30-min intracerebroventricular administration of interleukin-1α	Mid-follicular vs early-follicular phase	Increased	[41]
Women	Bilateral ovariectomy plus total hysterectomy	Mid- to late-follicular phase	Positive	[43]
		Early- to mid-luteal phase	No response	[42,43]
	Cholecystectomy	Early- to mid-follicular phase	Positive	[42]
	20-min progressive submaximal treadmill exercise	Mid-luteal phase vs early follicular phase	Increased	[44]
	90-min submaximal bicycle exercise	Mid-luteal phase vs mid-follicular phase	Increased	[46]
	90-min submaximal treadmill exercise	Mid-luteal phase vs early- and late-follicular phase	Increased	[47]
	60-min progressive submaximal treadmill exercise	Mid-follicular and luteal phase	No cycle effect	[49]
	Progressive maximal treadmill exercise to voluntary exhaustion or 40-min submaximal treadmill exercise	Early-follicular vs mid-luteal phase	No cycle effect	[50]
	20-min progressive submaximal aerobic treadmill	Early-mid-follicular, periovulatory and mid-late luteal phase	No cycle effect	[51]
	Psychological stress of remembering stressful situations in their lives and self-evaluation	Menstrual and periovulatory (late-follicular, ovulatory and early-luteal phases) phase	No cycle effect	[52]
	Psychological stress of self-evaluation	Ovulatory period vs premenstrual phase	Decreased	[48]

luteal (between 18-26 days after the start of menses) phases.

Concluding remarks

From the studies analyzed in this section, it seems that the ovarian cycle modulates the HPA axis response to acute stressors. In rats, it appears that proestrus morning is the more sensitive period. In rhesus monkeys, the HPA axis response is higher during the mid-follicular phase than during the early-follicular phase. And in women, the period more responsive seems to be during the mid-follicular and mid- and late-luteal phases. The absence of effect of the menstrual cycle on HPA axis responsivity to physical stress found in several studies may be explained by the presence of uncontrolled variables related to the training status of women and possibly to the training regimen followed in the days previous to the exercise test (cited by Williams et al. [53]).

Effect of hormone treatment

Table 2 shows the effect of hormone treatment on HPA axis response to acute stress in rats, rhesus monkeys and women.

Rats

It is known that immediately after exposure of ovariectomized rats to a 20-min restraint stress the plasma levels of ACTH are higher in estradiol-treated rats compared to the oil-treated control and to estradiol plus progesterone-treated rats. Moreover, during the 20-min period of restraint, the plasma levels of ACTH and corticosterone are always higher in the estradiol-treated group than in the oil-treated control and estradiol plus progesterone-treated rats [39]. Likewise, during the first 60 min after exposure to stress from a novel environment, ovariectomized estradiol- and estradiol plus progesterone-treated rats display an enhanced ACTH response compared to ovariectomized non-treated control and progesterone-treated rats. Ovariectomized estradiol plus progesterone-

Table 2: Effect of hormone treatment on HPA axis response to acute stress.

Species	Stressor	Hormone treatment	HPA axis response	References
Rats	20-min restraint	Estradiol vs oil and estradiol plus progesterone	Increased	[39]
	Exposition to a novel environment	Estradiol and estradiol plus progesterone vs no hormone treatment and progesterone	Increased	[40]
	5-sec footshock	Estradiol vs no hormone treatment	Increased	[54]
	1-min exposure to ether vapors	Estradiol vs no hormone treatment	Increased	[54]
Rhesus monkeys	30-min intracerebroventricular infusion of interleukin-1α	Estradiol at doses resulting in the typical high levels of plasma estradiol that reproduce the late follicular phase	Increased	[56]
		Estradiol at doses resulting in low levels of plasma estradiol	Increased	[57]
		Estradiol at doses that results in intermediate levels of plasma estradiol that reproduce the early-mid follicular phase	Inhibited	[57]
Women	Single intravenous injection of endotoxin	Estradiol vs no hormone treatment	No treatment effect	[35]
	Psychological stress (speech and math tasks)	Estrogens or estrogens plus progestogen vs no hormone treatment	No treatment effect	[58]
		Estradiol vs the same women before treatment	Decreased	[59]
	Psychological stress (mental arithmetic tasks accompanied by a repetitive annoying background noise	Estradiol vs placebo	Decreased	[60]

treated rats also display a higher corticosterone response than non-treated control and progesterone-treated rats during the same period of time [40].

Plasma levels of ACTH and corticosterone during the 2 h after a 5-sec footshock are higher in ovariectomized estradiol-treated rats than in ovariectomized non-treated control rats. At 20 min after 1-min exposure to ether vapors, plasma concentration of corticosterone is also higher in the group of estradiol-treated rats. Moreover, the ACTH and corticosterone secretory responses are less effectively suppressed by RU 28362 (a specific glucocorticoid receptor agonist) in estradiol-treated animals than in non-treated control rats [54]. This finding endorses other studies showing an inhibitory effect of estradiol on glucocorticoid receptor-mediated negative feedback [55].

Non-human primates

Studies in ovariectomized estrogen-replaced rhesus monkeys show that ACTH, cortisol and progesterone responses to a 30-min intracerebroventricular infusion of interleukin-1α are increased compared to the responses found in ovariectomized estrogen-replaced control monkeys infused intracerebroventricularly with a physiological saline solution. Experimental females received estrogen replacement therapy during 5 days at doses that elevated plasma estradiol concentration to 378 pmol/L (level that reproduces the typical high estradiol concentrations of the late follicular phase) [56]. The cortisol response to this immune-inflammatory challenge is also increased in ovariectomized 5-day-estrogen-treated monkeys with plasma concentrations of estradiol ≤ 73 pmol/L [57]. However, in 5-day-replaced rhesus monkeys with plasma levels of 114 pmol/L estradiol (concentration similar to that of the early-mid follicular phase), the cortisol response is inhibited [57].

Women

Studies in estrogen-replaced menopausal or ovariectomized women provide intriguing results. Some reports

evidence no effect of estradiol treatment on (1) the increases of plasma cortisol and progesterone observed in response to a single intravenous injection of endotoxin after 4 weeks of treatment [mean (± SEM) plasma estradiol levels: 23.5 ± 3.3 pmol/L in unreplaced women and 315.7 ± 36.7 pmol/L after estradiol replacement] [35]; or (2) the cortisol response to psychological stressors (speech and math tasks) after at least 2 years of estrogen alone (a mixture of 6 estrogenic substances) or estrogen and progestogen treatment (authors did not specify the plasma levels of estradiol of replaced and unreplaced women) [58]. Another study shows no changes in plasma ACTH concentration or even decreased peak levels of plasma cortisol and androstenedione in response to psychological stressors (speech and math tasks) after 6 weeks of treatment with estradiol compared to the increases evidenced in these women before estradiol replacement (plasma estradiol levels: ≤ 73.4 pmol/L before estradiol replacement and 587.4 ± 58.7 pmol/L after estradiol replacement) [59]. In perimenopausal women (within 2 years of their last period and actively experiencing vasomotor symptoms of menopause), estradiol supplementation for 8 weeks results in decreased plasma levels of ACTH and cortisol after a psychological stress (mental arithmetic tasks accompanied by a repetitive annoying background noise to induce difficulty in concentration) compared to placebo-treated perimenopausal women (plasma estradiol levels: 115.0 ± 19.0 pmol/L in placebo-treated women and 1118.0 ± 111.0 pmol/L in estradiol-treated women) [60].

As far as we know, there are no studies reporting increases in HPA axis reactivity to acute stressors in estrogen-treated perimenopausal, menopausal or ovariectomized women. Only one study in young men has found increased peak ACTH and cortisol responses to a brief psychosocial stress (free speech and mental arithmetic in front of an audience) after 24-48 h of estradiol treatment compared to a placebo group [61].

