Effects of cycloheximide or 6-dimethyl aminopurine on the parthenogenetic activation of pig oocytes using pulsatile treatment with nitric oxide donor

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ABSTRACT: Pig oocytes matured *in vitro* were parthenogenetically activated using nitric oxide donor SNAP (2mM). Continuous treatment successfully activated the oocytes only after more than 12 hours of exposure. Pulsatile treatments during which oocytes were repeatedly exposed to 2mM SNAP for a short time (10, 20 or 30 minutes) were more efficient with regard to the activation rate, even when the total exposure time did not exceed 4 hours. Parthenogenetic development was very limited after continuous treatment with 2mM SNAP. A significantly higher proportion of developing parthenogenetic embryos was observed after the pulsatile treatment (development to the morula stage 0 vs. 18%; development to the blastocyst 0 vs. 7%; $P < 0.05$). However, this developmental rate was significantly lower ($P < 0.05$) than the development induced by conventional activation treatment with calcium ionophore (development to the morula stage, 23%; development to the blastocyst stage, 18%). When we combined pulsatile SNAP-treatment with the effect of protein kinase inhibitor 6-dimethyl aminopurine (6-DMAP) (2mM 6-DMAP for 2 hours) or with the inhibitor of protein synthesis cycloheximide (CHX) (10 μM CHX for 2 hours), we observed a significant increase ($P < 0.05$) in the activation rate when compared to the respective pulsatile SNAP-treatment without 6-DMAP or CHX (63 vs. 78% of activated oocytes for 6-DMAP; 63 vs. 83% of activated oocytes for CHX). However, the development of parthenogenetic embryos was not enhanced when the pulsatile SNAP-treatment was combined with 6-DMAP or with CHX.

Keywords: activation; nitric oxide; oocyte; pig

In matured mammalian oocytes, meiosis is blocked at the stage of metaphase II. Further progress of meiosis beyond this spontaneously occurring block is dependent on the activating stimulus. Under natural conditions, this stimulus is brought into the oocyte by sperm (Yanagimachi, 1988). Phospholipase C zeta is thought to trigger activation, as it is known to trigger intracellular signalling of calcium ions in fertilized oocyte (Jones et al., 1998; Parrington et al., 2002; Fujimoto et al., 2004; Konig et al., 2006).

Oocyte activation is important in many areas, including cloning using the transfer of nuclei of somatic cells, because the development of clone embryos depends on an adequate activation stimulus. For this reason, current research focuses on the development of efficient activation treatments.

Supported by the Ministry of Agriculture of the Czech Republic (Grants No. MZE 0002701401), the Czech Science Foundation (No. 523/06/0295) and the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. MSM 6046070901).

Oocyte activation is generally thought to be a calcium-dependent process because the sperm is able to induce oscillations of levels of intracellular free calcium ions (Swann and Ozil, 1994). On the other hand, nitric oxide (NO) was recently shown to be an intracellular signal for activation in sea urchin eggs (Kuo et al., 2000). Nitric oxide is also able to induce parthenogenetic activation in mammalian oocytes (Petr et al., 2005a).

The mechanisms for activation of mammalian oocytes by the NO-dependent signalling cascade are not fully understood. Studies by Leckie et al. (2003) and Hyslop et al. (2001) showed that the NO-dependent signalling cascade is not the primary stimulus for activation because the increase in NO-levels only followed the intracellular increase of free calcium ions. However, the NO-signalling cascade is active in mammalian oocytes, and it is activated in fertilized or parthenogenetically activated oocytes (Hyslop et al., 2001). However, the NO-dependent signalling cascade is not sufficient for adequate oocyte activation. There was no exocytosis of cortical granules, and the development of parthenogenetic embryos did not proceed beyond the 4-cell stage (Petr et al., 2005a). Pig oocytes are activated by donors of nitric oxide only after continuous treatment lasting more than 10 hours (Petr et al., 2005a, 2007a) and the development of activated oocytes is seriously compromised (Petr et al., 2005a, 2007a).

The oscillatory (or pulsatile) but not continuous mode of signal is typical of living systems (Krsmanovic et al., 1996; Gudmundsson and Carnes, 1997; Terasawa et al., 2001; Porksen, 2002). Therefore, pulsatile treatments are thought to mimic physiological conditions more precisely (Hornick and Taylor, 1997; Medlicott and Tucker, 1999; Bussemer et al., 2001).

The activating stimulus brought by free calcium ions has a pulsatile nature because there are oscillations of intracellular levels of free calcium ions (Jones, 2005; Malcuit et al., 2005; Whitaker, 2006). The pattern of this oscillation, i.e. the duration and number of respective increases of intracellular levels of calcium ions and their summation, are very important because these parameters determine the development of activated mammalian oocytes (Ducibella et al., 2002; Ozil et al., 2006a; Toth et al., 2006). The pulsatile calcium signalling occurring at oocyte activation is very important for events occurring immediately after the penetration of sperm into the oocyte, e.g. for exocytosis of cortical granules, exit from the spontaneous meiotic block at the stage of metaphase II and entry into the first mitotic cleavage (Kline and Kline, 1992; Xu et al., 1994). However, pulsatile calcium signalling is important even for the subsequent development of preimplantation embryos and for their development to term (Malcuit et al., 2006a; Morozumi et al., 2006; Ozil et al., 2006b; Rogers et al., 2006).

