Calcium and pH Sensitivity of Myofibrils Isolated From Red and White Porcine Muscles

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The calcium and pH sensitivity of myofibrillar ATPase, primarily actin-activated myosin ATPase, plays an integral role in regulating postmortem muscle ATP utilization and may pace postmortem glycolysis. Furthermore, differences in postmortem myofibrillar ATPase activity due to myosin heavy chain isoforms have been hypothesized to contribute to differences in postmortem metabolism between muscles. The objective of this study was to determine the influence of pH on the basal and Ca^{2+} activated myofibrillar ATPase activity in myofibrils from porcine red (RST) and white semitendinosus (WST) muscles.

Myofibrils were isolated from RST and WST muscles removed immediately postmortem. To determine normal Ca^{2+} sensitivity, ATPase activity of myofibrils was measured at pH 7.0 at calcium levels pCa (-log[Ca^{2+}]) 4.0, 5.0, 6.0, 6.25, 6.5, 6.75, 7.0, 8.0, and 9.0 at 39°C. To determine the effect of low pH on Ca^{2+} sensitivity, the ATPase activity was measured at pH 5.5, 5.75, 5.875, 6.0, 6.125, 6.25, and 6.5 at pCa levels 4.0, 6.0, 6.25, 6.5, 6.75, 7.0, and 9.0 at 39°C. At pH 7.0, WST myofibrils had higher maximum activity but required more calcium for half-maximal activation (decreased pCa_{50}) than RST myofibrils.

At pH 7.0 both RST and WST myofibrils displayed maximum actin-activated ATPase activity at pCa ≤ 6.0 and basal myosin ATPase activity at pCa ≥ 6.75. At maximal Ca^{2+} activation levels (pCa 4.0), ATPase activity decreased with pH from pH 6.5 to 5.75 in both RST and WST myofibrils. At pCa 9.0, however, enzymatic activity of the myofibrils remained relatively constant from pH 6.5 to 6.0, but diminished below pH 6.0. Below pH 5.75, ATPase activity was negligible regardless of pCa level in both RST and WST myofibrils. Over pH range of 6.5 to 5.75, RST and WST myofibrils had similar activities at pCa 9.0, but WST myofibrils had approximately 20 to 50 percent higher ATPase activity than RST myofibrils at pCa 4.0. Between pH 6.125 and 6.5, RST and WST myofibrils both demonstrated a sigmoidal shaped Ca^{2+} activation curve with pCa titration, similar to that observed at pH 7.0. However, the maximum myofibrillar ATPase activity and the pCa required for half-maximal activation (pCa_{50}) decreased with pH from pH 6.5 to 6.125 in both RST and WST myofibrils. Calcium titration curves generated at pH levels between pH 6.125 and 6.5 demonstrated that RST and WST myofibrils had similar pCa_{50} values, but that WST myofibrils maintained a higher actin-activated myosin ATPase activity than RST myofibrils at submaximal pH and Ca levels. At pH ≤ 6.0, Ca^{2+} activation of the RST and WST myofibrils did not follow the sigmoidal shaped curves as observed at higher pH levels. In both the RST and WST myofibrils, at pH 6.0 there was a peak in activity at pCa 6.5. A similar trend was observed in the RST myofibrils at pH 5.875. At pH ≤ 5.75, pCa levels did not influence ATPase activity in WST or RST myofibrils.

These data suggest that myofibrils with predominantly fast myosin heavy chain have a higher actin-activated myosin ATPase activity than myofibrils with primarily slow myosin heavy chain isoforms at Ca^{2+} concentrations and pH values characteristic of postmortem muscle. This difference may partially account for the differences in postmortem metabolism between various muscles.

