Chemical manipulation of glucose metabolism in porcine oocytes: effects on nuclear and cytoplasmic maturation *in vitro*

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Abstract

The objectives of this study were to manipulate metabolism of glucose through glycolysis and the pentose phosphate pathway (PPP) in porcine oocytes during *in vitro* maturation, and determine the effects of this manipulation on meiotic progression, intracellular glutathione (GSX) concentrations and embryonic development. Cumulus-oocyte complexes isolated from abattoir ovaries were matured (40–44 h) in Purdue Porcine Medium for maturation alone (control) or supplemented with pyrroline-5 carboxylate (PC, 0.1 μ M; PPP stimulator), diphenyleneiodonium (DPI, 0.1 μ M; PPP inhibitor), dinitrophenol (DNP, 10 μ M; glycolytic stimulator), hexametaphosphate (HMP, 100 μ M; glycolytic inhibitor), PC + HMP or DNP + DPI. At the conclusion of *in vitro* maturation, cumulus cells were removed and oocytes were randomly allocated for analysis of GSX, metabolism and nuclear maturation, or *in vitro* fertilization and embryo culture. Both DPI and DNP + DPI decreased ($P \leq 0.05$) the activity of glycolysis and the PPP, increased ($P \leq 0.05$) the percentage of immature oocytes, and decreased ($P \leq 0.05$) the proportion of mature oocytes compared with control oocytes and oocytes from the other treatments. Embryonic development (cleavage and blastocyst stage) and the intracellular content of GSX were also decreased ($P \leq 0.05$) following exposure to DPI or DNP + DPI compared with control oocytes and oocytes from the other treatments. Oocyte metabolism, nuclear maturation, GSX content and embryonic development were unaffected ($P > 0.05$) following exposure to PC, DNP, HMP or PC + HMP. Our results suggest that metabolism of glucose through the PPP and/or glycolysis plays a key role in the control of nuclear and cytoplasmic maturation of porcine oocytes *in vitro*.

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Introduction

Oocyte maturation encompasses a variety of cellular changes necessary for normal fertilization and successful embryonic development. These changes can be roughly categorized as nuclear or cytoplasmic maturation (Eppig 1996). Nuclear maturation refers to the progression of the chromatin through meiosis from the germinal vesicle (GV) stage to arrest at the second metaphase (MII). In contrast, cytoplasmic maturation includes all of the other changes within the oocyte, such as accumulation of mRNA and protein, reorganization of the cytoskeleton and organelles, and changes in cellular metabolism. Although nuclear and cytoplasmic maturation are often discussed separately, the numerous processes involved are interrelated and critical for successful embryonic development.

Similarly to other cells, oocytes metabolize glucose via glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid cycle (mice, Downs & Utech 1999; cats, Spindler *et al.* 2000; cattle, Rieger & Loskutoff 1994,

Krisher & Bavister 1999; sheep, O'Brien *et al.* 1996; pigs, Durkin *et al.* 2001). Resumption of meiosis is associated with elevated activity of glycolysis and the PPP, as well as increased activity of hexokinase (glycolysis and PPP), phosphofructokinase (glycolysis), and glucose-6 phosphate dehydrogenase (PPP) within the cytoplasm of the oocyte (Tsutsumi *et al.* 1992, Downs *et al.* 1996, Downs & Utech 1999, Cetica *et al.* 2002). In mice, gonadotropin-induced meiosis is dependent on the presence of glucose (Fagbohun & Downs 1992, Downs & Mastropolo 1994). Increased metabolism of glucose through one or more metabolic pathways also occurs simultaneously with the progression of meiosis to MII in oocytes from cats (Spindler *et al.* 2000) and cattle (Rieger & Loskutoff 1994, Steeves & Gardner 1999). In primate oocytes, glucose is necessary for cytoplasmic maturation, although nuclear maturation can occur in the absence of carbohydrates (Zheng *et al.* 2001). In addition, elevated glucose metabolism in mature oocytes is correlated with, and predictive of, improved embryonic development in cats and cattle (Krisher & Bavister 1999,

Spindler *et al.* 2000, Spindler & Wildt 2002). Similarly, oocytes matured *in vitro* from cats and pigs metabolize significantly less glucose and have lower developmental potential than oocytes matured *in vivo* (Spindler *et al.* 2000, Durkin *et al.* 2001). These findings highlight the importance of glucose metabolism in oocyte maturation and the interactions between nuclear and cytoplasmic maturation.

Another important aspect of oocyte maturation is the accumulation of glutathione (GSX). This tripeptide (Glu-Cys-Gly) is involved in many aspects of oocyte physiology including cumulus cell expansion, sperm decondensation, male pronuclear formation and embryonic development (cattle, de Matos *et al.* 1995, Sutovsky & Schatten 1997, Furnus *et al.* 1998; sheep, de Matos *et al.* 2002; pigs, Yoshida 1993, Yoshida *et al.* 1993; hamsters, Zuelke *et al.* 2003). Both steps in the synthesis of GSX, addition of glutamate and cysteine and the addition of glycine to this dipeptide, are dependent on ATP, and therefore dependent on the metabolism of the oocyte (Stryer 1995). Once synthesized, GSX cycles between the reduced (GSH) and oxidized (GSSG) form through the actions of GSX reductase and GSX peroxidase. The peroxidase transfers electrons from GSH to oxidized molecules within the cytoplasm, minimizing the actions of various oxidative stressors and resulting in the production of GSSG (Guérin *et al.* 2001). The cellular pool of GSH is maintained by reduction of GSSG to GSH by GSX reductase, as well as further GSH synthesis. The reduction of GSSG requires NADPH, a product of the PPP. Therefore, intracellular concentrations of GSH, and the many processes affected by GSH, are tightly linked to the metabolic activity of the oocyte.