The pulsatile pattern is also typical of the NO-signalling generated by nitric oxide synthases (Salerno, 2008). However, the role of the pulsatile signalling of NO in mammalian oocytes is only poorly understood. We demonstrated in our previous study that so-called pulsatile treatment of pig oocytes by NO-donor (+) –S-nitroso-N-acetylpenicillamine (SNAP), i.e. repeated short exposure of oocytes to SNAP followed by short culture in a SNAP-free medium, is significantly more effective than the same length of continuous treatment with NO-donor (Petr et al., 2009). However, the pulsatile treatment did not induce embryonic development comparable to the standard treatments of parthenogenetic activation using calcium ionophore (Petr et al., 2008).

There are wide spectra of supportive treatments which are known to enhance parthenogenetic activation, e.g. treatment using the protein synthesis inhibitor cycloheximide (Petr et al., 1996; Jílek et al., 2000; Mori et al., 2008) or treatment using the inhibitor of protein kinases 6-dimethyl aminopurine (Nussbaum and Prather, 1995; Jílek et al., 2001). The objective of this study was to enhance the efficiency of pig oocyte activation using pulsatile treatment with NO-donor combined with the inhibition of protein synthesis or inhibition of protein kinases. Cycloheximide was selected for the inhibition of protein synthesis. 6-dimethyl aminopurine was chosen for the inhibition of protein kinases.

MATERIAL AND METHODS

Isolation and culture of oocytes

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the oestrous cycle and transported to the laboratory within 1 h in a saline solution (0.9% sodium chloride) at 39°C. Fully-grown oocytes were collected from follicles by aspirating follicles that were 2–5 mm in diameter with a 20-gauge needle. Only oocytes with com-

pact cumuli were chosen for further study. Before culture, the oocytes were washed three times in a maturation culture medium.

The oocytes were cultured in a modified M199 medium (GibcoBRL, Life Technologies, Paisley, Scotland) containing sodium bicarbonate (0.039 ml of a 7.0% solution per millilitre of the medium), calcium lactate (0.6 mg/ml), sodium pyruvate (0.25 mg/ml), gentamicin (0.025 mg/ml) HEPES (1.5 mg/ml), 13.5 IU eCG:6.6 IU hCG/ml (P.G.600 Intervet, Boxmeer, Holland) and 10% of foetal calf serum (GibcoBRL, Life Technologies, Germany, Lot No. 40F2190F).

The oocytes were cultured for 48 hours in 3.5 cm diameter Petri dishes (Nunc, Roskilde, Denmark) containing 3.0 ml of the culture medium at 39°C in a mixture of 5.0% CO_2 in air.

Culture of the embryos

The activated oocytes were cultured in NCSU23 (Petters and Wells, 1993) medium in four-well Petri dishes (Nunc, Roskilde, Denmark), each well containing 1.0 ml of the culture medium. The eggs were cultured at 39°C in a mixture of 5.0% CO_2 in air for 7 days.

Evaluation of oocyte activation and embryo cleavage

At the end of the culture, the oocytes or embryos were mounted on slides, fixed with acetic alcohol (1:3, v/v) for at least 24 h, and stained with 1.0% orceine. The oocytes and embryos were examined under a phase contrast microscope. Activation was considered to have occurred if the oocytes were in the pronuclear stage. Oocytes remaining at metaphase II or arrested at anaphase II or at telophase II were not considered as activated. The number of nuclei in parthenogenetic embryos was counted at the end of the culture (i.e. 7 days after activation).

Design of the experiments

We performed three control experiments to test *in vitro* maturation of oocytes, to exclude spontaneous parthenogenetic activation of oocytes matured under our culture conditions, and to determine the ability of oocytes cultured under our conditions to be activated by an artificial stimulus (calcium ionophore).

For the control experiments for *in vitro* maturation, the oocytes were cultured for 48 hours in a maturation medium, and the stage of nuclear maturation was assessed at the end of the culture.

For the control experiment performed to exclude the possibility that spontaneous activation of oocytes occurred under our culture conditions, the oocytes were cultured *in vitro* in a maturation medium for 48 hours. Then they were denuded of cumulus cells and cultured for another 24 hours in a maturation medium without P.G. 600. The stage of nuclear maturation was assessed at the end of the culture.

For the control experiment designed to demonstrate the ability of oocytes cultured under our culture conditions to be activated and develop after parthenogenetic activation, we cultured oocytes for 48 hours in a maturation medium. The oocytes were then denuded of cumulus cells and treated with 25 μM calcium ionophore A23187 for 5 minutes, then cultured with 2mM 6-dimethyl aminopurine in the culture medium for 2 hours, and subsequently in the NCSU23 medium for 7 days. The development of parthenogenetic embryos was checked at the end of the culture.

