Cellular and molecular responses of adult testis to changes in nutrition: novel insights from the sheep model

Yongjuan Guan[†] and Graeme B Martin

UWA Institute of Agriculture and School of Agriculture and Environment M087, University of Western Australia, Crawley, Australia

Correspondence should be addressed to G B Martin; Email: graeme.martin@uwa.edu.au

[†](Y Guan is now at Department of Biomedical Sciences, School of Veterinary Sciences, University of Pennsylvania, Philadelphia, Pennsylvania, USA)

Abstract

This review explores the cellular and molecular mechanisms that regulate spermatogenesis in the post-pubertal testis that is regressing in response to mild undernutrition, using the sexually mature male sheep as a model. Testis regression leads to reductions in daily sperm production and in the quality of ejaculated spermatozoa (poorer movement, DNA damage). There is also a reduction in spermatogenic efficiency that appears to be caused, at least partially, by increases in germ cell apoptosis. Sertoli cell number does not change with testis regression, although about 1% of Sertoli cells do appear to retain proliferative ability after puberty. On the other hand, Sertoli cell function is disrupted during testis regression, as evidenced by a disorganization of tight junctions and indications that cell differentiation and maturation are reversed. Disrupted Sertoli cell function can explain, at least partially, the increase in germ cell apoptosis and any decrease in the rate of spermatogenesis, the two major contributors to spermatogenic efficiency. These outcomes seem to be mediated by changes in two RNA-based processes: (i) the expression of small non-coding RNAs that are involved in the regulation of Sertoli cell function, spermatogenesis and germ cell apoptosis and (ii) alternative pre-mRNA splicing that affects the regulation of spermatogenesis but does not appear to affect germ cell apoptosis, at least during testis progression induced by undernutrition in the male sheep. These research outcomes can be extended to other animal models and are relevant to issues in human male fertility.

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Introduction

In this review, we present advances in our basic understanding of testis function that have been revealed by studies of the effects of nutrition in sexually mature male sheep. The boundaries of the experimental model are important: changes in the level of nutrition can induce a reversible, non-pathological process that leads to changes in testis mass, sperm output and the efficiency of spermatogenesis. These responses are expressed under normal field conditions, either in relation to the annual forage cycle or in response to acute nutritional supplementation. The fact that the animals are post-pubertal is critical because there is an inevitable focus on the function of Sertoli cells—cells that are thought to lose their ability to divide and become terminally differentiated at puberty. Finally, the model does not equate to fasting or to pathological effects caused by deficiencies in vitamins or trace elements, heavy metal toxicities or imbalances in essential amino or fatty acids. Rather, we constrain the model to variations in the amount of a balanced diet, using extremes that are

similar in magnitude and duration to those that would be experienced by animals grazing natural forage (review: [Martin et al. 1994b](#)).

The neuroendocrine and endocrine processes through which nutrition affects reproductive function in this model have been explored in depth, and this work has been reviewed elsewhere (e.g., [Martin et al. 1994b](#), [Blache et al. 2003](#)). In summary: (a) the responses involve metabolic inputs to the brain–gonadal axis (nutrients, metabolites, substrates) as well as endocrine signals from metabolic and storage tissues (leptin, insulin); (b) the metabolic and reproductive centres of the brain respond with changes in the output of gonadotrophin-releasing hormone (GnRH), and thus, the secretion of gonadotrophins, inhibin and sex steroids; (c) testis mass also seems to be affected by processes that are independent of changes in GnRH secretion. Clearly, this endocrine context is critical when considering the cellular and molecular responses within the testis.

