Animal Reproduction Science 207 (2019) 171–179

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Review article

Declining testicular function in the aging stallion: Management options and future therapies

Regina M. Turner

New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, 382 W. Street Rd., Kennett Square, PA 19348, USA

ARTICLE INFO

Keywords:
Stallion
Subfertility
Aging
Testicular degeneration

ABSTRACT

Declining fertility in association with declining testicular function is commonly seen as stallions age and can be the cause of significant economic losses in the equine breeding industry. This manuscript describes how to clinically recognize the signs of age-related declining testicular function (testicular degeneration) and also provides mare and stallion management strategies for improving reproductive outcomes. Finally, the current understanding of the pathophysiology of the disease is presented, including the results of recent studies that are beginning to uncover the underlying causes for age-related declines in testicular function in stallions. These new findings provide a basis for possible future treatments that could delay the effects of aging on the testis.

1. Introduction

Age-related declines in fertility are commonly seen in middle – aged and older stallions in association with declining testicular function. This is sometimes referred to as *testicular degeneration (TD)* or, more specifically, as *idiopathic or age-related testicular degeneration (ITD)* to differentiate it from testicular degeneration that occurs following a known insult to the testis (such as trauma, heat shock, administration of androgens, etc.). As the testis ages, its two main functions – production of gametes and production of hormones – are adversely affected. In stallions, where the animal's value is often defined by its ability to produce offspring, the focus is on declining gamete production and the associated decline in fertility, which typically long precede any noticeable declines in androgen production or libido. In stallions with large books, even slight decreases in reproductive efficiency can result in substantial economic losses stemming from the loss of breeding fees, fewer offspring, increased management costs and the loss of valuable male genetics. As the problem advances, reproductive inefficiency typically progresses to subfertility or infertility, rendering even stallions with smaller books unable to compete in the commercial market and eventually forcing the animal's retirement (Blanchard and Varner, 1993; Gehlen et al., 2001; Watson et al., 1994).

Animals in the early or middle stages of the condition often can be 'managed through' the problem. More intensive management +/− the use of assisted reproductive techniques can help to reduce the numbers and the quality of sperm that are required for fertilization and so can extend the breeding careers of affected stallions, sometimes for years. However, an actual treatment for the disease has not been identified. Although there are some manufacturers marketing products that claim to delay or prevent the onset of declining testicular function, there is little or no controlled data to support these claims. A better understanding of the pathophysiology of the disease will be the first step towards the development of effective methods for preventing or slowing the course of age-related TD. Recent studies are making progress towards these goals.

E-mail address: rmturner@vet.upenn.edu.

https://doi.org/10.1016/j.anireprosci.2019.06.009
Received 16 March 2019; Received in revised form 6 June 2019; Accepted 6 June 2019
Available online 08 June 2019
0378-4320/

2. Recognizing age-related testicular degeneration in the clinical setting

2.1. Signalment and history

The onset of age-related TD is most often appreciated in stallions in their late teens or early twenties, although some stallions remain highly fertile well into their twenties, while others begin to experience age-related declining fertility in their mid-teens or earlier. Therefore, in addition to maintaining excellent breeding records, we recommend that all breeding stallions, but especially those that are 15 years of age or older, be carefully monitored from year to year and eventually from month to month for clinical signs of TD. This should include regular assessment of semen characteristics and sperm numbers as well as physical examination and measurement of the testes.

Clinically and histologically, TD may be indistinguishable from testicular hypoplasia (Watson et al., 1994). Since, by definition, testicular degeneration is an acquired condition, while testicular hypoplasia is congenital, an accurate diagnosis of TD can be made only if the stallion has a history of declining reproductive efficiency, decreasing testicular size, decreasing semen characteristics, or some combination of these things (i.e., a history that indicates the stallion was once more fertile than it is now). As such, information on the stallion's past book sizes, seasonal pregnancy rates, average numbers of heat cycles per pregnancy, testicular measurements and past semen analyses all can be very helpful.

Because most cases of TD begin gradually, the earlier stages of the disease often manifest as a decline in reproductive efficiency that may at first go unrecognized. The average number of cycles per pregnancy (CPP, defined as the number of estrous cycles bred over the season divided by the number of mares pregnant) is a highly sensitive measure of breeding efficiency. As a result, it is likely to be a more sensitive indicator of declining fertility than, for example, the seasonal pregnancy rate (SPR, defined as the number of mares pregnant divided by the number of mares bred) (Love, 2011). Therefore, whenever possible, detailed breeding records should be maintained and CPP calculated to identify any downward trends. Particularly for stallions with smaller books, it is not uncommon to see an increase in CPP, even as SPR remains unchanged. Looking only at the SPR or foaling rate can cause more subtle declines in reproductive efficiency to go unnoticed.

Although age-related TD is typically a slowly progressive problem, keep in mind that some cases may present for an acute onset of infertility or subfertility. It is often unclear whether this 'acute' age-related decline in fertility is real, or whether it is only perceived because semen characteristics and testicular parameters were not being routinely monitored and the earlier stages of the disease therefore went unnoticed.

Because not all cases of testicular degeneration are idiopathic, it is important to establish from the history whether or not some inciting cause for the decline in testicular function might be present. Testicular trauma, recent fever, administration of anabolic steroids or other potentially damaging substances all can cause testicular degeneration. If any of these are part of the stallion's history, their resolution or removal may allow for restoration of full testicular function. In these cases, the prognosis for future fertility is typically much better than it is for age-related TD.