Experiment 1 was performed to investigate the effect of sustained treatment of *in vitro* matured pig oocytes with NO donor SNAP and the effect of cycloheximide or 6-dimethyl aminopurine on activation after sustained treatment with SNAP. Based on our previous experiments (Petr et al., 2005a), we selected 2mM SNAP for oocyte treatment. The oocytes were cultured for 48 hours *in vitro* in the maturation medium described above. They were denuded of cumulus cells by repeated pipetting through a narrow glass pipette and cultured in the maturation medium M199 without P.G. 600 and supplemented with 2.0mM of SNAP. The oocytes were exposed to SNAP for 1, 2, 3, 4, 8, 12, or 24 hours. After treatment with SNAP, the oocytes were cultured in a SNAP-free maturation medium for the remaining interval to complete an overall 24-hour treatment.

Further, we investigated whether the treatment with the inhibitor of protein kinases 6-dimethyl aminopurine had a beneficial effect on activation using continuous SNAP-treatment. The oocytes were cultured for 48 hours *in vitro* in the maturation medium described above. They were denuded

of cumulus cells by repeated pipetting through a narrow glass pipette and cultured in the maturation medium M199 without P.G. 600 and supplemented with 2.0mM of SNAP. The oocytes were exposed to SNAP for 1, 2, 3, 4, 8, 12, or 24 hours. The culture medium was supplemented with 2mM of 6-DMAP for the initial two hours of the respective treatment. After treatment with SNAP, the oocytes were cultured in a SNAP-free maturation medium for the remaining interval to complete an overall 24-hour treatment.

Further, we investigated whether the treatment with the inhibitor of protein synthesis cycloheximide had a beneficial effect on activation using continuous SNAP-treatment. The oocytes were cultured for 48 hours *in vitro* in the maturation medium described above. They were denuded of cumulus cells by repeated pipetting through a narrow glass pipette and cultured in the maturation medium M199 without P.G. 600 and supplemented with 2.0mM of SNAP. The oocytes were exposed to SNAP for 1, 2, 3, 4, 8, 12, or 24 hours. The culture medium was supplemented with 10 μM of cycloheximide for the initial two hours of the respective treatment. After treatment with SNAP, the oocytes were cultured in a SNAP-free maturation medium for the remaining interval to complete an overall 24-hour treatment.

The ratio of activated oocytes to the total number of oocytes was determined at the end of the culture.

Experiment 2 was performed to investigate the effect of pulsatile treatment on matured pig oocytes by the NO-donor SNAP. The oocytes were cultured for 48 hours *in vitro* in a maturation medium. They were denuded of cumulus cells by repeated pipetting through a narrow glass pipette and subjected to pulsatile treatment with SNAP. The oocytes were then exposed to 2mM SNAP in the maturation medium M199 without P.G. 600 for 10, 20 or 30 minutes. This respective treatment was followed by culture of the oocytes in a SNAP-free culture medium for the same length of time. Culture in a SNAP-supplemented medium followed by culture in a SNAP-free medium was repeated to reach total exposure to SNAP for 60, 120, 180 or 240 minutes. The schedule for all pulsatile treatments was as follows (Figure 1).

After the respective pulsatile treatment, the oocytes were cultured in a SNAP-free medium to complete the 24-hour culture interval, i.e. experiments were completed 72 hours from the beginning of the culture.

Control experiments were performed to exclude the possibility that activation after pulsatile treatment was due to manipulation of the oocytes. *In vitro* matured oocytes (cultured for 48 hours *in vitro*) were transferred 24 times for 10 minutes between the dishes with a SNAP-free medium and then cultured for another 20 hours in a SNAP-free medium to mimic the most effective pulsatile treatment (i.e. 12 times 10-minute exposure to a 2mM SNAP + 10-minute exposure to a SNAP-free medium). In another control experiment, oocytes matured *in vitro* (cultured for 48 hours *in vitro*) were transferred 48 times for 10 minutes between the dishes with a SNAP-free medium to mimic the most extensive manipulation performed (24 times 10-minute exposure to a 2mM SNAP + 10-minute exposure to a SNAP-free medium). We determined the ratio of activated oocytes to the total number of oocytes at the end of the culture.

In Experiment 3 we tested the improvement of oocyte activation using NO-donor after the combination of this treatment with the inhibitor of protein synthesis. The arrangement of the experiment was the same as in Experiment 2. However, the SNAP-supplemented and SNAP-free culture medium contained 10 μM of cycloheximide for the initial two hours of treatment. Then each treatment followed the same schedule as in Experiment 2, i.e. cycloheximide-free medium was used.

60	10	10	6 times
	20	20	3 times
	30	30	2 times
120	10	10	12 times
	20	20	6 times
	30	30	4 times
180	10	10	18 times
	20	20	9 times
	30	30	6 times
240	10	10	24 times
	20	20	12 times
	30	30	8 times

Figure 1. Total exposure duration of culture duration of subsequent number of treatment time (minutes) with SNAP (min) culture in SNAP-free repetitions medium (min)

In Experiment 4 we tested the improvement of oocyte activation using NO-donor after the combination of this treatment with the inhibition of protein kinases. The arrangement of the experiment was the same as in Experiment 2. However, the SNAP-supplemented and SNAP-free culture medium contained 2mM of 6-dimethyl aminopurine for the initial two hours of treatment. Then each treatment followed the same schedule as in Experiment 2, i.e. 6-DMAP-free medium was used.

