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Development of an Improved Porcine Embryo Culture Medium for Cloning, Transgenesis and Embryonic Stem Cell Isolation

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Abstract

Work in our laboratory for more than two decades has focussed on the production of genetically modified pigs for xeno transplantion research. More recent work has focussed on the isolation of porcine embryonic stem cells to facilitate this as well as and other research applications. Central to this research has been the production of *in vitro* Produced (IVP) embryos. These embryos are produced using a twostep culture system based on NCSU23. This culture system which was developed by modifying energy substrate availability and concentrations and by adding non-essential and essential amino acids in a sequential manner. As a result of this work we have developed a culture system that better suits the changing metabolic needs of the pig embryo and produces embryos with relatively high developmental competence compared to the original formulation. These embryos can be used for a range of research applications including the isolation of embryonic stem cells.

Keywords: Cloning; Transgenesis; Genetic Engineering

Introduction

For more than two decades, work in our laboratory has focussed on the production of genetically modified pigs for xeno transplantation research. More recent work has focussed on the isolation of porcine embryonic stem cells to facilitate this and other research applications. Central to this research has been the production of *in vitro* matured oocytes and *In vitro* Produced (IVP) embryos. However it has been previously demonstrated that any period of in vitro culture reduces the pig embryo's developmental potential particularly during the first 48 h after fertilisation [1]. Indeed cloned embryos are normally transferred at the one cell stage to minimize this effect. Hence there is an unmet need for a pig embryo culture medium that minimizes this loss of developmental potential and produces in vitro embryos of high developmental competence. One of the first embryo culture media developed to specifically meet the metabolic and nutrient needs of the pig embryo was North Carolina State University 23 (NCSU23)[2]. This was developed over two decades ago and until recently was widely used essentially unchanged from its original formulation. However, compared to in vivo derived pig embryos of a similar age, IVP embryos cultured in this medium display different energy substrate usage [3], exhibit delayed development and contain fewer cells [4]. Hence even though piglets have been produced following culture in NCSU23 there would appear to be considerable scope for improving this culture medium. There have been two overarching strategies to improving the culture medium, either developing a completely novel culture medium or modifying an existing culture medium. Here we review work undertaken by us previously aimed at improving NCSU23 in order to produce high quality embryos for research applications in particular cloning, transgenesis and embryonic stem cell isolation. A full description of the methods used is provided in the original papers [5,6].

Modifying Energy Substrates

Most studied mammalian embryos require pyruvate and lactate as the primary energy substrates during the first two of days of development [7,8]. Glucose exhibits an inhibitory effect on development during this time and can only be used by embryos during the later period of preimplantation development when the embryo's energy requirements increase dramatically [9-11]. Porcine embryos are unusual compared to other mammalian embryos in that they can successfully use glucose as an energy substrate for the entire period of preimplantation development [3]. Pig IVP embryos cultured in medium containing only glucose can also produce piglets after transfer [12,13].

While pig embryos do not appear to have an essential need for either pyruvate or lactate during early development, this does not necessarily mean that full developmental potential is achieved in the absence of these. The *in vivo* concentrations of lactate, pyruvate and glucose during the first two days of porcine preimplantation development were measured at 5.7 mM, 0.2 mM and 0.6 mM respectively [14] and these concentrations suggest a role for lactate and pyruvate and perhaps a diminished role for glucose during this early preimplantation period.

Several other studies have investigated changing porcine embryo culture medium by substituting glucose with pyruvate and lactate in various concentrations for either the first two days or the entire culture period [15]. Found that embryos grown only with pyruvate and lactate (pyruvate/lactate; 0.5 mM and 10 mM respectively) developed to the blastocyst at a higher rate than those embryos grown using glucose (5.6 mM) as the only energy substrate. However, changing the energy substrates during the in vitro culture period was not examined. In another study [16], tested various combinations of either pyruvate/lactate (0.17 mM and 2.73 mM respectively) or glucose (5.6 mM), changing the energy substrates present after 48 h in vitro culture. They found that including pyruvate/ lactate for days 0 to 2 and glucose for days 2 to 6 resulted in the highest rates of blastocyst development and the highest quality blastocysts as determined by cell number. Culturing with pyruvate/lactate for days 0 to 6 resulted in similar rates of blastocyst development compared to culturing with glucose for the same time period, in contrast to the result published by [15].