Glucose metabolism and GSX accumulation are important for successful oocyte maturation and embryonic development in pigs (Abeydeera *et al.* 2000, Durkin *et al.* 2001, Brad *et al.* 2003a,b, Herrick *et al.* 2003a). However, the interactions between nuclear maturation, glucose metabolism, GSX concentrations and embryonic development are unclear. The objective of this study was to elucidate these mechanisms in porcine oocytes. Chemical agents were used to alter the activity of glycolysis and/or the PPP during *in vitro* maturation of porcine oocytes, and then nuclear maturation, oocyte metabolism, GSX concentrations and embryonic development were evaluated. We hypothesized that increased glucose metabolism would lead to improved nuclear (MII) and cytoplasmic (intracellular GSX and developmental potential) maturation.

Materials and Methods

Chemicals and media preparation

Unless specified otherwise, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). All media contained 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 ng/ml amphotericin (GibcoBRL Products,

Gaithersburg, MD, USA). Maturation, fertilization, and culture media were freshly prepared from stock solutions for each replicate, filtered (0.22 µm, Millex GV; Millipore Corp., Bedford, MA, USA) immediately before use, and equilibrated at 38.7 °C in the appropriate gas atmosphere.

Oocyte recovery and *in vitro* maturation

Ovaries from pre-pubertal gilts were collected at a local abattoir and transported to the laboratory in 0.9% (w/v) NaCl (30–34 °C). Ovaries were washed with fresh saline and 3–8 mm follicles were aspirated with an 18 gauge needle attached to a vacuum pump. Oocytes surrounded by a minimum of two layers of cumulus cells with a uniform cytoplasm were selected and washed twice in Hepes-buffered synthetic oviductal fluid (SOF-Hepes) containing 0.3% (w/v) BSA, 1.0 mM glutamine, and both essential (1 ×) and nonessential (1 ×) amino acids (ICN Biomedicals, Costa Mesa, CA, USA). Cumulus-oocyte complexes (COCs) were then washed and cultured (5% CO₂ in air) for 40–44 h in 500 µl maturation medium under 500 µl mineral oil in a four-well plate (50–60 COCs/well; Nunclon, Nalge Nunc Intl, Rochester, NY, USA). Maturation medium was Purdue Porcine Medium (PPM) modified for oocyte maturation (PPM Mat; Stroble *et al.* 2002, Herrick *et al.* 2003b), containing 0.6 mM cysteine, 0.5 mg/ml hyaluronate (MAP5; Vetrepham Inc., Athens, GA, USA), 10 ng/ml epidermal growth factor, 0.01 U/ml porcine luteinizing hormone and follicle-stimulating hormone (FSH) (Sioux Biochemical, Sioux Center, IA, USA), and 1 × MEM vitamins (ICN Biomedicals). After oocytes were placed into maturation medium (PPM Mat), no further selection was performed throughout the experiment.

In vitro fertilization

At the conclusion of maturation, cumulus cells were removed by agitating COCs in 100 µl SOF-Hepes with 0.01% (w/v; 80–160 U/ml) hyaluronidase for 2–3 min using a vortex mixer. Cumulus-free oocytes were then washed twice in SOF-Hepes, and randomly allocated for fertilization, metabolism or GSX analysis. For fertilization, oocytes were washed twice in PPM fertilization medium (PPM Fert.2) containing 44 mM bicarbonate, 0.2% (w/v) fatty-acid free BSA, and 2.0 mM caffeine (Herrick *et al.* 2003b) and placed into 50 µl drops of PPM Fert.2 under 4 ml mineral oil (20 oocytes/drop; Falcon 1008; Becton Dickinson Labware, Franklin Lakes, NJ, USA).

Frozen, ejaculated sperm was thawed in 2.0 ml of Ca²⁺-free phosphate-buffered saline (PBS, Gibco), and processed through a 45%:90% gradient of Percoll by centrifugation for 20 min at 1000 g. The resulting sperm pellet was washed in 5.0 ml PBS containing 0.1% (w/v) Fraction V BSA by centrifuging for 5 min at 1000 g. Sperm were diluted (1 × 10⁶ sperm/ml) in fertilization medium (PPM Fert.2), and added (50 µl) to fertilization drops containing oocytes for a final sperm concentration of

5×10^5 sperm/ml. Gametes were coincubated for 5 h in 5% CO₂ in air.