Concluding remarks

The studies focused on the effects of hormone replacement on HPA axis response suggest that estrogen treatment in ovariectomized females or perimenopausal and menopausal women may modify the HPA axis response to acute stressors. In rats, it appears that estradiol treatment increases the responsivity of the HPA axis to different acute stressors. In rhesus monkeys, the HPA axis response to a 30-min intracerebroventricular infusion of interleukin-1α depends on the plasma level of estradiol after estrogen replacement. In particular, low and high plasma concentrations increase the response whereas intermediate concentrations are inhibitory. In contrast, estrogen replacement in women does not change or even may decrease the typical response of the HPA axis to acute stressors. Although it is difficult to compare among studies the absolute plasma levels of estradiol exhibited by women before being exposed to acute stressors because different measurement methods were used, it is worth mentioning that, in all the studies analyzed in this section, independently of the replacement regimen followed, the HPA axis always displayed a positive response when plasma levels of estradiol were relatively low (≤ 73.4 pmol/L) or intermediate (115.0-315.7 pmol/L) whereas the response was decreased when plasma levels of estradiol were relatively high (587.4-1118.0 pmol/L). Thus, it can be hypothesized that the response of the HPA axis to inflammatory or psychological stressors evidenced in perimenopausal, menopausal or ovariectomized women may depend on the range of plasma estradiol concentration present in women when exposed to these stressors.

Acute-stress-induced LH release

Effect of ovarian cycle

Table 3 shows the effect of ovarian cycle on acute-stress-induced LH release in rats, rhesus monkeys and women.

Rats

Brown-Grant et al. [62] reported that ≈ 50% of light-induced persistent-vaginal-estrus rats, which show elevated levels of estradiol (similar to the levels on the morning of proestrus in rats maintained under a controlled photoperiod [63]), can ovulate when placed in a mating cage without a male or when subjected to a more severe stress such as laparotomy. The laparotomy-associated stress can also advance the time of LH surge (zenith: ≈ 0.130 IU/L) in 5-day cycling rats if performed on the morning of proestrus [38]. On the contrary, in 4-day cyclers, laparotomy delays pituitary LH discharge and ovulation if carried out on the morning of diestrus-2 [64]. An inhibition of the primary LH surge and ovulation has also been found in 4-day cycling rats after intracerebroventricular injection of interleukin-1α or β on the morning of proestrus [65]. Furthermore, it has been reported that a rapid blood volume depletion from the external jugular veins under constant ether anesthesia is able to increase plasma LH concentrations (from ≈ 0.017 IU/L to 0.043 IU/L) during the first 10 min after the initial blood loss in 4-day cycling rats at diestrus (combined diestrus-1 and 2) [66].

Non-human primates

In cycling rhesus monkeys, Norman et al. [67] found that a prolonged (6 h) chair restraint resulted in suppression of LH release, but 1 out of the 9 females tested exhibited a 2-5-fold increase in plasma levels of LH within 30 min of the initiation of restraint in both the mid-follicular and mid-luteal phases, as well as an increase in frequency of LH pulses over that normally evidenced in the mid-luteal phase. Furthermore, a 30-min intracerebroventricular administration of interleukin-1α induces a 3-fold increase in LH by 5 h after interleukin-1α infusion if administered

Table 3: Effect of ovarian cycle on acute-stress-induced LH release.

Species	Stressor	Phase of the cycle	LH release	References
Rats	Placed in a mating cage without a male or laparotomy	Persistent-vaginal-estrus (estradiol levels similar to the levels on the morning of proestrus)	Positive (inferred by ovulation)	[63]
	Laparotomy	On the morning of proestrus	Advance of the time of the primary LH surge	[38]
		On the morning of diestrus-2	Delay of the time of the primary LH surge (and ovulation)	[64]
	Intracerebroventricular injection of interleukin-1α or β	On the morning of proestrus	Inhibition of the primary LH surge (and ovulation)	[65]
	Rapid blood volume depletion	At diestrus (combined diestrus-1 and 2)	Positive	[66]
Rhesus monkeys	30-min chair restraint	Mid-follicular and mid-luteal phases	Positive	[67]
	30-min intracerebroventricular administration of interleukin-1α	Mid-follicular phase	Positive	[41]
		Early follicular phase	No release	[41]
Women	Bilateral ovariectomy plus total hysterectomy	Mid- to late-follicular phase	Positive	[43]
		Early- to mid-luteal phase	No release	[43]
	Cholecystectomy	Early- to mid-follicular	Positive	[42]
	Progressive submaximal treadmill exercise to exhaustion	Mid-follicular and mid-luteal phase	No release	[69]
	90-min submaximal bicycle exercise	Mid-follicular and mid-luteal phase	Decreased plasma levels of LH	[46]
	60-min progressive submaximal treadmill exercise	Mid-follicular phase	Positive	[49]
		Mid-follicular phase	Positive	[53]
		Mid-luteal phase	No release	[70]

during the mid-follicular phase, but there is no LH response during the early follicular phase [41]. We should mention that 1 out of the 9 mid-follicular monkeys exhibited a sustained surge-like release of LH after interleukin-1α treatment reaching a maximum (≈ 0.130 IU/L) at 13 h after treatment [41]. These findings contrast with the inhibition of the primary LH surge in cycling rats after intracerebroventricular injection of interleukin-1α or β on the morning of proestrus (see above). Discrepancies between rhesus monkeys and rats may be explained by the higher capacity of the rat adrenal glands to synthesize and release progesterone compared to the primate adrenal glands [68]. Thus, administration of interleukin in rats may induce the release of adrenal progesterone at concentrations that become inhibitory to LH secretion, whereas in rhesus monkeys the activation of the HPA axis by interleukin administration may result in the release of adrenal progesterone at concentrations equivalent to those observed during the preovulatory midcycle rise (cited by Xiao and Ferin [68]).

Women

The surgical stress of bilateral ovariectomy plus total hysterectomy performed under general anesthesia during the mid- to late-follicular phase of the menstrual cycle induces a small increase in plasma levels of LH 36 h after surgery (from ≈ 6 IU/L to 8 IU/L), preceded by an elevation of adrenal progesterone [43]. Likewise, women undergoing cholecystectomy under general anesthesia during the early- to mid-follicular phase exhibit a small but significant increase in plasma levels of LH (from ≈ 6 IU/L to 12 IU/L) and FSH (from ≈ 6 IU/L to 9 IU/L) 12 h after the operation, coinciding with a peak in plasma progesterone [42]. However, no wave-like changes in plasma LH levels are observed in women undergoing bilateral ovariectomy plus total hysterectomy during the early- to mid-luteal phase [43].

Although some studies report that light, heavy or exhaustive exercise does not alter [69] or even decreases [46] plasma levels of LH at the mid-follicular or mid-luteal phases, other studies evidence small increases in

plasma levels of LH in response to acute physical exercise. In fact, 60 min of progressive submaximal treadmill exercise in the mid-follicular phase produces a small surge-like increase in plasma levels of LH (from ≈ 10 to 14 IU/L) in 24-h fasted eumenorrheic women [49] as well as a significant stimulatory effect on maximal peak amplitude of LH pulses (from 8.8 to 9.5 IU/L) in sedentary eumenorrheic women [53]. In contrast, the same stress applied in the mid-luteal phase [70] does not affect the LH pulse characteristics.

Concluding remarks

The studies analyzed in this section show conflicting results on the effects of ovarian cycle on acute-stress-induced LH release in rats. Some studies seem to indicate that the adenohypophysis is much more responsive to acute stressors on the morning of proestrus than at diestrus. In contrast, other studies suggest the opposite. It can be hypothesized that the degree of adrenal-progesterone response depends on the strength of the stressor applied. Thus, strong stressors such as intracerebroventricular injection of interleukin-1α or β or rapid blood volume depletion may induce an adrenal-progesterone response higher than that elicited by more moderate stressors such as laparotomy. Such an adrenal-progesterone response would inhibit the release of LH if plasma levels of estradiol are elevated (proestrus morning) but it would stimulate the release of LH under low estradiol backgrounds (e.g. the diestrus stage). Likewise, a moderate rise in adrenal-progesterone concentrations equivalent to that observed during the preovulatory midcycle rise would be stimulatory if it takes place under high plasma levels of estradiol (proestrus morning) but it would be unable to elicit a release of LH under low estradiol backgrounds (e.g. the diestrus stage).

In rhesus monkeys, females at the mid-follicular and mid-luteal phases, but not at the early-follicular stage, display appropriate plasma levels of estradiol for acute stressors to induce a release of LH.

Although there are some conflicting reports in women, it seems that the mid-follicular phase gathers more favorable conditions than the mid-luteal phase for the release LH by the adenohypophysis in response to acute stressors.