Comparison of NCSU23 and Gardner's G1.2/G2.2 Sequential Growth Medium for the Development of *In Vitro* Produced Porcine Embryos

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Porcine blastocyst development in vitro is relatively low compared to other livestock species. Currently, a single culture medium (NCSU23) is the standard for porcine in vitro systems. However, the G1.2/G2.2 sequential culture system has been found to be beneficial for embryo development in numerous other species. The purpose of this study was to compare porcine preimplantation embryo development and viability in vitro using single (NCSU23) and sequential (G1.2/G2.2) culture media.

Oocytes were matured 50 per 500 µL for 45-47 h in defined TCM199 maturation medium (GibcoBRL; Grand Island, NY) supplemented with 0.01 units/mL porcine LH and FSH (Sioux BCHM; Sioux Center; IA), 10 ng/mL EGF (Sigma; St. Louis, MO), 0.91 mM pyruvate (Sigma), 0.5 mM cysteine (Sigma), 3.05 mM glucose (Sigma), PSA (100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 ng/mL amphatercin, Gibco) and 0.01% w/v polyvinyl alcohol (Sigma) in 5% CO_2 in air at 39°C. Oocytes were denuded in HEPES with 0.01 µg/mL hyaluronidase (Sigma) and fertilized 20 per 100 µL drop with frozen-thawed sperm (5.0×10^6 sperm/mL) for 4 h in modified tris-buffered medium (mTBM) supplemented with 4 mg/mL fraction V BSA (Sigma) and 2 mM caffeine (Sigma). Embryos were cultured in 50 µL drops of either NCSU23 without hypotaurine supplemented with 4 mg/mL fraction V BSA (Sigma) for 144 h, or in G1.2 medium for 72 h followed by culture in G2.2 medium for an additional 72 h (both supplemented with 8 mg/mL crystallized BSA). Blastocyst development was evaluated (day 6 of culture) and total cell number counted by Hoescht staining. Data were analyzed using ANOVA. Results are reported as mean ± SEM, and are shown below.

These results suggest that NCSU23 is more effective in supporting porcine preimplantation embryo development to the blastocyst stage than G1.2/G2.2. However, porcine embryos can be cultured to the blastocyst stage in vitro in sequential culture media. Contrary to other species, porcine embryo development proceeds efficiently with high glucose and low pyruvate concentrations throughout preimplantation development. Further studies need to identify exact energy substrate requirements of porcine embryos during different developmental stages, allowing the formulation and optimization of sequential culture media for use in this species.

Treatment	n	Cleavage, %	Morula & Blastocyst Total, %	Blastocyst Total, %	Blastocyst Cell #
NCSU23	163	73% ± 2.94[a]	27% ± 0.06[a]	16% ± 3.73[a]	51.7 ± 8.8
G1.2/G2.2	158	62% ± 2.99[b]	7% ± 2.69[b]	4% ± 1.50[b]	52.4 ± 4.8

[a,b] Represents a statistical difference between treatments, $P < 0.01$.

Glutathione Content of *In Vivo* and *In Vitro* Matured Porcine Oocytes

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Glutathione (GSH) content in matured porcine oocytes is correlated with subsequent fertilization and developmental success. Glutathione is involved in scavenging free radicals and is an important factor in male pronucleus formation. The objective of this study was to compare GSH concentrations of in vivo and *in vitro* matured porcine oocytes.

