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Hypothesis

Proposed mechanism for sperm chromatin condensation/decondensation in the male rat

John C Chapman and Sandra D Michael*

Address: Dept. Biological Sciences, Binghamton University, Binghamton, NY 13902-6000

Email: John C Chapman - johnchapman1@juno.com; Sandra D Michael* - smichael@binghamton.edu

* Corresponding author

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Abstract

Condensation of sperm chromatin occurs after spermatozoa have left the caput epididymis and are in transit to the cauda epididymis, during which time large numbers of disulfide bonds are formed. The formation of these disulfide bonds requires the repeated oxidation of the cofactor, NAD(P)H. To date, the means by which this oxidation is achieved has yet to be elucidated. Spermatozoa lose the bulk of their cytoplasm prior to leaving the testis; and, as a result, any shuttle systems for removing and transferring reducing equivalents into the mitochondria are unlikely to be operational. In an apparent preparation for the loss of cytoplasm, however, the following events occur during spermatogenesis. First, androgen-binding protein (ABP) is produced by the Sertoli cells of the testis; second, high affinity binding sites for ABP are inserted into the membrane surrounding the nucleus; and third, a nuclear location is acquired for the enzyme, 3α-hydroxysteroid dehydrogenase (3α-HSD).

We propose that after the loss of cytoplasm, the nuclear region of spermatozoa is directly accessible to constituents contained in the lumen of the caput epididymis. As a consequence, luminal ABP attaches itself to the nuclear membrane via its binding sites, and is internalized. After internalization, ABP exerts its principle function, which is to bind to luminal 5α-dihydrotestosterone (5α-DHT), thereby ensuring its availability to the enzyme, 3α-HSD. In the conversion of 5α-DHT to 3α-androstanediol (3α-Diol), NAD(P)H is oxidized. Spermatozoa that reach the cauda epididymis have fully condensed chromatin. In addition, the nuclear region retains appreciable amounts of 5α-DHT and 3α-Diol, both bound to ABP. During fertilization, the bound 3α-Diol is converted back to 5α-DHT, reducing equivalents are transferred to $NAD(P)^+$, and disulfide bonds are broken.

IVF clinics report that spermatozoa with incompletely condensed chromatin have a low percentage of fertilization. If our proposed mechanism for chromatin condensation/decondensation is borne out by further research, IVF clinics might consider preincubating spermatozoa with 5α-DHT in order to increase the efficiency of fertilization.

Introduction

In eutherian mammals, the condensation of sperm chromatin has two main phases. The first phase, which occurs in the testis, involves the substitution of somatic histones by testis-specific protamines [1,2]. Protamines are small, only half the size of the core histones they replace, and are extremely basic. Between 55% and 70% of the amino acids are arginine. Sperm protamines also contain numer-

ous cysteine residues, which are used to generate disulfide cross-links between adjacent protamine molecules during chromatin condensation. Bull sperm protamine contains 47 amino acids, with 24 arginine and 6 cysteine residues [3]; and rat sperm protamine consists of 50 amino acids, with 32 arginine and 5 cysteine residues [4]. Both protamine molecules are of sufficient length to fill one turn of DNA, with adjacent protamines locked in place around DNA by multiple disulfide bridges [3].

The formation of large numbers of disulfide cross-links between protamine molecules describes what occurs in the second main phase of chromatin condensation. These cross-links are formed after the spermatozoa have exited the caput epididymis and are in route to the cauda epididymis [5–9]. In the rat, the head region of spermatozoa contains approximately 6.9 nMoles of sulfhydryl groups (SH) + disulfides (SS) per million sperm, a figure which remains constant throughout spermatogenesis [10]. Spermatozoa that are isolated from the caput epididymis contain 84% of total SH + SS groups in the head region as thiols; whereas, sperm heads from the cauda epididymis contain only 14% of total SH + SS groups as thiols. This difference indicates that during transit between the two epididymides, almost 1.5 billion disulfide bonds are formed per individual sperm. Therefore, it is not surprising that after chromatin condensation, sperm are highly resistant to a variety of agents such as strong acids, proteases, DNAse, and detergents [11]. The overall effect of chromatin condensation is a transient inactivation of the male genome [12].

