Author's response to reviews

Title: Maternal undernutrition does not alter Sertoli cell numbers or the expression of key developmental markers in the mid-gestation ovine fetal testis

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Author's response to reviews: see over
Dear Dr Brachvogel

**Re: MS: 1125264137797034 - Maternal undernutrition does not alter Sertoli cell numbers or the expression of key developmental markers in the mid-gestation ovine fetal testis**

With respect to your e-mail of October 24, we would like to thank the Journal of Negative Results in Biomedicine for providing an opportunity to respond to the referees’ comments. We have found the points raised to be very useful and now feel that our manuscript is greatly improved and, we hope, suitable for publication. The following is a point by point rebuttal of each point raised.

Yours sincerely

Richard G Lea
Associate Professor of Reproductive and Developmental Biology
Reviewer 1.

General comment
We would like to thank the referee for the suggested detailed revisions. We have addressed these points and feel that the scientific outcome of the paper is much improved.

Abstract
a) The text has been rephrased where appropriate to remove reference to the two studies.

b) The term “altered” has now been removed from the text. We have also rephrased when referring to “function”.

c) The reviewer is referring to the immunohistochemistry we have carried out and we have removed the word “immunoexpression” from the text and used “expression” or made reference to immunohistochemistry, directly, to avoid any ambiguity.

Background
a) We have added a section on the significance and role of the proliferation and apoptosis markers selected for this study.

b) We have reviewed the referenced background studies. Our previous work along with many studies carried out by others, have focussed on the in vivo effects of maternal/fetal undernutrition followed up with histological examination. We have made this clearer in the text and have also indicated which studies have been carried out in vitro. We feel that it is now clear in this section which cellular systems and tissues have been examined.

c) All references have been carefully reviewed for accuracy. The reference by Nef et al. 2000 has been removed.

Results
a) We have revised the results section so that numbers of samples analysed in all components of the study are clear. Numbers of animals in each of the 2 groups used for each study have been clarified in the results section. In the materials and methods, we have also provided a full rationale for use of a single male fetus from singleton and twin pregnancies. This point is addressed in more detail in response to referee 2.

b) We have added animal numbers to the appropriate figure legends.

Discussion
a) We have carefully restructured the start of the discussion and reported previous published findings on the effects of maternal undernutrition on fetal weight and feel that this better sets the scene for the remaining discussion.

b) The word “function” has been removed.
c) The discussion has been restructured so that it more effectively draws out several key points, based on a discussion of both current and previously reported work. Our data highlight for the first time that the genes involved in mediating the effects of undernutrition are different in the testis and ovary. We have presented the case for the selection of these testis genes for study, explaining why we believe them to be the most appropriate, and proposed that this new information may point to a possible effect at the hypothalamic-pituitary level.

Methods
a) The text has been revised and reduced.

b) The word micron has been replaced by the symbol.

c) The suppliers of equipment have been added to the text.

d) All references to “two studies” have been removed from the paper.

e) The reference to the name of the operator has been removed.
Reviewer 2.

**Major compulsory**

Point 1 and line 261:
The numbers of twin v single pregnancies have been added to the materials and methods section under “animal management and nutritional treatments”. There were 2 twin pregnancies in the control group (n=6) and 7 in the L group (n=9). Further subdividing on the basis of sex of the co-twin therefore produced very low n values. We do not consider this a problem because the design of the experiment ensured that the nutritional state of the fetuses was similar, irrespective of whether they were singles or twins (rations were adjusted according to litter size to ensure a uniform nutrient supply, irrespective of litter size). Relative to the size of effects associated with litter size (in the absence of compensatory feeding) effects of position in the uterus and sex of co-twin, if present, were considered likely to be very small and only demonstrable with very large numbers of animals and this study was not designed to address these issues. Furthermore, the types of measurements reported in the current study require large amounts of time and other resources; conducting similar measurements on the very large numbers of samples required to address the issues of uterus position and sex of co-twins would have been beyond our resources and that of most laboratories.

Line 341: This is largely dealt with above and this has been clarified in the materials and methods and the statistical analysis sections.

**Minor essentials**

L 60 – This has been altered.