The mature male sheep and its responses to nutrition have a long history as a model for research into testis function. The first rigorous investigation was by

Akira Mori who studied severely underfed rams in Japan during the Second World War. When his reports were published many years later (Mori 1959a,b), they showed that semen quality and sperm production were improved within a month or two by supplements of milk, pork and eggs! Subsequent studies included measures of testis mass, sperm output and 'spermatogenic efficiency', the number of sperm cells produced per unit mass of testis (Salamon 1964, Setchell *et al.* 1965, Braden *et al.* 1974, Oldham *et al.* 1978, Cameron *et al.* 1988, Guan *et al.* 2014b). The changes in spermatogenic efficiency are particularly relevant to the present review – for example, (Oldham *et al.* 1978) found that a 25% increase in testicular size led to an 81% increase in production of spermatozoa. Also important is the observation that it takes at least 7 weeks of nutritional treatment to affect the number of ejaculated spermatozoa (Parker & Thwaites 1972), suggesting that spermatogenic efficiency is affected after the last spermatogonial division.

Using the classical measures of semen and sperm quality, it was found that the deleterious effects of under-nutrition could be reversed by dietary supplementation (Mori 1959a, Salamon 1964, Tilton *et al.* 1964, Hiroe & Tomizuka 1965, Parker & Thwaites 1972) (Dana *et al.* 2000, Tufarelli *et al.* 2011). Not all studies agree (Fernandez *et al.* 2004) perhaps because the classical measures (sperm concentration, viability, morphology, subjective motility) lack accuracy and precision. Moreover, these techniques only assess the semen quality and do not address the quality of the spermatozoa. To resolve these issues, we used computer-assisted semen analysis and a sperm chromatin structure assay (Guan *et al.* 2014b). We found that sperm from rams that had been underfed for 65 days (testes regressing) swam with lower velocity and had more DNA damage than sperm from well-fed rams (testes growing). The amount of DNA damage was inversely correlated with change in testis mass, the percentage of motile sperm, and the numbers of sperm per gram of testis. It is therefore clear that, in adult rams, the effect of nutrition on spermatogenic efficiency is associated with a change in the quality of the spermatozoa as well as the number produced per day.

The effect of nutrition on testis morphology

Histological studies have shown that nutrition markedly affects the diameter of the seminiferous tubules, the relative proportion of testis occupied by the seminiferous tubules, the proportion of the seminiferous tubule occupied by the seminiferous epithelium, the relative proportion of interstitial tissue and the total volume of Leydig cells (Table 1). These observations were extended in a subsequent study (Guan *et al.* 2014a) that included the Johnsen Score, an indicator of spermatogenesis, again showing that spermatogenesis was impaired in underfed rams (testes regressing), but normal in both

Table 1 Morphometric analysis of the testicular tissue from mature male sheep ($n = 5$ per group) fed a supra-maintenance (high) or a sub-maintenance (low) diet for 69 days.

Variable	High diet	Low diet
Body weight (kg)	79 ± 3	48 ± 4*
Mean testis weight (g)	288 ± 14	117 ± 10*
Tubule diameter (µm)	229 ± 6	167 ± 12*
Lumen diameter (µm)	69 ± 3	66 ± 7
Tubule length (m)	3503 ± 104	2378 ± 329*
Leydig cells (×10 ⁶) per testis	75 ± 8	60 ± 11
Sertoli cells (×10 ⁶) per testis	120 ± 5	77 ± 6.7*

These data have been selected from the full set presented by Hötzel *et al.* (1998).

* $P < 0.05$.

maintenance-fed (testes stable) and well-fed rams (testes growing). The number of Leydig cells per testis was not affected by diet, but the total volume of Leydig cells was, indicating changes in the volume of individual cells. Due to the direct relationship between Leydig cells and testosterone secretion, therefore, an effect of diet on testosterone secretion and the peripheral concentrations of testosterone might be expected, and this is an important consideration because testosterone plays a major role in spermatogenesis. However, initial studies disagreed in terms of the outcome – for example, the early study showed a significant effect on testosterone secretion (Setchell *et al.* 1965) but recent work showed that nutritional treatments were not associated with changes in the amplitude of testosterone response to LH (Martin *et al.* 1994a). The disagreement could be due to differences between genotype, age or methodology, but the most likely explanation is the severity of the underfeeding (Hötzel *et al.* 1998).