Ovulated *in vivo* matured oocytes were flushed from the oviducts of gilts and sows 36 hours after the first sign of estrus. Oocytes were washed in glutathione assay buffer. Groups of 10-20 oocytes were transferred to a 1.5 mL tube in 5µL glutathione buffer and frozen at -80°C. *In vitro* oocytes were obtained from abattoir gilt ovaries (3-8 mm follicles) and matured 50 oocytes per 500 µL medium. Maturation medium was TCM199 (Gibco, Grand Island, NY) supplemented with 0.91 mM pyruvate (Sigma; St. Louis, MO) and 3.05 mM glucose (Sigma) with either 0.1% w/v polyvinyl alcohol (Sigma) or 0.5 mg/mL hyaluronic acid (Vetrepharm, Athens, GA), or NCSU23 with 10.0% porcine follicular fluid. Each media was supplemented with 0.01 units/mL each porcine LH and FSH (Sioux Biochemicals; Sioux Center, IA), 10 ng/mL EGF (Sigma), 0.5 mM cysteine (Sigma), 1.0 % PSA (100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 ng/mL amphotericin, Gibco) and matured in 5% CO_2 in air at 39°C. Oocytes were denuded 45-47 hours post maturation with hyaluronidase (0.01 µg/mL), washed in glutathione assay buffer and frozen as previously described. Glutathione content was determined by the dithionitrobenzoic acid-glutathione disulfide (DTNB-GSSG) reductase recycling assay. On the day of assay, samples were thawed and 5µl of 1.25 M phosphoric acid was added. Oocytes were ruptured by vortexing. The formation of 5-thio-2-nitrobenzoic acid was recorded every 0.5 min for 3.0 minutes on a spectrophotometer at 412 nm visible light, and glutathione content was determined from a standard curve. Statistical differences were determined using ANOVA.

Results indicate that oocytes matured *in vitro* in either a defined TCM199 with PVA or hyaluronic acid, or NCSU23 medium have significantly lower levels ($P < 0.05$) of GSH (n = 207, 9.82 ± 0.71 pmol/oocyte; n = 104, 9.73 ± 0.81 pmol/oocyte; n = 108, 7.89 ± 0.66 pmol/oocyte, respectively) as compared to *in vivo* matured oocytes (n = 217, 36.26 ± 11.00 pmol/oocyte). There were no significant differences in levels of GSH in any of the *in vitro* oocyte treatments. Lower levels of GSH in *in vitro* matured oocytes may contribute to reduced fertilization success and reduced development to the blastocyst stage typical of IVM oocytes. These results suggest that there is a need for improved medium for *in vitro* maturation of porcine oocytes. This research was supported by USDA-NRI grant 00-02143.

Development of a Sequential Medium for *In Vitro* Culture of Porcine Embryos

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Although current porcine in vitro culture medium supports embryonic development from the zygote to the blastocyst stage, efficiencies are very low. In this study, metabolic parameters were used to formulate a sequential culture medium to more precisely meet the energy requirements of porcine embryos. An initial formulation of this medium, Purdue porcine medium 1\2 (PPM1/PPM2), was compared to a standard culture medium for pig embryos (NCSU-23). The effect of varying sodium levels (in the absence of osmolarity fluctuations) and timing of transfer from PPM1 to PPM2 were examined.

Porcine oocytes were aspirated from 3-8 mm follicles of abattoir derived ovaries and matured for 48 hours in TCM-199 maturation medium supplemented with 0.01 units/mL porcine LH and FSH, 10 ng/mL epidermal growth factor, 0.91 mM pyruvate, 0.7 mM cysteine, 3.05 mM glucose, PSA (100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 ng/mL amphotercin) and 0.01% w/v polyvinyl alcohol. Oocytes were denuded and fertilized at 44 hours post maturation in modified tris-buffered medium (mTBM) supplemented with 3.5 mM caffeine and 0.2% fractionV BSA with frozen-thawed boar semen (1.0×10^6 sperm/mL). After 5 hours coincubation with sperm, zygotes were removed from fertilization medium, washed and randomly placed into culture medium. In Experiment 1, zygotes were distributed into PPM1 with high NaCl concentration (100mM), PPM1 with low NaCl concentration (70mM), and NCSU-23 (108.73 mM NaCl). Osmolarity was adjusted to 300 mOsm for all media. Embryos in sequential medium were transferred to PPM2 after 48 hours, and all embryos were cultured for 144 hours post insemination (hpi). Blastocysts were stained with 0.1 mg/mL Hoescht 33342 DNA stain, and cells were counted under ultraviolet light. In Experiment 2, zygotes were placed in PPM1 sequential medium or NCSU-23. Embryos were moved to PPM2 at 36, 48 or 72 hpi. All embryos were cultured for a total of 144 hpi, and blastocysts were stained to determine total cell number.