Embryo culture

Following coincubation, zygotes were washed three times in PPM culture medium 1 (PPM1) with 0.4% (w/v) crystallized BSA (Serologicals Proteins, Inc., Kankakee, IL, USA) to remove loosely bound sperm, and cultured in 50 μ l drops PPM1 (ten zygotes/drop; Falcon 1007) under 10 ml mineral oil in 5% CO₂, 10% O₂, 85% N₂ (Durkin *et al.* 2002, Stroble *et al.* 2002, Herrick *et al.* 2003b). After 3 days of culture, embryos were washed three times in PPM2 (0.4% crystallized BSA) and placed into 50 μ l drops of PPM2 for an additional 3 days of culture (5% CO₂, 10% O₂, 85% N₂). The number of embryos that cleaved was determined at the time of moving into PPM culture medium 1 (PPM2). On day 6 of culture, the proportion of oocytes that developed to the blastocyst stage was determined and all blastocysts were fixed and stained to determine total cell number. Briefly, blastocysts were counterstained with 0.1% (w/v) trypan blue in 2.3% (w/v) citrate for 1 min and then stained with 0.01 mg/ml Hoechst 33342 for 5 min (Pursel *et al.* 1985). Cells were counted under fluorescent illumination at $\times 400$.

Assessment of glucose metabolism and nuclear maturation

Following maturation and cumulus-cell removal, oocytes were washed twice in a simple, PPM-based metabolism medium containing 1.2 mM unlabelled glucose, 1.5 mM lactate, 0.1 mM pyruvate, and 0.5 mg/ml hyaluronate. Glycolysis and PPP were simultaneously measured using [5-³H]glucose (0.021 mM, 0.25 μ Ci/ μ l; Perkin-Elmer NEN Life Sciences Inc., Boston, MA, USA) and [1-¹⁴C]glucose (0.482 mM, 0.0265 μ Ci/ μ l; American Radiolabeled Chemicals Inc., St Louis, MO, USA) respectively (O'Fallon & Wright 1986, Rieger & Guay 1988, Krisher & Bavister 1999). Radiolabeled glucose was dissolved in metabolism medium and equilibrated at 38.7°C in 5% CO₂ in air. Oocytes were collected in 2 μ l metabolism medium, combined with 2 μ l medium containing radiolabeled glucose, and placed in the cap of a 1.5 ml microcentrifuge tube in a single, 4 μ l drop. The cap containing the oocyte was then replaced onto a 1.5 ml microcentrifuge tube filled with 1.5 ml 25 mM NaHCO₃ and the remaining air space gassed with 5% CO₂ in air as the lid was secured. Drops not containing an oocyte (blanks) were included in each assay to monitor nonspecific release of ¹⁴C and ³H. In addition, the total amount of radioactivity per tube was determined in each assay by thoroughly mixing metabolism drops without oocytes with the NaHCO₃ trap. After a 3 h incubation (38.7°C), the caps were removed, 1 ml NaHCO₃ trap was added to a scintillation vial containing 200 μ l 0.1 M NaOH, and the vials stored for 24 h

at 4°C. Ten milliliters of scintillation fluid (Ecolite; ICN) were added to each vial, vials were shaken briefly, and then held at 25°C for at least 24 h before measuring radioactivity on a liquid scintillation counter. When counts for one or both of the pathways were less than the mean value of the blanks, the oocyte was considered nonviable and not included in the analysis. Oocytes were recovered from the caps and fixed (6:3:1, ethanol:acetic acid:chloroform) for a minimum of 24 h. Chromatin was then stained with 1% (w/v) orcein in 45% (v/v) acetic acid and observed with differential interference contrast microscopy ($\times 400$) to evaluate nuclear maturation. Oocytes were classified as containing a GV, undergoing GV breakdown (GVBD), containing condensed chromatin (CC), or having reached metaphase I (MI), anaphase I (AI), telophase I (TI) or MII. Oocytes arrested at GV, GVBD or CC were considered immature (Motlik & Fulka 1976, Funahashi *et al.* 1997), while oocytes reaching AI, TI or MII were considered mature.

Determination of intracellular GSX concentrations

Groups of cumulus-free oocytes were washed three times in 0.4 M NaPO₄ buffer containing 10 mM EDTA, taken up in 5 μ l of this buffer, transferred to 1.5 ml microcentrifuge tubes, and stored at -80°C . Aliquots (5 μ l) of the buffer were also frozen in each replicate to serve as blanks. GSX assays utilizing the dithiobisnitrobenzoic acid (DTNB)-GSSG reductase recycling reaction were performed as described by Baker *et al.* (1990) and modified for use with embryos by Aréchiga *et al.* (1995). Briefly, 45 μ l water were added to each tube and the entire contents transferred to a well of a 96 well microtiter plate. GSX standards (50 μ l water containing 0.4–200 pmol GSH) were also added to individual wells. DTNB (1 mM), NADPH (1 mM) and GSX reductase (200 U/ml) were dissolved in assay buffer (200 mM NaPO₄, 1 mM EDTA, pH 7.5) and combined (5 ml DTNB, 5 ml NADPH, 5.75 ml assay buffer, and 0.1 ml or 20 U GSSG reductase). One hundred microliters of this reaction mixture were added to each well, and the plate immediately loaded into a microtiter plate reader. Formation of thionitrobenzoic acid was monitored by measuring absorbance at 410 nm at 30 s intervals (0–6 min of incubation). The rate of change of absorbance per minute was determined for each well and concentrations of GSX in the samples (pmol/oocyte) were computed from a standard curve (rate of change of absorbance vs pmol GSX/well) and the number of oocytes in each well. Medium blanks in this assay were equivalent to 0.55 ± 0.12 pmol GSX/well or 0.055 ± 0.012 pmol GSX/oocyte. Since these effects accounted for only a small portion of the measured GSX values and were not different ($P > 0.05$) between treatments, sample values were not corrected to compensate for blank effects. Due to the presence of NADPH and GSSG reductase, this assay measures both GSSG and GSH concentrations and all values are reported as pmol GSX/oocyte.