2.2. Examination of the testes

Serial examinations of the testes are important for identification of age-related TD. A decrease in testicular volume, sometimes associated with changes in the palpable texture of the testes, are hallmarks of the disease. Ideally, routine testicular measurements should be obtained on all breeding stallions beginning when the stallion is in its prime to establish baseline normal testicular volume. Testicular measurements must be performed accurately and ideally by the same experienced operator each time, so that outcomes are highly repeatable (Love et al., 1991). Because volume is a cubic measurement, even small errors in each linear measurement will have a significant effect on calculated volume. When measurement is performed incorrectly, false trends may be identified and real trends may be overlooked.

How frequently should the testes be examined? Monthly testicular examinations are performed at some farms as part of routine stallion care. When measurements are obtained monthly, keep in mind that it is normal to see small, seasonal decreases in testicular size during the fall and winter months and associated small, seasonal increases in testicular size during the spring and summer months (Clay and Clay, 1992). At a minimum, testicular measurements should be obtained annually, preferably at the same time of the year so that normal seasonal changes in testicular size do not confound the results. In general, the more frequently measurements are obtained, the easier it will be to identify a trending change in testicular size. Since age-related TD most typically affects middle aged and older stallions, it is reasonable to examine younger stallions less often and increase the frequency of examinations as the stallion reaches its mid to late teens.

Accurate measurements can be obtained either manually with calipers or ultrasonographically. Use care not to distort the shape of the testis by aggressive traction during measurement. Although not necessary in all cases, sedation of the stallion often facilitates accurate testicular measurement by allowing the testes to descend passively into the scrotum. Sedation also renders the situation safer for the examiner. The length, width and height of each testis should be measured, taking care not to include the epididymis. Total scrotal width should be obtained with both testes descended and side-by-side in the scrotum. Length, width and height measures then should be used to calculate testicular volume for the left and right testis using the following formula for the volume of an ellipsoid:

$$4/3\ \pi \times (\text{length of testis (cm)})/2 \times \text{width of testis (cm)})/2 \times \text{height of testis (cm)})/2$$

The volumes of the left and right testes are added together to determine total testicular volume (Love et al., 1991). Expected Daily Sperm Output (DSO) then is calculated using the following regression equation:

$$\text{Expected DSO (billions)} = (\text{Total Testicular Volume}) \times 0.024 - 0.76$$

The ultrasonographic appearance of the testicular parenchyma usually is not remarkable in stallions with TD. Nonetheless, ultrasonographic evaluation of the testes is recommended both to obtain accurate testicular measurements and to rule out the presence of less common testicular pathologies such as neoplasia, abscessation, hematoma formation, etc.

2.3. Examination of the ejaculate

Changes in semen characteristics may precede identifiable physical changes in the testes themselves. Specifically, studies on germ cell loss rates in stallions indicate that TD can be present before any clinically significant decrease in testicular size can be appreciated (Blanchard and Johnson, 1997). Therefore, serial semen analyses also are important for identifying TD. Both total sperm numbers and semen characteristics should be monitored. Because stallions can store large numbers of sperm in the epididymides and ampullae, total sperm numbers in the ejaculate can be highly variable when semen is evaluated after a period of sexual rest. This can make it more difficult to identify subtle downward trends in total sperm numbers. Similarly, semen characteristics can be affected by the length of sexual rest, thus making changing trends in the percentages of motile and morphologically normal sperm difficult to identify. Therefore, particularly when semen analysis is not performed frequently, it is recommended that the stallion's sperm numbers and semen characteristics be evaluated after the stallion's sperm reserves have been depleted and sperm numbers in the ejaculate are representative of DSO. This requires frequent semen collections to deplete stored sperm (2–3 ejaculations/day for 3–5 days or one ejaculate/day for 5 days; Dinger and Noiles, 1986). Once DSO is reached, ejaculated sperm numbers and sperm quality become more consistent, thus making subtle changes in numbers and quality more apparent. When semen characteristics is examined annually at DSO, declines in semen characteristics and sperm numbers as evidenced by a decline in the number of progressively motile, morphologically normal (PMMN) sperm may precede any measurable decrease in testicular volume and therefore may be one of the earliest indicators of the onset of the condition (Fig. 1) (Blanchard et al., 2013).

Although examination at DSO is preferred, meaningful data can still be obtained even with the stallion at sexual rest. If possible, multiple ejaculates should be examined throughout the year and annual means for semen parameters calculated. In our hands, evaluation of end-of-year averages has been a very efficient way of identifying early signs of TD in aging stallions, without the need for evaluation of the stallion at DSO (Fig. 2).

Another hallmark of age-related TD is the appearance of immature spermatogenic cells (round cells) in the ejaculate. In an unstained semen sample, these cells can sometimes be confused with white blood cells. However, because different stages of spermatogenic cells typically appear in a single ejaculate, spermatogenic cells usually vary in size while white blood cells are more