Effect of hormone treatment

Table 4 shows the effect of hormone treatment on acute-stress-induced LH release in rats, rhesus monkeys and women.

Rats

In contrast to ovariectomized non-estrogen-primed rats, ovariectomized estrogen-primed rats respond with a small but significant LH peak (≈ 0.017 IU/L) either 1 h after the onset of a prolonged (11 h) immobilization [71] or 10 min after a short (15 min) immobilization [71]. Likewise, ovariectomized rats treated with estradiol, progesterone and thyroxine respond with an LH surge (zenith: ≈ 0.052 IU/L) 10 min after undergoing a rapid blood volume depletion from the external jugular veins. This response, however, is not observed in ovariectomized non-treated rats [66]. We should note, however, that acute stressors such as the transfer of rats to a novel environment followed by a return to their original quarters 30 min later, or 15-min exposure to a strobe light have no effects on serum LH in 2-week ovariectomized estradiol-primed rats, but significantly enhance LH release (zenith: ≈ 0.015 IU/L and ≈ 0.020 IU/L, respectively) in 2-week ovariectomized non-estrogen-primed rats [72].

Table 4: Effect of hormone treatment on acute-stress-induced LH release.

Species	Stressor	Hormone treatment	LH release	References
Rats	1-h or 10-min immobilization	Estradiol vs no hormone treatment	Positive	[71,72]
	Rapid blood volume depletion	Estradiol+progesterone+thyroxine vs no hormone treatment	Positive	[66]
	Transfer to a novel environment followed by a return to the original quarters 30 min later, or 15-min exposure to strobe light	No hormone treatment vs estradiol	Positive	[72]
Rhesus monkeys	30-min intracerebroventricular infusion of interleukin-1α	Estradiol at doses that result in plasma estradiol levels that reproduce the late follicular phase	Positive	[56]
		Estradiol at doses that result in plasma estradiol levels that reproduce the early-mid follicular phase	No effect	[57]
Women	Single intravenous injection of endotoxin	Estradiol vs no hormone treatment	Positive	[35]

Non-human primates

In ovariectomized rhesus monkeys after 5 days of estrogen replacement therapy at doses simulating the late follicular phase, a 30-min intracerebroventricular infusion of interleukin-1α results in a small but significant increase in LH release during the first 5 h after treatment (from 0.014 IU/L to 0.019 IU/L) [56]. However, the response of LH is prevented in replaced monkeys with plasma levels similar to those of the early-mid follicular phase [57].

Women

Estrogen-treated menopausal or ovariectomized women respond to an inflammatory/immune-like stress, induced by single intravenous injection of endotoxin, with an ovulatory-like LH peak (50 IU/L) 7 h after injection. In contrast, non-estrogen treated women do not modify their basal LH levels after endotoxin injection [35].

Concluding remarks

Studies in ovariectomized rats show paradoxical results as far as the response of the adenohypophysis to different acute stressors is concerned. Whereas 2 studies [66,71] found a stimulatory effect of estradiol replacement on acute-stress-induced LH release, Briski and Sylvester [72] evidenced that several types of acute stress exerted different effects on pituitary LH release and that the steroid environment modulated in a different way (inhibiting or stimulating) the pattern of response of the HPG axis induced by these stressors.

Literature focused on rhesus monkeys shows a stimulatory effect of estradiol replacement on acute-stress-induced LH release at doses that result in plasma estradiol levels reproducing the late follicular phase. This stimulatory effect, however, is not found after estradiol treatment at doses that result in plasma estradiol levels simulating the early-mid follicular phase.

Finally, estrogen-treated menopausal or ovariectomized women respond with a clear ovulatory-like LH peak after a single intravenous injection of endotoxin [35].

Summary conclusions

The studies reviewed in this bioessay indicate that the ovarian cycle and, in particular, the female's estradiol background modulates the response of the HPA and HPG axes to acute stressors. The pattern of response, however, differs between the HPA and HPG axes. In fact, although the highest responses of the HPA axis within the ovarian cycle are observed when females display relatively high plasma levels of estradiol (proestrus morning in rats, mid-follicular phase in rhesus monkeys and mid-follicular and mid- and late-luteal phases in women), the HPA axis exhibits positive responses in practically all phases of the ovarian cycle. In contrast, it seems that there is only one specific period of time within the ovarian cycle during which the HPG axis response is possible. In particular, positive responses of the HPG axis are found under relatively high plasma levels of estradiol on the morning of proestrus in rats, during the mid-follicular and mid-luteal phase in rhesus monkeys and during the mid-follicular phase in women. This conclusion is endorsed by the fact that the HPG axis of estrogen-treated ovariectomized females and perimenopausal or menopausal women also exhibit a positive response to acute stressors.

Two studies [66,72] in the rat show that several types of acute stress may exert different effects on pituitary LH release and that the steroid environment may modulate in a different way (inhibiting or stimulating) the pattern of response of the HPG axis induced by acute stressors. In rhesus monkeys and women, the steroid environment may also modulate in a different way the pattern of response of the HPA axis (and therefore the response of HPG axis including release of LH) elicited by acute stressors. In particular, in estrogen-treated ovariectomized females and perimenopausal or menopausal women, some ranges of plasma estradiol concentration seem to prevent whereas others allow a response of the HPA axis to acute stressors.

In women, the pattern of LH release elicited by acute stressors may vary from small non-ovulatory rises in plasma concentrations and/or slight changes in pulse characteristics to the typical ovulatory surge that spontaneously takes place at the middle of the menstrual cycle. As women present waves of follicular development during an interovulatory interval (and likely during pregnancy and lactation), they may be induced to ovulate at any point of the menstrual cycle or even during periods of amenorrhea associated with pregnancy and lactation if exposed to an appropriate acute stressor under a right estradiol environment. We should bear in mind that the maximum diameters (range of 10-17 mm) of the dominant follicles from anovulatory major waves, which account for 21% of the anovulatory follicular waves, are compatible with potential ovulations if follicles were correctly stimulated by an ovulatory-like LH surge. Follicles large enough (≥ 15 mm of diameter) to ovulate may be found in the ovaries of all the regular-cycling women at the late-mid follicular phase (growing antral follicles from the ovulatory follicular wave). In addition, they may be present in the ovaries of women exhibiting (1) a major-major 2-wave pattern at the mid-luteal phase (10% of the overall population of regular-cycling women); (2) a minor-major-major 3-wave pattern at the early-mid-follicular phase (6% of the overall population of regular-cycling women); and (3) a major-major-major 3-wave pattern at the late-mid-luteal and early-mid follicular phases (6% of the overall population of regular-cycling women) [13]. We should note, however, that ovulation during the luteal phase or during pregnancy and lactation

are extremely rare due to the presence of negative feedbacks that prevent the LH surge.

We should bear in mind that an acute-stress-induced surge of LH is shortly preceded by an elevation of serum progesterone from the adrenal glands. This fact suggests that such an elevation of serum progesterone may advance the secretory transformation of the endometrium resulting in embryo-endometrium asynchrony and consequently reduced chances of implantation and pregnancy if ovulation and fertilization took place. However, a recent systematic review and meta-analysis [73] has not detected detrimental effects of elevated levels of progesterone on the day of human chorionic gonadotropin (hCG) administration on the probability of clinical pregnancy in women undergoing ovarian stimulation with GnRH analogues and gonadotropins for in vitro fertilization. Although this may not be the case for women treated with GnRH antagonists (for review, see Venetis et al. [73]), we should take into account that an elevation of plasma progesterone on the day of hCG administration should be sustained in order to impair endometrial receptivity. This is not the case in estrogen-treated menopausal or ovariectomized women after being exposed to single intravenous injection of endotoxin [35]. These women display a wave-like adrenal release of progesterone. In particular, plasma progesterone concentrations rise from ≈ 0.2 nmol/L 1-2 h after injection to ≈ 5 nmol/L 4-5 h after injection. Thereafter, plasma levels of progesterone drop to reach their baseline levels of ≈ 0.2 nmol/L 7 h after injection.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
JJT has been involved in conception and design, acquisition, analysis and interpretation of data and drafting the article. TH and AC have been involved in analysis and interpretation of data, and revising the article critically for important intellectual content. All authors read and approved the final manuscript.