The effect of nutrition on Sertoli cell number

Sertoli cells provide nutritional and structural support for germ cells and each Sertoli cell has a fixed capacity for the number of germ cells it can support (Sharpe *et al.* 2003); therefore, changes in production of spermatozoa may result from alterations in Sertoli cell number. This question was first addressed by Hötzel and coworkers (1998) who reported that total volume of Sertoli cell nuclei and the Sertoli cell number per testis were both higher in well-fed than in underfed adult Merino rams (Table 1). This finding might seem logical considering the differences in sperm production, but it contradicts the dogma that Sertoli cells stop proliferating at puberty, leaving the number fixed during adult life (Kluin *et al.* 1984, Monet-Kuntz *et al.* 1984, Hochereau-de Reviers *et al.* 1987). We therefore decided to repeat the study by using modern techniques (Guan *et al.* 2014a). We adopted GATA4 as a marker for Sertoli cells (Ketola *et al.* 2000) and combined stereological cell counts with the assessment of Sertoli cell activity by analysis of immunoreactivity to proliferation cell

nuclear antigen. We found that there was no effect of nutritional treatment on Sertoli cell numbers, so the earlier observations of Hötzel and coworkers (1998) can be attributed to histological artefact, perhaps caused by effects of nutrition on Sertoli cell functionality (see below) that affected the ease and accuracy with which their nuclei could be counted. Interestingly, about 1% of Sertoli cells in all animals appeared to retain their ability to proliferate (Guan *et al.* 2014a). The possibility that Sertoli cells can proliferate after puberty suggests that, in future we might find ways to replenish damaged testes and restore germ cell production.

The effect of nutrition on Sertoli cell function

Around the onset of puberty, Sertoli cells undergo radical changes as they switch from an immature, proliferative state to a mature, non-proliferative state. Adjacent Sertoli cells form tight junctions with each other to create a unique adluminal compartment within which the meiotic and post-meiotic steps of spermatogenesis can proceed, as well as allowing formation of a fluid-filled lumen. As a result, the germ cells developing in the adluminal compartment are effectively sealed off from direct access to many nutrients, so the mature Sertoli cell takes on new functions that are lacking in fetal, proliferating Sertoli cells (McLaren *et al.* 1993).

In sexually mature sheep, we have shown that Sertoli cell function is clearly affected by nutritional treatment (Guan *et al.* 2014a), as evidenced by changes in molecular regulators of critical processes that are specific to Sertoli cells. Underfeeding-induced testis regression was associated with: (a) disrupted distribution of the tight junction protein CLDN11, increased expression of *CLDN11* and decreased expression of zonula occludens protein 1 (*ZO1*); (b) reduced expression of *GATA1*, a marker for mature Sertoli cells (Beau *et al.* 2000), but an increase in the expression of *AMH*, a marker for immature Sertoli cells (Rey 1998), (Kliesch *et al.* 1998, Steger *et al.* 1999, Sharpe *et al.* 2003); (c) reduced expression of three Sertoli cell-specific genes (*KLM*, *SOX9*, *MSI1*). Therefore, in underfed rams experiencing a reduction in testis mass and spermatogenesis, it seems that tight junctions are degraded and differentiation and maturation are reversed in the Sertoli cells, reducing their efficiency as supporters of the germ cells. These observations are consistent with the reduction in the quality of ejaculated spermatozoa from the underfed rams.