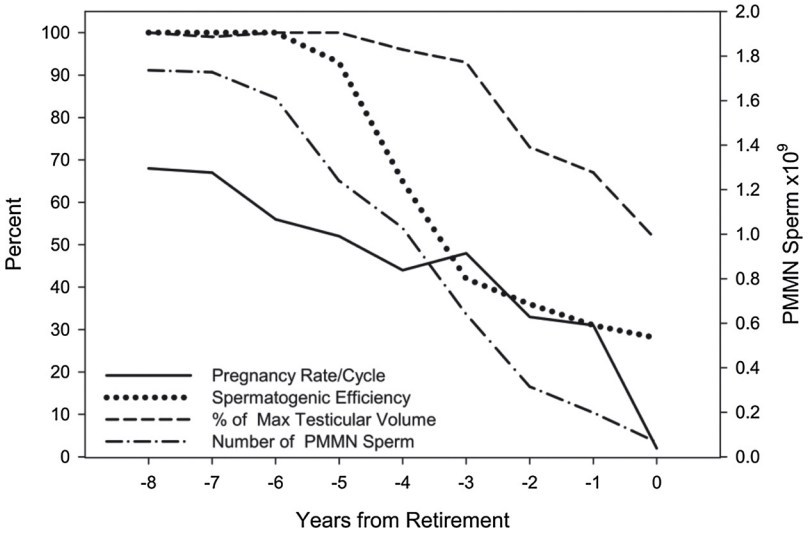

Fig. 1. Pregnancy rate per cycle (%), spermatogenic efficiency (%), percentage of initial (maximum) testicular volume recorded at first examination, and total number of progressively motile morphologically normal (PMMN) sperm in ejaculates at daily sperm output in three aging Thoroughbred stallions. Data are normalized to year of retirement because of infertility for each stallion and averaged among stallions. Declines in the number of PMMN sperm, spermatogenic efficiency and pregnancy rate per cycle all appear to precede a measurable decline in testicular volume.
Reprinted directly from Blanchard, T.L., Brinsko, S.P., Varner, D.D., and Love, C.C., 2013. Progression of Reproductive Changes Accompanying Testicular Dysfunction in Aging Thoroughbred Stallions: case Studies. Proceedings of the 59th Annual Convention of the American Association of Equine Practitioners, (59): 532 – 536. Reprinted with permission from the American Association of Equine Practitioners.

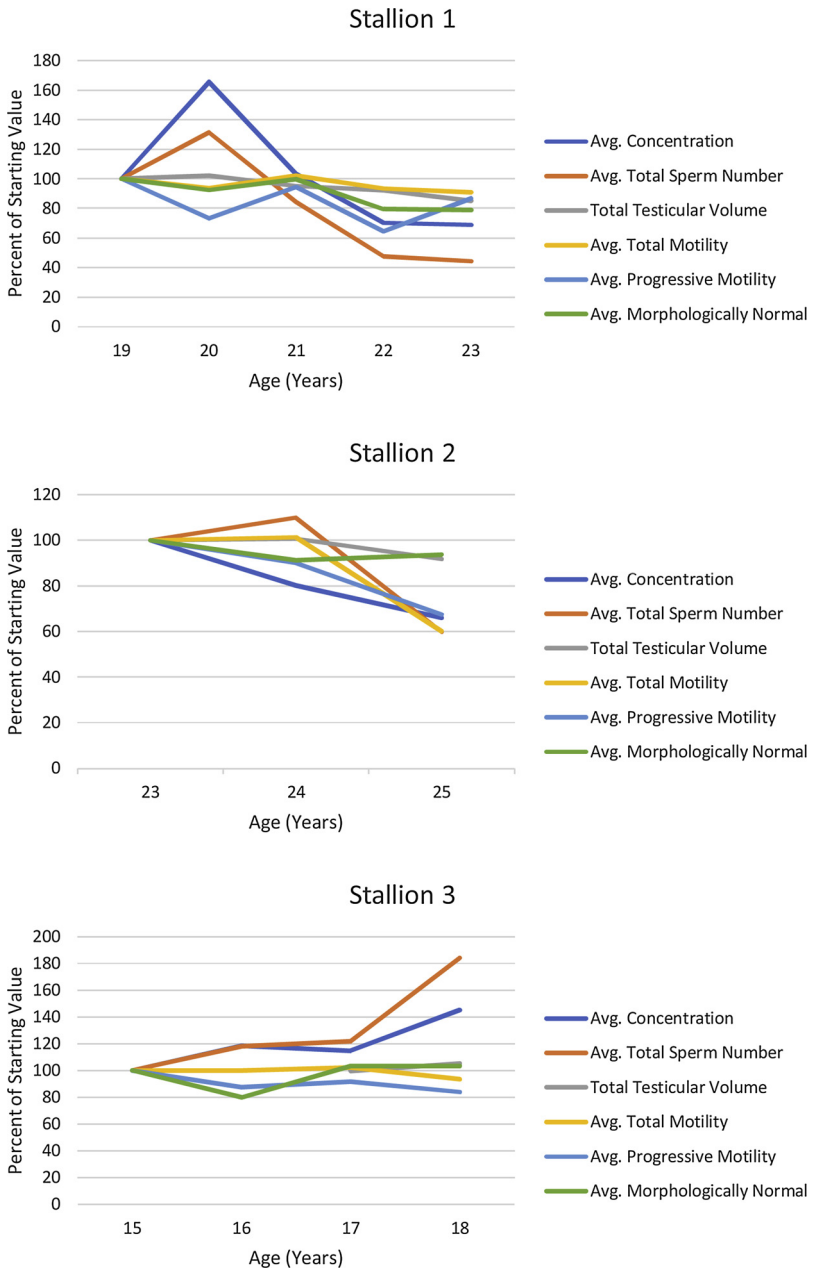

Fig. 2. Trends in semen characteristics, sperm numbers and testicular volume in representative aging stallions. These stallions were used in cooled, transported semen programs and were bred to small to moderate books of mares each year. As a result, several ejaculates were collected and evaluated each year, but ejaculates were obtained sporadically, after varying periods of sexual rest. Graphs show annual averages for sperm concentration, total sperm number, total motility (%), progressive motility (%), and morphologically normal sperm (%) as well as a single annual measurement of total testicular volume. Data are shown as percent of starting averages calculated in the year the stallion was first examined. Although none of these ejaculates represented daily sperm output, trends are still obvious when observed over time. The graphs for Stallions 1 and 2 both show clear downward trends in most parameters, indicative of the early, progressive TD. In contrast, none of the parameters measured for Stallion 3 are in decline, indicating that this animal's testicular function remains robust.