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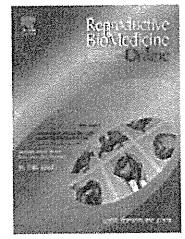

COMMENTARY

Spermatozoal RNA profiling towards a clinical evaluation of sperm quality

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Abstract Human spermatozoal RNAs were recently profiled using microarrays and explored as clinical markers of male infertility. An appropriate study design with a considerable number of biological replicates (sperm samples) is necessary to validate the accuracy and reproducibility of these microarray data. If the genes identified as sperm quality markers by microarray studies are successfully attributed to the pathogenesis of male infertility, then the microarray strategy may be used as a clinical diagnostic tool for male infertility. On the other hand, spermatozoal RNAs may contain not only remnant RNAs after spermatogenesis, but also RNAs that may contribute extragenomically to early embryonic development. Therefore, spermatozoal RNA profiling may enable a better understanding of what is contributed to the oocyte by sperm, in addition to their genome, to facilitate early embryonic development.

KEYWORDS: ICSI, male fertility, RNA profiling, sperm, spermatozoa, transcriptome

Assessments of male reproductive fitness have typically relied upon microscopic evaluation using semen parameters including morphology, motility, sperm concentration, presence of any cell types other than mature spermatozoa, and semen volume. The observation of normal semen features using these parameters does not necessarily guarantee male fertility. Recently, morphological real-time observation at high magnification (> ×6000) has been used to select sperm for intracytoplasmic sperm injection (ICSI). The morphological normalcy of the sperm nucleus is defined by both its shape (smooth, symmetric and oval) and its chromatin content (homogeneity of the chromatin mass containing no extrusion or invagination and no more than one vacuole involving less than 4% of the nuclear area) (Bartoov et al., 2002). Most publications have reported better rates of implantation and clinical pregnancy as well as a reduction in the rate of abortion where sperm cells were strictly morphologically selected at high magnification (Souza Setti et al., 2010). Prospective randomized clinical studies are still needed to confirm the preliminary findings on the efficacy of intracytoplasmic morphologically selected sperm injection (IMSI) over conventional ICSI. The further improvement of diagnosis and treatment of male infertility will need a new method to evaluate sperm quality based on molecular analysis, rather than on morphological observation.

Mature spermatozoa have little cytoplasm and a highly condensed chromatin architecture that is enriched in protamines. These structural features led to the long held view that mature spermatozoa are inert cells but both transcription and translation occur in the mitochondria, and not in the cytoplasm, of mature spermatozoa (Miller and Ostermeier, 2006). Spermatozoal nuclei, containing RNA polymerase and abundant transcription factors, are capable of transcribing RNA from endogenous templates (Hecht and Williams, 1978). Although mature spermatozoa do not contain some of the essential components of the 80S cytoplasmic ribosomes such as 28S and 18S rRNAs; 55S mitochondrial ribosomes are present in spermatozoal polysomal fractions (Gur and Breitbart, 2006). The incorporation of labeled amino acids into polypeptides occurs during sperm

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capacitation, and is completely inhibited by mitochondrial translation inhibitors but not by a cytoplasmic translation inhibitor (Gur and Breitbart, 2006). Therefore, it is apparent that mitochondrial ribosomes are actively involved in protein translation in spermatozoa.

The first mRNA that was identified in human mature spermatozoa was the c-Myc mRNA (Kumar et al., 1993). The existence of a complex population of mRNAs in ejaculated human mature spermatozoa was shown by expression profiling using oligo DNA microarrays (Ostermeier et al., 2002). Although these mRNAs were previously thought to be non-functional remnants of stored mRNAs that are synthesized at earlier stages of spermatogenesis, Ostermeier et al. proposed that a specific set of functional RNAs may be delivered into oocytes and support early embryonic development (Ostermeier et al., 2004). Although the specific functional significance of these mRNAs in mature ejaculate spermatozoa remains poorly investigated; they have been demonstrated to influence the phenotypic traits of offspring (Miller and Ostermeier, 2006). The poor developmental ratios relative to normal of both parthenogenetic embryos and cloned embryos obtained from somatic-cell nuclear transfer, are consistent with a developmental role for spermatozoal mRNAs.

Garcia-Herrero et al. used microarrays to investigate spermatozoal RNAs (this issue; Garcia-Herrero et al., 2011). They compared the profile gene of expression in spermatozoa that achieved pregnancy (group P) through an ICSI cycle in an oocyte donation program with the profile of those that did not achieve pregnancy (group NP) (Garcia-Herrero et al., 2011). In order to reduce female infertility as a bias factor, all of the oocytes originated from young female donors. Furthermore, the coupled pairs of women (pregnant and non pregnant) received the oocytes from the same donor. The total number of expressed transcripts detected in fresh sperm samples was 19,229. Of those transcripts, 16,035 (83.4%) were expressed in both groups, P and NP. Among these commonly expressed transcripts, only 44 sequences were overexpressed in group P versus NP and five in group NP versus P. Notably, the 44 differentially-expressed genes in group P included four cathepsins and six metallothioneins. Cathepsins are a family of cysteine proteases and are likely to prevent atrophy of seminiferous tubules and support spermatogenesis to pachytene spermatocytes (Gye and Kim, 2004; Wright et al., 2003). Metallothioneins function as detoxicants to prevent damage of the testes by heavy metals (Kusakabe et al., 2008). An ontology analysis by DAVID (http://david.abcc.ncifcrf.gov/home.jsp) of 1358 exclusively-expressed transcripts in group P, found the term 'embryo development ending in birth or egg hatching' as one of the highest-ranked gene ontology (GO) terms. The exclusively-expressed genes corresponding to this GO term include adducin 1 (*ADD1*), activin A receptor type-II like 1 (*ACVRL1*), androgen receptor (*AR*), and aryl-hydrocarbon receptor nuclear translocator (*ARNT*). All of those genes are potential pregnancy success markers rather than potential fertilization makers. In fact, because ICSI removes the physiological process of sperm entry, spermatozoal fertilization factors may not be necessary for pregnancy after ICSI. These highly expressed RNAs in group P may partially represent spermatozoal extra-genomic components that are required for successful pregnancies. In addition, DAVID also analyzed frozen spermatozoa used in ICSI in the same way, and demonstrated that the RNA profile of frozen spermatozoa was considerably changed by the sperm cryopreservation procedure.

Several other studies have profiled spermatozoal RNAs in clinical samples with the aim of finding a marker RNA or a distinctive expression pattern to represent sperm quality. A cross-platform microarray strategy was used to assess the profile of human spermatozoal transcripts from 13 fertile males who had fathered at least one child compared to those from eight teratozoospermic individuals (Platts et al., 2007). This analysis successfully distinguished between the normal and teratozoospermic groups using unsupervised hierarchical clustering. The teratozoospermic group lacked the RNAs of genes related to the ubiquitin-proteasome pathway and those genes transcribed at late stages of spermatogenesis including; an egg-activating sperm factor, *PLCZ1*; acrosomal proteins, *ACRV1* and *SPAM1*; and non-tubulin components of sperm tails, *ODF1-4*. These changes in gene expression are indicative of the failure of late-stage spermatogenesis in teratozoospermia. In a study by Lalancette et al., the spermatozoal RNAs of 24 healthy donors were expression profiled and a series of invariable transcripts were consistently present in all of the donor samples (Lalancette et al., 2009). Based on the expression of these consistently-expressed genes, only a single donor sample was not well correlated with the other 23 samples, suggesting that spermatozoal RNA profiling could be clinically applied to mark outliers. Furthermore, Garcia-Herrero et al. compared the transcriptomic profiles of sperm samples that achieved pregnancy after the first IUI to those that did not (Garcia-Herrero et al., 2009). They identified 756 genes that were significantly preferentially expressed in the pregnant group, and 194 genes that were significantly preferentially expressed in the non-pregnant group (Garcia-Herrero et al., 2009). Interestingly, these 756 genes include 20 of the 44 genes that were overexpressed in group P (pregnant after an ICSI in oocyte donation program) in the current study by the same group described in this volume of *Reproductive BioMedicine Online*. These 20 genes out of the 756 genes could be considered as potential pregnancy success markers rather than potential fertilization makers.

