Porcine Satellite Cells from Large and Small Siblings Respond Differently to *In Vitro* Conditions

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Abstract

In order to detect the effects of intra-uterine variation on the performance of neonatal satellite cells, the following experimental model was devised. Satellite cells were removed from the *m. semitendinosus* of the largest and smallest siblings in six litters of pigs and cultured under standardised, high glucose, conditions to reach approximately 85% confluence.

As the smallest piglet in a litter generally has a decreased growth rate in comparison to its larger littermates, it was expected that satellite cells derived from these piglets would also perform at a lower level. This was not found; satellite cells from small siblings proliferated at a significantly higher level than from larger siblings in all trials.

This result implies that, instead of the satellite cells being detrimentally affected by early developmental events, they are in fact primed to react positively to any additional nutrition present in the surrounding environment.

Key words: intra-uterine variation, growth rate, porcine, satellite cells.

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Current thought holds that early developmental events and environments have profound effects on future growth and development. Proponents of this fetal origins hypothesis [1, 2], have shown that the incidence of coronary heart disease and *diabetes mellitus* is increased in individuals who were exposed to undernutrition during specific stages of gestation. The hypothesis is borne out by studies on the Dutch famine of the 1940s [19]; in which cases of diabetes and hypertension were correlated with the stage of pregnancy at which famine was experienced. Other reasons for believing that early environment plays a significant role comes from agricultural *in vitro* fertilisation (IVF) studies and nutritional trials. It has been found that, when IVF techniques are used in cattle, the fetal oversize condition is more common [15]. This phenomenon frequently results in maternal death and affects growth planes and muscle development.

Studies on the effect of porcine somatotropin (pST- a growth hormone) on porcine development have shown that pST injection into pregnant sows in early gestation results in an increased birthweight and muscle fibre number in the offspring [20, 21, 22]. pST is a useful tool as it is hypothesised to stimulate growth via a release of additional nutrition. Actual nutrition studies on the pig confirm the pST findings and show that increased levels of nutrition from days 25 to 50 of gestation result in improved secondary fibre number (those formed during the second wave of fibre formation) and a faster, more efficient growth rate [9]. This, again, shows the importance of nutritional events early in gestation.

An important point in postnatal growth studies is that muscle fibre number is already fixed at birth. Pig muscle fibres have been studied in depth and Handel and Stickland [11] have shown that low birthweight is associated with a reduced total fibre number. A study by Dwyer et al. [8], enlarged on these findings, and showed that total muscle fibre number, not birth weight, was a more important determinant of growth rate after pigs reached 25 kg. Pigs with large muscle fibre numbers, given good health, tend to grow faster than those pigs with a low muscle fibre number. Weight is generally only a good indicator of performance up to weaning, after which, the influences of competition has been eliminated. It can be argued that intra-litter variation in body weight is due to local differences in nutritional supply along the uterine horn (e.g. 17). Wigmore and Stickland [24] have shown that this variation in body weight can be linked to a variation in secondary fibre number.

As fibre number is fixed, postnatal growth occurs by hypertrophy of the extant fibres. The satellite cells, located on the edges of fibres, are important in this growth process. By joining with myofibres, they can help to

regulate the growth and repair of fibres. The resulting increase in nuclear number also provides an opportunity for increased protein synthesis [3]. The impact of early developmental events may affect the efficacy of satellite cell performance in addition to affecting muscle fibre number. Indeed, Mathison et *al.* [14] showed that slow and fast growth Targhee rams exhibited different levels of Insulin-like Growth Factor (IGF) receptors. This result suggests that the pattern of development of satellite cells differed between strains and was possibly impaired in the slow-growing strain.

It is hypothesised that the satellite cells of the smallest piglet of a litter will perform at a lower level to those of its largest sibling when grown *in vitro* under the same conditions.