Chemicals for metabolic manipulation

A variety of chemicals were tested to find a stimulator and inhibitor of glycolysis and the PPP. The electron acceptors methylene blue (1000, 100, 10, 1, 0.1, 0.01 μM), phenazine ethosulfate (10, 1, 0.1, 0.01, 0.001 μM), and pyrroline-5 carboxylate (PC; 250, 100, 10, 1, 0.1 μM) were tested as potential PPP stimulators. Diphenyleneiodonium (DPI; 100, 10, 1, 0.1, 0.01, 0.001 μM), an inhibitor of NADPH oxidase, and 6-aminonicotinamide (25, 10, 1, 0.1, 0.01 μM) an inhibitor of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were tested as possible PPP inhibitors. Sodium azide (10 μM) and 2,4-dinitrophenol (DNP; 10 μM), an inhibitor and uncoupler of oxidative phosphorylation respectively were evaluated as possible glycolytic stimulators. Hexametaphosphate (HMP; 5, 1, 0.5, 0.1, 0.01 mM (Zollner 1999)) and EDTA (10 μM) were tested as glycolytic inhibitors.

Initially, COCs were exposed to four doses (ten COCs/dose) of each chemical, except sodium azide, DNP and EDTA, during maturation. A second replicate was then performed with an additional four doses (ten COCs/dose) of each chemical that overlapped the most promising doses from the first replicate. Sodium azide, DNP and EDTA were tested (two replicates of ten COCs/chemical/replicate) at concentrations used previously in murine (Lane & Gardner 2001) or bovine (Thompson *et al.* 2000, Rieger *et al.* 2002) embryos. At the conclusion of maturation, cumulus cells were removed and the metabolic activity of each oocyte was assessed. Oocyte metabolism was evaluated instead of COC metabolism because it is the metabolism of the oocyte, not the COC or cumulus cells, that has been correlated with developmental competence (O'Brien *et al.* 1996, Krisher & Bavister 1999, Spindler *et al.* 2000, Spindler & Wildt 2002). The concentration of each chemical that had the most profound and consistent effect on metabolism was selected (data not shown). Stock solutions of each chemical were prepared in culture grade (18.2 MΩ) water, and 1-20 μl were added to 2 ml PPM Mat prior to equilibration and addition of COC. Final concentrations used and intended effects of each chemical are shown in Table 1.

Table 1 Dose and intended effect of chemicals used to alter metabolism of glucose through the pentose phosphate pathway and glycolysis in porcine oocytes.

Treatment	Dose	Intended effect
Control	NA	None
PC	0.1 μM	↑ PPP
DPI	0.1 μM	↓ PPP
DNP	10 μM	↑ Glycolysis
HMP	100 μM	↓ Glycolysis
HMP + PC	100 μM/0.1 μM	↓ Glycolysis/↑ PPP
DNP + DPI	10 μM/0.1 μM	↑ Glycolysis/↓ PPP

Statistical analysis

Due to the limited number of oocytes available on a single day (approximately 150), three experimental groups (treatments or control) were randomly selected to be tested each day. In each replicate (day = replicate), 50 COCs were assigned to each of the three selected groups. At the conclusion of maturation, 20 oocytes were fertilized and cultured, 10 oocytes were assessed for metabolic activity and nuclear maturation, and 20 oocytes (two groups of 10) were used for GSX determination in each replicate. All experimental groups were replicated six or seven (control only) times.

All data were analyzed using ANOVA in the PROC MIXED procedure of SAS. For continuous data (metabolism and GSX), treatment was considered a fixed factor and replicate effect and the treatment by replicate interaction were treated as random factors. The largest and smallest variances among the treatments were used to calculate an F-value, which was used to determine if variance was homogeneous (nonsignificant F-test, *P* > 0.20) across treatments (Hoshmand 1994). Variances for both metabolic pathways and GSX concentrations were found to be heterogeneous, so a separate variance for each treatment was used for analysis. For binomial data (nuclear maturation, cleavage and blastocyst development) each oocyte or embryo was assigned a 1 if it had achieved the desired stage of maturation or development or a 0 if it had not. These data were analyzed using the generalized linear mixed model (GLIMMIX) macro with treatment as a fixed factor, and replicate and the treatment by replicate interaction as random factors in the initial model (Littell *et al.* 1996). The error was designated as having a binomial distribution and the probit link function was used. Counts of blastocyst cell numbers were also analyzed with the GLIMMIX macro with treatment as the only factor in the model statement. The error was designated as having a Poisson distribution and the log link function was used. In some cases these models required simplification in order for all parameters to be estimated. Random effects were removed from the model individually, beginning with the treatment by replicate interaction, until all factors could be estimated. For blastocyst development, data for the DNP + DPI treatment were excluded from analysis by the SAS program in order to achieve convergence for the GLIMMIX model. When the ANOVA was significant, Fisher's least significant difference test was used for the comparison of individual treatments. The PROC REG procedure was used to assess correlation between oocyte maturation, embryonic development (dependent variables, transformed by arcsine of the square root of the proportion), or GSX (dependent variable) and metabolism or GSX content (independent variable) within treatments using data paired by replicate (*n* = 6 or 7). *P* values ≤ 0.05 were considered significant. Means are presented ± S.E.M.