Thus far, microarray technologies have been used to assess the profiles of human spermatozoal RNAs and the utility of spermatozoal RNAs as clinical markers of male infertility. An appropriate study design with a considerable number of biological replicates (sperm samples) is necessary to validate the accuracy and reproducibility of these microarray data. If the genes identified as sperm quality markers by microarray studies are successfully attributed to the pathogenesis of male infertility, then the microarray strategy may be used as a clinical diagnostic tool for male infertility. On the other hand, spermatozoal RNAs may contain not only RNAs left over from failed or abnormal spermatogenesis, but also RNAs that may contribute extragenomically to early embryonic development. Therefore, spermatozoal RNA profiling may enable a better understanding of what is contributed to the oocyte by sperm, in addition to their genome, to facilitate early embryonic development.

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ORIGINAL ARTICLE

Early metaphase II oocytes treated with dibutyryl cyclic adenosine monophosphate provide suitable recipient cytoplasm for the production of miniature pig somatic cell nuclear transfer embryos

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ABSTRACT

We investigated the effects of *in vitro* maturation duration and treatment with dibutyryl cyclic adenosine monophosphate (dbcAMP) on the blind enucleation efficiency and developmental competence of miniature pig somatic cell nuclear transfer (SCNT) embryos. Oocytes were cultured for 22 h in NCSU-23 medium with or without 1 mM dbcAMP and then additionally cultured in dbcAMP-free NCSU-23 for 14, 18, or 22 h. Regardless of dbcAMP treatment, the rate of nuclear maturation reached a plateau at 36 and 40 h. However, mitochondrial distribution, a marker for cytoplasmic maturation, differed between the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h. The metaphase II chromosomes were adjacent to the first polar body in 68.8% and 63.5% of the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h, respectively. Furthermore, the blind enucleation efficiency by removing a small volume of cytoplasm was significantly higher in the dbcAMP-untreated oocytes at 36 h (82.9%) and dbcAMP-treated oocytes at 40 h (89.9%) than other groups. The rate of blastocyst formation was highest in the dbcAMP-treated oocytes at 40 h. Hence, this study demonstrated that dbcAMP-treated early metaphase II oocytes are suitable for the production of miniature pig SCNT embryos.

Key words: *enucleation, miniature pig, nuclear transfer, oocyte maturation.*

INTRODUCTION

Miniature pig cloning by somatic cell nuclear transfer (SCNT) has many potential biomedical applications such as xenotransplantation and the development of animal models for human diseases (Dai *et al.* 2002; Lai *et al.* 2002) because characteristics such as anatomy, physiology, and body size of the miniature pigs are similar to humans (Tucker *et al.* 2002). Despite recent successes in the miniature pig cloning technology, the efficiency of the procedure remains low (Lai & Prather 2003). One of the probable reasons for the low efficiency is the poor understanding of the factors determining the developmental ability of miniature pig SCNT embryos. Recently, we demonstrated that the factors determining *in vitro* developmental competence included the culture conditions for oocytes (Wakai *et al.* 2008), activation protocol (Yamanaka *et al.* 2007), and the presence of cytoskeletal inhibitors (Sugimura *et al.* 2008).

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For miniature pig cloning in particular, improving the *in vitro* maturation (IVM) of domestic pig oocytes is essential for ensuring a high production of SCNT embryos. Thus, the culture condition for oocytes that function as recipient cytoplasts is the main factor for a successful miniature pig cloning (Wakai *et al.* 2008). The enucleation of the IVM oocyte is a crucial step for subsequent development in cloned embryos (Liu *et al.* 2002; Simerly *et al.* 2004; Lee & Campbell 2006; Byrne *et al.* 2007). During enucleation, the position of the chromosomes is either indirectly determined by determining the location of the first polar body (PB) or directly determined by staining the oocytes with a DNA-specific dye (e.g., Hoechst 33342) and observing them under UV light (Li *et al.* 2004). However, the first PB often migrates from its place of origin and does not always remain in proximity to the chromosomes. This is because of oocyte aging that occurs as IVM progresses and/or the removal of cumulus cells by pipetting (Li *et al.* 2004; Miao *et al.* 2004). Recently, it has been speculated that Hoechst staining and the exposure of oocytes to UV light may disrupt functions in the cytoplast, especially the function of mitochondria, thus affecting subsequent development (Li *et al.* 2004; Byrne *et al.* 2007). Hence, a simple, facile and highly efficient enucleation technique, which does not require the visualization of DNA by exposure to UV light, is important for the high production of SCNT embryos exhibiting high developmental competence. A previous study revealed that the culture of activated oocytes in demecolcine resulted in efficient enucleation without the visualization of DNA by exposure to UV light. However, further studies on the reagent toxicity and further development are necessary for this method (Yin *et al.* 2002). Additionally, other previous studies have shown that the efficiency of 'blind' enucleation (without the visualization of DNA by exposure to UV light) of anaphase I (AI)-telophase I (TI) and AII-TII oocytes is very high because the chromosomes of these oocytes are located near the PB (Bordignon & Smith 1998; Nour & Takahashi 1999; Lee & Campbell 2006). However, these oocytes have low levels of maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK), which may be the main factors involved in remodeling events, including nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) of G_0/G_1 phase donor somatic cells (Bordignon & Smith 1998; Lee & Campbell 2006). On the other hand, the distance between oocyte chromosome and first PB increases as the oocyte ages (Miao *et al.* 2004). Hence, it is hypothesized that metaphase II (MII) oocytes from which the first PB has just been extruded, which are termed as early MII oocytes, may exhibit high enucleation efficiency and high occurrence of NEBD and PCC after SCNT.

Other than enucleation, the homogeneity in the oocyte quality with respect to the cytoplasmic maturation status is important for the high production of SCNT embryos exhibiting high developmental competence. The heterogeneity of porcine oocytes is caused by spontaneous maturation, which is associated with a decrease in the intracellular concentration of cyclic adenosine monophosphate (cAMP) and subsequent inactivation of protein kinase A (PKA), may adversely affect the coordination between nuclear and cytoplasmic maturation, resulting in decreased developmental potential (Kim & Menino 1995; Bagg *et al.* 2006).

Previously, the treatment of prepubertal porcine oocytes for 20–22 h with dibutyryl cAMP (dbcAMP), a membrane permeable analog of cAMP, has been shown to inhibit GV breakdown (GVBD) and increase the incidence of blastocyst formation after IVF (Funahashi *et al.* 1997; Somfai *et al.* 2003). Thus, treatment with dbcAMP is considered helpful because it may improve the synchronization between nuclear and cytoplasmic maturation. However, little information is available on the beneficial effects of dbcAMP on cytoplasmic maturation.

In the present study, to gain information on the optimal IVM conditions of oocytes, we examined the effect of IVM duration and dbcAMP treatment for the first 22 h of IVM on the efficiency of blind enucleation, developmental competence after SCNT and mitochondrial distribution.

MATERIALS AND METHODS

Donor cells

Miniature pig fetuses (CSK, Suwa, Japan) were collected from sows at day 56 of pregnancy. Each fetus was decapitated and eviscerated. The remaining tissues were washed in Dulbecco's phosphate buffered saline (PBS; Sigma Chemical Co., St. Louis, MO, USA) and then digested with 0.1% (w/v) trypsin-EDTA (Sigma) for 45 min at 38.5°C. After digestion, fetal fibroblast cells were collected and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma). The culture medium was changed every 2 days until confluence was attained. Then the cells were harvested by treatment with 0.1% (w/v) trypsin in PBS containing 0.5 mmol/L EDTA for 5 min at 38.5°C, frozen with a cryoprotectant (Cellbanker; Zenyaku, Tokyo, Japan), and stored in liquid nitrogen (passage 0). Before starting the experiments, the cells derived

from a single fetus were thawed and cultured in DMEM supplemented with 10% (v/v) FBS and used between passages 4 and 9.