The developmental competence of parthenogenetic embryos was tested in Experiment 5. We selected treatments which resulted in an activation rate above 50% in Experiments 1, 2, 3 and 4.

The oocytes were cultured for 48 hours *in vitro*. Then they were denuded of cumulus cells by repeated pipetting through a narrow glass pipette and then treated with the respective treatment. Subsequently, oocytes were cultured in NCSU as mentioned above. Activation of oocytes using calcium ionophore (25 μM calcium ionophore A23187 for 5 min) and 6-DMAP (2mM 6-DMAP in the culture medium for 2 hours) (Jílek et al., 2001) was used as a positive control. After the respective activation treatment, the oocytes were cultured in the medium NCSU 23 for another 7 days.

Statistical Analysis

The data from all the experiments were subjected to statistical analysis. Each experiment was performed four times. Data were evaluated by ANOVA (Statistica 6.0). A *P* value of less than 0.05 was considered significant.

RESULTS

During the test of *in vitro* maturation, we observed that 95% of the oocytes had reached the stage of metaphase II at the end of 48-hour culture. The remaining oocytes were at the stage of metaphase I, anaphase I or telophase I. It is clear from another control experiment that *in vitro* matured oocytes are capable of being activated. After treatment of *in vitro* matured oocytes (cultured for 48 hours) by calcium ionophore A23187 and 6-dimethyaminopurine (see Material and Methods for details), the result was activation of 81.7% of the oocytes (n = 120). Control experiments demonstrated that under our culture conditions the aging of oocytes did not result in spontaneous parthenogenetic activation of oocytes in the first 24-hour culture of

Table 1. Effect of CHX and 6-DMAP on the activation of pig oocytes using continuous treatment with NO-donor SNAP; the oocytes were cultured for 48 hours and then exposed to SNAP for various lengths of time; after respective SNAP-treatment, the oocytes were cultured in a SNAP-free medium to complete the overall culture time of 24 hours; for the first two hours, the oocytes were treated according to the protocol mentioned above (medium supplement 0) or were cultured with cycloheximide (medium supplement CHX) or with 6-dimethyl aminopurine (medium supplement DMAP)

Medium supplement	Duration of SNAP treatment (hours)	Activated oocytes (%)
0	1	8.7 ± 2.6^{a}
0	2	14.3 ± 3.0^{ab}
0	3	16.1 ± 3.4^{abc}
0	4	21.2 ± 3.8^{abcd}
0	8	46.5 ± 3.6^{gh}
0	12	68.6 ± 2.9^{j}
0	24	70.4 ± 2.3^{j}
CHX	1	18.1 ± 2.5^{abcd}
CHX	2	34.2 ± 3.8^{cdefg}
CHX	3	43.7 ± 3.9^{fgh}
CHX	4	25.7 ± 3.1^{bcd}
CHX	8	42.3 ± 2.5^{efgh}
CHX	12	51.9 ± 2.7^{hi}
CHX	24	73.8 ± 2.2^{j}
DMAP	1	26.1 ± 4.1^{bcde}
DMAP	2	40.7 ± 3.9^{efgh}
DMAP	3	44.9 ± 3.1^{fgh}
DMAP	4	32.6 ± 3.5^{cdef}
DMAP	8	50.5 ± 3.6^{hi}
DMAP	12	63.8 ± 2.8^{ij}
DMAP	24	69.2 ± 3.7^{j}

^{a,b,c,d,e,f,g,h,i,j}statistically significant differences in the activation rate of the oocytes are indicated by different superscripts; the total number of oocytes for each experimental group was 120

in vitro matured oocytes. Oocytes cultured previously for 48 hours, then denuded of cumulus cells and cultured for another 24 hours did not exhibit a significant activation rate (0% of activated oocytes, n = 120).

In Experiment 1, the oocytes were activated using continuous treatment with NO-donor SNAP. The activation rate was 71.7% after 24-hour culture of oocytes with 2mM SNAP. When an exposure to SNAP was shortened to 12 hours, 68.6% of the oocytes were activated. When the oocytes were cultured continuously with SNAP for 8 hours, the activation rate was significantly reduced (46.5%). Further shortening of the time of continuous exposure of the oocytes to SNAP (4 h) resulted in a further significant reduction of the activation rate (24% of activated oocytes) compared to an 8-hour continuous treatment with SNAP (Table 1).

A significant increase in the activation rate was also observed when continuous treatment with NO-donor combined with CHX or 6-DMAP was prolonged. In oocytes subjected to continuous SNAP-treatment, cycloheximide treatment for the initial two hours of culture increased the activation rate only in oocytes treated with SNAP for 2 or 3 hours. The addition of cycloheximide increased the activation rate from 14 to 34% or from 16 to 43%, respectively.

A similar increase of activation rate was observed when continuous treatment with NO-donor was combined with the effects of protein kinase inhibitor 6-DMAP. In this case, the activation rate increased significantly only in the shortest SNAP-treatments. In a 1 hour culture with SNAP, the 6-DMAP increased the activation rate of oocytes from 9 to 26%. After 2-hour culture with NO-donor, the activation rate increased from 14 to 40% and after 3-hour culture with NO-donor the activation rate increased from 16 to 45%.

