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Inhibition of c-Jun N-terminal kinase (JNK) suppresses porcine oocyte ageing *in vitro*

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ABSTRACT: Oocyte ageing is a complex of processes that occur when matured *in vitro* oocytes are, after reaching the metaphase II stage, exposed to further *in vitro* culture. Aged oocytes remaining at the metaphase II stage undergo spontaneous parthenogenetic activation, or cellular death, through apoptosis (fragmentation) or lysis. The key factor in apoptotic pathway regulation is c-Jun-N-terminal kinase (JNK), stress kinase from the mitogene-activated protein kinase (MAPK) family. To investigate the effect of JNK inhibition on porcine oocytes ageing, cleavage rate, and embryonic development after parthenogenetic activation, DNA fragmentation, and pro-apoptotic factor Bax expression, we cultured *in vitro* matured oocytes for another 1–4 days in the presence of a JNK inhibitor. The inhibition of JNK significantly protected the oocytes from fragmentation (0% of fragmented oocytes under JNK inhibition vs. 13.4% of fragmented oocytes in the control group, 2nd day of ageing) and increased the percentage of parthenogenetically activated oocytes (82 vs 57.7%, 2nd day of ageing). The embryonic development of oocytes parthenogenetically activated after 24 h of ageing was influenced by JNK inhibition as well. The percentage of oocytes at the morula stage, after seven days of cultivation, was significantly increased when oocytes aged in the presence of a JNK inhibitor (42.5%) by comparison to the percentage of oocytes exposed to ageing in an inhibitor-free medium (23.3%). DNA fragmentation was significantly suppressed by JNK inhibition from the 1st day of ageing, but the expression of pro-apoptotic factor Bax in the oocytes was not influenced. On the basis of our experiments, we can conclude that JNK inhibition suppresses apoptosis and DNA fragmentation of aged oocytes and improves their embryonic development following the parthenogenetic activation. However, to completely eliminate all ageing related processes is insufficient.

Keywords: MAPK; DNA fragmentation; apoptosis; Bax

The effectiveness of biotechnology in the types of reproduction like *in vitro* fertilization or cloning, depends on the quality of *in vitro* matured oocytes. The quality of matured oocytes decreases when they are exposed to prolonged *in vitro* cultivation. Undesirable changes in oocyte quality, called ageing, hinder the utilization of aged oocytes.

Oocyte ageing is partly due to the changes in the M-phase promoting factor (MPF) and mitogene-activated protein kinase (MAPK) activity, which are necessary to maintain the meiotic arrest in metaphase II. Furthermore, ageing is associated with abnormalities of chromosomes, defects of the meiotic spindle, mitochondrial disorders, partial exocytosis

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of cortical granules, hardening of the zona pellucida, etc. (Kikuchi et al., 2000; Miao et al., 2009; Mammucari and Rizzuto, 2010 and others). Only a small percentage of oocytes remain in metaphase II arrest, while the majority of them gradually undergo parthenogenetic activation, apoptosis or lysis (Petrová et al., 2004). Spontaneous parthenogenetic activation and release from metaphase II is related to MPF inactivation, cyclin B degradation, and a decrease in MAPK activity (Sun and Nagai, 2003; Takakura et al., 2005). Ageing decreases MAPK phosphorylation and the cleavage rate of parthenogenetically activated oocytes (Ebeling et al., 2010). Conversely, a high level of MAPK activity induces apoptosis of oocytes (Sadler et al., 2004).

A key factor in apoptotic pathway regulation is c-Jun-N-terminal kinase (JNK) from the MAPK kinase family (Dickens et al., 1997; Bagowski et al., 2003). JNK is activated in somatic cells by stress factors such as ionizing radiation, DNA damage, and responses to inflammation cytokines. Activated JNK plays a key role in the initiation of apoptosis as a stress response of the cells (Kyriakis and Avruch, 2001; Verma and Datta, 2012) and has been observed in the oocytes of *Xenopus* (Mood et al., 2004), starfish (Sadler et al. 2004), mice (Baatout et al., 2007), and swine (Petrová et al., 2009).

The downstream molecules of JNK are pro-apoptotic factors from the Bcl-2 family of proteins (Bax, Bak, Bad) (Vlahopoulos and Zoumpourlis, 2004; Chu et al., 2009; Du Pasquier et al., 2011). The activated pro-apoptotic proteins permeabilize the mitochondrial outer membrane, thereby releasing cytochrome c which, through the activation of Apaf-1, regulates the activity of efector caspase 3, and this results in DNA fragmentation (Budihardjo et al., 1999; Antignani and Youle, 2006). The involvement of factors from the Bcl-2 protein family in apoptosis and DNA fragmentation was described in cattle (Yang and Rajamahendran, 2002), mouse and human (Guillemin et al., 2009), and *Xenopus* (Du Pasquier et al., 2011) oocytes; however, there is no evidence for it in porcine oocytes.

