

## Perspectives and Editorials

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### Editorial Commentary

Culty M, Luo L, Yao Z, Chen H, Papadopoulos V, Zirkin B. Cholesterol transport, peripheral benzodiazepine receptor, and steroidogenesis in aging Leydig cells. *J Androl.* 2002;23:439–447.

Using the specific ligand [<sup>3</sup>H]Ro5-4864 (4'-chloro-diazepam, a convulsant benzodiazepine), peripheral benzodiazepine binding sites/receptors (PBRs) have been localized to rat central and peripheral endocrine tissues. These unique sites have been shown to consist of 3 distinct proteins (ie, subunits) of 18, 30, and 32 kd, which have been identified as the isoquinoline carboxamide-binding protein (IBP), adenine nucleotide carrier (ADC), and the voltage-dependent anion channel (VDAC), respectively. Although numerous functions such as cell proliferation, apoptosis, and chemotaxis were attributed to PBR, its major physiological role has not yet been disclosed. One of the likely candidates for this position is the active participation of PBR in steroidogenesis in endocrine organs and glia. Indeed, Ritta et al (1987) demonstrated the pharmacological activity of Ro5-4864 in Leydig cells, where basal testosterone production more than doubled by this agent, and human chorionic gonadotropin (hCG)-induced androgen synthesis was slightly increased. Subsequently, another specific ligand for PBR, the isoquinoline carboxamide derivative PK 11195, exhibited similar activity in nonstimulated, isolated rat Leydig cells.

Although specific binding of PBR ligands was reported to be present on cell membranes (Weissman et al, 1990), copious studies associated these receptors with the outer mitochondrial membranes and linked them to one or more mechanisms that are involved in cholesterol trafficking. Because the transfer of cholesterol to P450<sub>sec</sub> for conversion to pregnenolone is the limiting step in hormone-induced steroid biosynthesis, there is no need to emphasize the relevance of PBR in this process. The concentration of the final product of this pathway in testicular tissue (ie, testosterone), is controlled by the trophic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH). It turns out that in the testis, PBR density is subject to both LH/FSH and testosterone influence. Thus, elevation of the levels of the latter hormone caused a marked decrease in the binding capacity of [<sup>3</sup>H]PK 11195 (Amiri et al, 1991). However, whereas these observations

seem to portray a clear picture of regulation and feedback mechanisms, the picture is muddled due to findings reported by the same authors demonstrating that the anti-androgen cyproterone mimics the effects of testosterone.

The article by Culty et al in this issue of the *Journal of Andrology* reports on reductions in PBR messenger RNA and protein expression as well as a decrease in the binding capacity of [<sup>3</sup>H]PK 11195, observed in isolated Leydig cells from aging rats. The latter finding is in accord with an earlier publication describing the ontogenesis of this ligand binding to rat testis (Mercer et al, 1992). These changes in receptor characteristics were correlated to diminution of cholesterol transport from the outer to the inner mitochondrial membrane in old Leydig cells. Lower cholesterol transport is translated into diminished testosterone output by steroidogenic enzymes. Moreover, it is also notable that unlike other endocrine organs (eg, adrenal gland), the density of [<sup>3</sup>H]PK 11195 binding sites in the testis exhibited developmental alterations, culminating in a distinct decline at the age of 24 months. Because cholesterol transport is of the utmost importance, another candidate for its trafficking should be discussed, namely, steroidogenic acute regulatory (StAR) protein. There is no winner in the contest between PBR and StAR for physiological supremacy yet, and in this respect, the paper by West et al (2001) is of particular interest. According to the proposed model by West et al, cholesterol-loaded StAR associates with PBR at the outer mitochondrial membrane. Nevertheless, although a recipe for collaboration was offered, evidence for actual cholesterol transfer among the two entities was not presented.

Notwithstanding that remarkable effort was invested in a search for the roles played by the 3 subunits of PBR, basic questions such as possible interactions between subunits are unanswered. Furthermore, an additional topic that clearly deserves attention is the physiological significance of the putative endogenous ligand, diazepam binding inhibitor (DBI). Endozepine could modulate cholesterol transfer by PBR or affect the proposed interaction

between StAR and PBR; other actions of this peptide cannot be excluded at this stage. Studies using PBR-knockout mice, for example, could shed light on questions such as the implication of PBR and hCG as partners that act through a shared pathway. Although current knowledge offers reasonable clues as to the function of PBR in steroidogenesis, no categorical definition is available. If only for their phenomenal abundance in endocrine organs, they should get our utmost attention.