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In a follow up study [17], confirmed the result of Kikuchi et al. [1], and also determined that the optimum time to change from pyruvate/lactate to glucose was a rather precise 58 h post fertilization. A later study [18] also found that culturing with pyruvate/lactate (using similar substrate concentrations to (Kikuchi et al. [1]) for the first 58 h and then with glucose considerably improved the rate of blastocyst development compared with those embryos cultured with glucose for the entire time. In contrast to this result [19], found that culturing with pyruvate/lactate for either days 0 to 2 or days 0 to 7 resulted in similar rates of blastocyst formation and cell numbers compared to culturing with glucose for days 0 to7. However, the concentrations of pyruvate/lactate that [19] and [15] used (0.5 mM and 5.0 mM respectively) were quite different from that used in the other studies. This difference in the substrate concentrations used may partially explain the difference in results.

For our pyruvate/lactate containing medium, we decided to base our concentrations on those estimated to be *in vivo*, namely 5.7 mM lactate and 0.2 mM pyruvate [14]. As glucose can be utilized by early preimplantation pig embryos and is present in the oviduct during this period, we also decided to include it in the pyruvate/lactate containing medium at the low concentration estimated in vivo (0.6 mM). The concentration of glucose in the glucose only medium was unchanged at 5.6 mM.

Our initial investigations into modifying the available energy substrates were conducted using electrically activated parthenogenetic embryos which we have used previously as a model for somatic cell nuclear transfer or cloned embryos [5]. Using the substrate concentrations given above we compared three treatments; (1) medium containing glucose for days 0 to 6 (G/G), (2) medium containing pyruvate, lactate and glucose for days 0 to 6 (PLG/PLG), and (3) pyruvate/lactate/glucose for days 0 to 2 and glucose only for days 2 to 6 (PLG/G). The results of these experiments are summarized in (Table 1). Both the rate of blastocyst development and blastocyst cell number were compared using methods described previously [5].

Interestingly we found even though the PLG/G treatment provided the highest rates of blastocyst development, it was not statistically different from the other two treatments. The greatest effect was on the quality of the blastocyst as determined by cell number with the blastocysts cultured in the PLG/G treatment containing approximately 15% more cells than those cultured only in glucose. Culturing in pyruvate/lactate/glucose mixture for the entire culture period tended to be better than culturing in glucose only. These results were the same whether the parthenotes were generated from oocytes from sows or gilts.

These results are similar to those studies discussed above that used concentrations of 0.17 mM pyruvate and 2.73 mM lactate. The study by [19] which did not find any beneficial effect of changing the energy substrates used comparatively high concentrations of pyruvate and lactate (0.5 mM and 10 mM respectively). This suggests that such high concentrations of pyruvate and lactate are not optimal for development and the lower concentrations used by us are preferable. Having established the benefits of using pyruvate and lactate in a two-step culture system, the next modification we examined was the addition of amino acids.

Treatment[1]	No. oocytes activated	No. blastocysts on day 6 (%)	Blastocyst cell number (n)
G/G	189	97 (51)	47.1 ± 2.1[a] (52)
PLG/PLG	188	92 (49)	49.9 ± 2.0[a,b] (62)
PLG/G	185	102 (55)	53.8 ± 1.7[b] (77)

Morphological data from 5 replicates, cell number data from 4.
[1] G, glucose 5.6 mM; PLG, pyruvate 0.2 mM, lactate 5.7 mM and glucose 0.6 mM, medium changed on day 2 of culture. Cell number data are means ± SEM. Percentages calculated by number at blastocyst divided by total number activated.
Values with different superscripts in the same column are significantly different; [a,b] $p<0.05$. (Developmental data analysed by Chi Square .Total Cell number by ANOVA) [5].