In our previous study (Petrová et al., 2009) we determined that JNK inhibition protected the porcine oocytes from apoptosis. Conversely, it increased the percentage of spontaneously parthenogenetically activated oocytes during the entire ageing period. The aim of the present study is to examine in detail the influence of JNK inhibition on porcine oocytes ageing, particularly on DNA integrity, pro-apoptotic factor Bax expression, cleavage rate, and early embryonic development after parthenogenetic activation.

MATERIAL AND METHODS

Isolation and culture of oocytes

Pig ovaries were obtained from a local slaughterhouse from pre-pubertal gilts (mostly a crossbreed of Landrace × Large White) of local farms at an unknown stage of the estrous cycle and transported to the laboratory in a saline solution (0.9% NaCl, 39°C) within 1 h. Fully-grown oocytes were collected from follicles by aspirating those measuring 2–5 mm in diameter. Only oocytes with compact cumuli were selected for further study. Before the cultivation, the oocytes were washed three times in a maturation culture medium.

The oocytes were cultured in Petri dishes (Nunc, Roskilde, Denmark) for 48 h (39°C, 5% CO_2) with 3.0 ml of modified M199 medium Gibco BRL (Life Technologies, Carlsbad, USA) containing sodium bicarbonate (32.5mM), calcium L-lactate (2.75mM), gentamicin (0.025 mg/ml), HEPES (6.3mM), 13.5 IU eCG: 6.6 IU hCG/ml (P.G. 600) (Intervet International B.V., Boxmeer, the Netherlands), and 10% (v/v) foetal calf serum Gibco BRL (Life Technologies). For further experiment, only oocytes with a maturation rate of at least 85% were used. Matured oocytes were denuded from cumulus cells, transferred to P.G. 600 free culture medium, and subjected to culture in the presence of an active form of JNK inhibitor (1,9-parazoloanthrone, 20μM) (Calbiochem, Darmstadt, Germany) or an inactive form of JNK inhibitor (N1-methyl-1,9-pyrazoloanthrone, 20μM) (Calbiochem) for another 1–4 days.

The active and inactive forms of JNK inhibitors were dissolved in DMSO. The final DMSO concentration in the culture medium was 0.2% (v/v). The effect of DMSO (0.2%) was examined, and no significant differences were found between the groups cultured in a free medium, those cultured in the medium with DMSO, and the group cultured in the medium with the inactive form of JNK inhibitor. The data are not shown.

Evaluation of oocytes

At the end of the cultivations, the oocytes and/or embryos were mounted on slides, fixed with acetic

alcohol (1 : 3, v/v; 24 h), and stained with 1.0% (w/v) orcein. Then they were examined under a phase-contrast microscope and classified into four groups: intact oocytes (oocytes at metaphase II, anaphase II or telophase II), activated oocytes (oocytes with pronuclei or embryos), fragmented oocytes (oocytes were designated as fragmented when fragmented "vesicles" were observed under the zona pellucida), and lysed oocytes (rupture of the cytoplasmic membrane and loss of the integrity of the oocyte were the criteria for lysis) (Petrová et al., 2009).

DNA integrity assessment

DNA integrity was evaluated by the alkaline version of the Comet assay (Singh et al., 1988) modified for porcine oocytes. Briefly, the oocytes were centrifuged at 14 000 *g* for 5 min to relocate the lipids to one pole of the oocytes, and then treated with pronase (5 min, 0.1% w/v) (Sigma-Aldrich, St. Louis, USA) at 38°C to remove the zona pellucida. Subsequently, the oocytes were quickly washed in M199 culture medium, mixed with low melting point (LMP) agarose (Amresco, Solon, USA), and spread over a layer of normal melting point (NMP) agarose attached to microscopic slides SuperFrost Plus (Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG., Braunschweig, Germany) precoated with 2% agarose (for details see Novotná et al., 2010). The slides were washed with acetone (2 × 5 min) to remove the high content of lipids typical of porcine oocytes and treated in a lysing solution (2.5M NaCl, 100mM EDTA, 10mM Tris, 0.16M DMSO, 0.016mM Triton X-100, 1% lauryl sarcosinate, pH 10 – all Sigma-Aldrich) for 1.5 h.