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## Editorial Commentary

### Another Piece in the Maddening Puzzle of Declining Steroidogenesis in Aging Leydig Cells

Culty M, Luo L, Yao Z, Chen H, Papadopoulos V, Zirkin B. Cholesterol transport, peripheral benzodiazepine receptor, and steroidogenesis in aging Leydig cells. *J Androl.* 2002;23:439–447.

Firm evidence for the age-related decrease in testosterone secretion was provided by Hollander and Hollander in 1958, when they determined that spermatid testosterone levels were significantly lower in elderly men compared with young men (Hollander and Hollander, 1958). However, it has long been appreciated that there is a decrease in male reproductive function with age, now referred to as “andropause.” Indeed, Plato (ca. 430–350 BCE) even commented on the subject in *The Republic*. “Cephalus the elder, talking with Socrates, about the advantages of old age, ‘. . . I remember someone asking Sophocles, the poet, whether he was still capable of enjoying a woman.’ ‘Don’t talk in that way,’ he answered; ‘I am only too glad to be free of all that; it is like escaping from bondage to a raging madman.’ I thought that a good answer at the time, and I still think it is, for certainly a great peace comes when age sets us free from the passions of that sort. When passions weaken and relax their hold, most it certainly means, as Sophocles said, a release from servitude to many forms of madness.”

The modern man may prefer not to resort to philosophy in his older age, but may instead appreciate the psychological and physiological benefits of a youthful dose of testosterone. To be sure, there is a burgeoning industry

devoted to androgen replacement therapy, the consequences of which are a subject of active debate in the current literature. The article by Culty et al in this issue of *Journal of Andrology* addresses the more fundamental question: What are the cellular mechanisms underlying the decrease in testosterone production that accompanies aging?

This article from Dr Zirkin’s laboratory represents the next logical step in their analysis of age-related changes in steroidogenesis in Leydig cells from Brown Norway rats. The Brown Norway rat is an excellent model for studying aging in males because it presents primary testicular deficits similar to that observed in humans. Leydig cell numbers do not change with aging; instead, the steroidogenic capacity of Leydig cells in old individuals declines. Previous work from the Zirkin group has established that the activity, protein, and messenger RNA level of P450<sub>scc</sub> and each of the enzymatic reactions distal to this mitochondrial step are reduced in aged Leydig cells. Steroidogenic acute regulatory protein (StAR) is responsible for mediating the first hormonally regulated and rate-limiting step in testosterone biosynthesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane to P450<sub>scc</sub>, which catalyzes the first enzymatic

step in the pathway (Stocco, 2001). Zirkin's group, as well as Azhar and coworkers (Leers-Sucheta et al, 1999) have shown that Leydig cell StAR expression decreases with aging. Although the preponderance of evidence supports the essential role that StAR plays in mediating the acute and hormonally regulated transfer of cholesterol into the mitochondrion, it is clear that StAR alone cannot account for cholesterol transfer across the mitochondrial membranes, especially under basal or unstimulated conditions. Moreover, considerable controversy exists as to the molecular mechanisms through which StAR mediates hormonally stimulated cholesterol transfer (for a review see Christenson and Strauss, 2000). The peripheral benzodiazepine receptor (PBR) is a high-affinity mitochondrial cholesterol-binding protein that also participates in cholesterol transport to the steroidogenic pathway. How StAR and PBR may interact, or how they both participate in cholesterol transport in steroidogenic tissues remains a subject of some controversy. It is evident, however, that both StAR and PBR participate in and are essential to the process of substrate delivery across the mitochondrial membranes (Waterman, 1998).

Because the rate-limiting step of steroidogenesis has been shown to be the transport of cholesterol to P450<sub>sc</sub>, alterations in cholesterol availability to this first enzymatic step in the pathway would affect the capacity for testosterone biosynthesis. The report by Zirkin and coworkers assesses the availability of cholesterol stores for steroidogenesis in old vs. young Leydig cells, and is the first study that examines age-related changes in PBR expression and activity.