Chromatin condensation is directly related to the capacity of sperm to fertilize the ovum. For example, spermatozoa from both the caput epididymis and the proximal corpus epididymis lack the ability to fertilize; whereas, spermatozoa from the distal corpus epididymis and the cauda epididymis have this ability [8,13,14]. Human spermatozoa in which the chromatin is not completely condensed are also reported to have a low percentage of fertilization [15]. In a recent study, human sperm that were incompletely condensed failed to fertilize, even after their injection directly into the ovum [16]. Incomplete chromatin condensation is independent of other causes of infertility, such as abnormalities in sperm morphology (teratozoospermia), low sperm count (oligospermia), or poor sperm motility (asthenozoospermia) [17]. It has been suggested that incompletely condensed sperm constitute a significant factor in the assessment of male fertility [17].

In contrast to spermatogenesis, the process of fertilization requires that disulfide bonds between protamine molecules be broken. This occurs before chromatin decondensation, pronucleus formation, and DNA synthesis [18–21]. It has been proposed that glutathione, which is present in the egg cytoplasm, provides the reducing equivalents for the reduction of the disulfide bonds [18]. Under *in vitro* conditions, heparin-reduced glutathione does cause sperm decondensation [22]. The possibility that mitochondria, located in the middle piece of the spermatozoan, might be involved in decondensation via a lactate/pyruvate shuttle system [23] has also been considered. However, when spermatozoa were treated with cyanide, there was no effect on chromatin decondensation [24]. It has also been suggested that chromatin decondensation is the result of a trypsin-like, acrosomal protease that causes a proteolytic degradation of sperm protamine [25].

There is no question that the oxidation and reduction of sulfhydryl groups is critical to sperm chromatin condensation/decondensation. However, very little is known about the processes, or whether each utilizes the same mechanisms. The usual recipient for reducing equivalents is $NAD(P)^+$, which is reduced to NAD(P)H. Unless the nuclear region contains an unlimited supply of $NAD(P)^+$, it is critical that NAD(P)H transfer its reducing equivalents to some other molecule. During the early stages of spermatogenesis, reducing equivalents can be transferred from the cytoplasm into the mitochondria via shuttle systems [26,27]. However, spermatozoa lack cytoplasm, and their mitochondria are located in the middle piece [28]. Without cytoplasm it is unlikely that spermatozoa can transfer reducing equivalents from the head region to the middle piece. It has been reported that spermatozoa contain a membrane-bound NADPH oxidase for the transfer of reducing equivalents [29,30]. However, it was also reported that this NAD(P)H oxidase activity is insignificant [31], which limits the likelihood of it being a substitute for the shuttle systems. This leaves the question unanswered of how reducing equivalents are transferred in spermatozoa. The unique structure of spermatozoa, relative to that of a typical cell, suggests that their pathway for oxidizing NAD(P)H is unique as well. We previously reported that the head region of sonication-resistant spermatids converts endogenous 5α-dihydrotestosterone (5α-DHT) to 3α-androstanediol (3α-Diol) [32], a reaction in which NAD(P)H transfers its reducing equivalents to 5α-DHT. In our study, no cofactor was added to the incubation, indicating that endogenous NAD(P)H was the source of the reducing equivalents. Evidence that spermatozoa are also capable of this transfer is indicated by the report that bovine spermatozoa convert ^{3}H-DHT to ^{3}H-3α-Diol without added cofactor [33]. The remainder of this paper will use published data to develop and describe the putative role of the principle constituents in this unique pathway for reducing equivalents.

Hypothesis

Proposed mechanism for sperm chromatin condensation/decondensation

The first constituent in the proposed mechanism is androgen binding-protein (ABP). ABP is one of the major secretory products of the Sertoli cells of the mammalian testis [34–37]. In the rat, Sertoli cells secrete 20% of their ABP across the basal membranes into the interstitial compartment and 80% into the lumen of the seminiferous tubules [38,39]. ABP is then transported via the rete testis and efferent ductales into the caput and caudal epididymides [40–44]. During transit, the levels of ABP increase, and then fall in the cauda epididymis [45]. For example, in seminiferous tubule fluid the level of ABP is 40 nM; in rete testis fluid it is 60 nM; in the lumen of the caput epididymis it is 265 nM; and, in the lumen of the cauda epididymis it is 65 nM. Biologically active ABP can be detected in the serum of the male rat at 15 days of age [46,47]. However, after 40 days of age, the serum of the adult male rat contains less than 0.2% of the ABP measured in testis and caput epididymis [45]. *In vitro* and *in vivo* studies have demonstrated that the synthesis and secretion of ABP is regulated by androgens and FSH [48–50]. Testicular ABP has been found in all species that have been examined. The best characterized are rat, rabbit, and human ABP/SHBG (steroid hormone binding globulin) [51]. Despite testicular ABP being produced by the Sertoli cells and plasma SHBG originating from hepatocytes [52], it is now known that ABP and SHBG are encoded by the same gene and share a number of identical amino acid sequences [53–55].