After lysis of the oocytes, the slides were equilibrated in alkaline buffer (0.3M NaOH, 1mM EDTA, pH 13) for 40 min to allow the DNA to unwind. For distribution of DNA fragments, electrophoresis was performed in a fresh alkaline buffer (20 min, 1.2 V/cm, 300 mA). Finally, the slides were neutralized in 0.4M Tris (pH 7.5), fixed in methanol (15 min), stained with 0.005% ethidium bromide (Sigma-Aldrich) for 7 min, washed in distilled water, and dried at room temperature. To prevent artificial damage to DNA, all steps preceding the lysis were performed under yellow light. Slides rehydrated in distilled water were observed under a VANOX BHS fluorescence microscope (Olympus, Tokyo, Japan). As a relatively high number of oocytes (up to 30–40%) still retained cytoplasm (Figure 1) which prevented quantifying the percentage of fragmented DNA from the total amount of nuclear DNA, we used a qualitative evaluation of DNA damage according to Mattioli et al. (2003), i.e. visual analysis of nuclei for the presence or absence of tails of DNA fragments escaping from the nucleus (Figure 1). Images were captured with CCD 1300B camera (VDS Vosskühler GmbH, Stadtroda, Germany) and saved using Lucia G 4.81 software.

Parthenogenetic activation of oocytes

In vitro matured oocytes were denuded from cummulus cells and subjected to further culture (24 h) in the presence of an active form of JNK inhibitor or its inactive form. The oocytes were later parthenogenetically activated (calcium ionophore A23187, 25μM, 20 min and 6-dimethyl aminopurin, 2mM, 2 h (both Sigma-Aldrich) (Jílek et al., 2001) and, after being washed, cultured for another 24 h to evaluate the activation rate, 48 h to evaluate the cleavage rate, and 7 days to evaluate embryonic development. The oocyte control group was activated immediately after maturation, without a prolonged culture.

Bax localization

After cultivation, the zona pellucida of oocytes was removed by pronase (0.1% w/v) (Sigma-Aldrich – here and after unless otherwise stated) and fixed (paraformaldehyde, 2.5% w/v, 60 min). After membrane permeabilization (PBS-BSA-Triton X-100, 2 h), the oocytes were incubated with a mouse anti-Bax antibody (1 : 100, 4°C overnight), and with goat anti-mouse IgG-FITC (1 : 100, PBS-BSA-Tween 20, 60 min, RT). Nonspecific staining was determined through incubation without a primary antibody. The chromosomes were stained with Hoechst 33258 (0.5% w/v, PBS-BSA, 10 min, RT). The oocytes were mounted on slides using a SlowFade antifade kit (Invitrogen, Carlsbad, USA) and observed under a laser-scanning confocal microscope Leica TCS SPE (Leica Microsystems, Wetzlar, Germany). Signal intensity was evaluated in cytoplasm, cortical region, and nucleus (activated oocytes) using digital image analysis program NIS-Elements (Version 4.0, 2011). No significant differences were found