To assess availability of cholesterol and to determine whether alterations in the cholesterol pool during aging may account for decreased steroidogenic response in old Leydig cells, the P450<sub>sc</sub> inhibitor aminoglutethimide (AMG) was employed. Leydig cells were stimulated with human chorionic gonadotropin and treated with AMG, which led to an accumulation of hormonally recruited cholesterol into mitochondrial membranes. This approach provides an indirect measure of the steroidogenic cholesterol pool. After removal of AMG and reactivation of P450<sub>sc</sub>, there was a significant decrease in mitochondrial steroidogenesis, far greater than could be accounted for by the overall decrease in P450<sub>sc</sub> in Leydig cell mitochondria from the older rats. This observation indicated that there was a reduction in the transport of cholesterol into the mitochondria in old Leydig cells. This could be due to a decrease in the available cholesterol pool, or to a defect in the cholesterol transport machinery. To test the second possibility the expression and function of PBR was examined. The transfer of cholesterol across the mi-

tochondrial membranes is a complex process. The mechanism through which PBR participates as a component of the cholesterol transfer machinery is not fully understood. PBR is an 18-kd outer mitochondrial membrane protein that was discovered as a peripheral binding site for benzodiazepines, and is distinct from the neurotransmitter receptors. PBR isoquinoline-binding activity provides a measure of its cholesterol transfer activity. In this report by Culty et al, PBR messenger RNA and protein levels were reduced in Leydig cells from old rats. In addition, Zirkin and coworkers also determined, in receptor binding assays, that isoquinoline PK 11195 binding to PBR was decreased, indicating that the activity of PBR was reduced as a result of aging. The use of a radiolabeled isoquinoline binding assay provides an independent and highly specific assay for PBR activity, independent of changes in its protein expression.

The senescence of Leydig cell steroidogenic function with aging involves a host of factors. The elegant study from the Zirkin group clearly shows that changes in cholesterol transport into the steroidogenic pathway contribute in a significant way to the age-related reduction in steroidogenic capacity. Their demonstration that a reduction in PBR expression and receptor binding activity parallels this reduction identifies another component of the steroidogenic apparatus that declines with age, and it reinforces the important role that PBR plays in the process. The present data add to a further understanding of one Socratic dilemma, and proves that there is more than a Platonic relationship between StAR and PBR.

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## Response to Commentaries

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The mechanism by which steroidogenesis declines as males age is indeed a “maddening puzzle.” In Brown Norway rats, as in humans, serum testosterone concentration is reduced with aging, the result of the reduced ability of individual Leydig cells to produce testosterone. For the past 5 years, we have conducted studies addressing 2 major issues: 1) What are the deficits in aging Leydig cells that might explain age-related reductions in their ability to produce testosterone? and 2) What causes these deficits?

We now know a great deal about age-related Leydig cell deficits. Aging Leydig cells are characterized by reductions in luteinizing hormone receptor number, cyclic adenosine monophosphate (cAMP) production, steroidogenic acute regulatory (StAR) protein, peripheral benzodiazepine receptor (PBR), cholesterol transport, and conversion of cholesterol to testosterone by enzymes residing in the mitochondria and smooth endoplasmic reticulum. Is there any one among these changes that leads to the rest? Recently obtained results strongly suggest that the reduced ability of Leydig cells to produce cAMP is likely to represent a critical, initiating event that leads to downstream deficits in the steroidogenic machinery; and, consistent with this, that it may be possible to restore steroidogenic function to the old Leydig cells by bypassing cAMP. Just how deficits in signal transduction affect cholesterol transport, and the exact relationship of the latter to PBR and StAR, remain to be determined.

Although the causes of age-related Leydig cell deficits remain elusive, support is emerging for the idea that re-

active oxygen is likely to play a major role in the reduced ability of old Leydig cells to produce testosterone. The risk of damage from reactive oxygen species is particularly high for steroidogenic cells because reactive oxygen is produced both via the electron transport chain and the P450 enzymes. Recent studies from our laboratory indicate that the production of superoxide increases with Leydig cell aging (Chen et al, 2001), and that the expression of genes for SOD1, catalase, and glutathione peroxidase, the products of which protect Leydig cells from oxidative stress, are reduced in aging Leydig cells. We speculate that aging may alter the ability of the Leydig cell to respond to stress, and that this leads to damage to the cell's steroidogenic machinery and ultimately to reduced steroidogenesis. The “maddening puzzle” appears to be on the edge of becoming less maddening.