L 109 - A clear working hypothesis has been added to the final paragraph of the background section. This section reads as follows:

“In view of previous reports of compromised testicular structure in adult sheep born to nutritionally-restricted dams, we hypothesised that an early driver of these changes may be a reduction in fetal testis Sertoli cell numbers and/or changes in developmental gene expression similar to those observed in the ovaries of fetuses from undernourished ewes. Consequently we examined day 110 fetal testes from undernourished ewes for Sertoli cell numbers, cell proliferation and the expression of developmental genes shown, previously, to be altered in fetal ovaries at the same stage of gestation.”
Reviewer 3

1. The aim has now been re-written as a hypothesis, in line with the comments of referee 2.

2. The aim was not to compare gonadal development in the sexes per se. The work on the females was cited because it demonstrated that some of the genes known to be involved in gonadal development are sensitive to maternal undernutrition at the same stage of gestation. We have made this clear in the revised introduction.

3. We suggest that the 30-90 day window is unlikely to be the only critical window since maternal undernutrition may alter the developing testis before day 30, prior to sexual differentiation, or may act through perturbation of the fetal HPG axis which is not completely developed until after 80 days; indeed the results of our study seem to point to hypothalamic-pituitary involvement in expression of the effects of undernutrition. We chose to look at the effects of undernutrition from 0 to 110 days since this encompasses the key developmental stages of testis development, including the first part of the period of proliferation of Sertoli cells which is reported to occur from 70 days through to parturition.

4. We did not want to demonstrate that undernutrition has a different impact on the testis compared to the overnutrition/placental insufficiency model. A comparison of the effects of over- and undernutrition would have required the two contrasting treatments to be applied in a single study. A sentence has now been added to the introduction, explaining the importance of adverse effects on ram function for sheep flock productivity, to add more context to the work.

5. We are not entirely sure what the referee means by the question and by primary and secondary factors. If nutrition is considered to be a ‘primary factor’, it is well known that it does induce alterations. If the term ‘secondary factors’ refers to the changes in gene expression that might be involved in such a process, inducing an effect at the time of study or later in development, then that is indeed what we want to investigate.

6. In studies such as this, maternal body weights are not a useful measure because they are heavily influenced by gut fill. Accordingly, mean condition scores provide a better index of long-term nutritional state and that is why they, and not liveweights, were given; this is now further explained in the methods section.

7. Mean fetal weights are now stated.

Minor revisions

8. An explanation of body condition scoring has now been added to the methods section and reads as follows:

“At the time of mating, ewes were allocated randomly, within body condition score class (range 2.25 – 3.00, on a scale of 0 to 5 [33]), to one of two nutritional
treatment groups. This widely used measure comprises a subjective assessment of fat cover on the back of the animal and is highly correlated with body fat reserves. Since the live weight of animals is greatly influenced by gut fill in studies involving nutritional manipulations such as this, body condition scores were considered to be a more useful measure of medium term changes in nutritional state.”

9. The authors have extensive experience of this type of study (Rhind has published extensively on the subject of nutritional effects on reproduction in sheep, usually using housed animals). Subjective assessment of the animals in this and other studies suggest that the animals overcome the stress associated with movement and housing within hours, as measured by food intake, vocalisation and general behaviour. Studies in the past by Rhind have shown that it is actually difficult to induce a chronic stress in housed sheep through normal management procedures, as measured by a combination of behaviours, cortisol profiles, heart rates and immune function (Rhind, S.M., Doney, J.M., Gunn, R.G. and Leslie, I.D. 1984. Effects of body condition and environmental stress on ovulation and embryo survival rates and associated plasma FSH, LH, prolactin and progesterone profiles in Scottish blackface ewes. *Animal Production* 38 201-209; Rhind, S.M., Reid. H.W., McMillen, S.R. and Palmarini, G. 1998. The role of cortisol and β-endorphin in the response of the immune system to weaning in lambs. *Animal Science* 66 397 – 402.

10. Gestation (d0) begins on the day when most ewes are observed standing for the ram. Since oestrus was synchronised, the mating time is restricted to a period of approximately 48h with the majority being mated within a 24h period. Thus, the nutritional intervention lasted almost exactly 110 days. Reference is now made to d0 as the day of synchronised oestrus.