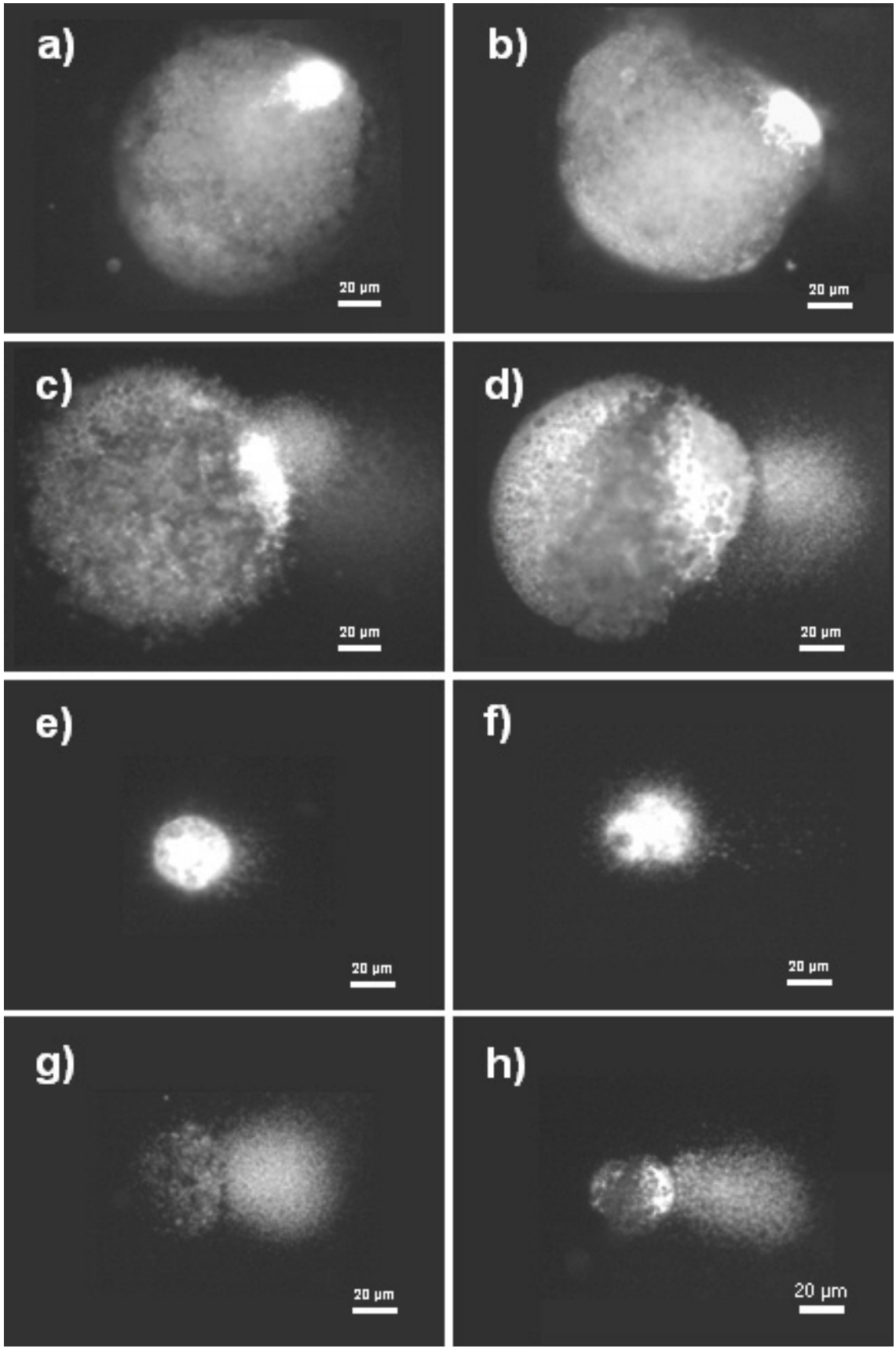

Figure 1. Porcine oocytes processed by the comet assay: (**a**–**d**) images with and (**e**–**h**) images without persisting cytoplasmic material assessed as negative (a,b,e,f) and positive (c,d,g,h) for DNA fragmentation

between signal intensity of cortical region and cytoplasm, thus the average signal intensity of each group was expressed as intensity of the whole cytoplasm and as a value relative to the average intensity of the MII oocyte group. No significant differences were found in nucleus signal intensity of activated oocytes between groups. The data are not shown. There were a minimum of 8 oocytes in each group.

Statistical analysis

Each experiment was performed at least four times. The differences among oocyte groups (days of ageing, control and experimental groups of oocytes) were evaluated by the Analysis of Variance (Scheffé's test) using the statistical software package STATISTICA (Version 8.0, 2007).

In the case of DNA integrity analysis, the data from all experiments were pooled in order to get a sufficient number of oocytes within each experimental group. Inter-group differences in the percentage of nuclei with fragmented DNA were then tested using the chi-squared test. The *P*-value of less than 0.05 was considered significant.

Experimental design

Effect of JNK inhibition on pig oocytes aged *in vitro*. *In vitro* matured oocytes were denuded of cumulus cells, placed in M199 culture medium, and cultured for another 1–4 days with a specific JNK inhibitor (1,9-pyrazoloanthrone, 20μM). The oocyte control group was cultured in the presence of an inactive form of JNK inhibitor (N1-methyl-1, 9-pyrazoloanthrone, 20μM). At the end of culture, the ratio of intact, activated, fragmented, and lytic oocytes was evaluated as described above.

Effect of JNK inhibition on the DNA fragmentation of ageing oocytes. Before the proper comet assay, the matured oocytes were cultured for 1–3 days in the presence of the active form of JNK inhibitor or its inactive form. In parallel, another group of oocytes was cultured in an inhibitor-free medium as a control.

Effect of JNK inhibition on cleavage rate and embryonic development of aged oocytes. *In vitro* matured oocytes were cultured for one more day in the presence of the active form of JNK inhibitor or its inactive form. Then, the oocytes were parthenogenetically activated and the activation rate was evaluated after 24 h. The cleavage rate was examined after additional 24 h in the culture. Embryonic development was evaluated after 7 days of culture. All assessed parameters were compared with the parameters of the oocyte control group, which was not exposed to ageing prior to parthenogenetic activation.