It is generally accepted that one mole of ABP binds one mole of steroid. Although there is some variation in steroid specificity, ABP from most species binds DHT, T, estradiol-17-β (E_2), and 5α-diol with high affinity. The dissociation constant (K_d) of ABP for DHT is between 1.6×10^{-9} M and 0.8×10^{-9} M. Testosterone, E_2, and 5α-diol bind with lower affinities, but generally within one order of magnitude of that of DHT [42,56–59]. ABP does not appear to have any binding affinity for either androstenedione or progesterone.

Despite the extensive amount of data that have accumulated on the ABP molecule *per se*, very little definitive information on its role in male reproduction has been obtained. The underlying problem contributing to the lack of knowledge is that there are no known natural mutants in humans or animals where ABP is totally absent. This suggests that ABP is extremely important for mammalian development (i.e., mutants are lethal), or conversely, it is of little or no importance. The latter would appear unlikely considering the homology of sequence and activity of mammalian ABP, irrespective of species [51,59]. Early studies indicated that there is a correlation between decreased levels of ABP and infertility in: the pregnenolone-treated rat [60], the restricted rat [61], and hamsters exposed to altered photoperiods [62]. A series of papers by Huang et al. [48,63,64] indicated that the ability of spermatogenesis to produce viable sperm is closely related to ABP levels.

Sertoli cell cultures enriched with germ cells (spermatogonia, primary spermatocytes) undergo a doubling in the secretion of ABP [65,66]. The increased secretion of ABP requires FSH stimulation and the direct contact of the Sertoli cells with the spermatocytes. In 1984, Steinberger's group [67] reported that rat spermatocytes contain specific binding sites for ABP. Pelliniemi et al. [68], using anti-ABP antibodies, reported the presence of positive granules within the cytoplasm of spermatocytes and spermatids. Further use of the technique of immunocytochemistry has shown that the intensity of immunoreactive ABP staining and its intracellular localization in rat testis are dependent on the stage of the spermatogenic cycle [69].

Using purified ABP complexed to ^{3}H-testosterone, Gerard et al. [70] demonstrated the endocytosis of ABP/SHBG by coated vesicles in monkey germ cells. The ligand-ABP complex was taken up by spermatogonia, spermatocytes, and early spermatids. This group reported that late spermatids and sperm did not internalize the ABP/SHBG. In a second study, using transmission electron microscopy and autoradiography, Gerard et al. [71] examined the internalization of ABP by rat germ cells and found that ABP was internalized by spermatocytes, round spermatids, and elongated spermatids. It was also noted that the intracellular site of ABP accumulation changed as the sperm matured. For example, labeling was most intense in nuclei having the less condensed form of chromatin. A nuclear location for ABP during the early stages of spermatogenesis suggests that it might play a role in transcription. Reports that stage XI elongated spermatids contain the androgen receptor [72], and synthesize mRNA [28], tend to bear this out.

ABP has also been shown to be associated with sperm during the later stages of spermatogenesis. For example, in an investigation of the endocytosis of ABP by sperm, Felden et al. [73], and Gerard [74] reported that rat germ cells each have 12,000 to 13,000 binding sites for ABP. The binding sites are a single class with a dissociation constant (K_d) for ABP of 0.78 nM [74]. It was proposed that the endocytosis of ABP is receptor mediated and related to the ABP binding activity previously identified on germ cell plasma membranes [71]. This suggests that ABP may also function in spermatogenesis as a steroid trans-membrane carrier. While this has yet to be demonstrated, it has been reported that tubules of the caput epididymis accumulate ^{3}H-testosterone more efficiently from the luminal surface

in the presence of ABP [75]. Others have suggested that the function of ABP is to establish and maintain the high androgen levels required for sperm maturation in the epididymides [45]. High levels of immunoreactive ABP have been found in the lumen of the initial segment of the caput epididymis, where the protein is seen to be coating both the spermatozoa and the brush border of the principal cells. Considerable staining is also observed in the epithelial cells, with the heaviest staining in the apical portion of the cells. The heavy staining of spermatozoa and epithelial cells in this region suggests a considerable degree of endocytosis of luminal ABP. Very little immunoreactive ABP is detected in the epithelial cells of the distal caput, corpus, and cauda epididymides [68].