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## Perspectives and Editorials

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### From *Androlog*

*Note:* Postings to *Androlog* have been lightly edited for grammar and usage before publication.

In the study of andrology, laboratory assessment constitutes one of the cornerstones of our investigative efforts. Nevertheless, there must be times when each of us contemplates the usefulness of some of the studies that we order or perform. We wonder, for example, whether the assay is being handled correctly, and whether the test itself is relevant to the clinical problem at hand. Grace Centola recently set in motion an *Androlog* discussion to address these questions in assessing semen pH. A number of experienced clinicians and basic scientists joined the effort, and they have cast considerable light on this topic. In their own words, let's see what they had to say . . .

Dr Centola asked:

Does anyone still measure semen pH as part of routine semen analysis? We currently assess pH by using pH strips, but I am finding that the overwhelming majority of specimens have a pH of 8–8.5. According to World Health Organization (WHO) standards, the normal pH is at 7.2. Would anyone care to comment or offer suggestions on methods and the significance of measuring pH?

Clearly, Steven Schrader had been giving this matter some thought as well:

We find that the average pH is around 8.0. I cannot remember ever seeing a semen sample as low as 7.2. pH is sensitive to time since ejaculation. The older the semen sample, the higher the pH.

We use a pH meter, and have been discussing whether to continue to measure pH. I would like to hear the views of others on the importance and usefulness of semen pH measurements.

Juergen Liebermann indicated that he too had concerns regarding the WHO standard:

We measure semen pH as part of routine semen analysis by using pH strips, and have found that 75% of specimens have a pH of higher than 8.0 (average 8.2) in contrast to the WHO normal of 7.2 to 8.2. Measurement of pH should be done shortly after the specimen has liquified, because after liquefaction the equilibrium of the specimen changes and the pH increases with time. In addition, there is a positive correlation between pH and concentration of fructose!

Karen Seifarth reported better luck with this assay and provided some technical information:

We measure semen pH with pH strips from Microessential Laboratory, Brooklyn, NY, which are distributed through Allegiance Healthcare. The name of the strips is pHydriion Microfine 6.0 to 8.0. The average pH range for our patients is 7.2 to 7.8. We check each batch of pH strips before use, using standard solutions (4.0, 7.0, and 10.0). We have not had any problems. The average pH of our specimens is probably about 7.4.

At this point, things really started to take shape! Rune Eliasson submitted the following contribution:

It is my impression that many laboratories still officially measure pH. One reason is that it is included as a standard method in the WHO manual on semen analysis. However, in a routine semen analysis, the pH of the seminal plasma does not contribute any information of value.

Dr Centola's observation is absolutely correct and so are her questions. Everyone who measures pH in seminal plasma is a victim of one of the many unscientific paradigms that strongly influence the performance and interpretation of semen analysis.

The "normal value" for the pH presented in the WHO manual is only one example of many unscientific statements in that document. In addition, the "normal" values have changed over the years from 7.2 to 7.8 in 1987, 7.2 to 8.0 in 1992, and 7.2 in 1999. There is no reference to any publication to motivate these changes.

When I started to work with human semen in 1961, I decided to start from scratch because it was obvious that almost everything published in the field was useless from a scientific point of view. The only person who published critical views on the methodology at that time (1964 to 1968) was Dr Matthew Freund, in the United States. I can recommend his articles for more than historical reasons.

In my laboratory, we needed only a few weeks of work in 1964 to understand that measurement of seminal pH did not give any information of value. I was one of the initial promoters of the first WHO manual on semen analysis (1980), and I convinced the other members of the editorial committee that pH measurements were useless. It was therefore not included in the first edition. In the preparation of the second edition, a majority of the editorial committee wanted pH to be included and I lost that vote. I was not involved in the third edition, but I was unable to remove pH measurements from the 4th edition of the WHO manual. It is always a problem when a scientific matter is decided by a majority vote, and only too often the prevailing opinion wins.