In Experiment 1, there was no difference between treatments in the percent of cleaved embryos (NCSU 62.9 $\pm$ 5.1%, PPM high 68.8 $\pm$ 3.3%, PPM low 58.6 $\pm$ 6.4%) or the percent of embryos reaching the morula or blastocyst stage of total inseminated oocytes (NCSU 27.6 $\pm$ 4.1%, PPM high 32.8 $\pm$ 3.6%, PPM low 11.9 $\pm$ 4.0%). Fewer oocytes of the total inseminated tended to reach the blastocyst stage in PPM low (9.9 $\pm$ 3.4%) than in PPM high (28.5 $\pm$ 3.1%) or NCSU (22.7 $\pm$ 3.6%). Blastocysts had higher ($P < 0.05$) mean cell numbers in PPM high (53.5 $\pm$ 2.8) than NCSU (42.8 $\pm$ 2.4) or PPM low (24.7 $\pm$ 2.7). In Experiment 2, there was no effect on development to the morula or blastocyst stage of total inseminated oocytes due to the time of transfer to PPM2 (36 hpi = 25.0 $\pm$ 3.3, 48 hpi = 20.4 $\pm$ 3.4, 72 hpi = 23.7 $\pm$ 3.6), or on mean blastocyst cell number (36 hpi = 42 $\pm$ 2.7, 48 hpi = 37.2 $\pm$ 2.9, 72 hpi = 38.8 $\pm$ 6.2).

These results indicate that in vitro derived porcine embryos prefer higher sodium concentrations during in vitro culture, independent of osmolarity. Blastocysts with greater viability were produced, as indicated by increased cell number, using the sequential medium. Porcine embryos do not appear to be sensitive to manipulation at any of the time points examined. These results illustrate the efficacy of our sequential medium for in vitro culture of pig embryos. However, further optimization is necessary to continue to improve efficiency in porcine *in vitro* embryo production.

Metabolism of Glucose, Pyruvate and Glutamine by Porcine Oocytes Matured *in Vitro* and *In Vivo*

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Oocyte metabolism has been associated with increased developmental competence and may provide insight into efficiency of *in vitro* maturation (IVM) conditions. Metabolic activity of *in vivo* derived porcine oocytes was determined and compared to metabolism of porcine oocytes matured under standard IVM conditions.

Our findings indicate *in vivo* derived porcine oocytes utilize glucose as their primary energy substrate via both glycolysis and the pentose phosphate pathway (PPP). Oxidative metabolism of pyruvate occurred at intermediate levels, while glutamine oxidation was minimal. Unovulated oocytes metabolized more glucose via glycolysis and PPP and more pyruvate via the Kreb's Cycle ($P < 0.05$) compared to ovulated porcine oocytes. *In vivo* derived metaphase I oocytes oxidize significantly more glutamine and pyruvate compared to metaphase II oocytes. *In vitro* matured porcine oocytes preferentially utilized glucose via the glycolytic pathway over all other substrates examined. Oocytes matured *in vitro* utilize significantly less glucose via PPP and pyruvate via the Kreb's Cycle than *in vivo* matured oocytes. *In vitro* matured oocytes tended to use less glucose via glycolysis and glutamine via the Kreb's Cycle than *in vivo* matured oocytes. Overall, *in vitro* matured oocytes were less metabolically active than those matured *in vivo*. Altered metabolic activity of *in vitro* matured porcine oocytes suggests suboptimal IVM conditions, which likely compromise subsequent embryonic development.

Assessment of a Novel Media System for *In Vitro* Porcine Embryo Production

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In vitro production of porcine embryos is historically difficult. Culture systems based on the metabolic needs of the oocyte and embryo should result in increased viability. The objective of this study was to compare porcine *in vitro* embryo production in the standard system (STND) and a newly developed single medium system (Purdue porcine media; PPM) modified for maturation (mat), fertilization (fert), early embryonic cleavage (1) and blastocyst development (2).