There was no significant positive effect of 6-DMAP or CHX after continuous treatments longer than 4 hours.

In Experiment 2, we tested pulsatile treatment of oocytes with 2mM NO-donor SNAP. The results are shown in Table 2. To reduce the overall exposure of the oocytes to the nitric oxide donor, we chose pulsatile regimens during which the oocytes were exposed to SNAP for 60, 120, 180 or

Table 2. The effects of pulsatile treatment with NO-donor SNAP on oocyte activation; oocytes were cultured for 48 hours and then exposed to the respective pulsatile treatments; after completion of the pulsatile treatment, the oocytes were cultured in a SNAP-free medium to complete the overall culture time of 24 hours

Total time of exposure (min)	Number of treatments	Medium supplement	Duration of respective treatment	Activation rate (%) mean ± SEM
60	2	0	30 min	23.3 ± 3.8^{abcd}
60	3	0	20 min	20.0 ± 3.6^{a}
60	6	0	10 min	26.7 ± 4.0^{abcd}
120	4	0	30 min	53.3 ± 4.6^{fg}
120	6	0	20 min	45.8 ± 4.5^{def}
120	12	0	10 min	63.3 ± 4.3^{g}
180	6	0	30 min	40.8 ± 4.5^{def}
180	9	0	20 min	54.2 ± 4.5^{fg}
180	18	0	10 min	48.3 ± 0.5^{ef}
240	8	0	30 min	37.5 ± 4.4^{bcdef}
240	12	0	20 min	32.5 ± 4.2^{abcde}
240	24	0	10 min	35.0 ± 4.3^{bcde}

^{a,b,c,d,e,f,g}statistically significant differences in the activation rate of the oocytes are indicated by different superscripts; the total number of oocytes for each experimental group was 120

240 minutes. Continuous treatments for 60, 120, 180 or 240 minutes were shown to be very ineffective in Experiment 1, where optimal treatment had to last at least 12 hours. However, in Experiment 2, we demonstrated that the pulsatile pattern of treatment could compensate for the short exposure of the oocytes to SNAP, and this mode of treatment appeared to be very effective. The effect of pulsatile treatment with SNAP was remarkable in groups exposed to SNAP for 120 or 180 minutes. When compared to continuous treatment with SNAP lasting for 120 or 180 minutes, all the checked pulsatile treatments were significantly more effective ($P < 0.05$). No significant difference was observed between the activation rate of oocytes treated with SNAP continuously for 240 minutes and oocytes treated with the pulsatile SNAP regimen with a total exposure of oocytes to NO-donor for 240 minutes. Pulsatile treatment with SNAP with a total time of exposure of 60 minutes was superior to continuous 60 minute treatment only with 6 pulses each lasting 10 minutes or 2 pulses each lasting 30 minutes.

Control experiments were performed to exclude the possibility that activation after pulsatile treatment was due to the manipulation of oocytes. We performed a mock treatment of *in vitro* matured oocytes in a SNAP-free medium. For this mock-manipulation we chose the most effective treatment (12 times 10-minute exposure to 2mM SNAP + 10-minute exposure to a SNAP-free medium, which resulted in 63.3% of activated oocytes) and the most extensive manipulation (24 times 10-minute exposure to 2mM SNAP + 10-minute exposure to a SNAP-free medium, which resulted in 35.0% of activated oocytes). None of the mock treatments induced activation in oocytes matured *in vitro* (0% activated, $n = 120$).

In Experiment 3, we tested the pulsatile treatment of oocytes with 2mM nitric oxide donor SNAP combined with the protein synthesis inhibitor cycloheximide. Pulsatile regimens identical to Experiment 2 were accompanied by cycloheximide treatment for the first two hours. The results are shown in Table 3.

Table 3. The effects of pulsatile treatment with NO-donor SNAP combined with cycloheximide on oocyte activation; the oocytes were cultured for 48 hours and then exposed to the respective pulsatile treatments; for the first two hours, the oocytes were cultured with cycloheximide (medium supplement CHX); after completion of the pulsatile treatment, the oocytes were cultured in a SNAP-free medium to complete the overall culture time of 24 hours

Total time of exposure (min)	Number of treatments	Medium supplement	Duration of respective treatment	Activation rate (%) mean ± SEM
60	2	CHX	30 min	38.3 ± 2.9^{ab}
60	3	CHX	20 min	83.0 ± 4.1^{e}
60	6	CHX	10 min	32.7 ± 3.0^{a}
120	4	CHX	30 min	49.3 ± 4.4^{bc}
120	6	CHX	20 min	70.8 ± 4.2^{d}
120	12	CHX	10 min	59.3 ± 4.1^{cd}
180	6	CHX	30 min	43.8 ± 3.6^{ab}
180	9	CHX	20 min	45.2 ± 2.9^{abc}
180	18	CHX	10 min	72.3 ± 1.8^{de}
240	8	CHX	30 min	38.5 ± 3.5^{ab}
240	12	CHX	20 min	44.5 ± 4.4^{ab}
240	24	CHX	10 min	39.0 ± 3.7^{ab}

^{a,b,c,d,e}statistically significant differences in the activation rate of the oocytes are indicated by different superscripts; the total number of oocytes for each experimental group was 120

Overall, the positive effect of cycloheximide treatment on the activation rate of oocytes treated under the pulsatile SNAP-regimen was significant ($P < 0.05$).