Materials and Methods

Piglet selection and preparation

The largest and smallest piglets of six commercial litters were selected at four days old. Selection at this age ensures that the piglets used are healthy and viable; most neonatal mortality occurs within the first two days. Piglets were given a 5mi intramuscular (*im*) injection of pentobarbitone sodium (Euthatal, Rhône Mérieux Ltd, Harlow, U.K.). At this point, weights and crown-rump lengths were taken. Finally, the hind limbs were sprayed liberally with 70% alcohol and removed to a class two, microflow culture hood.

The *m. semitendinosus* was removed from each leg. The left muscle was kept in cold (4°C) Phosphate Buffered Saline (P.B.S. (GIBCO BRL, Paisley, U.K., supplier of all chemicals unless stated)), and from Pair 2 onwards, the muscle from the right leg was snap frozen in liquid nitrogen for further analysis.

Histology

10 mm thick transverse sections were taken from the mid-belly of the frozen *m. semitendinosus* by means of a cryostat (Bright, U.K.) and thaw mounted on 3'aminopropyltriethoxysilane coated slides (Sigma, U.K.) for better adhesion. Sections were allowed to air dry for 2-3 hours before being stained with haematoxylin and eosin (H and E). Nuclei and fibre number were counted utilising a Kontron KS300 image analysis package (image Associates for Zeiss, U.K.).

Cell extraction

Cells were extracted for primary cell culture using a modification of Bischoff's original method [4]. Muscles were finely chopped and extraneous non-muscle tissues removed wherever possible. The tissue was incubated, with constant agitation, for an hour at 37°C in collagenase/DNAase solution (1 mg/ml and 10 mg/ml respectively, Sigma, U.K.). This solution was made up in Dulbecco's Modified Eagle's Medium (DMEM) + HEPES containing 1% Penicillin Streptomycin Fungizone® (PSF) solution. Following incubation, muscle fibres were further teased apart. Serial centrifugation (5 minutes at 3000 rpm, 5°C) and trituration were carried out to reduce the tissue to a single cell solution. Media was changed after every centrifugation step to remove contaminating debris. When the solution could be passed through a Pasteur pipette easily, it was filtered through single layers of 45 and 30 mm Nitex. The resulting solution was layered over 20% Percol in order to set up a diffusion gradient [13], and spun for 5 minutes at 13000 rpm, 8°C. This allowed muscle cells to be separated from the rest of the muscle debris (fat, connective tissue etc: 12). The pellet was retrieved and diluted five times in DMEM Glutamax 1 (4500 mg glucose/L) containing 10% fetal calf serum (other serum may be toxic to porcine satellite cells: 5) and 1% PSF.

After preplating for ten minutes to remove fibroblasts, cells were plated at a density of 1.28 x 10^6 cells per six-well plate and incubated at 37°C, 5% CO_2 in DMEM Glutamax 1 as above.

Care of cells and collection

Twenty-four hours after plating, the cells were washed with cold PBS to remove excess debris and unattached cells. Attached cells were then returned to the DMEM Glutamax 1 solution used previously. This media was replaced every forty-eight hours.

On reaching 80-90% confluence (4-5 days), the washed wells were incubated with 1 x Trypsin EDTA for two minutes to remove the cells from the plates. The contents of each well were then transferred to 10 mls media containing 10% fetal bovine serum per well. Counting of cells was done by means of a haemocytometer after staining in Toluidine blue (Sigma, U.K.) for 5 minutes. Total numbers were calculated from this and the results analysed by use of a Mann-Whitney U-test.

Freezing and thawing of cells

Cell suspensions (1-1.5 ml per cryotube) were centrifuged as before. The supernatant was removed and the pellet resuspended in 91% serum and 9% DMSO (Sigma, U.K.). Tubes of cell suspension were frozen slowly overnight at -70°C before being transferred to liquid nitrogen for storage.

When required, cells were defrosted quickly at 37°C. Contents were centrifuged and cells resuspended in media to remove DMSO. Density was checked by means of a haemocytometer.

Results

Proliferation

Five of the six pairs of piglets used provided useful information. Pair number three was abandoned due to heavy, irreversible contamination in the majority of wells although muscle data was collected to ensure that

selection of large and small fetuses generally gave the expected muscle fibre number.