homogeneous. Analysis of Diff-Quik stained semen sample can facilitate identification of neutrophils and lymphocytes and so, can aid in the identification of spermatogenic cells. Multinucleated giant cells also may be present (Blanchard et al., 2000, 2001; McEntee, 1990; Watson et al., 1994). Keep in mind that low numbers of immature spermatogenic cells may be found in the ejaculates of normal stallions, particularly at the beginning of the breeding season (Swerczek, 1975).

As degeneration progresses, the above clinical signs of decreasing sperm numbers, decreasing semen characteristics and decreasing testicular size all become apparent. Other signs include palpable softening of the testicular parenchyma, and low DSO per ml

of testis (calculated by dividing the total number of sperm in the ejaculate at DSO by the total testicular volume) (Love et al., 1991). In advanced cases, stallions may become azoospermic. Because the size of the epididymis usually does not change in cases of TD, the epididymis may seem to be disproportionately large with respect to testicular size (Blanchard and Varner, 1993). In severe, end-stage TD, the testicles may become overly firm (Varner et al., 1991).

More recently, Anti-Mullerian Hormone (AMH) has been suggested to be a marker for gonadal function in horses. In this regard, an increase in AMH has been reported in stallions with medically-induced acute testicular degeneration (Pozor et al., 2018). It will be interesting to determine if AMH concentration also proves useful as a biomarker for age-related testicular degeneration as well.

2.4. Endocrinologic evaluation

Although changes in hormone levels are frequently discussed in the context of age-related TD, in our experience, evaluation of circulating hormone levels is an unrewarding diagnostic tool. There are significant daily and seasonal variations in plasma hormone levels even in normal, fertile stallions that can make it very difficult to distinguish 'normal' from 'pathologic' levels in early to moderate TD (Blanchard and Johnson, 1997). In severe cases of TD, elevated FSH and LH as well as low plasma estradiol may be seen. Similarly, persistently low testosterone is usually observed only in very advanced cases. By the time these hormonal changes become consistent and apparent, the condition is typically advanced and can be diagnosed based on testicular size and character as well as semen characteristics and sperm numbers without the need for hormonal assays.

2.5. Histopathologic evaluation

Histopathology of affected testes reveals a common group of spermatogenic abnormalities including cytoplasmic vacuolization, the absence of later stages of germ cells, and a loss of the normal architecture of the seminiferous epithelium (McEntee, 1990). The diameter of the seminiferous tubules may be decreased and immature spermatogenic cells may be shed into the lumen of the seminiferous tubule, eventually appearing in the ejaculate. In the most extreme cases, fibrous tissue may be present and tubules can become almost devoid of spermatogenic cells and be left with only Sertoli cells and few spermatogonia. Fibrosis and calcification of the testicular parenchyma also may be seen (Humphrey and Ladds, 1975).

Because histopathologic findings can help to define TD (and testicular hypoplasia), evaluation of a testicular biopsy sample does provide definitive evidence of these conditions. However, in practice, testicular biopsy is rarely indicated. Once the clinician has obtained an adequate history and has performed a complete physical and reproductive examination, a diagnosis of TD can usually be made with some confidence and a biopsy sample is not necessary. Additionally, there is some concern that a single biopsy sample may not be representative of the condition of the entire testis and thus may not be of significant prognostic value. If a biopsy sample is to be taken, the testes should be examined ultrasonographically prior to obtaining the biopsy (Turner, 1998). The ultrasonographic appearance of the parenchyma can help the clinician to choose a representative site for sampling. Several reports have indicated that obtaining testicular biopsy samples in the stallion can be done safely and with minimal permanent damage to the remaining testicular parenchyma (DelVento et al., 1992; Faber and Roser, 2000). However, many of these studies were performed on normal stallions and thus the risk to an already compromised testicle (e.g. a degenerating testicle) is more difficult to ascertain. The additional diagnostic benefit that a testicular biopsy sample provides often does not outweigh the potential risks and therefore is rarely warranted in cases of age-related TD.

3. Managing the aging stallion with declining fertility

Current research has shown that the pathology underlying TD lies within the testis itself, and not with the extratesticular environment. As would be expected for a tissue autologous disease, treatments designed to drive testicular function have proven ineffective. Although there are some reports of the successful use of GnRH therapy as a treatment for infertility in stallions (Evans and Finely, 1990; Shiner et al., 1993) these successes have not been duplicated in controlled studies (Blue et al., 1991; Douglas and Umphenour, 1992; Roser and Hughes, 1994). GnRH therapy has been highly successful in treating men with hypogonadotropic-hypogonadism, but this condition has not been clearly documented in stallions and our studies strongly suggest that a lack of gonadotropins is not the underlying cause of age-related TD in the horse. In addition, our xenografting studies of degenerate testes have shown no improvement in the condition of the testes following treatment of host mice with exogenous gonadotropins or provision of the mice with a source of endogenous hormones from normal, functional testis xenografts (Turner et al., 2006). If all of this information is taken together, the use of GnRH implants or pulsatile administration of GnRH as a treatment for stallion infertility in general or age-related TD specifically becomes highly questionable. If this therapy is to be attempted, it has been suggested that treatment must start early, before the testis has reached a severe state of degeneration (Brinsko, 1996).