Table 1: Effect of altering substrate concentrations in the culture medium on the in vitro development of parthenogenetic embryos made from sow oocytes

Including Non-essential and Essential Amino Acids

Amino acids used by mammalian cells have been divided into two groups namely essential (EAA) and non-essential amino acids (NEAA) [20]. For convenience, they can be purchased and added to culture media as prepared concentrated stock solutions at a dilution of 1:50 for EAA and 1:100 for NEAA. Apart from their obvious role in protein anabolism, amino acids are thought to have a variety of roles during in vitro embryo culture. These include being utilized as energy substrates [21] and osmolytes [22], protecting the embryo from oxidative stress [23] and assisting in pH regulation [24].

There has been considerable research conducted on the effect of EAA and NEAA on the *in vitro* embryo development of various species. Early cleavage development has been shown to be stimulated by the addition of NEAA to the culture medium but inhibited by EAA in both the mouse and cow [25-27]. When added to a protein free culture medium containing porcine embyros, the presence of EAA during the first 48 h of culture inhibited the development, but stimulated development when added after 48 h [28]. Embryonic requirements and toleration of different amino acids appear to change during preimplantation development, with some amino acids present in the EAAs being inhibitory during the first days of development and then becoming stimulatory after this time. Furthermore [29], also found that mouse embryo development was improved if the concentration of EAA was half of the recommended concentration of the stock solution (a dilution of 1:100 instead of 1:50).

We decided to investigate the effect of adding NEAA during days 0 to 2 and both NEAA and EAA during days 2 to 6 and the interaction with modified energy substrates on the in vitro development of porcine electrically activated parthenogenetic embryos. Initially, we added the amino acids, both at a dilution of 1:100 (as suggested by Lane et al. [29], in a sequential way to both the standard NCSU23 and to our modified system discussed above. The results of these experiments are summarized in (Table 2).

Firstly, it should be noted, even without any added amino acids, the modified culture system still increased the blastocyst cell number compared to the standard glucose containing medium, confirming the benefit of this modification as discussed above. When the standard NCSU23 medium (G/G) was compared to NSCU23 with added amino acids (G/G-AA), the result was a modest 15% (and not statistically significant) increase in blastocyst cell number. This result was perhaps surprising given the significant benefits amino acids were reported to have on the *in vitro* culture of mouse and cattle embryos [25-27]. However, NCSU23 contains three amino acids in its standard formulation, glutamine, taurine and hypotaurine. Taurine and hypotaurine, either individually or in combination, were demonstrated to improve the development of pig embryos in culture [30]. These two amino acids have also been shown to protect mouse embryos from osmotic stress [22] and together with glutamine may protect the embryos from oxidative stress [21,31]. Thus, several of the

Treatment[1]	No. Oocytes Activated	No. Cleaved at 48h (%)	No. blastocysts on D6 (%)	Blastocyst Cell number (n)
G/G	130	112 (86)	61 (47)	49.7 ± 2.3[a] (57)
G/G-AA	130	115(89)	67 (51)	51.3 ± 2.2[a,b](62)
PLG/G	131	118 (90)	71 (54)	55.4 ± 2.1[b](69)
PLG/G-AA	133	120 (90)	81 (61)	61.3 ± 2.0[c](77)

Data were from 4 replicates.
[1] G, glucose 5.6 mM; PLG, pyruvate 0.2 mM, lactate 5.7 mM and glucose 0.6 mM, medium changed on day 2 of culture. Cell number data are means ± S.E.M. Percentages calculated by number of blastocysts divided by total number activated.
Values with different superscripts in the same column are significantly different; [a,b,c] P<0.05. (Developmental data analysed by Chi Square.Total Cell number by ANOVA) [5].

Table 2: Effect of adding amino acids to the culture medium on the in vitro development of parthenogenetic embryos made from sow oocytes.

physiological roles of amino acids thought to be important during in vitro culture are already being fulfilled in the basic NSCU23. This may partially explain why adding NEAA and EAA to NCSU23 only had a modest impact, at least on the parameters we examined.

