

## Understanding and managing age-related subfertility in the stallion

Regina M. Turner

New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, PA

### Abstract

Age-related or idiopathic testicular degeneration is a common cause of subfertility and infertility, particularly in middle aged and older stallions. This manuscript describes the problem of idiopathic testicular degeneration in the equine breeding industry and summarizes what is known about the pathophysiology of the disease. Additionally, the clinical signs of idiopathic testicular degeneration are reviewed so that the clinician can more quickly and accurately arrive at a diagnosis. The practitioner is provided with practical information on how to more effectively manage affected stallions and what, if anything, can be done to improve reproductive performance of these animals both in the field and in a referral setting. Finally, the findings of current research on the disease are presented with a discussion of potential future therapies.

**Keywords:** Stallion, subfertility, aging, testicular degeneration

### Introduction

Aging is evident in most tissue and organ systems, and the testis is not immune. The mechanisms of aging are difficult to identify and poorly understood. Idiopathic testicular degeneration (ITD) refers to the adverse effects of aging on the testis and is a common cause of acquired and often progressive infertility in stallions.<sup>1,2</sup> Idiopathic testicular degeneration most often affects middle aged or older animals, but can be seen in much younger animals as well.<sup>3</sup> Regardless of the age of onset, ITD is typically progressive and results in a steady decline in fertility, sometimes ending in sterility. Economic losses resulting from this disease in the equine breeding industry are substantial and stem from losses of breeding fees, increased management costs and loss of valuable male genetics. In the past, the pathophysiology of the disease was poorly understood and even today, ITD is clinically characterized largely by the appearance of a common set of progressive clinical signs including, but not limited to, an increase in palpable softness and a decrease in size of the testicular parenchyma and a decline in semen quality. This manuscript will review the clinical approach to ITD and our current understanding of the disease. Finally, new research that sheds light on the pathophysiology of ITD and potential therapeutic approaches will be presented.

### Clinical approach

#### History

A complete history is necessary before one can make an accurate diagnosis of ITD since both clinically and histologically, ITD may be indistinguishable from testicular hypoplasia. Since testicular degeneration is an acquired condition, while testicular hypoplasia is congenital, a firm diagnosis of ITD can be made only if the stallion has a history of declining reproductive efficiency, decreasing testicular size, decreasing semen quality, or some combination of these things. 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. It should be kept in mind that many animals with testicular hypoplasia often are affected by degeneration as well.<sup>2</sup>

Not all cases of testicular degeneration are idiopathic. Testicular trauma, recent fever, administration of anabolic steroids or other potentially damaging substances all can cause testicular degeneration. As such, when obtaining a history, one should also attempt to determine if the animal has any history of, for example, testicular trauma, recent illness associated with fever, or drug administration. In cases of traumatic or thermal injury to the testes, the onset of infertility is generally sudden and closely associated with the inciting incident. If steroids or other harmful agents are involved, the progression of the problem may be acute or more gradual. In cases in which a history reveals a likely inciting cause,

removal of the cause can allow for restoration of full testicular function. Thus, unless the spermatogonial stem cells have been permanently damaged, the prognosis for future fertility is much better than for cases of ITD.

If a stallion is presented with a history of declining fertility over time with no apparent inciting incident then ITD should be suspected, particularly in older animals. Although ITD is considered to be a slowly progressive problem, some cases may present for a perceived acute onset of infertility or subfertility, particularly if semen quality and testicular parameters were not being routinely monitored.

#### Clinical signs and diagnosis

Cases of ITD can present with a range of clinical signs. Mild cases of ITD may not be associated with any noticeable change in testicular character. Specifically, studies on germ cell loss rates in stallions indicate that ITD can be present before any clinically significant decrease in testicular size can be appreciated.<sup>4</sup> As such, early signs of ITD may only be noticed if semen quality is being frequently and carefully monitored. A gradual decline in overall semen quality (including a decline in total sperm numbers and/or declines in the percentages of motile and morphologically normal sperm) may be the only clinical signs early in the disease. As the disease progresses, clinical signs become more apparent and include decreasing testicular size (most often affecting both testes similarly, but infrequently affecting one more than the other), palpable softening of the testicular parenchyma, decreasing sperm numbers, low daily sperm output (DSO) per ml of testis, the appearance of increasing numbers of immature round spermatogenic cells and/or multinucleate giant cells in the ejaculate and an overall decline in semen quality.<sup>2,5-7</sup> In advanced cases, stallions may become azoospermic. Because the size of the epididymis usually does not change in cases of ITD, the epididymis may seem to be disproportionately large with respect to testicular size.<sup>1</sup> If a stallion's fertility is not regularly monitored, some cases of ITD present for what is perceived to be an acute onset of subfertility or infertility. In fact, in many of these cases, the problem was more likely progressive over time but went unnoticed until it had become a severe problem. In severe, end-stage ITD, the testicles may become overly firm.<sup>8</sup>