There is support in the literature for beneficial effects of fat-soluble antioxidants (e.g., docosahexaenoic acid (DHA)) on semen characteristics in stallions (Brinsko et al., 2005). However, proven benefits so far have been limited to improved longevity of sperm motility in cooled, stored semen samples and in cryopreserved semen samples. Whether or not this improvement translates into increased pregnancy rates, particularly in subfertile stallions, has not been tested. To the authors knowledge, there is no evidence that fat-soluble antioxidants improve the function of a diseased testis, nor is there evidence that their administration improves fertility in subfertile individuals. In this regard, we used the xenografting model to study the effects of DHA supplementation on the histological appearance of degenerate stallion testes and have identified no benefit (Turner et al., 2006). In spite of the lack of evidence for any benefit to age-related TD, it is a common practice to supplement subfertile stallions with nutraceuticals containing DHA or similar

substances.

In cases of unilateral testicular damage or degeneration, some have recommended removal of the affected testis. The reasoning behind this recommendation is that the damaged testicular tissue could result in the production of anti-sperm antibodies that hypothetically might adversely affect sperm produced by the normal testis (Zhang et al., 1990). Additionally, removal of one testis often results in hypertrophy of the remaining testis and a resultant increase in sperm numbers. However, this compensatory hypertrophy most likely occurs only in a normal testis. Whether a testis already on the path to degeneration would undergo compensatory hypertrophy is highly debatable and seems unlikely. The practice of unilateral castration is therefore questionable when there is any suspicion that the remaining testis might be abnormal. Additionally, there are numerous instances of acceptable fertility in stallions with unilateral TD in which the affected testis was not removed (Blanchard and Varner, 1993).

In breeds allowing artificial insemination, semen cryopreservation is recommended for any genetically valuable stallion when the animal is at the peak of its fertility. This provides a genetic 'insurance policy' against reproductive loss should a stallion's fertility be compromised by ITD as it ages.

In spite of attempts to develop a 'cure for aging' in the testes, there currently is no proven method that will mitigate the effects of aging on the testes of the horse. We therefore are left only with the option of managing our way through the associated decline in testicular function. The veterinarian first should determine the number of progressively motile, morphologically normal sperm that the stallion is capable of producing while on a breeding schedule similar to what is expected during the breeding season. The stallion's mare book then should be adjusted accordingly to ensure that the stallion's sperm numbers are not depleted to the point that mares receive a less-than-optimal breeding dose. If possible, the sperm numbers and semen characteristics of each ejaculate should be monitored. Addition of a semen extender to the ejaculate may help improve longevity of sperm motility in some cases. For stallions breeding by natural cover, reinforcement breeding is highly recommended to maximize the number of sperm delivered to the mare (Blanchard et al., 2006). The use of deep horn reinforcement breeding may further increase the chances of pregnancy when dealing with small numbers of sperm.

Semen from stallions with age-related TD should be handled with particular care. Mares should be inseminated as quickly as possible after semen collection and close to ovulation. In many cases of moderate to severe TD, sperm longevity of motility is poorly maintained and pregnancy rates may be significantly reduced in mares bred with cooled stored semen. If this is the case, it may be prudent to discontinue the use of shipped semen and only breed mares on site with fresh, extended semen or by natural cover.

Semen processing techniques can be used in an attempt to boost semen characteristics. For stallions with poor quality seminal plasma, centrifugation of semen with removal of seminal plasma and subsequent resuspension of the sperm pellet in semen extender can increase sperm longevity. In cases where a high percentage of sperm morphologic defects are present, gradient separation of sperm can result in a higher quality insemination dose. However, sperm numbers are typically greatly reduced. In our clinic, we recommend that one or more laboratory trials of gradient separation be performed prior to breeding mares with gradient separated sperm. This allows us to determine if the process appears to benefit the semen characteristics of that individual stallion and determine if the reduction in total sperm numbers is likely to be outweighed by these benefits.

More intensive mare management also can be used to improve pregnancy rates. With judicious use of ovulation induction agents, mares can reliably be bred very close to the time of ovulation, and in extreme cases within 6 h post ovulation, thus minimizing the requirement for sperm longevity. The routine use of deep horn, low volume insemination can increase pregnancy rates when sperm numbers are limited. A final option for management is the use of assisted reproductive techniques such as Intracytoplasmic Sperm Injection (ICSI). ICSI allows for the production of offspring even from severely oligospermic stallions. However, the expense is significant and not all breed registries approve of this technique.

4. Current research on aging in the stallion testis

We have used a testis tissue xenografting model to show that restoring the aged, degenerate stallion testis to a 'young' extratesticular environment does not result in any improvement in testicular function. This, together with earlier endocrinologic studies, is strong evidence that the primary defect in these aged, degenerate testes lies within the testis itself, and not with the hypothalamus, the pituitary or the extratesticular environment (Turner et al., 2010a; Stewart and Roser, 1998). The nature of the testicular defect is almost certainly complex and involves degenerative changes affecting multiple cell types as well as the factors that comprise each cell's local niche.