Adding NEAA and EAA in a sequential manor had the greatest stimulatory effect when it was combined with the energy substrate modified system (PLG/G-AA). The PLG/G-AA treatment increased blastocyst cell number by 23% compared to the standard NCSU23 (G/G) and by 11% compared to the modified culture medium without amino acids (PLG/G). The amino acids also appeared to increase the rate embryonic development as more blastocysts were present in the PLG/G-AA treatment on day five of culture compared to the other treatments [5]. Although by day six all the treatments had similar rates of blastocyst development. As such we suggest that day five of culture would be a better time point for assessing the effect of treatments on the rate of embryonic development as culture beyond this time allows the slower growing embryos to form blastocysts, masking any treatment effects on embryonic development. This is also when blastocyst formation occurs *in vivo*.

As mentioned, these initial experiments were conducted using parthenogenic embryos as such it was also necessary to confirm the results we had seen with IVP embryos to see if the embryo source could affect the result. When we compared the modified culture including NEAA and EAA with unaltered NSCU23 we found the new culture system significantly increased day 6 blastocyst cell number (76 vs. 64) [5], similar to when parthenothes were used. However, we also saw a significant increase in the day 6 blastocyst rate (59 vs. 41%) [5], in contrast to the result seen using parthenotes. This suggests that perhaps the rate of day 6 blastocyst formation of parthenotes may be determined by factors other than the modifications we made to the culture system, such as oocyte quality. However, this result also clearly demonstrates the benefits of our newly developed culture system over the standard NCSU23 medium for porcine IVP embryos.

As mentioned above, the initial concentration of EAA used in our experiments was a 1:100 dilution of the purchased stock solution instead of the 1:50 dilution that is recommended. This was based on research on mouse and cattle embryos that found that using EAA at 1:100 increased the rate of blastocyst development and blastocyst cell number when compared to a 1:50 dilution [27,29]. We investigated whether this was also the case using porcine IVF embryos [6]. Theporcine embryo culture medium, PZM, developed by [32] contains the equivalent of both NEAA and EAA for the entire culture period and can produce very high quality embryos. So we also needed to determine if it was necessary to exclude EAA from our culture medium for the first couple of days of culture.

The results of this experiment are shown in Table 3. What we found was when EAA were included at the higher concentration (1:50), the rate of blastocyst development of IVP embryos declined significantly, consistent with the findings with cattle and mouse embryos [27,29]. However, when EAA were added at the lower concentration (1:100), the blastocyst rates and the cell number of the blastocysts were similar whether the EAA were included for the entire culture period or for just latter period of culture. This suggests that for pre implantation pig embryos, low concentrations of EAA are not overly toxic during the first couple of days of development, in contrast to mouse and cattle embryos. There is evidence that certain amino acids present in the EAA formulation can be inhibitory to early pig embryos [28]. However, [33] reported that reducing the concentration of selected essential amino acids can change the amino acids effect on hamster embryos from inhibitory to stimulatory. A similar situation may also be occurring with pig embryos.

The decrease in the EAA concentration may also reduce the build-up of ammonium, which results from the spontaneous breakdown of amino acids as well as embryo metabolism [34]. Ammonium accumulation in the culture medium has been shown to be detrimental to the in vitro development of mouse embryos [35]. The decrease in ammonium ion accumulation has been suggested as being partially responsible for the improved mouse embryo development seen when the concentration of EAA in the culture medium is reduced [29]. However, further research is required to determine the specific causes in pig embryo culture. Even though including EAA at a low concentration for the first two of days of culture did not appear to delay embryonic development and given that there is some evidence that specific essential amino acid can be inhibitory and that the EAA were not demonstrably stimulatory during this time, we decided to exclude EAA from the culture medium during early development.

The Timing of the Culture Medium Change

We initially chose day two as the time to change the medium as it was the first time point we would normally assess embryonic development. Karja et al. [17] found the optimum time to change from a culture medium containing pyruvate and lactate to a culture medium containing glucose was 58 h after fertilization, however, we did not know what the optimum time to change the our formulation of media was.