#### Monitoring and diagnostic techniques

In stallions that can be followed over time, it is recommended that testicular measures be obtained at least annually. In general, the more frequently the measurements can be obtained, the better as this allows more precise identification of any trends towards a decrease in testicular size. At some top farms, the testes of valuable stallions are measured monthly. Measurements can be obtained either with calipers or ultrasonographically and ideally should be performed by the same individual to minimize variation. Use care not to distort the shape of the testes by overly aggressive manipulation during measurement. Although not absolutely necessary, sedation of the stallion can help facilitate testicular measurement by allowing the stallion to relax and the testicles to descend passively, thus minimizing the need to pull the testicles down into the scrotum and potentially distorting measurements. Measurements should include total scrotal width, as well as length, width and height of each testis individually. Length, width and height measures then should be used to calculate testicular volume and to determine if the stallion is producing appropriate sperm numbers for its testicular size. The volume of a single testis can be calculated using the following formula:

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

And total testicular volume equals:

$$\text{volume of the left testis} + \text{volume of the right testis.}^9$$

Additionally, DSO per ml of testis can be calculated by dividing the total number of sperm in the ejaculate at DSO by the total testicular volume. Low DSO/ml of testis, together with a low percentage of

morphologically normal sperm in the ejaculate have been recommended as good indicators of the possible presence of ITD.<sup>9</sup>

Frequent examination and measurement of the testes facilitate early identification of trends suggestive of ITD (e.g., decreasing testicular size/volume). Semen analysis (preferably with the stallion at DSO) also should be performed at least annually and, when possible, much more frequently (i.e., for stallions breeding by artificial insemination, each ejaculate should be analyzed). However, this may not be practical for Thoroughbred stallions breeding exclusively by natural cover.

Stallions at sexual rest typically have large epididymal stores of sperm. Therefore, until this sperm reserve is essentially exhausted or stabilized, total sperm numbers at sexual rest can be highly variable. Therefore, gradual downward trends in total sperm numbers and semen quality will be more difficult to identify if ejaculates are only examined when the stallion is at sexual rest. A better option is to examine the stallion's semen quality after sperm reserves are depleted (i.e., when the stallion has reached DSO). This requires serial semen collections (2 to 3 ejaculations/day for 3 to 5 days). Once DSO is reached, ejaculated sperm numbers become more consistent, thus making subtle changes in sperm numbers and semen quality more apparent. Idiopathic testicular degeneration might be suspected if a stallion's total sperm numbers or semen quality are declining progressively over time or if a stallion at DSO is producing low sperm numbers for his testicular volume.

Another hallmark of ITD 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 homogeneous. Analysis of a modified Romanowsky (Diff-Quik®) stained semen sample can facilitate identification of neutrophils and lymphocytes and so, by process of elimination, can aid in the identification of spermatogenic cells. Multinucleated giant cells also may be present.<sup>2,5</sup> Keep in mind that low numbers of immature spermatogenic cells may be found in the ejaculates of normal stallions.<sup>10</sup>

Because of the variation in plasma hormone levels seen in normal and subfertile stallions, circulating hormone levels may not be a good predictor of mild to moderate ITD.<sup>4</sup> In severe cases, elevated follicle stimulating hormone (FSH) and luteinizing hormone (LH) as well as low plasma estradiol are consistent with a diagnosis of ITD. However, by the time these hormonal changes become consistent and apparent, the disease is typically advanced and can be diagnosed based on testicular size and character as well as semen quality and sperm numbers.

Ultrasonographic evaluation of testes affected by ITD is often unrewarding since the ultrasonographic appearance usually is not remarkable. Nonetheless, ultrasonographic evaluation of the testes is recommended both to obtain accurate testicular measurements and to rule out possible inciting causes of ITD or other testicular pathologies such as testicular neoplasia.

Histopathology of affected testes reveals a common group of spermatogenic abnormalities including cytoplasmic vacuolization and a loss of the normal architecture of the seminiferous epithelium.<sup>5</sup> The diameter of the seminiferous tubules may be decreased and immature spermatogenic cells may be shed into the lumen of the seminiferous tubule. In more severe cases, these immature (or 'round') spermatogenic cells may appear in the ejaculate in increasing numbers, as described above. As ITD progresses, there is an increased loss of germ cells from the seminiferous tubule. In the most extreme cases, fibrous tissue may be present and tubules can become almost devoid of spermatogenic cells and may be left with only Sertoli cells and few spermatogonia. Fibrosis and calcification of the testicular parenchyma also may be seen. (11) Keep in mind that even normal testes can have some focal areas of abnormal spermatogenesis. Thus, the percentage of the testicular parenchyma that is affected as well as the severity of the histological lesions should be taken into account before a diagnosis of TD is made.