Oocytes from abattoir ovaries were matured in either modified TCM-199 (STND) or PPMmat, each supplemented with 0.01 units/mL porcine LH and FSH, 0.5 mM cysteine and 10 ng/mL EGF. PPMmat also contained 5mM glucose, 2 mM lactate, 1 mM pyruvate, 1 mM glutamine, 5 mM taurine, 1X vitamins and 0.5 mg/mL hyaluronate. After maturation, oocytes were denuded and fertilized in mTBM (STND) or PPM fert, both containing 2 mM caffeine, 2 mg/mL BSA and 7.5 mM calcium. Oocytes were co-incubated with sperm for 5 h, then placed into NCSU23 (STND) or PPM1. Embryos were moved from PPM1 to PPM2 after 72 h. In a second experiment, oocytes were matured in PPMmat, fertilized in either PPMfert or mTBM and cultured in PPM1/2 to specifically examine the effects of fertilization conditions. Data were analyzed by Chi-square, significance, $P < 0.05$.

There was no difference in penetration rate (PPM, 75%; STND, 76%), but more oocytes were normally fertilized (2 pronuclei; PN) in PPM (23%) than in STND (14%). More embryos cleaved in PPM (64%) than STND (44%). There was no difference in d 6 blastocyst development (PPM, 8%; STND, 11%). In the second study, oocytes matured and cultured in PPM but fertilized in mTBM had higher polyspermy (40%) and a lower percentage of 2PN per oocytes penetrated (41%) than those fertilized in PPMfert (15% and 75%, respectively). Fertilization in mTBM resulted in lower cleavage (64%) and blastocyst development (6%) than in PPM (79% and 16%, respectively). These results demonstrate that a single medium system based upon the metabolic needs of the oocyte and embryo result in an increase in normal fertilization and embryonic cleavage, possibly as a result of more correct cytoplasmic maturation and appropriate fertilization conditions.

Changes in Pyruvate and Glucose Metabolism by Developing Porcine Embryos Produced *In Vitro*

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Embryo metabolism is a valuable indicator of embryo viability and may provide insight into efficiency of the in vitro culture system. Currently, there is a lack of data concerning porcine embryo metabolism and porcine in vitro culture systems result in poor development compared to other domestic species. This study measured glycolysis and Kreb's Cycle activity in porcine embryos at the 2-cell, 8-cell, morula and blastocyst stages.

Oocytes were collected from 3-8 mm antral follicles and matured 50/500 µL for 45-47 h in defined TCM 199 maturation medium (Gibco; Grand Island, NY) supplemented with 0.01 units/mL porcine LH and FSH (Sioux; Sioux Center, IA), 10 ng/mL EGF (Sigma; St. Louis, MO), 0.91 mM pyruvate (Sigma), 0.5 mM cysteine (Sigma), 3.05 mM glucose (Sigma), PSA (100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 ng/mL amphotercin, Gibco) and 0.01% polyvinyl alcohol (PVA; Sigma) in 5% CO_2 in air at 39^0 C. Oocytes were denuded with hyaluronidase (0.01 µg/mL) and fertilized 20/100 µL drop with frozen sperm (5.0×10^6 sperm/mL) for 4 h in modified tris-buffered medium (mTBM) supplemented with 0.2% w/v fraction V BSA (Sigma) and 2 mM caffeine (Sigma). Embryos were cultured 10/50 µL drop in NCSU23 without hypotaurine supplemented with 0.4% w/v fraction V BSA (Sigma) in 5% CO_2, 10% O_2 balance N_2 for 21, 79, 119 and 144 h. Embryo metabolism was measured with 0.0114 mM 5-^{3}H glucose (Amersham; Arlington Heights, IL) and 0.25 mM 2-C^{14} pyruvate (ARC; St. Louis, MO) using a modified hanging drop technique over a 25 mM sodium bicarbonate sink. Morula and blastocyst stage embryos were stained with Hoescht 33342 and cells counted under ultraviolet light. Metabolism data was analyzed using ANOVA and Kruskal-Wallace multiple comparison z-value test. Results are reported below as a mean ± SEM.