Effects of nutrition on small RNAs in the testis

Small RNA molecules have recently emerged as potent regulators of gene expression at the post-transcriptional or translational level, with diverse biological outcomes (Plasterk 2006, Liang *et al.* 2014). There are three major categories of small RNAs: small interfering RNA (siRNA);

microRNA (miRNA) with about 22 nucleotides in single-strand non-coding molecules and piwi-interacting RNA (piRNA). In this review, we will focus only on miRNAs and piRNAs where we have direct evidence for their roles in our model. MicroRNAs were discovered in 1993 in a nematode where they regulate the expression of complementary mRNA (Lee *et al.* 1993, Wightman *et al.* 1993). In 2011, miRNAs were also identified in mammals (Lagos-Quintana *et al.* 2001, Liang *et al.* 2015). miRNAs are highly conserved across species, and importantly, appear to regulate up to 30% of all genes in the human genome (Lewis *et al.* 2005). Thousands of miRNAs have now been discovered (miRBase Release 21.0, as of July, 2015, <http://www.mirbase.org/index.shtml>). The biogenesis of miRNAs is a multi-step process and has been well documented (Hutvagner *et al.* 2001, Kim *et al.* 2009, Liang *et al.* 2015), so will not be detailed here. The piRNAs form a relatively newly identified class of slightly longer molecules (26–32 nt) that bind to 'PIWI', a spermatogenesis-specific protein belonging to the Argonaute protein family (Aravin *et al.* 2006, Girard *et al.* 2006). The synthesis of piRNAs is not clear yet, although a 'ping pong' mechanism has been suggested (Liu *et al.* 2012). They are essential for germ cell maintenance and spermatogenesis in *Drosophila* and mammals (Thomson & Lin 2009), and so are of interest in our quest to understand the effects of nutrition on spermatogenesis.

Nutrition, miRNAs and spermatogenesis

During spermatogenesis, the spatial and temporal regulation of gene expression is vitally important – indeed, transcription is periodically silenced in germ cells by miRNAs (Papaioannou & Nef 2010). The importance of miRNAs for spermatogenesis is also indicated by, for example, the infertility in male mice that follows removal of *Dicer1*, a gene necessary for the synthesis of miRNAs (Maatouk *et al.* 2008). Specifically, in *Dicer1* knockout mice, only a few tubules contain elongating spermatids and any germ cells that did differentiate to elongating spermatids exhibited abnormal morphology and motility. Similar findings were reported in human (Hayashi *et al.* 2008).

It thus became clear that miRNAs could play a role in eliciting changes in spermatogenesis following a change in nutrition. We identified 44 miRNAs, including miR-10b and miR-23b, which were differentially expressed in well-fed and underfed male sheep, and the predicted functions of these miRNAs were related to reproductive system development and sperm production and quality (Guan *et al.* 2015).

There is also a strong relationship between miRNAs and apoptosis – for example, three miRNAs (miR-15, miR-16, miR-31) are able to induce apoptosis by targeting the major anti-apoptotic factor, *BCL2* (Cimmino *et al.* 2005, Korner *et al.* 2013). It has also become clear that apoptosis in male germ cells is regulated by

miRNAs – for example, miR-34c was detected in mouse pachytene spermatocytes and highly expressed in spermatids; when it was silenced, the *Bcl-2/Bax* ratio increased, preventing the induction of germ cell apoptosis by testosterone deprivation (Liang *et al.* 2012). In another study, transient inhibition of miR-21 in spermatogonial stem cell-enriched germ cell cultures increased the number of germ cells undergoing apoptosis (Niu *et al.* 2011).

Clearly, miRNAs could play a major role in germ cell apoptosis in the regressing testis of underfed rams. We found that 12 genes involved in apoptosis could be targeted by 9 miRNAs that were expressed differentially between well-fed and underfed sheep, with novel-miR-144 targeting four of the apoptosis-related genes (*FASL*, *CASP3*, *BCL2L1*, *TP53*; Guan *et al.* 2015). These findings led us to conclude that the decline in sperm production and sperm quality induced by undernutrition in the sexually mature sheep are mediated at least partly by increased apoptosis in germ cells, and this process is mediated by changes in the expression of miRNAs (Guan *et al.* 2015).

piRNAs affect spermatogenesis

A role for piRNAs in spermatogenesis is primarily supported by the functions of their partner Piwi proteins, including MIWI, MIWI2 and MILI, in stem cell self-renewal and the development of male germ cells (Cox *et al.* 1998). In MILI-knockout mice, spermatogenesis is disordered at the pachytene spermatocyte stage (Kuramochi-Miyagawa *et al.* 2004) and, in Miwi-deficient mice, no elongated spermatids or mature spermatozoa are observed (Deng & Lin 2002).