Using IVF pig embryos, we initially decided to compare changing the medium after two, three or four days of culture by examining blastocyst rate and cell number [6]. All embryos were pooled up until the second day of culture to reduce any group variation up until that

Treatment [1]	No. Fertilized	No. Cleaved on Day 3 (%)	No. Blastocysts on Day 6 (%)
1	282	190[a,b] (67)	109[c] (39)
2	279	178[a] (64)	78[d] (28)
3	283	196[a,b] (69)	102[c] (36)
4	283	204[b] (72)	80[d] (28)

Data from 4 replicates
[1]The treatments represent the addition of essential amino acids to modified NCSU23 containing NEAA: 1) at a 1:100 dilution from day 3 to 6; 2) at a 1:50 dilution for day 3 to 6; 3) at a 1:100 dilution for days 1 to 6; 4) at a 1:50 dilution for days 1 to 6. Percentages calculated by number at particular embryonic stage divided by total number fertilized.
Values with different superscripts within the same column are significantly different, P<0.05. (Developmental data analysed by Chi Square) [6].

Table 3: Effect of adding essential amino acids on the morphological development of porcine *in vitro* produced embryos.

time. The blastocyst rate and cell number for the embryos whose media was changed on day two or three were almost identical. However, although not statistically significant, there was a trend for the embryos whose media was changed on day 4 to have a reduced blastocyst rate and cell number, or for the embryos to be of slightly lower quality, compared with the other treatment groups. This suggests that embryos become sensitive to the stimulatory effect of EAAs sometime on the third day of culture. If EAAs are not added to the culture medium by this time, embryonic growth may become slowed compared to those embryos who received EAA earlier. However, more research is needed to confirm this finding. Due to concerns about ammonium ion accumulation in the culture medium we decided to change the medium on day three of culture as standard practice as this would mean the embryos spend one less day with both EAA and NEAA in the culture medium and their potential breakdown products.

In Vivo Development

While blastocyst development and cell numbers can provide an indication of an embryos quality or developmental competence, the only true test of any culture system is to examine development to term following the transfer of cultured embryos to recipient animals. The final media formulation contained 0.2 mM pyruvate, 5.7mM lactate, 0.6 mM glucose and NEAA (1:100 dilution) up to day 2 of culture and 5.6 mM glucose and both NEAA and EAA (both at 1:100 dilution) for the remaining culture period. As a test of the in vivo competence of the embryo produced in our culture system we transferred day 6 IVP blastocysts surgically into the top of one uterine horn of day 5 recipient animal. Gilts that were on their second to fourth (average 3) oestrous cycle were used as recipients and synchronised with regumate [36]. A total of 6 transfers were conducted, with between 23 to 34 blastocysts transferred into each recipient (average 31). Four of these recipients became pregnant (67%) and delivered a total of 21 piglets. This represents a total *in vivo* embryo survival rate of 11% (21 piglets from 186 embryos transferred) and an embryo survival rate in those recipients that farrowed piglets of 17% (21 piglets from 118 transferred embryos). This clearly demonstrates that our new culture system produces developmentally competent porcine embryos.

The developmental competence of the blastocysts produced by our *in vitro* culture system was further demonstrated as part of ongoing research in our laboratory to characterise putative embryonic stem cell lines isolated from IVP produced blastocyst using this same culture medium [36,37]. In these studies ESC were injected into IVP produced blastocysts to produce chimaeras [37].In a subsequent study (unpublished results) day 5 IVP blastocysts were used as host blastocyst which were then transferred into recipients The recipients were synchronised to be approximately 48 hours behind the embryos, as suggested by [38]. Six recipients had between 23 and 44 ESC injected blastocysts (average 28 per recipient) transferred to them. Of these five became pregnant (83%) and farrowed a total of 20 piglets. The overall embryo survival rate was similar to the previously discussed experiment (12%). Together these results further highlight that our new culture system produces developmentally competent porcine embryos.

The Improved Quality of Embryos Produced with our New Culture System

In conclusion by modifying the energy substrate availability and concentrations and by adding NEAA and EAA in a sequential manner, we have produced a modified culture system based onNCSU23 which we suggest better suits the changing metabolic needs of the pig embryo and as such represents an improvement over the original formulation . Embryos produced using this system have a high developmental competence and can produce piglets at relatively high efficiencies following their transfer. Furthermore embryonic stem cells can be isolated from these embryos which when injected back into IVP blastocysts produced using this medium can produce chimaeric pigs. However It is important to remember than the culture medium is only one aspect to producing high quality IVP pig embryos and further research is still required across all aspects of porcine IVP in order to produce embryos equivalent to in vivo derived embryos [39,40].

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