Effect of JNK inhibition on the subcellular localization of the pro-apoptotic factor Bax. Oocytes matured *in vitro* were cultured for another 1–3 days in the presence of the active form of

Table 1. Effect of JNK inhibition on porcine oocytes ageing

In vitro ageing (days)	Group of oocytes	Type of oocytes (% ± SEM)				
		metaphase II	activated	fragmented	lysed	*n*
1	inactive JNK inhibitor	97.7 ± 2.6^{A}	2.3 ± 2.6^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	120
	active JNK inhibitor	98.3 ± 2.6^{A}	1.7 ± 2.6^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	120
2	inactive JNK inhibitor	17.5 ± 4.4^{A}	57.7 ± 9.0^{A}	13.4 ± 3.8^{A}	11.3 ± 3.5^{A}	97
	active JNK inhibitor	14.0 ± 3.9^{A}	82.0 ± 11.2^{B}	0.0 ± 0.0^{B}	4.0 ± 2.0^{B}	100
3	inactive JNK inhibitor	4.9 ± 2.2^{A}	45.6 ± 7.6^{A}	19.4 ± 4.5^{A}	30.1 ± 5.9^{A}	103
	active JNK inhibitor	2.9 ± 1.7^{A}	79.6 ± 10.9^{B}	2.9 ± 1.7^{B}	14.6 ± 3.9^{B}	103
4	inactive JNK inhibitor	3.1 ± 1.8^{A}	38.8 ± 7.0^{A}	24.5 ± 5.3^{A}	33.7 ± 6.4^{A}	98
	active JNK inhibitor	2.0 ± 1.4^{A}	80.4 ± 11.0^{B}	1.0 ± 1.0^{B}	16.7 ± 4.2^{B}	102

oocytes were matured *in vitro* for 48 h and then further cultured for another 1–4 days in the presence of the active form of JNK inhibitor 1,9-parazoloanthrone (active JNK inhibitor) or an inactive form of JNK inhibitor N1-methyl-1,9-pyrazoloanthrone (inactive JNK inhibitor)

^{A,B}statistically significant differences between oocytes of control group (inactive JNK inhibitor) and the experimental group (active JNK inhibitor) within the same type of oocytes and day of ageing are indicated by different superscripts ($P < 0.05$)

JNK inhibitor, the inactive form of JNK inhibitor or in an inhibitor-free medium. The localization of Bax in oocytes was assayed as described above.

RESULTS

JNK inhibition showed an effect through the whole period of ageing. The percentage of parthenogenetically activated oocytes in the group cultured with an active form of JNK inhibitor was significantly higher than that in the group cultured with an inactive form of JNK inhibitor from the 2nd day of ageing. Simultaneously, the active form of JNK inhibitor suppressed fragmentation and lysis of the aged oocytes to a greater extent than did the inactive form of JNK inhibitor. The results are shown in Table 1.

In the next experiment, the effect of JNK inhibition on oocyte DNA fragmentation was studied during ageing within *in vitro* conditions. Figure 2 demonstrates that the presence of the active form of JNK inhibitor in the medium significantly decreased the percentage of oocytes with fragmented DNA when compared to oocytes ageing in the presence of the inactive form of JNK inhibitor or in a free medium (21.8% vs. 57.6% or 73.2%, respectively). However, this effect was observed only after the 1st day of prolonged culture, while no inter-group differences were detected after two and three days of prolonged culture (Figure 2).

The effect of JNK inhibition on the quality of ageing oocytes was tested by evaluating the activation rate, cleavage rate, and embryonic development of oocytes aged in the presence of the active form of JNK inhibitor. With respect to MII oocytes cultured for 1 day after maturation, the percentage of parthenogenetically activated oocytes was

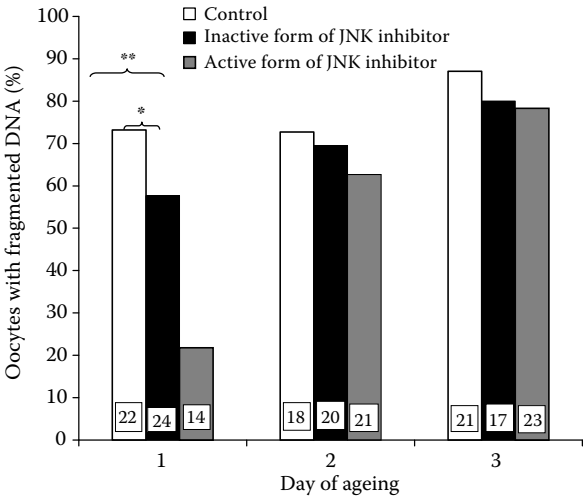

Figure 2. Effect of JNK inhibition on DNA fragmentation (%) in porcine oocytes ageing *in vitro.* Oocytes were matured *in vitro* for 48 h and then further cultured for 1–3 days, either in the pure medium (control) in the presence of an active form of JNK inhibitor (1,9-parazoloanthrone) or of an inactive form of JNK inhibitor (N1-methyl-1,9-pyrazoloanthrone)