Because histopathologic findings can help to define ITD (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 ITD 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.<sup>12</sup> 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.<sup>13,14</sup> 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. Clinicians must carefully weigh the diagnostic benefits of obtaining a biopsy sample against the risk of damaging some portion of an already marginally functional testicular parenchyma.

### Treatment

If there is a known or suspected cause of the degeneration (e.g., fever, toxin), successful treatment or removal of the inciting cause should at least prevent further progression of the disease and may allow for complete recovery.

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 might adversely affect sperm produced by the normal testis.<sup>15</sup> Additionally, removal of one testis often results in hypertrophy of the remaining testis and a resultant increase in sperm numbers. The practice of unilateral castration is debatable, however, as there are reports of acceptable fertility in stallions with unilateral ITD in which the affected testis was not removed.<sup>1</sup>

As would be expected for a tissue autologous disease, clinical treatments designed to drive testicular function have proven ineffective. Although there are some reports of the successful use of gonadotropin releasing hormone (GnRH) therapy as a treatment for infertility in stallions<sup>16,17</sup> these successes have not been duplicated in controlled studies.<sup>18-20</sup> Gonadotropin releasing hormone 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 severe ITD in the horse. In addition, our studies on xenografts of equine testes severely affected by ITD have identified 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.<sup>21</sup> 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 ITD 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.<sup>22</sup>

There is support in the literature for beneficial effects of fat-soluble antioxidants (e.g., docosahexaenoic acid (DHA)) on semen quality in stallions.<sup>23</sup> However, proven benefits so far have been limited to improved longevity of sperm motility in cooled, stored semen samples. Whether or not this improvement translates into increased pregnancy rates, particularly in subfertile stallions, has not been tested. We have used our xenografting model to study the histological effects of DHA supplementation on degenerate stallion testes and have identified no benefit.<sup>21</sup> Nonetheless, it is a common practice to supplement subfertile stallions with nutraceuticals containing DHA or similar substances (e.g., ProSperm, Minitube of America, Verona, WI).

### Management

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.

Since there is no proven treatment for ITD, the basis of dealing with this problem centers around stallion and mare management. The veterinarian first should determine the number of progressively

motile, morphologically normal sperm that the stallion is capable of producing while on a schedule set up to mimic what would be required during the breeding season. The stallion's mare book then should be adjusted accordingly to insure that the stallion is not overused. If a stallion with low or marginal sperm numbers is required to serve too frequently, it is not uncommon for the animal's sperm numbers to drop below what would be required for a minimum insemination dose. Limiting the animal's book so that it has one or more days of sexual rest between each ejaculate often can help boost sperm numbers and improve pregnancy rates in mares. If possible, the semen quality of each ejaculate should be monitored to be certain that each mare is receiving a minimum insemination dose. Addition of an 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.<sup>24</sup> 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 ITD should be handled with particular care. Mares should be inseminated as quickly as possible after semen collection. Semen should be carefully evaluated as to its suitability for cooled transport. However, in many cases of moderate to severe ITD, sperm longevity of motility is poorly maintained and pregnancy rates may be significantly reduced in mares bred with cooled 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 quality. For stallions with poor quality seminal plasma, centrifugation of semen with removal of seminal plasma and subsequent resuspension 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. One or more laboratory trials of gradient separation are recommended before semen is needed to breed mares. These laboratory trials allow one to tailor the technique to each stallion and to determine in advance if the improvement in semen quality is likely to outweigh the loss of sperm numbers.

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 six hours after 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). Intracytoplasmic sperm injection allows for the production of offspring even from severely azoospermic stallions. However, the expense is significant and not all breed registries approve of this technique.