To explore further the functions of piRNAs in spermatogenesis, undernutrition-induced testicular regression is an attractive experimental paradigm. We identified 35 putative piRNAs, including oar-piR-12568 and oar-piR-9006, which were differentially expressed in well-fed and underfed males and were associated with sperm production and quality (Guan *et al.* 2015). Interestingly, there was a positive correlation between the proportions of miRNAs and putative piRNAs in testicular tissue, for both underfed and well-fed males, indicating a synergistic relationship between these classes of small RNAs, a hypothesis that should be tested in further studies (Guan *et al.* 2015). Moreover, unlike the miRNAs, piRNAs are not conserved among species, so their functions in the sheep testis require further study.

Effects of nutrition on alternative pre-mRNA splicing

Alternative pre-mRNA splicing (AS) is an important mechanism for regulating gene expression and for increasing transcriptome plasticity and proteome diversity (Liang *et al.* 2016). It has been reported that

around 60% of human gene products undergo alternative splicing (Modrek & Lee 2002).

The process that generates alternative splicing has been reviewed in detail (Schwerk & Schulze-Osthoff 2005), so will be addressed only briefly here. In a typical multiexon mRNA, the splicing pattern can be altered in many ways and, to date, eight types have been reported, of which the most common is a cassette exon that can be included in the mRNA or skipped, inserting or deleting a portion of internal sequence (Gurskaya *et al.* 2012). Two special cases of paired-cassette exons are mutually exclusive splicing (only one exon is included) and coordinate cassette exons (both exons are included). The fourth and fifth patterns are alternative 5' or 3' splice sites, in which exons can be extended or shortened in length (Fu *et al.* 1992). The sixth pattern is alternative first exon, in which transcriptional initiation at different promoters generates alternative 5'-terminal exons that can be joined to a common 3' exon downstream (Mironov *et al.* 1999). Similarly, for the seventh pattern, alternative last exons, with alternative polyadenylation sites, can be joined to a common upstream exon (Wang *et al.* 2008). Finally, we have intron retention to leave the retained intronic sequence in the mRNA (Galante *et al.* 2004).

Nutrition, alternative pre-mRNA splicing and spermatogenesis

It has been reported that spermatogenesis is regulated by alternative pre-mRNA splicing that generates multiple transcript species from a common mRNA precursor. For example, some specific *CREB* mRNA isoforms generated by alternative splicing are expressed at a high level in the adult testis, and these isoforms are expressed after spermatogenesis has started (Ruppert *et al.* 1992). In addition, transcripts from several testis-specific genes that regulate gene expression are themselves alternatively spliced. For instance, a testis-specific splice of the Sry-related transcription factor, *Sox17*, which lacks the exon containing a single high mobility group box near the NH₂-terminus, replaces the normal message during male meiosis, and results in an inactive N-terminal truncation that lacks the DNA-binding domain in spermatids (Kanai *et al.* 1996). Another example is prolactin receptor, a pivotal factor for spermatogenesis in the mouse – one of its isoforms lacks two exons leading to downregulation of the expression of the full-length prolactin receptor, with the potential for explaining the role of prolactin in the annual cycles of testis growth in seasonal breeders such as red deer (Jabbour *et al.* 1998). These examples are probably the tip of an iceberg, and we expect many more candidates to be discovered experimentally by, for example, knock-out of testis-specific splicing factors (Feng *et al.* 2002).