Results

Both DPI and DNP + DPI decreased ($P \leq 0.05$; Table 2) the activity of glycolysis (DPI, 0.73 pmol/3 h per oocyte and DNP + DPI, 0.58 pmol/3 h per oocyte) and the PPP (DPI, 0.34 pmol/3 h per oocyte and DNP + DPI, 0.31 pmol/3 h per oocyte) relative to control oocytes (glycolysis, 1.56 pmol/3 h per oocyte and PPP, 0.57 pmol/3 h per oocyte; Table 2). The other treatments (PC, DNP, HMP, HMP + PC) failed to significantly ($P > 0.05$) alter the activity of glycolysis or the PPP in the oocytes relative to control oocytes and metabolic rates were not different ($P > 0.05$) between these treatments. Exposure to DPI alone or in combination with DNP increased ($P \leq 0.05$) the percentage of immature (GV, GVBD or CC) oocytes (71.9–79.9%) and decreased ($P \leq 0.05$) the proportion of mature (AI to MII) oocytes (7.2–11.1%) compared with control oocytes (11.9% immature and 74.6% mature; Table 3) and oocytes from the other treatment groups. Maturation after exposure to the other chemicals (2.8–11.0% immature, 63.5–73.3% mature) was not different ($P > 0.05$) between treatments or from maturation of control oocytes.

Embryonic development was also inhibited ($P \leq 0.05$) by DPI, with or without DNP, compared with control oocytes and oocytes from the other treatments (Table 4). Development was not different ($P > 0.05$) between control oocytes and oocytes treated with PC, DNP, HMP or HMP + PC. A high proportion (75.7–82.3%) of the oocytes from the other treatment groups, as well as the control, cleaved compared with 5.8–10.0% of oocytes exposed to DPI (Table 4). Similarly, development to the blastocyst stage was low (0.0–0.8%) among oocytes treated with DPI or DNP + DPI, compared with the other treatment groups and control oocytes (7.1–15.1%, Table 4). Blastocyst cell numbers were similar among all treatments and controls (27.3–38.3 cells/blastocyst, Table 4).

The intracellular content of GSX was not affected ($P > 0.05$) by exposure to PC, DNP, HMP or PC + HMP (7.42–8.68 pmol/oocyte) compared with controls (7.37 pmol/oocyte, Table 5). Exposure to DPI decreased ($P \leq 0.05$) intracellular GSX (5.35 pmol/oocyte) relative to control oocytes and oocytes from the other treatments,

and addition of DNP further decreased ($P \leq 0.05$) GSX concentrations (3.37 pmol/oocyte, Table 5).

Among control oocytes and oocytes treated with DNP + DPI, there was a positive correlation between the proportion of mature oocytes and glycolysis ($P \leq 0.05$, $r^2 = 0.71$ –0.81; Table 6). Similarly, there were negative correlations ($P \leq 0.05$, $r^2 = 0.70$ –0.84) between the proportion of immature oocytes and the activity of glycolysis or the PPP among oocytes treated with DPI, DNP or DNP + DPI (Table 6). In contrast, the proportion of immature oocytes after exposure to PC was positively correlated ($P = 0.03$, $r^2 = 0.74$) with glycolytic activity (Table 6). The proportion of mature oocytes was positively correlated ($P = 0.04$, $r^2 = 0.69$) with intracellular GSX content among DNP + DPI-treated oocytes (Table 6), and there were positive correlations between GSX content and the glycolytic ($P = 0.05$, $r^2 = 0.65$) and PPP ($P = 0.02$, $r^2 = 0.78$) activity of DPI-treated oocytes. Embryonic cleavage ($P = 0.01$, $r^2 = 0.81$) and GSX content ($P = 0.01$, $r^2 = 0.84$) were also positively correlated with glycolytic activity after exposure to DNP + DPI (Table 6). Finally, embryonic cleavage was positively correlated with GSX content ($P = 0.04$, $r^2 = 0.69$) after treatment with DNP + DPI. There were no significant correlations ($P > 0.05$) between oocyte metabolism or GSX content and development to the blastocyst stage in any treatment group.

Discussion

The objective of this study was to manipulate the activity of glycolysis and the PPP in order to determine the interactions between nuclear maturation, cytoplasmic maturation (metabolism and GSX content), and embryonic development in porcine oocytes. Exposing oocytes to DPI, either alone or in combination with DNP, during maturation significantly inhibited glycolysis and activity of the PPP. In addition, DPI-exposed oocytes contained less GSX and fewer of these oocytes matured, cleaved, or developed to the blastocyst stage. These results provide the first evidence of a functional link between glucose metabolism and nuclear maturation in porcine oocytes. Similarly, decreased GSX content following inhibition of glucose metabolism demonstrates for the first time in any species

Table 2 Effects of chemical exposure on the activity (pmol/oocyte per 3 h) of glycolysis and pentose phosphate pathway in porcine oocytes.