Similarly to Experiment 2, we demonstrated that a pulsatile pattern of treatment combined with cycloheximide treatment is superior to continuous treatment with NO-donor and cycloheximide, and this mode of treatment appeared to be very effective. The effect of pulsatile treatment with SNAP was remarkable in groups exposed to SNAP for 60, 120 or 240 minutes. In these groups, all checked pulsatile treatments were significantly more effective ($P < 0.05$) than continuous treatment. A significant difference was, however, observed between the activation rate of oocytes treated with SNAP continuously for 180 minutes and oocytes treated with a pulsatile SNAP regimen with total exposure of oocytes to the nitric oxide donor for 180 minutes after only 18 pulses each lasting 10 minutes.

When compared to pulsatile treatment without cycloheximide (i.e. the results of Experiment 2), the most effective treatment resulting in 83% of activated oocytes (treatment with 3 pulses of SNAP each lasting 20 minutes accompanied by a 2-hour cycloheximide treatment) was significantly higher than the best result of pulsatile treatment with SNAP only (63% of activated oocytes after 12 pulses of SNAP, each lasting 10 minutes). This further confirmed that cycloheximide had a positive effect on the activation rate after pulsatile treatment with the nitric oxide donor SNAP.

In Experiment 4, we tested pulsatile treatment of oocytes with 2mM SNAP combined with an inhibitor of protein kinase 6-DMAP. Pulsatile regimens identical to Experiment 2 were accompanied by 6-DMAP treatment for the first two hours. The results are shown in Table 4.

Overall, the positive effect of 6-DMAP treatment on the activation rate of oocytes treated under the pulsatile SNAP-regimen was significant ($P < 0.05$).

We demonstrated again that the pulsatile pattern of combined treatment is superior to continuous treatment with nitric oxide donor. The effect of pulsatile treatment with SNAP and 6-DMAP was

Table 4. The effects of pulsatile treatment with NO-donor SNAP combined with 6-DMAP on oocyte activation; the oocytes were cultured for 48 hours and then exposed to the respective pulsatile treatments; for the first two hours, the oocytes were cultured with 6-DMAP (medium supplement DMAP); after completion of the pulsatile treatment, the oocytes were cultured in a SNAP-free medium to complete the overall culture time of 24 hours

Total time of exposure (min)	Number of treatments	Medium supplement	Duration of respective treatment	Activation rate (%) mean ± SEM
60	2	DMAP	30 min	42.3 ± 2.7^{ab}
60	3	DMAP	20 min	78.0 ± 2.9^{d}
60	6	DMAP	10 min	46.7 ± 3.1^{b}
120	4	DMAP	30 min	44.3 ± 4.2^{ab}
120	6	DMAP	20 min	69.8 ± 4.1^{cd}
120	12	DMAP	10 min	62.3 ± 4.0^{c}
180	6	DMAP	30 min	47.8 ± 3.8^{b}
180	9	DMAP	20 min	41.2 ± 4.4^{ab}
180	18	DMAP	10 min	49.3 ± 2.9^{b}
240	8	DMAP	30 min	43.5 ± 3.3^{ab}
240	12	DMAP	20 min	39.5 ± 3.7^{ab}
240	24	DMAP	10 min	32.0 ± 3.9^{a}

^{a,b,c,d}statistically significant differences in the activation rate of the oocytes are indicated by different superscripts; the total number of oocytes for each experimental group was 120

remarkable in the group exposed to SNAP for 60 minutes. In this group, all checked pulsatile treatments were significantly more effective ($P < 0.05$) than continuous treatment. A significant difference was observed between the activation rate of oocytes treated with SNAP continuously for 120 minutes and oocytes treated with pulsatile SNAP regimen with total exposure of oocytes to NO-donor for 120 minutes after 6 pulses each lasting 20 minutes or 12 pulses each lasting 10 minutes.

When compared to pulsatile treatment without 6-DMAP (i.e. the results of Experiment 2) the most effective treatment resulting in 77% of activated oocytes (treatment with 3 pulses of SNAP each lasting 20 minutes accompanied by 2 hour 6-DMAP treatment) was significantly higher than the best result of pulsatile treatment with SNAP only (63% of activated oocytes after four pulses of SNAP each lasting 30 minutes). This indicated that 6-DMAP had a positive effect on the activation rate after pulsatile treatment with the nitric oxide donor SNAP.

Experiment 5 was performed to test cleavage and parthenogenetic development after the most effective treatments from Experiments 1, 2, 3 and 4. The modes of continuous or pulsatile treatment with an activation rate above 50% were selected. Oocytes activated by calcium ionophore and 6-DMAP were used as controls.

Continuous treatment with SNAP for 12 hours resulted in very poor cleavage, and none of the oocytes developed beyond the 4-cell stage. Additional treatment with cycloheximide or 6-DMAP did not result in any significant improvement. Cleavage rate and progression to the morula or blastocyst stage were significantly lower than in oocytes activated by ionophore and 6-DMAP.