Data from the mouse model system shows that aging has adverse effects on the spermatogonial stem cell (SCC) itself (Schmidt et al., 2011), and also on the SSC niche. The niche describes the complex and ever-changing local microenvironment of the SSC and it is comprised of a variety of cellular and molecular components. In rodents, the niche is influenced in part by the presence of interstitial blood vessels, by input from both the Leydig cells and Sertoli cells, and by a host of other factors that have yet to be been determined (Oatley et al., 2009). Studies by Ryu et al. have shown that, if murine SSCs were maintained in a 'young' somatic niche environment by serial retransplantation into young animals, their function (including self-renewal and potentially differentiation through spermatogenesis) could be maintained well beyond what would be defined as old age in the donor (Ryu et al., 2006). These studies show that, if the effects of aging on the niche can be avoided, the SSC can continue to function normally well beyond the natural lifespan of the host.

Studies on testicular aging in the horse are much more difficult as we must deal with significant amounts of genetic variation, differences in management, a naturally long lifespan, and a dearth of molecular tools. We again resorted to a testis tissue xenografting model as a crude method of determining if the exposure of aged testicular tissue (including aged SSCs) to young testicular tissue might improve the function of the aged tissue and the associated aged SSCs. Small fragments (1-2 mm^3) of normal young equine

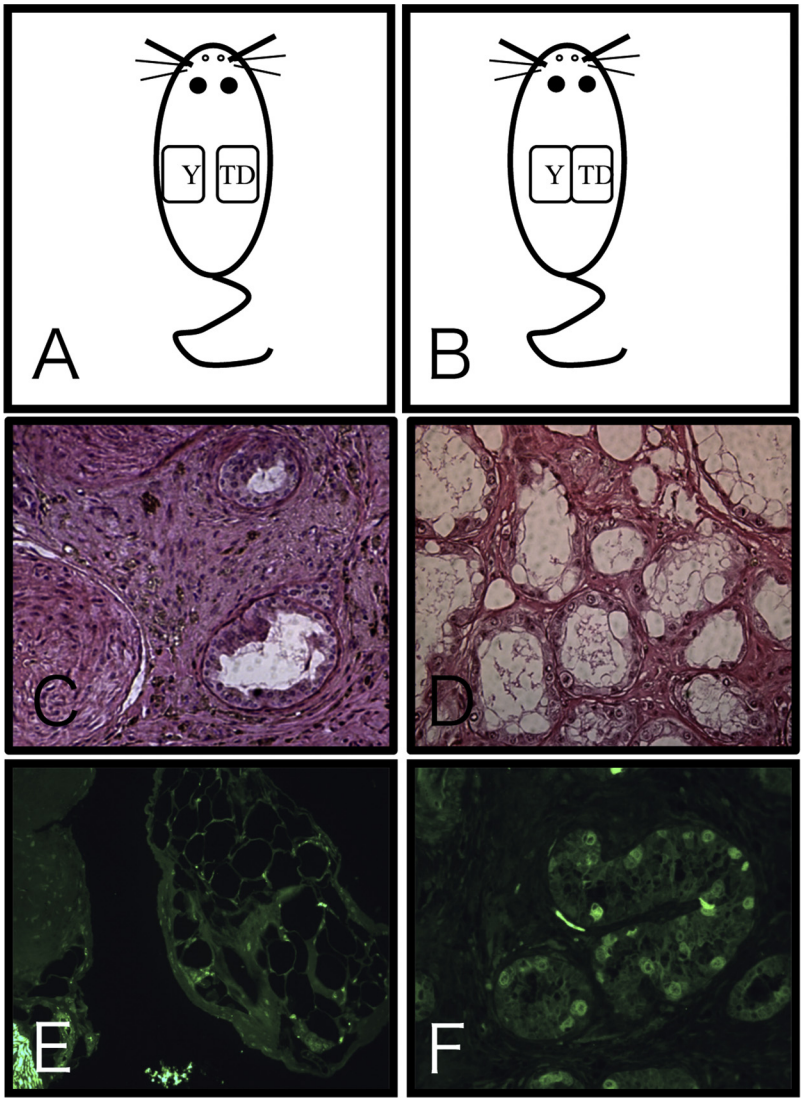

Fig. 3. Physical contact with young testicular tissue improves the condition of aged, degenerate testicular tissue and supports survival of aged spermatogonia. (A) and (B): Schematic diagrams illustrating the experimental model. Small pieces of healthy, young testicular tissue (Y) and aged degenerate testicular tissue (TD) were grafted under the back skin of immunocompromised mice. In the control mice (A), the young and degenerate tissue pieces were physically separated. In the experimental mice (B), the young and degenerate tissue pieces were grafted in close physical contact. Panels (C) and (D) show the histologic appearance of the TD grafts after several months of incubation on the mouse hosts. In the TD grafts that were not in physical contact with young tissue, degeneration had progressed such that very few seminiferous tubules remained (C). Additionally, a fluorescent antibody marker for spermatogonia (anti-PGP 9.5) identified no spermatogonia (E). In contrast, the TD grafts that were in contact with young tissue contained numerous seminiferous tubules (D). Although most of these tubules were degenerate, spermatogonia were still present along the basement membrane (F).