Treatment (n) ¹	Glycolysis (pmol/oocyte per 3 h)	Pentose phosphate pathway (pmol/oocyte per 3 h)
Control (55)	1.56 ± 0.12 ^a	0.57 ± 0.06 ^c
PC (57)	1.99 ± 0.14 ^a	0.69 ± 0.04 ^c
DPI (39)	0.73 ± 0.09 ^b	0.34 ± 0.03 ^d
DNP (55)	1.93 ± 0.14 ^a	0.67 ± 0.05 ^c
HMP (49)	1.58 ± 0.12 ^a	0.59 ± 0.06 ^c
HMP + PC (49)	1.61 ± 0.12 ^a	0.65 ± 0.05 ^c
DNP + DPI (43)	0.58 ± 0.06 ^b	0.31 ± 0.04 ^d

¹Total number of oocytes evaluated.

^{a,b,c,d}Treatments (mean ± S.E.M.) within the same column with different superscripts differ ($P \leq 0.05$).

Table 3 Summary of nuclear maturation in porcine oocytes following chemical exposure during *in vitro* maturation.

Treatment	GV ¹	GVBD/CC ²	MI ³	A/TI ⁴	MII ⁵	Total ⁶	Immature ⁷ (%)	Mature ⁸ (%)
Control	0	7	7	3	35	52	11.9 ± 6.5 ^a	74.6 ± 8.0 ^c
PC	0	1	12	0	32	45	2.8 ± 2.8 ^a	67.8 ± 10.8 ^c
DPI	4	31	9	0	4	48	71.9 ± 7.9 ^b	7.2 ± 3.7 ^d
DNP	0	4	12	0	35	51	8.3 ± 5.3 ^a	68.3 ± 8.6 ^c
HMP	0	6	9	1	40	56	11.0 ± 3.9 ^a	73.3 ± 4.7 ^c
HMP + PC	1	2	14	1	29	47	6.0 ± 2.7 ^a	63.5 ± 6.7 ^c
DNP + DPI	2	31	4	0	6	43	79.9 ± 12.4 ^b	11.1 ± 11.1 ^d

¹Germinal vesicle.²Germinal vesicle breakdown or condensed chromatin.³Metaphase I.⁴Anaphase or telophase I.⁵Metaphase II.⁶Total number of oocytes assessed for nuclear maturation.⁷Mean ± S.E.M. of the percentages of GV, GVBD or CC oocytes in 6 or 7 replicates.⁸Mean ± S.E.M. of the percentages of AI, TI or MII oocytes in 6 or 7 replicates.^{a,b,c,d}Treatments within the same column with different superscripts differ ($P \leq 0.05$). Binomial data was analysed using the GLIMMIX macro in PROC MIXED of SAS.

that these two critical aspects of oocyte maturation are interrelated.

The effects of DPI on PPP activity are attributed to its ability to inhibit NADPH oxidase, the enzyme that produces the NADP necessary for PPP activity (Stryer 1995, Downs *et al.* 1998), but the possibility of toxic effects must be considered due to the severity of the effects of DPI exposure. However, meiotic arrest due to DPI is almost completely reversible in mouse oocytes, with approximately 84% of DPI-exposed oocytes resuming meiosis (Downs *et al.* 1998). In addition, the effects of 0.1 µM DPI appear to be reversible in porcine oocytes. At the conclusion of maturation (40 h) in the presence of DPI (0.1 µM), approximately 75% of oocytes were still at the GV or GVBD stage of meiosis, as seen in the present study (Krisher 2005). After an additional 40 h of culture in the absence of DPI, only 9.0% of oocytes remained at the GV or GVBD stages (Krisher 2005). Although only a small percentage of exposed oocytes (10.1%) reached MII, the increased proportion of oocytes at MI indicates that >60% of DPI-exposed (0.1 µM, 40 h) porcine oocytes were still viable and capable of resuming meiosis (Krisher 2005).

The likelihood that the effects of DPI are specific to altered glucose metabolism is also supported by the fact that maturation of porcine oocytes in the absence of glucose, but with pyruvate and lactate, results in no embryonic cleavage, similar to what we found in the presence of DPI (Tubman *et al.* 2005). Therefore, we conclude that the effects of DPI can be attributed to altered glucose metabolism, and the severity of these effects is probably due to the importance of glucose metabolism in porcine oocyte maturation rather than its toxicity.

Treatment with DPI had the intended effect of inhibiting PPP activity, but glycolysis was also decreased. This finding suggests that the mechanisms controlling the PPP and glycolysis are related in porcine oocytes. It may be that increased activity of the PPP is one of the initial stimuli for the resumption of meiosis, while increased glycolysis is a result of the progression of meiosis. In mice, ribose 5-phosphate produced by the PPP is converted to phosphoribosyl pyrophosphate (PRPP), which participates in purine production, via *de novo* synthesis or the salvage of hypoxanthine, and resumption of meiosis (Downs 1993, 1997). In addition, stimulation of the PPP with either

Table 4 The effects of chemical exposure during *in vitro* maturation of porcine oocytes on *in vitro* embryonic development following *in vitro* fertilization.