IVM of oocytes

Ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory within 2 h in a container containing warm saline. Porcine oocytes were aspirated from antral follicles of diameter 3–6 mm using a 5-mL disposable syringe attached to an 18-gauge needle. Cumulus-oocyte complexes (COCs) with uniform ooplasm and compact cumulus cell mass were selected in PB1 (Quinn *et al.* 1982). After washing in PB1, the COCs were cultured in bovine serum albumin (BSA)-free NCSU-23 medium containing 10 IU/mL pregnant mares serum gonadotropin (PMSG; Serotropin; Teikokuzouki, Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (hCG; Puberogen; Sankyo, Tokyo, Japan), 0.1 mg/mL cysteine (Sigma), 1 mmol/L dbcAMP (Sigma) and 10% (v/v) porcine follicular fluid (Miyoshi *et al.* 2000) supplemented with or without 1 mmol/L dbcAMP (Sigma) (Funahashi *et al.* 1997) for 22 h at 38.5°C in a highly humidified atmosphere of 5% CO_2 in air and then transferred into the same medium, but without hormonal supplements and dbcAMP, for an additional 10, 14, 18 or 22 h of culture. After culturing, expanded cumulus cells of the COCs were removed by vortexing in PB1 containing 1 mg/mL hyaluronidase (Sigma) and oocytes showing the first PB under a stereomicroscope were selected as mature oocytes. The mature oocytes were transferred to PB1 and used for the experiments.

Oocyte enucleation

The cumulus-free oocytes were transferred into PB1 containing 1 mmol/L sucrose and 2.5 μg/mL cytochalasin D (Sigma). Enucleation of oocytes was performed by the blind method (without the visualization of DNA by exposure to UV light). Oocyte enucleation was performed by aspirating the first PB and the adjacent cytoplasm with a pipette using a piezo-drive unit (Primetech, Tokyo, Japan). The enucleated oocytes were washed thrice and transferred to a droplet of NCSU-23 medium for the subsequent microinjection of the donor nucleus.

Nuclear transfer

The cells were thawed and cultured for 1 week after reaching confluence, and a single-cell suspension was prepared prior to SCNT. Most of these cells were identified as being the G0/G1 phase of the cell cycle by analysis with a flow cytometry (Sugimura *et al.* 2008). The prepared donor cells were transferred to a 50-μL droplet of PB1, and their plasma membranes were damaged by repeated gentle aspirations using an injection pipette of 10 μm diameter. The denuded nucleus was microinjected into the cytoplasm of the enucleated oocyte.

Activation and culture of SCNT embryos

The embryos were activated using the protocol reported by Yamanaka *et al.* (2007). At 3 h after microinjection, SCNT embryos were activated by ionomycin (Sigma) and cycloheximide (Sigma). In order to activate the SCNT embryos with ionomycin, they were treated with 15 μmol/L ionomycin in NCSU-23 medium for 20 min at 38.5°C in a humidified atmosphere of 5% CO_2 in air and then washed 5 times with NCSU-23 medium. After the treatment, the SCNT embryos were cultured in NCSU-23 medium containing 5 μg/mL cycloheximide and 2.5 μg/mL cytochalasin D for 5 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air and then washed 5 times with cycloheximide and cytochalasin D-free NCSU-23 medium. Finally, the embryos were transferred to NCSU-23 medium and cultured at 38.5°C in an atmosphere of 5% CO_2 in air.

Experimental design

In **Experiment 1**, we studied the effect of dbcAMP treatment during IVM on the time-dependent changes in the nuclear maturation rate of oocytes. Oocytes were cultured in NCSU-23 medium with or without dbcAMP for 22 h and then without dbcAMP for an additional period of 10, 14, 18, or 22 h. In order to evaluate the nuclear maturation rate, after the culture for each period, the oocytes were mounted, fixed for 48 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and observed under a phase-contrast microscope.

In **Experiment 2**, we determined the location of MII chromosomes with respect to the location of the first polar body in untreated oocytes and oocytes treated with dbcAMP. Oocytes were examined at 36, 40 or 44 h of IVM. After staining the samples with 0.1 mg/mL Hoechst 33342 for 5–10 min, the location of first polar body relative to that of the MII chromosome was determined.

In **Experiment 3**, we determined the efficiency of blind enucleation in porcine oocytes treated with dbcAMP. The success rates for enucleation were assessed at 36, 40, and 44 h of IVM. Either 10% or 30% of the cytoplasm surrounding the first PB was removed by an enucleation pipette. Then, cytoplasts were stained with 0.1 mg/mL Hoechst 33342 for 5–10 min, and the enucleation efficiency was determined.

In **Experiment 4**, we studied the effect of the maturation period and treatment of dbcAMP on the *in vitro* development of SCNT embryos. Oocytes from which 10% cytoplasm was removed along with the nuclear material at 36, 40, or 44 h of IVM were used as recipient cytoplasts. PCC and pseudo pronucleus (pPN) formation were examined by orcein staining as described in Experiment 1 at 3 h after SCNT and 6 h of *in vitro* cultivation, respectively. The number of cleaved SCNT embryos was determined on day 2 of *in vitro* cultivation, and the number of SCNT embryos that developed to the blastocyst stage was determined on days 7 of *in vitro* cultivation. The total cells number of blastocysts stained with 0.1 mg/mL Hoechst 33342 for 5–10 min was determined on day 7.

In **Experiment 5**, we studied the mitochondrial distribution in untreated and dbcAMP-treated porcine oocytes. Oocytes were examined at 36, 40, or 44 h of IVM. First, the oocytes were stained with 10 μg/mL rhodamine 123 (Molecular Probes, Eugene, OR, USA) in PB1. Then, the oocytes were mounted on glass slides and immediately observed under a confocal scanning laser microscope (Hara *et al.* 2005). The fluorescence intensity depended on the mitochondrial membrane potential.

Statistical analysis

There were at least three replicates for each experiment. The data for the rate of pronuclear formation, cleaved and blastocyst formed embryos, and mitochondrial distribution were analyzed by the chi-square test. Other data were analyzed by the analysis of variance (ANOVA); post hoc analysis was performed by the Bonferroni/Dunn test ($P < 0.05$).

RESULTS

Experiment 1. The time-dependant changes in the nuclear maturation rate in untreated and dbcAMP-treated oocytes

We examined the time-dependent changes in the nuclear maturation rate. The rates of nuclear maturation were examined at 32 (22 + 10), 36 (22 + 14), 40 (22 + 18), and 44 (22 + 22) h (Fig. 1). In the dbcAMP-untreated group, the rate of nuclear maturation was significantly lower (47.3 ± 4.6%) at 32 h IVM than other maturation periods and reached a plateau at 36 h (73.3 ± 8.1%). On the other hand, in the dbcAMP-treated group, the rate was significantly lower at 32 h (3.8 ± 0.8%) and 36 h (24.2 ± 3.1%) than other periods and reached a plateau at 40 h (88.8 ± 3.2%). The rate of MII did not differ significantly between the dbcAMP-untreated and dbcAMP-treated groups at 40 and 44 h. These results indicate that dbcAMP treatment does not affect the proportion of oocytes that attain nuclear maturation, but it affects the time required to attain maturity.

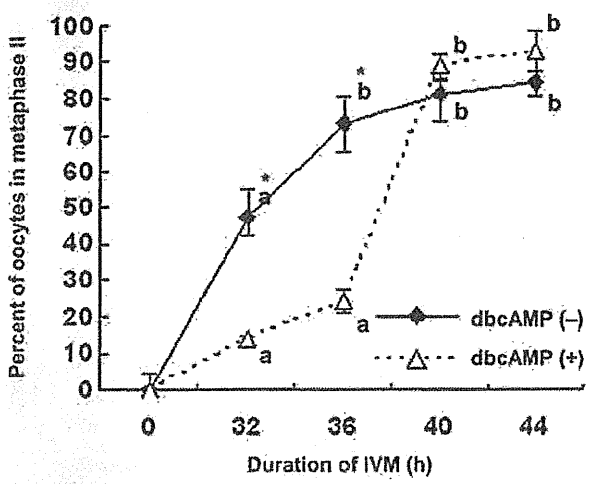

Figure 1 Incidence of meiotic progression to the MII stage during IVM in untreated and dbcAMP-treated porcine oocytes. The number of oocytes examined for each IVM period ranged from 79 to 150 in 3 replicate experiments. dbcAMP (–), dbcAMP-untreated groups; dbcAMP (+), dbcAMP-treated groups. Values are mean ± S.E. Values marked with different alphabets are significantly different ($P < 0.05$).