To date, very few studies have touched on the effects of environmental factors on alternative splicing within the testis and, again, undernutrition-induced testicular

regression is an attractive experimental paradigm. In our transcriptome analysis in the testicular tissue from well-fed and underfed sheep, we found that nutrition did not affect the total number of alternative splicing junctions, but affected more than 200 alternative splicing events (Fig. 1). A total of 159 genes, including *CREM* and *DDX4*, were differentially spliced between dietary treatments, with functions related to RNA splicing and spermatogenesis (Guan *et al.* 2017). Therefore, changes in alternative pre-mRNA splicing can help explain the effects on nutrition on spermatogenesis, but the effects of specific alternative types of splicing on spermatogenesis require further study.

Nutrition, alternative pre-mRNA splicing and apoptosis

Alternative splicing also plays a major role in the control of apoptosis, as evidenced by its effects on the expression of many of the proteins directly involved in the apoptotic pathways. Moreover, the proteins belonging to each family of apoptotic factors are alternatively spliced and, normally, the different isoforms produced in this process have distinct and even opposing functions during apoptosis. For example, by alternative splicing, *C. elegans CED-4* is expressed in two isoforms, *CED-4L* and *CED-4S*, that have opposite functions in apoptosis. Interestingly, splice site mutations in *CED-4* lead to increased expression of anti-apoptotic *CED-4L* (Shaham & Horvitz 1996). In addition, alternative splicing inhibits apoptosis by removing the intracellular domain and part of the extracellular domain from *FasL* (Ayroldi *et al.* 1999).

In the testes affected by nutrition, we explored alternative splicing events using RNA-Seq and found that the alternatively spliced genes that differed between underfed and well-fed sheep were not related to apoptosis (Guan *et al.* 2017), although one gene (*HIPK3*) was reported to be a member of a gene family that has been implicated in apoptosis. This result was surprising, because as indicated above, apoptosis is strongly associated with alternative splicing in other models in which nutrition was not the factor driving change in the testis. Clearly, the effects of nutrition need to be tested in other species.

Extension to other species and human fertility

The effect of nutrition on spermatogenesis has also been explored in other animal models and humans. For example, in a recent study of Holstein bulls, it was claimed that enhanced early-life nutrition increases sperm output potential without decreasing semen quality (Dance *et al.* 2016). Similarly, for the human, it was reported that sperm concentration, total sperm number, progressive motility were all positively associated with a healthy dietary pattern (Oostingh *et al.* 2017). This finding is supported by the positive correlations between

the supplements of Vitamins D, B6 and B9 and sperm and semen parameters in humans (Abbasihormozi *et al.* 2017, Najafipour *et al.* 2017). It seems likely that changes in Sertoli cell function, such as those we have observed in the sheep, mediate such effects.

In recent years, there has been growing interest in the transgenerational effects of paternal malnutrition on subsequent offspring health, many of which are causally associated with epigenetic effects of nutrition on germ cells (Sinclair *et al.* 2016). Most intriguing in the context of the role of Sertoli cells is the finding that chronic paternal folate deficiency alters sperm DNA methylation and delays the onset of spermatogenesis, an effect associated with decreased pregnancy rates and birth defects (Bentivoglio *et al.* 1993, Lambrot *et al.* 2013). Such transgenerational effects suggest that nutritional factors alter the epigenetic landscape of sperm, leading to the transmission of altered epigenetic signatures to the next generation (Lambrot *et al.* 2013, Siklenka *et al.* 2015).

It is important to clarify the difference between well-fed animals that are the main focus of our studies and diet-induced obesity. In our studies, the animals were limited to a 10% increase in body weight over 3 months, similar to the normal annual pattern driven by the natural forage cycle (Martin *et al.* 1994b). Our animals never reached a body condition score that would be classified as 'obese', so cannot be compared to, for example, the extreme condition scores described by Zhu *et al.* (2010) or a human adult body mass index >30 (Centres for Disease Control and Prevention). Obesity in humans is associated with a decrease in male fertility as evidenced by the prevalence of artificial reproductive technologies (Sunderam *et al.* 2017). Many such patients have diabetes, a disease also linked to reduced sperm count and sperm motility and increased numbers of abnormal sperm. In one study, only 8.5% of diabetic patients had normal testis function (Inih *et al.* 2017). This phenomenon seems to apply across species because similar outcomes are observed in mice, with male obesity altering sperm microRNA content (Ghanayem *et al.* 2010, Fullston *et al.* 2016). The adipose-tissue hormone, leptin, might play a role in obesity-induced male infertility or subfertility (Barash *et al.* 1996), perhaps through effects on Sertoli cell acetate production and thus the nutritional support of spermatogenesis (Martins *et al.* 2015). In our sheep model, the dietary treatments are relatively mild but nevertheless affect circulating leptin concentrations (Blache *et al.* 2003), suggesting the need to investigate this mechanism.