Pulsatile treatment with SNAP improved the cleavage of oocytes. It induced development beyond the 4-cell stage and allowed the develop-

Table 5. Effect of pulsatile SNAP-treatment with cycloheximide (CHX) or 6-dimethyl aminopurine (DMAP) on the cleavage and development of parthenogenetic embryos; the oocytes were cultured for 48 hours and then exposed to the respective pulsatile treatments; after completion of the activation treatment, the oocytes were cultured in medium NCSU23 for 7 days

Type of treatment	Parthenogenetic embryos (%)		
	cleavage	morula	blastocyst
Calcium ionophore	51.2 ± 5.6^{a}	18.1 ± 2.8^{ab}	21.3 ± 4.3^{a}
SNAP – 12 hours continuously	6.7 ± 2.3^{c}	0.0	0.0
SNAP – 12 hours continuously + CHX	9.2 ± 3.1^{c}	0.0	0.0
SNAP – 12 hours continuously + DMAP	7.6 ± 4.3^{c}	0.0	0.0
SNAP – 4 pulses, 30 min each	37.5 ± 4.4^{ab}	18.3 ± 3.5^{a}	6.7 ± 2.3^{b}
SNAP – 12 pulses, 10 min each	40.8 ± 4.5^{ab}	10.0 ± 2.7^{a}	2.5 ± 2.7^{b}
SNAP – 9 pulses, 20 min each	38.3 ± 4.3^{ab}	5.8 ± 2.1^{b}	0.0
SNAP – 3 pulses, 20 min each + CHX	32.5 ± 3.2^{b}	9.2 ± 3.2^{ab}	0.0
SNAP – 6 pulses, 20 min each + CHX	41.1 ± 5.5^{ab}	12.3 ± 2.8^{ab}	3.1 ± 1.8^{b}
SNAP – 12 pulses, 10 min each + CHX	37.9 ± 3.9^{ab}	14.7 ± 2.4^{ab}	4.3 ± 1.5^{b}
SNAP – 18 pulses, 10 min each + CHX	39.8 ± 4.6^{ab}	8.6 ± 3.5^{ab}	2.6 ± 1.2^{b}
SNAP – 3 pulses, 20 min each + DMAP	31.3 ± 4.9^{b}	11.4 ± 3.2^{ab}	0.0
SNAP – 6 pulses, 20 min each + DMAP	35.4 ± 5.8^{b}	13.5 ± 3.3^{ab}	1.5 ± 1.4^{b}
SNAP – 12 pulses, 10 min each + DMAP	32.6 ± 4.9^{b}	8.7 ± 3.7^{ab}	3.6 ± 1.7^{b}

^{a,b}statistically significant differences between embryos at the same stage of development originating from the oocytes after different treatments (i.e. differences within columns) are indicated by different superscripts; the total number of oocytes for each experimental group was 120

ment of a limited number of morulae and blastocysts.

The regimens combining pulsatile SNAP-treatment with cycloheximide did not result in improved development. This was the same in the case of pulsatile SNAP-treatment combined with 6-DMAP. The results are shown in Table 5.

We can conclude that the addition of cycloheximide or 6-DMAP has positive effects on the activation rate after pulsatile treatment with the nitric oxide donor SNAP. However, it was ineffective with regard to parthenogenetic embryo cleavage and development.

DISCUSSION

In the present study, we demonstrated that the combination of pulsatile treatment by NO-donor with protein kinase inhibitor 6-DMAP or with protein synthesis inhibitor cycloheximide enhances the activation rate of *in vitro* matured pig oocytes. On the other hand, we did not confirm our hypothesis that additional treatment with cycloheximide or 6-DMAP could significantly increase the cleavage rate or could enhance development to the blastocyst stage. We confirmed our previous finding demonstrating that the pulsatile mode of treatment with NO-donor is more efficient than continuous treatment despite the same exposure time of both treatments (Petr et al., 2009). Continuous treatment did not induce the development of parthenogenetic embryos beyond the stage of 4-cell (Petr et al., 2005a). However, we observed that pulsatile treatment with NO-donor increases the cleavage rate of embryos, and some of these embryos continue their development up to the morula or blastocyst stage (Petr et al., 2009).

Changes in intracellular signalling by free calcium ions seem to be important for oocyte activation using NO-donor, and these changes are at least partially triggered by the cGMP-dependent signalling cascade (Kuo et al., 2000; Petr et al., 2005b, 2006, 2007a). These effects on calcium signalling could mimic the intracellular oscillation of free calcium ions which is induced by phospholipase C-zeta after fertilization of the oocyte by sperm (Jones et al., 1998; Parrington et al., 2002; Fujimoto et al., 2004; Konig et al., 2006).

These effects of NO-donor on calcium signalling were demonstrated even after the continuous treatment with low efficiency for the induction of embryo development (Petr et al., 2005b). On the other hand, our previous experiments showed that the activation of oocytes using a nitric oxide donor did not trigger all the signalling cascades that are necessary for oocyte parthenogenetic activation by calcium-dependent artificial stimuli. For example, the activation of oocytes using calcium ionophore depends on the active delta isotype of protein kinase C, but the oocyte activation using NO-donor is independent of this isotype of protein kinase C (Petr et al., 2007b).