testicular tissue and aged degenerate equine testicular tissue were grafted under the back skin of anesthetized, castrated, male NCR nu/nu mice through small incisions as previously described (Honaramooz et al., 2002). In control mice, the young and aged grafts were placed such that there was no physical contact between the grafts. In the experimental mice, the young and aged grafts were placed in close physical contact. The exact location of each graft was carefully recorded so that the identity of each tissue piece could be determined at the time of graft recovery. Grafts were incubated on the mouse hosts for 3 to 6 months. At the time of graft recovery, the host mice were killed by CO_2 inhalation. The skin tissue containing the grafts was dissected and grafts from all experiments were fixed, paraffin embedded and processed for either H&E staining or for indirect immunofluorescence. We found that aged, degenerate testis grafts that were in physical contact with young testicular tissue contained more seminiferous tubules per unit weight than did aged, degenerate testis grafts that were not in physical contact with young testicular tissue. Additionally, indirect immunofluorescence using a marker antibody for spermatogonia showed that seminiferous tubules in the aged grafts in contact with young testicular tissue contained more spermatogenic cells per tubule than did aged grafts that were not in contact with young tissue (Fig. 3)

(Turner et al., 2010a; Turner et al., 2010b). We concluded that contact with young testicular tissue (i.e., a very crude attempt to reconstruct a young somatic cell environment) supports the survival of aged spermatogonia in the equine testis. These findings suggest that the SSC itself may be relatively resistant to aging and raise the possibility that cell-based therapies could be developed as a means for mitigating the adverse effects of aging on spermatogenesis in the equine testis.

Although cell-based therapies can provide transforming results for some disease processes, these therapies are fraught with difficulties including isolation and storage of therapeutic cells, complicated and sometimes invasive cell delivery techniques, and maintenance of the therapeutic cell population in the recipient (immunologic rejection, cell death, etc.). If the specific proteins that are responsible for the beneficial effect of the young cell population on the aged testis could be identified and isolated, it may be possible to move towards a more standard method of treatment potentially involving, for example, periodic systemic protein or hormone administration. In addition to leading to potential therapeutic approaches to TD, these studies also lead us to a better understanding of the pathophysiology of TD and thus provide means of identifying the condition in its early stages and/or developing methods to prevent its occurrence and progression.

In this regard, we recently applied RNA transcript profiling technology to the study of age-related TD in the horse. RNA sequencing (RNA-seq) is a whole-transcriptome profiling technique that offers numerous advantages over microarray technology (t Hoen et al., 2008). By direct sequencing of reverse-transcribed, amplified cDNA, RNA-seq represents the next generation of transcriptomics. Unlike microarrays, RNA-seq is not limited by the quality of the microarray, the ability of each probe to hybridize to sequences on the microarray, or the number of sequences on the microarray. These advantages are particularly notable in the horse where, to our knowledge, only 2 proprietary microarrays currently are available, and one of these arrays is based on a mare genome - a significant limiting factor when studying male infertility, since many male fertility genes are located on the Y chromosome.

By comparing the transcriptome of healthy adult stallion testicular tissue to that of aged, degenerate stallion testicular tissue, we identified several individual genes and genetic pathways that appear to be altered in the aging horse testis. Several of these pathways are promising targets for therapies that could slow the progression of the condition. In this regard, our future studies will be aimed at modifying these altered pathways and determining whether or not these treatments prolong robust testicular function in aging stallions.

Funding

Portions of this work were supported by the Grayson Jockey Club Research Foundation, Inc. [grant number 30014629]