Those who still routinely measure the pH of seminal plasma should ask themselves why they do so, and then one hopes they would be aware of its zero value. Such a critical view of one's own routine analysis of semen could be an opening to a more critical view of many methods described in the WHO manual on semen analysis.

It would be an advantage to the science of andrology if more people followed Dr Centola's example and questioned recommended methods for pH, sperm count, sperm motility, and sperm morphology. If you are interested in more comments on the 4 editions of the WHO manual, *Human Semen and Sperm-Cervical Mucus Interaction*, I can send you a PDF file of a poster on this subject, which was presented at the first European Congress of Andrology in L'Aquila, Italy, in 2000.

Conversely, Robert Oates pointed out that in selected cases, assessment of semen pH may be clinically helpful:

In response to Grace Centola's question about semen pH and to Dr Eliasson's comments, semen pH can be an important part of an evaluation. As we all know, the alkalinity of the seminal fluid derives from the seminal vesicles, and acidity derives from the prostate. In evaluation of patients with azoospermia, the semen volume and pH are important for determining the differential. In low volume, acidic, azoospermic samples (volume 0.6cc, pH 6.5, for example), the differential is congenital bilateral absence of the vas deferens (CBAVD) or bilateral complete ejaculatory duct obstruction (EDO). A fructose assay is not needed because the volume coupled with pH indicates no contribution from the seminal vesicles. On the other hand, if the volume is normal and pH is alkaline, the seminal vesicles are functional and the ejaculatory ducts are patent (again, no need for fructose), and the differential includes spermatogenic failure or an obstruction at the level of the more proximal vas or epididymis—it does not include CBAVD or bilateral EDO. In cases in which pH is alkaline but the volume is low and azoospermic, the seminal vesicles are present and functional, and at least one ejaculatory duct is open. Palpation of the scrotal anatomy helps to clarify the exact etiology, whereas transrectal ultrasonography and hormonal assays may also help. Therefore, I agree with Dr Eliasson that pH is of no value when sperm are present in the semen (the ejaculatory ducts must be open), but I disagree in cases of azoospermia when attention to the details of semen volume and pH may be quite helpful in making the diagnosis. The "normal" pH is irrelevant. If laboratories want only to measure pH in cases of azoospermia, I think that would be just fine.

To this, Dr Eliasson replied:

In all clinical laboratory work, one should have a reason for an analysis. One must know what to look for, and one must know the relevant reference limits. A biochemical analysis can be used to investigate the functional capacity of an organ or to assist in the diagnostic work. pH in seminal plasma does not give any information on the functional status or capacity of the organs producing semen, nor is it equal to or better than any other method(s) for diagnosis of a disease or dysfunction in the reproductive organs, including "azoospermia."

That is why measurement of pH in the seminal plasma has no place in modern medicine. The various "reference limits" given in the three latest editions of the WHO Manual also support this view.

Trine Haugen provided additional technical information:

In my study, "pH of Human Semen" (*Int J Androl.* 1998; 21:105–108), we used both a pH meter with a special electrode and pH paper, and measured 30 minutes and 60 minutes after ejaculation. Mean values were: meter (30 minutes) 8.2, paper (30 minutes) 8.4, meter (60 minutes) 8.3, paper (60 minutes) 8.5.

As the last comment in this discussion, Fernando Vasquez provided additional technical insight and some clinical correlation:

In our city, which is located at sea level on the coast, we have observed that pH, as measured in the laboratory, is alkaline (above 8.0), whereas other laboratories in non-coastal cities generally report pH values of 7.2–8.0. We thought the higher values may be caused by the pH strips (Spezialindikator; Merck). For that reason, we did a comparison between the pH meter and the strips, which gave results similar to those presented by Dr Trine B. Haugen. We have found that measurements given by the pH meter gave values that were lower than those of the strips. On many occasions, we have obtained high pH values that were associated with an increase in seminal viscosity. We now have the impression that the pH is associated with an inflammatory infectious process, and so has a clinical value in the functioning of the prostate and seminal vesicles.

Clearly, the topic of assessment of semen pH remains controversial. Still, this discussion brought out many helpful observations. Our thanks to all those involved for letting us "listen in" on this *Androlog* interaction.

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