At the end of each trial, cell number per well was ascertained. From these results, a mean number of cells per millimetres was calculated. This was then used in a Mann-Whitney U-Test to determine whether the means from the small and large piglets differed significantly. At approximately 85% confluence, it was found that cells derived from the smallest piglets did perform better than those from their larger littermates (Fig. 1). Pair number four also had a degree of contamination in some of the wells. This is thought to be the reason for the impaired performance seen in Figure 1.

In one trial, cells were frozen on extraction and kept for future work. On thawing, it was found that there was little cell death in the sample as the volume of cells calculated post-thawing was similar to the known volume that was originally frozen (determined by use of Toluidine Blue staining as used previously for calculating cell number per volume). However, after the cells had been plated and allowed to proliferate, it was found that the proliferative capacity of cells from both piglets, but especially the smaller piglet, had been impaired (Fig. 2). In a direct comparison, results showed a trend towards greater cell number from the smaller piglet, yet the difference between cells derived from the large and small piglets was no longer significant (Students T-test, P = 0.1).

Muscle data

Complete transverse sections were taken from the opposite *m. semitendinosus* (mid-belly region) and stained in order that cell number could be estimated. Total nuclei and fibre numbers were determined for a representative proportion of the cross-sectional area. From these figures, a count per mm^2 was calculated and used to work out the total number per section (Table 1). A paired t-test showed that there was a difference between the large and small piglets in terms of fibre and nuclear number. However, the low sample number for the muscle data precludes strong statistical differences.

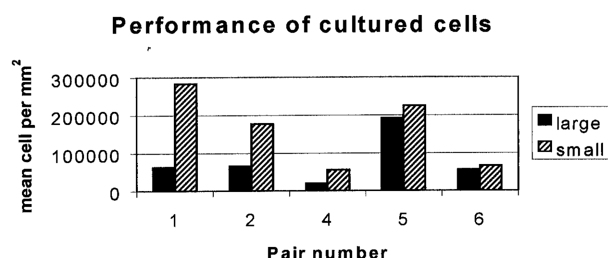

Figure 1. Mean number of cells after 4-5 days of culture. In each trial performed, the cells from the smallest piglet proliferated at a greater rate. Variations in degree of proliferation can be ascribed to the presence of contamination (as in pairs 4 and 6).

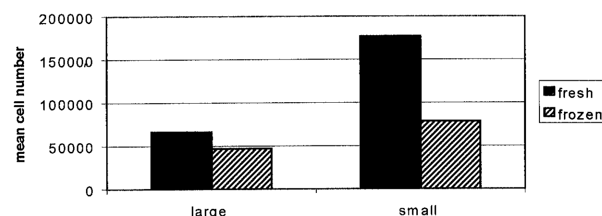

Figure 2. The effect of freezing on cell proliferation. The data shown here illustrates the effect of freezing on cell performance. After storage in liquid nitrogen, proliferative capacity is impaired. Freezing did not appear to kill cells but it may have affected cell structure or the ability to interact with the environment.

Table 1. Total fibre and nuclear numbers for large and small siblings. Table 1a displays the data for the estimated total fibre number in a 10 mm thick cross-section of the m. semitendinosus. *Table 1b gives similar data for the number of nuclei present in the same section. Values were calculated as follows; counts per mm^2 were multiplied by the cross-sectional area to give an estimated total number for the section. Data shown includes numbers from a trial which did not produce* in vitro *results due to contamination of culture wells (pair number 3). This muscle data is included to demonstrate that selection of the smallest piglet was generally an effective way of choosing a slow growing animal.*

Table 1a. Fibre number

Trial no.	Small piglet	Large piglet
2	406656	605896
3	234374	624592
4	644815	560604
5	437363	571625
6	195433	259083

Table 1b. Nuclei number

Trial no.	Small piglet	Large piglet
2	480028	629778
3	182340	417572
4	617864	524293
5	290912	510477
6	194815	268551

Discussion

Results indicate that, contrary to the initial hypothesis, porcine satellite cells are not detrimentally affected by a decreased level of nutrition *in utero.* On the contrary, it would appear that *in vitro* cell behaviour is in fact boosted by the deprivation encountered. The cells from

the smallest sibling consistently showed an elevated degree of proliferation when compared to cells from the larger sibling. The reasons for this change in behaviour have not been fully investigated in this study but possible explanations will be discussed here.