References

Blanchard, T., Johnson, L., Roser, A.J., 2000. Increased germ cell loss rates and poor semen quality in stallions with idiopathic testicular degeneration. J. Equine Vet. Sci. 20, 263–265.
Blanchard, T., Varner, D., 1993. Testicular degeneration. In: McKinnon, A.O., Voss, J.L. (Eds.), Equine Reproduction. Lea & Febiger, Philadelphia, pp. 855–860.
Blanchard, T.L., Brinsko, S.P., Varner, D.D., Love, C.C., 2013. Progression of reproductive changes accompanying testicular dysfunction in aging thoroughbred stallions: case studies. Proc. 59th Annual Convention of the American Association of Equine Practitioners 532–536.
Blanchard, T.L., Johnson, L., 1997. Increased germ cell degeneration and reduced germ cell:sertoli cell ratio in stallions with low sperm production. Theriogenology 47, 655–677.
Blanchard, T.L., Johnson, L., Varner, D., Rigby, S., Brinsko, S., Love, C.C., et al., 2001. Low daily sperm output per ml of testis as a diagnostic criteria for testicular degeneration in stallions. J. Equine Vet. Sci. 21, 11–35.
Blanchard, T.L., Love, C.C., Thompson, J.A., Ramsey, J., O'Meara, A., 2006. Role of reinforcement breeding in a natural service mating program. Proc. 52nd Annual Convention of the American Association of Equine Practitioners 384–386.
Blue, B.J., Pickett, B.W., Squires, E.L., McKinnon, A.O., Nett, T.M., Amann, R.P., et al., 1991. Effect of pulsatile or continuous administration of GnRH on reproductive function of stallions. J. Reprod. Fertil. Suppl. 44, 145–154.
Brinsko, S.P., 1996. GnRH therapy for subfertile stallions. Vet. Clin. North Am. Equine Pract. 12, 149–160.
Brinsko, S.P., Varner, D.D., Love, C.C., Blanchard, T.L., Day, B.C., Wilson, M.E., 2005. Effect of feeding a DHA-enriched nutriceutical on the quality of fresh, cooled and frozen stallion semen. Theriogenology 63, 1519–1527.
Clay, C.M., Clay, J.N., 1992. Endocrine and testicular changes associated with season, artificial photoperiod, and the peri-pubertal period in stallions. Vet. Clin. North Am. Equine Pract. 8, 31–56.
DelVento, V.R., Amann, R.P., Trotter, G.W., Veeramachaneni, D.N., Squires, E.L., 1992. Ultrasonographic and quantitative histologic assessment of sequelae to testicular biopsy in stallions. Am. J. Vet. Res. 53, 2094–2101.
Dinger, J.E., Noiles, E.E., 1986. Prediction of daily sperm output in stallions. Theriogenology 26, 61–67.
Douglas, R.H., Umphenour, N., 1992. Endocrine abnormalities and hormonal therapy. Vet. Clin. North Am. Equine Pract. 8, 237–249.
Evans, J.W., Finely, M., 1990. GnRH therapy in a stallion of low fertility. J. Equine Vet. Sci. 10, 182.
Faber, N.F., Roser, J.F., 2000. Testicular biopsy in stallions: diagnostic potential and effects on prospective fertility. J. Reprod. Fertil. Suppl. 56 (31), 42.
Gehlen, H., Bartmann, C.P., Klug, E., Schoon, H.A., 2001. Azoospermia due to testicular degeneration in a breeding stallion. J. Equine Vet. Sci. 21, 137–139.
Honaramooz, A., Snedaker, A., Boiani, M., Scholer, H., Dobrinski, I., Schlatt, S., 2002. Sperm from neonatal mammalian testes grafted in mice. Nature 418, 778–781.
Humphrey, J.D., Ladds, P.W., 1975. A quantitative histological study of changes in the bovine testis and epididymis associated with age. Res. Vet. Sci. 19, 135–141.
Love, C.C., 2011. Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 76, 547–557.
Love, C.C., Garcia, M.C., Riera, F.R., Kenney, R.M., 1991. Evaluation of measures taken by ultrasonography and caliper to estimate testicular volume and predict daily sperm output in the stallion. J. Reprod. Fertil. Suppl. 44, 99–105.
McEntee, K., 1990. Reproductive Pathology of Domestic Animals. Academic Press, Inc., San Diego.
Oatley, J.M., Oatley, M.J., Avarbock, M.R., Tobias, J.W., Brinster, R.L., 2009. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136, 1191–1199.
Pozor, M., Conley, A.J., Roser, J.F., Nolin, M., Zambrano, G.L., et al., 2018. Anti-Mullerian hormone as a biomarker for acute testicular degeneration caused by toxic insults to stallion testes. Theriogenology 116, 95–102.
Roser, J.F., Hughes, J.P., 1994. Use of GnRH in stallions with poor fertility: a review. Proc. 40th Annual Convention of the American Association of Equine Practitioners 23–25.
Ryu, B.Y., Orwig, K.E., Oatley, J.M., Avarbock, M.R., Brinster, R.L., 2006. Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. Stem Cells 24, 1505–1511.

Schmidt, J.A., Abramowitz, L.K., Kubota, H., Wu, X., Niu, Z., Avarbock, M.R., et al., 2011. In vivo and in vitro aging is detrimental to mouse spermatogonial stem cell function. Biol. Reprod. 84, 698–706.
Shiner, K.A., Pickett, B.W., Juergens, T.D., 1993. Clinical approaches to diagnosis and treatment of subfertile. Stallions Proc. 39th Annual Convention of the American Association of Equine Practitioners 149.
Stewart, B.L., Roser, J.F., 1998. Effects of age, season, and fertility status on plasma and intratesticular immunoreactive (IR) inhibin concentrations in stallions. Domest. Anim. Endocrinol. 15, 129–139.
Swerczek, T.W., 1975. Immature germ cells in the semen of thoroughbred stallions. J. Reprod. Fertil. Suppl. 23, 135–137.
t Hoen, P.A., Ariyurek, Y., Thygesen, H.H., Vreugdenhil, E., Vossen, R.H., de Menezes, R.X., et al., 2008. Deep sequencing-based expression analysis shows major advances in robustness, resolution and inter-lab portability over five microarray platforms. Nucleic Acids Res. 36, e141.
Turner, R.M., 1998. Ultrasonography of the genital tract of the stallion. In: Reef, V.B. (Ed.), Equine Diagnostic Ultrasound. W.B. Saunders Company, Philadelphia, pp. 446–479.
Turner, R.M., Rathi, R., Honaramooz, A., Zeng, W., Dobrinski, I., 2010a. Xenografting restores spermatogenesis to cryptorchid testicular tissue but does not rescue the phenotype of idiopathic testicular degeneration in the horse (Equus caballus). Reprod. Fertil. Dev. 22, 673–683.
Turner, R.M., Rathi, R., Zeng, W., Honaramooz, A., Dobrinski, I., 2006. Xenografting to study testis function in stallions. Anim. Reprod. Sci. 94, 161–164.
Turner, R.M., Zeng, W., Li, Y., Modelski, M., Dobrinski, I., 2010b. Paracrine factors from normal equine testicular tissue improve the condition of xenografts of degenerate testicular tissue. Anim. Reprod. Sci. 121S, S198–S199.
Varner, D., Schumacher, J., Blanchard, T., Johnson, L., 1991. Diseases and Management of Breeding Stallions. American Veterinary Publications, Goleta, CA.
Watson, E.D., Clarke, C.J., Else, R.W., Dixon, P.M., 1994. Testicular degeneration in 3 stallions. Equine Vet. J. 26, 507–510.
Zhang, J., Ricketts, S.W., Tanner, S.J., 1990. Antisperm antibodies in the semen of a stallion following testicular trauma. Equine Vet. J. 22, 138–141.