Finally, we need to remember that males rarely live in an environment where only one factor varies (Martin *et al.* 1994b). In addition to variation in nutrition, free-ranging animals are often confronted with stressors that will likely affect Sertoli cell function. In mice, for example, it has been shown that heat stress induces germ cell apoptosis and chronic restraint stress

Figure 1 A working hypothesis of the mechanisms through which undernutrition affects testis function in the sexually mature sheep, indicating roles could be played by small, non-coding RNAs. Stimulation is indicated by '+ve' and inhibition is indicated by '-ve'. Effects mediated by regulatory factors are indicated by vertical arrows, and effects mediated by alternative RNA splicing are indicated by 'X'. Solid lines denote pathways supported by strong evidence, whereas broken lines indicate pathways where the evidence is still accumulating. Nutritional and metabolic signals do not seem to affect the proliferation of Sertoli cells but they do affect Sertoli cell function, notably the organization of tight junctions and cellular maturation. The Sertoli cell responses, combined with nutrition-induced changes in germ cell apoptosis, affect the quantity and quality of spermatozoa produced. Modified from Guan *et al.* (2015).

decreases testosterone secretion (Lopez-Calderon *et al.* 1991, Yin *et al.* 1997, Paul *et al.* 2009). Similar findings have been reported for the human (Fenster *et al.* 1997, Clarke *et al.* 1999).

Conclusions and future directions

In sexually mature male sheep, the loss of testis mass and reductions in sperm production, sperm quality and spermatogenic efficiency caused by mild undernutrition are not accompanied by changes in Sertoli cell number, although a few Sertoli cells do appear to retain proliferative ability after puberty. On the other hand, testis regression is associated with disruption of Sertoli cell function, including a loss of tight junction integrity and reversal of differentiation and maturation. These effects would be expected to reduce the nutritional and structural support for the developing germ cells, thus explaining, at least partially, the increase in germ cell apoptosis and reduction in spermatogenesis, two contributors to spermatogenic efficiency. These outcomes seem to be mediated by changes in two RNA-based processes: the expression of small non-coding RNAs that are involved in the regulation of Sertoli cell function, spermatogenesis and germ cell apoptosis, and alternative pre-mRNA splicing that affects regulation of spermatogenesis but does not appear to affect germ cell apoptosis, at least during testis regression induced by mild undernutrition in the male sheep.

Our observations of nutritional effects on spermatogenesis in the sheep need to be confirmed in other animal models, such as the mouse, if only because ruminants and monogastrics might be expected to differ significantly in their physiological responses to nutritional treatments. Moreover, piRNAs are not conserved among species so the changes that we observed require further study. In addition, our profiling of small RNAs and the transcriptome, and our observations of the relationship between RNA expression and protein function, need to be tested in other models. Finally, we need to test whether the molecular mechanisms that are responsible for the reversible and non-pathological change in testis function induced by under-nutrition in male sheep can be applied to other environmental challenges or pathological disruptions of sperm production. On the other hand, the differentially expressed miRNA (novel-miR-144), the differentially spliced genes (*CREM* and *DDX4*), and the testis phenotype related genes (*CFLAR*, *PTPRC*) that we have identified in sheep could be potential biomarkers for apoptosis and spermatogenesis in humans. The predicted functions of these genes need to be verified using *in vivo* and *in vitro* experimentation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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