The number in each column represents the total analyzed oocytes count within a given group; $^{*}P < 0.05$, $^{**}P < 0.01$

Table 2. Effect of JNK inhibition on porcine oocytes ageing *in vitro* and their subsequent activation, cleavage, and embryonic development (n = 120 per each group)

Stage of development/duration of culture after activation	Group of oocytes (%)		
	control (without ageing)	inactive JNK inhibitor (24 h of ageing)	active JNK inhibitor (24 h of ageing)
Activation/24 h	91.7^{A}	79.2^{B}	88.3^{A,B}
Cleavage/48 h	90.0^{A}	43.3^{B}	51.6^{B}
Stage of morula/7 days	16.7^{A}	23.3	42.5^{B}
Stage of blastocyst/7 days	30.0^{A}	0.0^{B}	0.0^{B}

oocytes were matured *in vitro* for 48 h to the MII stage and then cultured for further 24 h in the presence of an active form of JNK inhibitor 1,9-parazoloanthrone (active JNK inhibitor) or an inactive form of JNK inhibitor N1-methyl-1,9-pyrazoloanthrone (inactive JNK inhibitor). Both groups of oocytes were then parthenogenetically activated by calcium ionophore and 6-DMAP. Control group (control) was activated without ageing

^{A,B}statistically significant differences between oocytes of control groups (control and inactive JNK inhibitor) and the experimental group (active JNK inhibitor) within the same time of cultivation (in the same row) are indicated by different superscripts ($P < 0.05$)