Experiment 2. Chromosome location of MII oocytes relative to that of the first PB in untreated and dbcAMP-treated oocytes

We determined the location of the MII chromosome relative to the first PB (Fig. 2 and 3). As shown in Figure 3, the chromosome location of MII oocytes was classified into 1 of 3 groups according to the angle formed by 2 lines: 1 line from the oocyte center to the first PB and 1 line from the center to the MII chromosome (0°–5°, 6°–30°, and 31°–180°). In both the dbcAMP-untreated and dbcAMP-treated groups, the percent of oocytes with angles of 0°–5° decreased as the IVM duration increased, and the percent was significantly higher in the dbcAMP-untreated oocytes at 36 h (69.0 ± 4.9%) and dbcAMP-treated oocytes at 40 h (63.5 ± 9.8%) than other groups ($P < 0.05$). In contrast, the percent of oocytes with angles of 6°–30° and 31°–180° increased as the IVM duration increased and these percents were significantly lower in the dbcAMP-untreated oocytes at 36 h (6°–30°, 21.3 ± 5.4% and 31°–180°, 9.7 ± 0.5%) and dbcAMP-treated oocytes at 40 h (6°–30°, 28.7 ± 6.0% and 31°–180°, 5.3 ± 1.0%) than other groups.

Experiment 3. Efficiency of blind enucleation in untreated and dbcAMP-treated oocytes

We determined the efficiency of blind enucleation (Fig. 4). In both the untreated and dbcAMP-treated groups, the enucleation efficiency decreased as the IVM duration increased. On removing 10% cytoplasm with MII chromosomes, the enucleation efficiency was significantly higher in the dbcAMP-untreated oocytes at 36 h (82.9 ± 8.2%) and dbcAMP-treated oocytes 40 h (89.9 ± 3.3%) than in the other groups (dbcAMP-untreated oocytes at 40 h: 58.0 ± 10.5%; dbcAMP-untreated oocytes at 44 h : 45.1 ± 2.2%; dbcAMP-treated oocytes at 44 h: 64.3 ± 3.4%). Moreover, the efficiency did not differ significantly with difference in the volume of the cytoplasm removed for the dbcAMP-untreated oocytes at 36 h, dbcAMP-treated oocytes at 40 h and dbcAMP-treated oocytes at 44 h.

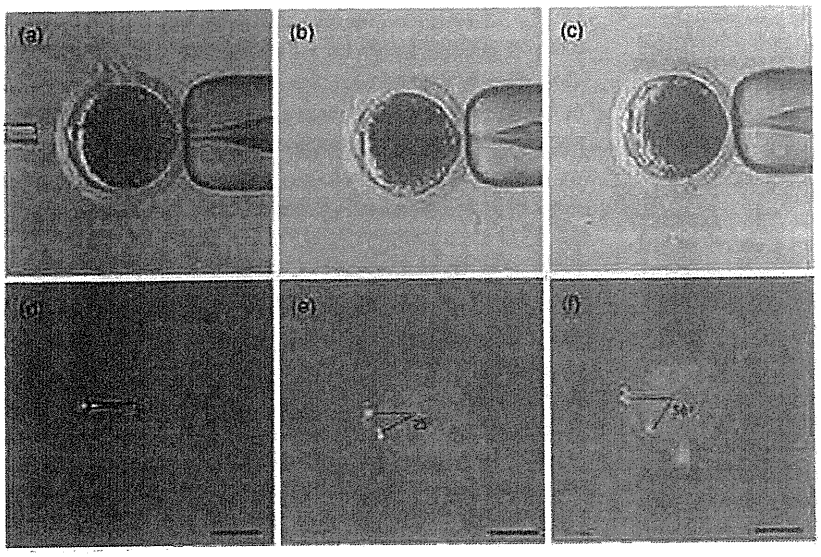

Figure 2 Time-dependent changes in the location of the MII chromosome relative to that of the first PB in untreated and dbcAMP-treated porcine oocytes. The number of oocytes examined for each IVM period ranged from 106 to 145 in 3 replicate experiments. dbcAMP (–), dbcAMP-untreated groups; dbcAMP (+), dbcAMP-treated groups. Values are mean ± S.E. Values marked with different alphabets (a and b) are significantly different in the same group of chromosome location ($P < 0.05$).

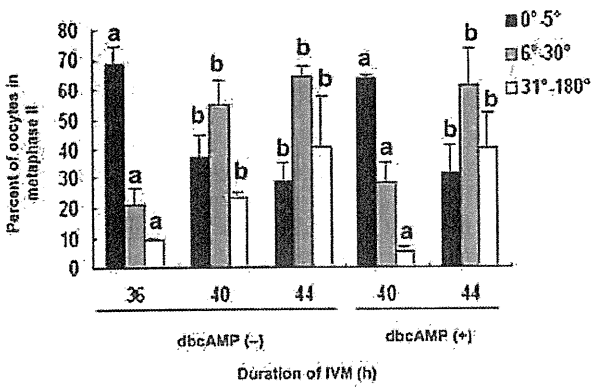

Figure 3 Representative photographs of porcine MII oocytes with different locations of the meiotic chromosome relative to that of the first PB. The chromosomes were visualized by staining with Hoechst 33342. These locations, determined by measuring the angle between a line connecting the oocyte centre with the meiotic nucleus and a line connecting the oocyte centre with the first PB, are 2° in (A) and (a), 25° in (B) and (b), and 50° in (C) and (c). These images represent the 3 oocyte groups with respect to the chromosome location (0–5°, 6–30°, and 31–180°, respectively). Bar = 60 μm.

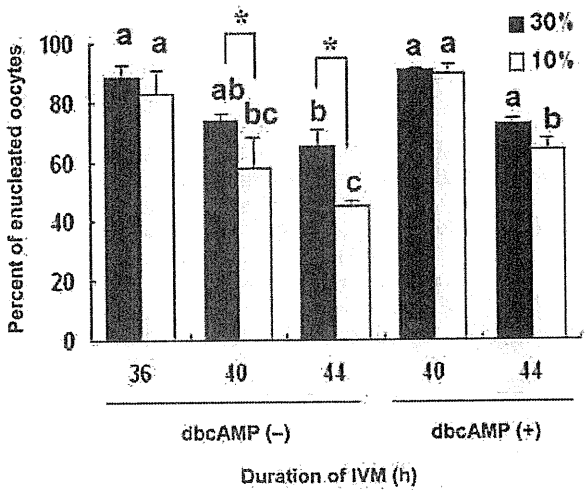

Figure 4 The efficiency of bind enucleation in untreated and dbcAMP-treated porcine oocytes. The number of oocytes examined for each IVM period ranged from 116 to 143 in 3 replicate experiments. In oocytes of the 30% and 10% group, 30% and 10% of the cytoplasm beneath the first PB was removed using an enucleation pipette of 20-μm diameter, respectively. dbcAMP (–), dbcAMP-untreated groups; dbcAMP (+), dbcAMP-treated groups. Values marked with different alphabets (a–c) are significantly different in the same groups (10% or 30% groups) and those marked with an asterisk (*) are different between the 2 groups ($P < 0.05$).

Experiment 4. Effect of maturation period and dbcAMP treatment on the in vitro development of SCNT embryos

First, we examined the occurrence of PCC at 3 h after SCNT (Fig. 5). The occurrence of PCC did not differ significantly in the dbcAMP-untreated oocytes at 36 h, dbcAMP-untreated oocytes at 40 h, dbcAMP-treated oocytes at 40 h and dbcAMP-treated oocytes at 44 h, but it was significantly lower in the dbcAMP-treated oocytes at 44 h (50.2 ± 5.4%) than other treatment groups (dbcAMP-untreated oocytes at 36 h and

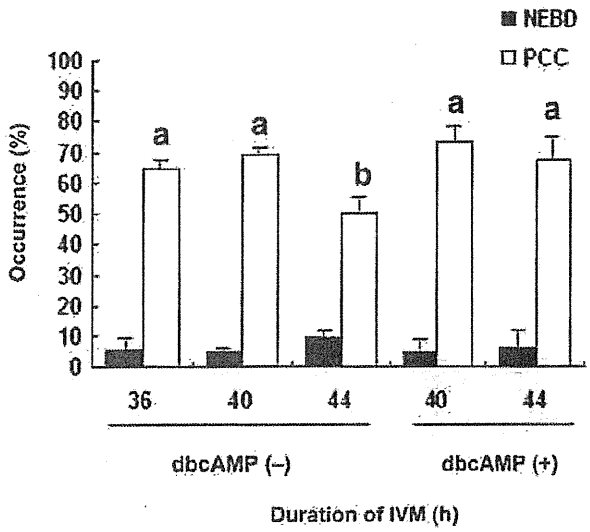

Figure 5 The occurrence of PCC and NEBD in SCNT embryos. At 3 h after nuclear injection, PCC and NEBD were observed. The number of SCNT embryos examined for each IVM period ranged from 60 to 116 in 3 replicate experiments. dbcAMP (–), dbcAMP-untreated groups; dbcAMP (+), dbcAMP-treated groups.Values are shown as the mean ± S.E. Values marked with different alphabets (a and b) are significantly different ($P<0.05$).

dbcAMP-untreated oocytes at 40 h: 64.4 ± 2.6% and 69.2 ± 2.3%, respectively; dbcAMP-treated oocytes at 40 h and dbcAMP-treated 44 h: 73.5 ± 5.3% and 67.5 ± 6.9%, respectively).