Treatment (n) ¹	Cleavage on day 3 (%)	Blastocyst on day 6 (% of oocytes)	Blastocyst cel# (n) ²
Control (140)	77.1 ± 4.1 ^a	7.1 ± 3.1 ^c	30.9 ± 3.8 ^e (9)
PC (119)	78.1 ± 5.1 ^a	13.4 ± 3.6 ^c	32.9 ± 4.2 ^e (8)
DPI (119)	10.0 ± 6.5 ^b	0.8 ± 0.8 ^d	NA
DNP (119)	82.3 ± 1.8 ^a	15.1 ± 4.6 ^c	38.3 ± 4.0 ^e (18)
HMP (120)	77.5 ± 6.7 ^a	13.3 ± 3.1 ^c	28.8 ± 2.9 ^e (8)
HMP + PC (119)	75.7 ± 5.5 ^a	14.3 ± 3.3 ^c	27.3 ± 3.5 ^e (13)
DNP + DPI (120)	5.8 ± 5.8 ^b	0.0 ± 0.0 [*]	NA

¹Total number of oocytes cultured in 6 or 7 (control only) replicates.²Number of blastocysts analyzed for cell number.^{a,b,c,d,e}Treatments within the same column with different superscripts differ ($P \leq 0.05$). Binomial data was analysed using the GLIMMIX macro in PROC MIXED of SAS.^{*}Excluded from statistical analysis due to lack of blastocysts.

Table 5 Intracellular GSX content (pmol/oocyte) of porcine oocytes following chemical exposure during *in vitro* maturation.

Treatment (n) ¹	GSX (pmol/oocyte)
Control (7)	7.37 ± 0.71 ^a
PC (6)	7.42 ± 0.78 ^a
DPI (6)	5.35 ± 0.58 ^b
DNP (6)	7.90 ± 0.85 ^a
HMP (6)	8.18 ± 0.37 ^a
HMP + PC (6)	8.68 ± 0.36 ^a
DNP + DPI (6)	3.37 ± 0.55 ^c

¹Number of replicates. Two pools of 10 oocytes were analysed in each replicate (6 or 7 replicates per treatment).

^{a,b,c}Treatments (mean ± S.E.M.) within the same column with different superscripts differ ($P \leq 0.05$).

chemical agents (PC or phenazine ethosulfate) or FSH induces a significant increase in GVBD in murine oocytes (Downs *et al.* 1998). Glycolysis has been associated with the maintenance of meiotic arrest in mouse oocytes, but also increases coincidentally with maturation in mice, cattle and cats (Downs & Mastropolo 1994, Downs & Utecht 1999, Steeves & Gardner 1999, Spindler *et al.* 2000). In this study, we found that the glycolytic activity of oocytes was positively correlated with the proportion of mature oocytes in control and DNP + DPI-treated oocytes. In addition, increased activity of the PPP and glycolysis was associated with a decrease in the proportion of immature oocytes after DPI, DNP or DNP + DPI exposure. These results suggest that activity of both the PPP and glycolysis are higher in mature porcine oocytes. It is possible that inhibition of the PPP in porcine oocytes by DPI, prevented nuclear maturation and the associated increase in glycolytic activity. However, whether low metabolism is a cause or a consequence of inhibited maturation remains to be determined. In a follow-up experiment to the present study, the inhibitory effects of DPI (50 nM) on the maturation of porcine oocytes could be overcome by supplementing DPI-containing media with PRPP, ribose 5-phosphate or NADP (Krisher 2005). These findings

further support our hypothesis that the PPP is a controlling factor in porcine oocyte maturation, and provide experimental conditions that could be used to determine how DPI affects glycolysis independently of PPP activity and nuclear maturation.

Exposure to DPI during maturation also decreased intracellular GSX concentrations compared with control oocytes and oocytes exposed to the other treatments. In addition, an increased concentration of GSX was associated with a higher proportion of mature oocytes in the presence of DNP + DPI and with increased activity of glycolysis and the PPP in the presence of DPI-exposed oocytes. The concentration of GSX within the oocyte increases throughout maturation in porcine (Yoshida *et al.* 1993), bovine (Miyamura *et al.* 1995) and hamster oocytes (Perreault *et al.* 1988, Zuelke *et al.* 2003), but it is not known whether this increase is caused by meiotic progression or the associated changes in cellular metabolism. Perhaps the low rates of glycolysis, and presumed decrease in ATP production, in the presence of DPI reduced the synthesis of GSX. Since tricarboxylic acid cycle activity is known to be low in porcine oocytes (Durkin *et al.* 2001, Sturmey & Leese 2003), glycolysis may be the primary source of ATP. This hypothesis is supported by the fact that addition of DNP, which disrupts the mitochondrial H⁺ gradients and inhibits mitochondrial ATP production (Stryer 1995), to DPI further decreased GSX concentrations relative to the DPI group. In contrast, DNP alone did not affect GSX content, possibly due to sufficient ATP production from glycolysis in the absence of DPI. Alternatively, meiosis is associated with changes in RNA transcription and translation of various proteins (Paynton & Bachvarova 1994, Wu *et al.* 1996, Dalbiès-Tran & Mermillod 2003). By inhibiting maturation, DPI may have disrupted these processes and altered the synthesis of GSX independently of changes in metabolic activity or substrate (amino acid and/or ATP) availability. Finally, decreased PPP activity may have altered the REDOX potential of the cytoplasm

Table 6 Linear correlations¹ between nuclear and cytoplasmic maturation in porcine oocytes.