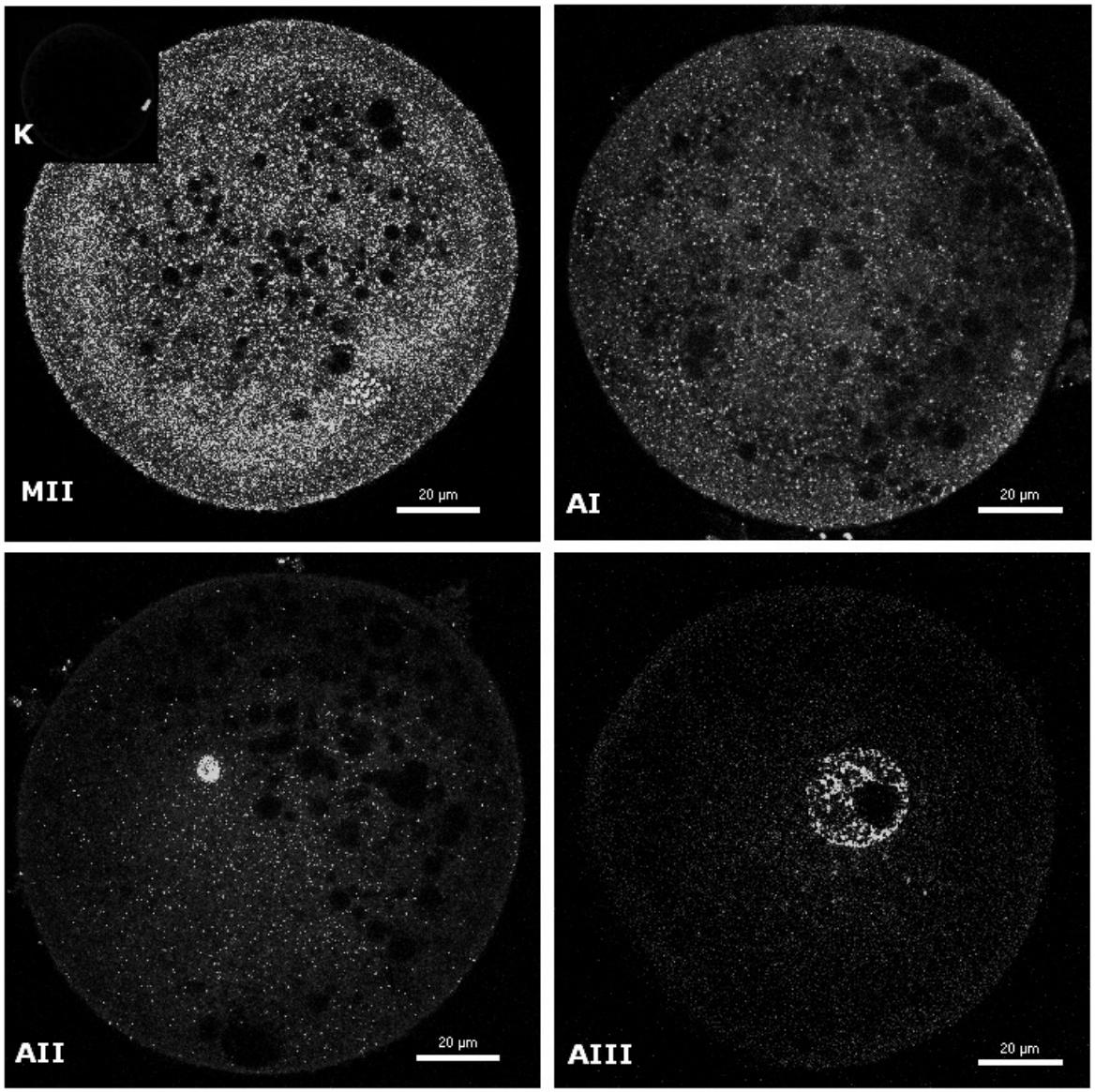

Figure 3. Subcellular localization of Bax in aged pig oocytes

K = matured MII oocyte (control oocyte exposed only to the second antibody), MII = matured MII oocyte, AI = aged oocyte (1 day after ageing), AII = aged oocyte (2 days of ageing), AIII = aged oocyte (3 days of ageing)
oocytes were stained with mouse anti-Bax and anti-mouse IgG-FITC (protein) and Hoechst 33258 (DNA)

lower in comparison with oocytes cultured for a prolonged period (Table 2). The percentage of activated oocytes in the group cultured during ageing in the presence of an inactive form of JNK inhibitor was significantly lower than that of the control group of oocytes, which were not too exposed to prolonged culture. However, the percentage of parthenogenetically activated oocytes in the group aged in the presence of the active form of JNK inhibitor was not significantly different from the percentage of activated oocytes in the control group, nor was it significantly different from the group aged in the presence of an inactive form of JNK inhibitor.

The cleavage rate of the oocytes after 24 h of ageing was significantly lower than that of the control group without ageing (51.6% – active form of JNK inhibitor or 43.3% – inactive form of JNK inhibitor vs. 90% – control). Compared to the inactive form, the active form of JNK inhibitor in culture medium did not increase the oocyte cleavage rate during ageing (43.3 vs. 51.6%); however, it significantly increased the percentage of the oocytes that reached the morula stage after

Table 3. Effect of JNK inhibition on subcellular localization of the pro-apoptotic factor Bax in ageing porcine oocytes

In vitro ageing (days)	Group of oocytes ($\bar{x}$ ± SEM)					
	control	*n*	inactive JNK inhibitor	*n*	active JNK inhibitor	*n*
0	$1.0 \pm 0.1^{A,a}$	10	–	–	–	–
1	$1.4 \pm 0.2^{A,b}$	11	$1.4 \pm 0.2^{A,b}$	9	$1.4 \pm 0.1^{A,b}$	13
2	$0.7 \pm 0.1^{A,c}$	9	$0.9 \pm 0.2^{A,c}$	9	$1.1 \pm 0.1^{A,c}$	10
3	$0.4 \pm 0.0^{A,c}$	8	$0.3 \pm 0.1^{A,d}$	9	$0.3 \pm 0.1^{A,d}$	9

oocytes were matured *in vitro* for 48 h to the MII stage (0 days of ageing) and then further cultured for another 1–3 days in the presence of an active form of inhibitor JNK 1,9-parazoloanthrone (active JNK inhibitor) or an inactive form of JNK inhibitor N1-methyl-1,9-pyrazoloanthrone (inactive JNK inhibitor) or in the inhibitor free culture medium (control). The signal intensity is expressed as a mean relative intensity based on MII oocytes (control, 0 days of ageing)

[A]statistically significant differences between oocytes of control groups (control and inactive JNK inhibitor) and the experimental group (active JNK inhibitor) within the same day of ageing ($P < 0.05$)

[a–d]statistically significant differences between oocytes of the same group during ageing are indicated by different superscripts ($P < 0.05$)

7 days of cultivation (Table 2). Interestingly, only control group oocytes that were not exposed to prolonged culture reached the blastocyst stage.

The intracellular expression of the pro-apoptotic factor Bax, influenced by the active form of JNK inhibition, expressed as mean fluorescence signal intensity in the whole cytoplasm of oocytes, was evaluated by picture analysis (Table 3, Figure 3). The highest mean intensity in the oocytes was found after the 1st day of ageing. The mean signal intensity gradually diminished during the following 2 days of ageing. However, the presence of the active form of JNK inhibitor in the culture medium did not influence the expression of the pro-apoptotic Bax factor.