Glucose utilization through glycolysis was active throughout porcine embryo development and increased significantly after the 8-cell stage, reaching a maximum at the blastocyst stage. Metabolism of pyruvate through the Kreb's Cycle was low in all stages examined with no significant increase until the blastocyst stage. Although both glucose and pyruvate utilization increased with development on a per embryo basis, substrate utilization per cell declined. This data indicates that unlike other species, porcine embryos rely heavily on glucose metabolism throughout preimplantation development.

		Glycolysis, Glucose		Kreb's Cycle, Pyruvate		
stage	**n**	**pmol/emb/3h**	**pmol/cell/3h**	**pmol/emb/3h**	**pmol/cell/3h**	**cell #**
2-cell	12	2.24 ± 0.46^a	1.12 ± 0.23^a	0.30 ± 0.0065^a	0.15 ± 0.0032^a	2
8-cell	21	3.81 ± 0.43^a	0.47 ± 0.0053^b	0.38 ± 0.0073^a	0.047 ± 0.00091^b	8
Morula	32	7.19 ± 0.82^b	0.29 ± 0.0033^c	$0.47 \pm 0.0065^{a,b}$	0.0018 ± 0.00025^c	25.25 ± 2.86
Blastocyst	41	12.47 ± 1.05^c	0.30 ± 0.0031^c	0.57 ± 0.0046^b	0.0013 ± 0.00010^c	47.24 ± 0.68

[a, b, c] Represent statistical differences within columns, $P < 0.05$.

Effects of Leptin on Porcine Oocyte Maturation and Embryo Development *In Vitro*

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Leptin is a 16 kDa protein secreted by mature adipocytes, which has been shown to affect normal reproductive function in several species. Interestingly, leptin and it's receptors have been found in a variety of reproductive structures, ranging from the oocyte and cumulus cells to the embryo. However, it's mode of action is unknown. The objective of this study was to determine the effects of leptin supplementation on porcine oocyte maturation and embryo development *in vitro.*

Oocytes were collected from abattoir ovaries and matured in a defined TCM199 based medium for 45-47 h. Oocytes were fertilized in a modified tris-buffered medium (mTBM) for 5 h and presumptive zygotes cultured in NCSU23 for 146 h. In a 2×2 factorial design, oocytes were matured in the presence or absence of 20 ng/mL of leptin and subsequent embryos were cultured in the presence or absence of 20 ng/mL of leptin. Randomly selected oocytes were examined for nuclear maturation, embryos were examined for development and total blastocyst cell numbers counted. Developmental data were subjected to an arc sin transformation before analysis and are reported as a mean ± SEM. Data were analyzed with ANOVA and differences determined using the Bonferroni Multiple Comparison Test, $P < 0.05$.

Results indicate the presence of leptin during only embryo culture resulted in similar blastocyst development and cell numbers compared to controls (27% vs. 29% and 42.3 ± 2.6 vs. 44.0 ± 3.6, respectively). Blastocyst development and cell numbers resulting from leptin supplementation during only oocyte maturation were lower, but not significantly so compared to controls (22%, 38.3 ± 2.7). However, the presence of leptin during both oocyte maturation and embryo culture resulted in significantly lower rates of blastocyst development (13%) with similar blastocyst cell numbers (41.5 ± 4.3). The addition of leptin to oocyte maturation medium had no effect on completion of nuclear maturation (71%) compared to controls (67%). Decreased blastocyst development suggests leptin may exert negative effects on porcine embryo development through synergistic actions during oocyte maturation and embryo culture, perhaps inhibiting cytoplasmic maturation.