Next, pPN and pseudo polar body (pPB) was observed at 6 h of *in vitro* culture (Table 1). The rate of activated oocytes did not differ significantly between the groups. However, 2pPN0pPB formation rate was significantly higher in the dbcAMP-untreated oocytes at 36 h (46.3%) and dbcAMP-treated oocytes at 40 h (47.2%) than in the dbcAMP-untreated oocytes at 44 h (22.9%).

Finally, we examined the *in vitro* developmental competence (Table 2). The rate of cleaved embryos did not differ significantly among all groups. However, the rate of blastocyst formation was significantly higher in the dbcAMP-treated oocytes at 40 h (26.4%) than in the dbcAMP-untreated oocytes at 36 h (10.3%) and dbcAMP-untreated oocytes at 44 h (11.2%).

Experiment 5. Mitochondrial distribution in untreated and dbcAMP-treated oocytes

We examined the mitochondrial distribution as a marker of cytoplasm maturation (Table 3). The pattern of mitochondrial distribution was categorized into I of the 4 types shown in Figure 6: peripheral (type I), semiperipheral (type II), diffused (type III), and weak (type IV). The distribution was more homogenous in oocytes of the dbcAMP-treated groups than in oocytes of the dbcAMP-untreated groups. The proportion of MII oocytes exhibiting the type II distribution pattern was significantly higher in the dbcAMP-untreated oocytes at 36 h (34.0%) than in the dbcAMP-untreated oocytes at 44 h (13.2%), dbcAMP-treated oocytes at 40 h (14.3%) and dbcAMP-treated oocytes at 44 h (12.9%). The proportion of MII oocytes exhibiting the type III distribution pattern was significantly higher in the dbcAMP-treated oocytes at 40 h (82.9%) and dbcAMP-treated oocytes 44 h (87.1%) than in the untreated oocytes at 36 h (54.0%) and untreated oocytes at 44 h (65.8%).

DISCUSSION

In the present study, in order to produce homogenous and high quality recipient cytoplasts, we evaluated the effects of IVM duration and dbcAMP treatment for the first 22 h of IVM on the following factors: the rate of nuclear maturation, mitochondrial distribution, chromosome location of MII oocytes relative to that of the first PB, efficiency of blind enucleation, and developmental competence of miniature pig SCNT embryos.

Enucleation is the key step in the preparation of a large number of high quality recipient cytoplasts. A highly efficient enucleation method, which does not involve the use of UV radiation and DNA-specific dyes, has been in demand. In the present study, we demonstrated that dbcAMP-untreated oocytes at 36 h of IVM and dbcAMP-treated oocytes at 40 h of IVM exhibit a high efficiency of blind enucleation. On the other hand, previous studies have indicated that the efficiency of blind enucleation is high in the AI-TI (Lee & Campbell 2006) and AII-TII (Bordignon & Smith 1998) oocytes compared to MII oocytes. However, it has been reported that the MPF and MAPK levels in these oocytes are lower than the levels in MII oocytes (Lee & Campbell 2006). It has been suggested that MPF and MAPK are important for the successful occurrence of nuclear remodeling events such as NEBD and PCC, which may be essential for the development of SCNT embryos with donor cells in the G_0/G_1 phase (Tani *et al.* 2003). Therefore, AI-TI or AII-TII stage oocytes were considered unsuitable for the induction of proper nuclear remodeling events, including NEBD and PCC, in the G_0/G_1 donor cells. In

Table 1 Effect of IVM duration and dbcAMP treatment on pseudo-pronucleus formation of miniature pig SCNT embryos

dbcAMP	IVM duration	No. of oocytes used	No. (%) of activated oocytes	No. (%) of SCNT embryos having		
				1 pPN† and 1 pPB‡	1 pPN and 0 pPB	2 pPN and 0 pPB
Untreated	36	60	41 (63.3)	1 (2.4)	21 (51.2)	19 (46.3)[a]
	40	52	38 (73.1)	1 (2.6)	21 (55.3)	16 (42.1)[ab]
	44	50	35 (74.0)	2 (5.7)	24 (68.6)	8 (22.9)[b]
Treated	40	55	36 (65.5)	0 (0)	19 (52.8)	17 (47.2)[a]
	44	46	31 (67.4)	1 (3.2)	18 (58.1)	12 (38.7)[ab]

[a,b]Values with different superscripts are significantly different ($P < 0.05$). †Pseudo pronucleus. ‡Pseudo polar body.

Table 2 Effect of IVM duration and dbcAMP treatment on the development of miniature pig SCNT embryos

dbcAMP	IVM duration (h)	No. (%) of cultured oocytes	No. (%) of cleaved oocytes	No. (%) of blastocysts formed	Total cell number (mean ± SD)
Untreated	36	117	54 (46.2)	12 (10.3)[a]	26.6 ± 4.9
	40	93	63 (67.7)	17 (18.3)[ab]	30.1 ± 8.3
	44	98	50 (51.0)	11 (11.2)[a]	26.5 ± 4.8
Treated	40	110	72 (65.5)	29 (26.4)[b]	30.5 ± 3.5
	44	92	59 (64.1)	17 (18.5)[ab]	27.3 ± 5.0

[a,b]Values with different superscripts are significantly different ($P < 0.05$).

Table 3 Mitochondrial distribution in untreated and dbcAMP-treated porcine oocytes

dbcAMP	IVM duration (h)	No. of oocytes used	No. (%) of oocytes having a distribution pattern of			
			Type I	Type II	Type III	Type IV
Untreated	36	50	5 (10.0)	17 (34.0)[a]	27 (54.0)[a]	1 (2.0)
	40	38	1 (2.6)	8 (21.1)[ab]	29 (76.3)[ab]	1 (2.6)
	44	38	4 (10.5)	5 (13.2)[b]	25 (65.8)[a]	4 (10.5)
Treated	40	35	1 (2.9)	5 (14.3)[b]	29 (82.9)[b]	0 (0)
	44	31	0 (0)	4 (12.9)[b]	27 (87.1)[b]	0 (0)

[a,b]Values with different superscripts are significantly different in same type of mitochondrial distribution ($P < 0.05$).

contrast, we used the dbcAMP-untreated oocytes at 36 h of IVM and dbcAMP-treated oocytes at 40 h of IVM and found that the frequency of oocytes undergoing PCC was high, which may be induced by high MPF and MAPK activities in the cytoplasts (Kawahara *et al.* 2005; Lee & Campbell 2006; Wakai *et al.* 2008). Additionally, it has been suggested that oocytes having high MPF activity may effectively induce the formation of 2 pPN from PCC with normal bipolar spindle, and SCNT embryos having 2 pPN induce proper mitotic division, resulting in high developmental potential (Ng *et al.* 2004; Wakai *et al.* 2008). In the present study, several SCNT embryos having 2 pPN were observed when the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM were used, indicating that the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM may be suitable for the induction of proper nuclear remodeling events, including NEBD, PCC, and pPN formation.

Moreover, we found that the use of the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM as recipients enabled blind enucleation by removing a smaller volume of cytoplasm. It has been suggested that appropriate cytoplasm volume is crucial for further normal development after reconstruction (Lee & Campbell 2006). Additionally, the removal of a smaller volume of cytoplasm may result in the loss of a smaller percentage of oocyte proteins that are associated with the meiotic spindle (Lee & Campbell 2006). A previous study on non-human primates suggested that proteins associated with the chromatin and spindle may vary between oocytes arrested in the early MII phase (pre-MII oocytes) and