Muscle histology data confirmed that the smallest piglet usually has a smaller fibre number at birth. However, two pairs of piglets showed different results. Both piglets in pair number six had a low fibre number. This can be explained by the very small size of both the piglets compared to other pairs used in the trial. Their small size is reflected in their cross- sectional muscle areas (79.79 cm^2 and 50.93 cm^2 compared to an average of 146.79 cm^2 and 97.89 cm^2 for the other pairs). Pair 4 has a reversal of the expected results with the large piglet actually having a smaller number of fibres. However, the litter that these piglets came from was unusual in that, in a total of 12, 4 were dead and three were mummified. Interestingly, the cells from the small pig, even with more fibres, still performed better than the cells from the larger sibling. The higher fibre number probably meant that this pig was better nourished in early gestation but received less nutrition that its larger sibling in late gestation. It is therefore possible that nutrition in late gestation is more important in priming satellite cells.

The response of cells from different strains of animals has been studied in several species; turkeys [18, 23], mice [10] and sheep [14]. In each case, it was found that the fastest growing strain produced satellite cells, which responded better to culture conditions and manipulations than the corresponding slow-growth samples. Mathison *et al.* [14], showed that there was a variation in the number of IGF-1 receptors, with more in cells from the faster growing strain. This provides us with a mechanism whereby the divergence of growth patterns may occur. However, inter-strain studies, whilst helping to explain how genetic changes lead to a different plane of growth and its associated body forms, do not elaborate on how intra-litter variation occurs.

The effects of removing cells from their natural environment and placing them in culture may be transient and wear off after multiple cell divisions as the cell acclimatises to new conditions. However, further work on turkey cultures [16, 25] has looked at the performance of clones from fast- and slow- growing turkey cultures. By removing and cloning fast- and slow- growing satellite cells from the same animal, this work has shown that cell behaviour is retained and that fast- growing clones are seemingly permanently more responsive to Growth Factors and differentiation repressors.

Some studies have shown that the eventual fate of the satellite cell is not fixed. For example, Dunglison *et al.* [6] showed that adult myoblasts are capable of fusing with all fibre types. However, it appears that there may be a critical time, during development, that determines the extent of cellular response to the immediate environment In the current study, it could be suggested that satellite cell performance is not impaired by early events *in utero.* On the contrary, it would appear that a deprived start leads to the development of a cell primed to react very positively to any increase in resources. In placental studies in the guinea-pig [7] it has been found that decreased levels of nutrition, early in development, results in an increased placental area in order that maximum benefit may be gained from whatever level of nutrition is available. In this experiment, a similar scenario may also exist at the cellular level. It may be that cells from the largest piglet are exhibiting a normal response to a typical level of local nutrition. However, in the case of the smaller piglet, cells that have been subject to low levels of nutrition have developed a highly efficient response to the local environment. Given an increase in available resources, the smaller cells are primed to react and thus give a boosted rate of proliferation. Overall growth rates might not be significantly affected by the cell's ability to respond to increased levels of nutrition as the basic framework for growth (the fibre number) has been impaired earlier in development. Previous studies [8] have shown that there is a correlation with fibre number and growth rates after pig weight has reached 25 kilograms. This correlation does not exist until this point, an observation which may help to explain the satellite cells' role in early catch up growth and the failure to maximise on this potential in later development.

This study shows that satellite cell performance is not solely controlled by the genetic factors involved in strain differences. In line with the findings by Dunglison *et al.* [6], this study demonstrates the plasticity of these cells and their ability to interact with the local environment.

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