Pulsatile treatment using NO-donor is clearly more effective than continuous treatment. The mechanisms which allow higher efficiency of pulsatile treatment are poorly understood. We cannot exclude the direct effect of NO on the oocyte activation. A decrease in the activity of mitogen activated protein kinases (MAP) and a decrease in the activity of the complex cyclin B-cdc2 are necessary for the exit of the oocyte from metaphase II and its activation (Dekel, 2005). Nitric oxide has direct effects on the activity of cyclins or cyclin-dependent kinases (Guo et al., 1998), and it also has a direct effect on the activity of MAP kinases in mammalian oocytes (Huo et al., 2005).

With regard to the fact that calcium ions are generally thought to be the primary signal for the exit from metaphase II and the activation of the mammalian oocyte (Yanagimachi, 1988; Hyslop et al., 2001; Leckie et al., 2003), we can conclude that pulsatile treatment with NO-donor significantly changes intracellular signalling with calcium ions, and this results in the improved parthenogenetic activation of oocytes. Coupling of NO-dependent and calcium-dependent signalling cascades can occur on many levels. Calcium ions activate calmodulin, which is known as an activator of nitric oxide synthases. At the same time calmodulin activates calmodulin-dependent kinase II, which plays a key role in oocyte activation (Lorca et al., 1993), and it also mediates the effects of pulsatile signalling of calcium ions on their target systems (Dupont and Goldbeter, 1998).

Generally, the combination of activation stimuli (ethanol, calcium ionophore, electrical pulses, ultrasonic waves) with cycloheximide or 6-DMAP increases the activation rate of oocytes and also increases the portion of oocytes developing to the blastocyst stage (Pressice and Yang, 1994; Nussbaum and Prather, 1995; Petr et al., 1996; Jílek et al., 2000, 2001; Mori et al., 2008). It is not clear why under our experimental design the effect of both inhibi-

tors increased the activation rate of oocytes but did not increase the proportion of oocytes developing to the blastocyst stage.

The beneficial effects of cycloheximide on parthenogenetic activation are usually attributed to interruption of the synthesis of short-lived proteins which are involved in the stabilization of the second meiotic block in oocytes maturing to the stage of metaphase II.

The beneficial effects of 6-DMAP on the parthenogenetic activation of mammalian oocytes probably have a more complex background. On the one hand, these effects are thought to be due to the inhibition of kinases responsible for the maintenance of the second meiotic block in oocytes maturing to the stage of metaphase II. (Collas et al., 1993; Pressice and Yang, 1994; Soloy et al., 1997; Swann and Lai, 1997). On the other hand, Im et al. (2007) demonstrated a very strong influence of 6-DMAP on intracellular calcium signalling in activated pig oocytes. In our study, we did not measure *de novo* protein synthesis, intracellular concentrations of free calcium ions or the activity of kinases which are responsible for the maintenance of the second meiotic block in oocytes matured to the stage of metaphase II. Therefore we can only speculate on which of these mechanisms may be involved in the positive effects of 6-DMAP on the activation rate of oocytes treated with pulsatile treatment of NO-donor.

We were disappointed by the very low proportion of embryos developing to the blastocyst stage after pulsatile treatment with NO-donor combined with cycloheximide or 6-DMAP. The reason for this failure is not clear.

The developmental competence of embryos is significantly influenced by intracellular signalling of free calcium ions which may be sufficient for oocyte activation but may be insufficient for the induction of all the events that are necessary for embryo development, reactivation of the embryonic genome or development to term (Malcuit et al., 2006b; Morozumi et al., 2006; Ozil et al., 2006b; Rogers et al., 2006). It is possible that the pulsatile treatment with NO-donor will not induce an adequate pattern of intracellular calcium signalling necessary for the successful preimplantation development. Neither can we exclude that pulsatile treatment with NO-donor has detrimental effects which are expressed during embryonic development, e.g. at morula compaction or differentiation of embryonic cells into the trophoblast and inner cell mass. Nitric oxide influences the expression of many genes in various cell types (Sparkman and Boggaram, 2004; Garban et al., 2005; Campbell et al., 2007) and may also affect the activity of many important molecules, e.g. by its ability to change the activity of many proteins by its direct nitrosylation (Xu et al., 1998). An excess of nitric oxide is able to affect or even to block embryonic development (Athanassakis et al., 2000; Tranguch et al., 2003; Kim et al., 2006). Depending on the circumstances, nitric oxide can act as an anti-apoptotic or pro-apoptotic factor (Stefanelli et al., 1999). However, our unpublished results demonstrated that pig oocytes exposed to pulsatile treatment with NO-donor express reduced symptoms of apoptosis when compared to oocytes exposed to continuous treatment with NO-donor, and more interestingly, even when compared to oocytes parthenogenetically activated by standard treatment using calcium ionophore. This indicates that at least with regard to DNA integrity, the pulsatile treatment with NO-donor is very gentle.

Acknowledgment

We thank Mrs. Lois Russell for her editorial assistance with this manuscript.

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Received: 2008–12–10
Accepted after corrections: 2009–03–13

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