Treatment	Dependent variable ²	Independent variable ³	Direction	P-value	r ²
Control	% Mature	Glycolysis	+	0.02	0.71
PC	% Immature	Glycolysis	+	0.03	0.74
DPI	% Immature	PPP	–	0.04	0.70
	GSX	Glycolysis	+	0.05	0.65
	GSX	PPP	+	0.02	0.78
DNP	% Immature	Glycolysis	–	0.01	0.84
HMP	% Cleavage	Glycolysis	+	0.02	0.79
DNP + DPI	% Immature	Glycolysis	–	0.02	0.76
	% Mature	Glycolysis	+	0.01	0.81
	% Mature	GSX	+	0.04	0.69
	GSX	Glycolysis	+	0.01	0.84
	% Cleavage	Glycolysis	+	0.01	0.81
	% Cleavage	GSX	+	0.04	0.69

¹Data paired by replicate ($n = 6$ or 7).

²Percentages were transformed using arcsine of the square root of the proportion.

³Rates of glycolysis and PPP activity are pmol/oocyte per 3 h and GSX values are pmol GSX/oocyte.

(NADPH:NADP and/or GSH:GGSG), interfering with REDOX-regulated processes (Harvey *et al.* 2002) that could be involved in meiotic resumption, metabolic control, and/or GSX synthesis.

Treatment with DPI, alone or in combination with DNP, also inhibited embryonic cleavage and development to the blastocyst stage. However, the inhibition of cleavage and blastocyst development was most probably associated with the low rate of nuclear maturation induced by DPI. The similarity between the proportion of mature oocytes (7.2–11.1%) and the proportion of oocytes that cleaved (5.8–10.0%) supports this hypothesis.

In contrast to preliminary observations made in our laboratory (data not shown), PC, DNP and HMP were not effective in significantly altering glucose metabolism in porcine oocytes. Due to the relatively small sample sizes in the dose–response experiments it is possible that the concentrations of chemical agents used were simply insufficient to produce significant effects. However, the lack of significant effects of PC, DNP and HMP on metabolism also may be related to variability in oocyte quality that affects the response to the chemicals. The use of abattoir-derived oocytes introduces significant variability within and between replicates due to differences in the histories of the females. In addition, oocytes from pre-pubertal animals are known to have reduced developmental potential (Pinkert *et al.* 1989, Peters *et al.* 2001). This variability between oocytes is evident within the metabolic data from control oocytes, where rates (pmol/oocyte per 3 h) of glycolysis ranged from 0.02 to 3.46 and those of the PPP ranged from 0.07 to 1.75. It seems likely that the effects of the chemicals, and the effective dose of each chemical, may be dependent on the initial quality of the oocyte. Metabolic stimulators may fail to have an effect on oocytes with high metabolic activity or poor quality oocytes containing insufficient amounts of metabolic enzymes or substrate transport proteins to support higher levels of metabolism. Similarly, increased concentrations of inhibitors may be necessary to affect high quality oocytes with elevated metabolic activity, while low concentrations of inhibitors might be effective in poor quality oocytes with already depressed metabolic activity. Although COC morphology influences developmental potential (Blondin & Sirard 1995, Wood & Wildt 1997, Chauhan *et al.* 1998), dramatic differences in both metabolism and developmental potential exist among oocytes from morphologically identical COCs matured under identical conditions (Spindler & Wildt 2002). Development of better methods for selection of a homogeneous group of COCs may be more useful in future studies of this type, rather than prolonged dose–response experiments aimed at identification of a single effective dose. Despite the potential impact of oocyte quality on the metabolic response to chemical exposure, both PC and DNP elicited numeric, nonsignificant increases in mean PPP and glycolytic activity respectively, as predicted. This fact argues against the possibility of an insufficient dose,

and supports our hypothesis of an interaction between oocyte quality and chemical response.

Our objective was to alter glucose metabolism using various chemical agents in order to determine how glucose metabolism affects porcine oocyte maturation. Inhibition of glycolysis and the PPP by DPI, alone or in combination with DNP, resulted in more immature oocytes, fewer mature oocytes, lower intracellular concentrations of GSX, and decreased embryonic development. In addition, significant, linear correlations between maturation, metabolism and GSX demonstrated interactions between these cellular functions. Use of alternative chemicals and culture conditions to manipulate metabolism or supplementation of culture media with substrates of reactions downstream of the point of inhibition will be useful in further elucidating the mechanisms of these actions. In conclusion, this study provides the first evidence for a role of glucose metabolism in the control of both nuclear and cytoplasmic maturation of porcine oocytes.

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