### **Pathophysiology and future avenues for treatment**

In both moderate and severe cases of ITD, the primary defect resides within the testis itself, and not with the hypothalamus, the pituitary or the extratesticular environment.<sup>25</sup> Earlier endocrinologic work<sup>26</sup> suggested that the primary defect in equine ITD resides within the Sertoli cell. However, more recent work in mice suggests a somewhat more complicated basis for age-related declines in male fertility. While there is now evidence for some direct effects of aging on the spermatogonial stem cell (SCC) itself,<sup>27</sup> a highly significant contributor to infertility in old male mice was shown to result from failure of the spermatogonial stem cell (SSC) niche, a specific testicular microenvironment comprised of a variety of cellular and molecular components. In vivo cell retransplantation experiments demonstrated that SSC self-renewal could continue normally for several years past the normal life span of the donor animal if the SSCs were maintained by a young 'niche'.<sup>28</sup> In other words, if SSCs can be maintained in a 'young' somatic niche environment, 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. In rodents, there is evidence that this niche is based within the interstitial compartment, in proximity to interstitial blood vessels and involving input from the Leydig cells.<sup>29</sup> Sertoli cell function also is likely to be part of the niche, but the role of Sertoli cells in the niche may be influenced by input

from the interstitial compartment. If these observations also hold true for the stallion, then declines in Sertoli cell function remain a central factor responsible for age-related declines in fertility. However, the full picture likely involves much more than Sertoli cell function alone, and probably is heavily influenced by input from the interstitial compartment. Taking all this together, it appears that numerous cell types and cell interactions are required to create a 'young' testicular environment. In support of this hypothesis, we have shown that exposing aged spermatogonia to young testicular somatic cells results in improved survival of the spermatogonia compared to aged spermatogonia exposed only to aged testicular somatic cells.<sup>30</sup>

Given the evidence that aging of somatic cells and aging of the SSC niche play large parts in compromising the function of aging SSCs, studies have been undertaken to determine what cellular functions are altered in aging testicular somatic cells. In Brown Norway rats, one of the best laboratory models for testicular aging, it has been determined that Leydig cells from aged rats produce less testosterone than do Leydig cells from young rats.<sup>31-33</sup> Since then, researchers have identified several differences in aged Leydig cells that could adversely affect the function of the steroidogenic pathway (differences in LH-receptor number, cAMP production, PK-A activity, cholesterol transport, activities of steroidogenic enzymes, etc.).<sup>33-38</sup> Additionally, a factor originating from both Leydig and peritubular myoid cells (colony stimulating factor 1 (Csfl)) has been implicated as a stimulator of spermatogonial stem cell self-renewal in mice.<sup>29</sup> Thus, changes in function of even peritubular myoid cells could adversely affect the SSC niche and be involved in age-related declines in spermatogenesis. Finally, an inability of the Sertoli cells to respond to signals (e.g. changes in FSH responsiveness) or to produce factors required to regulate SSC self-renewal and differentiation (e.g. glial cell-derived neurotrophic factor; GDNF) has been implicated as part of the aging process.<sup>29</sup> Thus, the functions of numerous cell types are affected by aging and the list of cell functions that are altered is long and growing. Although until recently none of these studies have focused on the horse, the work that has been done in other species can be used as a 'short cut' to identifying age-related problems in the stallion testis.

We have used a xenografting model to show that testicular function in tissue from stallions affected with ITD is not rescued by grafting the tissue into a young extratesticular environment.<sup>25</sup> These findings confirm that, in cases of ITD, a primary testicular defect is present. However, if healthy, prepubertal tissue is co-grafted in physical contact with degenerate testicular tissue, we observe an improvement in the degenerate grafts.<sup>39</sup> These findings suggest that there is a beneficial paracrine effect of the young tissue on the old tissue. In other words, as might be expected from a tissue autologous disease, improvement in the disease is seen only when the 'treatment' originates from within a normal testis. This finding suggests that cell-based therapies (e.g. forms of stem cell therapy) may be the best approach to arriving at a treatment for the adverse affects of aging in the equine testis.

Cell-based therapies can provide transforming results for some disease processes. However, 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 old cells 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 injections. In addition to leading to potential therapeutic approaches to ITD, these studies also lead us to a better understanding of the pathophysiology of ITD and thus provide means of identifying the disease in its early stages and/or developing methods to prevent its occurrence and progression.

We have recently completed experiments designed to identify whole-transcriptome differences in gene expression between cell populations from adult, fertile testes vs. gene expression in cell populations from age-matched, degenerate testes. We now are in the process of mining a large dataset comprised of thousands of genetic sequences in order to identify those genes whose expression are significantly altered between the two tissue types. Within this dataset, we have identified numerous genes and signaling pathways that are affected by aging and that are likely to influence testicular function in the horse. By developing therapies targeted to affect the function of these pathways, we may be able to develop a

simpler approach to the treatment of ITD. In addition to potential therapeutic approaches to ITD, these studies also lead us to a better understanding of the pathophysiology of ITD and thus provide means of identifying the disease in its early stages and/or developing methods to prevent its occurrence and progression.

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