DISCUSSION

In our study, we observed the dynamics of the effect of JNK inhibition on the morphological manifestation of ageing of porcine oocytes under *in vitro* conditions. The inhibition of JNK suppresses the apoptosis and lysis of oocytes and the manifestation of the effect is statistically significant from the 2nd day of oocyte ageing and persists in oocytes which were subjected to ageing for the duration of four days as well. The inhibition of JNK in our experiments significantly suppressed the fragmentation of DNA in porcine oocytes, but only during the 1st day of ageing. From the 2nd day of ageing, the differences were not statistically significant.

JNK is one of the main regulatory factors of apoptosis in somatic cells (Dhanasekaran and Reddy, 2008; Plotnikov et al., 2011). Caspase-3 and caspase-9 belong among the downstream molecules and their activation leads to DNA fragmentation (Chen et al., 2003). The results of our experiments suggest that JNK-regulated signalling is also involved in the control of apoptosis in porcine oocytes.The JNK inhibition did not manage to completely suppress the DNA fragmentation. JNK-induced signalling is probably not the only one involved in oocyte apoptosis induction.

One of the activating stimuli of JNK is oxidation stress and the increase of oxygen-free radicals (ROS) level (Levkovitz et al., 2005; Antignani and Youle, 2006). Some authors consider oxidation stress to be one of the main causes of ageing (Cui et al., 2011) and the ROS level continously increases during the ageing process (Miao et al., 2009). ROS induce apoptosis not only by activating JNK and the subsequent release of cytochrome c from mitochondria, but also through endoplasmic reticulum (ER) (He et al., 2008) which is closely related to maintaining intracellular calcium homeostasis (Petr et al., 2001). Increased ROS level affects calcium signalling and leads to the increase of cytoplasmic concentration of calcium cations (Takahashi et al., 2003).

During our experiments, the ratio of spontaneously parthenogenetically activated oocytes increased in the group of oocytes ageing in the presence of the active form of JNK inhibitor. For

the MII block output, the increase of cytoplasmic concentration of calcium cations is crucial (Wassarman, 1988; Wang et al., 1999; Sedmíková et al., 2003, 2006). Therefore, the increase of parthenogenetically activated oocytes ratio may be caused by the fact that blocking the apoptotic signalling controlled by JNK induces spontaneous parthenogenetic activation of the oocytes by increasing the levels of calcium cations.

JNK signalling leads to DNA fragmentation through pro-apoptotic factors Bax and Bad which, by mitochondrial membrane permeabilization, releases cytochrome c (Vlahopoulos and Zoumpourlis, 2004; Levkovitz et al., 2005; Antignani and Youle, 2006; Chu et al., 2009). By means of Bax (but not Bad) it takes part in controlling the JNK apoptosis in *Xenopus* oocytes. In the oocytes the JNK-induced apoptosis is manifested by overexpression of Bax protein (Du Pasquier et al., 2011). We found no differences in the expression of Bax in porcine oocytes dependent on an active JNK signalling pathway in our experiments.

It seems that Bax does not take part in apoptosis induction in porcine oocytes. It is also possible that in porcine oocytes, in contrast to *Xenopus* oocytes, the main downstream molecule of JNK is the pro-apoptotic factor Bad, similarly to some somatic cells (Bhakar et al., 2003). The presence of the active form of JNK inhibitor during porcine oocytes ageing improves their development competence, even though the percentage of parthenogenetically activated oocytes in the cleavage stage is lower. The ratio of parthenogenetically activated oocytes among ageing oocytes decreases proportionally to the duration of ageing. The ability of embryonic development in activated oocytes also decreases (Ebeling et al., 2010) and JNK apparently no longer has an influence.

The lower ratio of parthenogenetically activated oocytes may also be influenced by the function of ER affected by oxidation stress. Suppression of ER stress improves the embryonic development of porcine oocytes, due to a blockage of apoptosis induced by ER stress (Zhang et al., 2012). Greater development competency, when exposed to the effects of a JNK inhibitor, is apparently influenced by the suppression of DNA fragmentation by means of caspase activation. The course of early embryonic development is closely related to apoptosis (Antunes et al., 2010) and the effect of caspase-3 inhibitor on a higher ratio of blastocysts in parthenogenetically activated embryos has been described in porcine (Coutinho et al., 2011). The inhibition of JNK seems to be a possible way of preventing detrimental processes accompanying the ageing of mammalian oocytes, thus it could be used for improving the quality of *in vitro* matured oocytes in biotechnologies.

CONCLUSION

The results of our experiments show that JNK inhibition suppresses apoptosis manifestation in ageing porcine oocytes and improves their early embryonic development following the parthenogenetic activation. However, sole JNK inhibition does not manage to eliminate all the processes connected with oocyte ageing during prolonged cultivation under *in vitro* conditions.

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