The second constituent in the proposed pathway for transferring reducing equivalents is the enzyme, 3α-hydroxysteroid dehydrogenase (3α-HSD). This enzyme is believed to exist to limit the levels of DHT and to aid in the metabolic clearance of potent androgens. The clearance pathway for androgens is understood to be: testosterone → 5α-DHT → 3α-Diol glucuronide [76,77]. In humans, there are 4 isoforms of 3α-HSD, all within the aldo-keto reductase (AKR) superfamily [77]. The isoforms are NAD(P)(H)-dependent and convert 5α-dihydrotestosterone (17beta-hydroxy-5alpha-androstan-3-one [5α-DHT]) to yield 5α-androstane-3α, 17β-diol; [3α-Diol]). At present, different isoforms of 3α-HSD have been found in liver, prostate, mammary glands, uterus, brain, and skin [78,79]. In the oxidation direction, only AKR1C2, which is found in the brain, is able to convert 3α-Diol to 5α-DHT.

In the rat, there is a single form of 3α-HSD [80]. The reported locations for 3α-HSD in the male rat are liver, scrotal skin, muscle, prostate, epididymis, and sonication-resistant spermatids [32,80–82]. The epididymis of the adult male rat converts 3α-diol to 5α-DHT [82], indicating that the single form of 3α-HSD is reversible.

The head region of sonication-resistant rat spermatids converts endogenous bound 5α-DHT to 3α-Diol in the absence of added NAD(P)H cofactor [32]. In addition, bovine epididymal spermatozoa and ejaculated bovine sperm convert ^{3}H-DHT to ^{3}H-Diols, also without added cofactor [33]. This suggests that spermatozoa contain the enzyme, 3α-HSD, and that it utilizes endogenous NAD(P)H. It was also reported that ^{3}H-DHT and ^{3}H-Diols become bound to bovine spermatozoa during incubation [33].

The large numbers of disulfide bonds that are formed during chromatin condensation necessitate the repeated oxidation of NAD(P)H. We believe oxidation occurs via the transfer of reducing equivalents to 5α-DHT. The overall process requires the concerted efforts of the enzyme 3α-HSD, and ABP. Unlike 5α reductase, an enzyme found primarily in spermatocytes [83,84], 3α-HSD is found in spermatids [32], as well as in spermatozoa [33]. With 3α-HSD localized in the nuclear region of the sperm [32], the only limitation on the oxidation of NAD(P)H is the availability of 5α-DHT, which is the responsibility of ABP. The nuclear region of each spermatozoan is enclosed by a membrane that has 12,000 to 13,000 high affinity binding sites for ABP [73,74]. These ABP binding sites come into play after the loss of cytoplasm and the spermatozoa have entered the caput epididymis. Here, immunoreactive ABP can be seen covering the spermatozoa [69]. The binding and internalization of ABP, as well as the subsequent delivery of 5α-DHT, is facilitated by the extremely high levels of both ABP and 5α-DHT, which in the caput epididymis are 265 nM and 200 nM, respectively [45]. The observation that ABP is not found in epithelial cells of the distal caput, corpus, and cauda epididymides [68], suggests that the internalization of ABP is limited to the proximal caput epididymis.

We have examined the head region of spermatozoa, isolated from the cauda epididymis for the presence of bound androgens, and detected 3α-Diol (1344 pg/mg DNA) and 5α-DHT (385 pg/mg DNA) [85]. Both androgens were tightly bound to the head region and remained there even when spermatozoa were isolated from the cauda epididymis of male rats that had been castrated three days previously. The obvious tenacity of the head region for these two androgens is very likely due to ABP. Evidence for the continued presence of ABP in mature spermatozoa can be provided by calculating the theoretical levels of bound androgen, and comparing this figure with the actual levels of bound androgen. Since the nuclear region of each spermatozoan contains 3.5 pg DNA [85], and has between 12,000 to 13,000 binding sites for ABP [73,74], the theoretical level of bound androgen would be between 1650 pg/mg DNA and 1790 pg/mg DNA. The measured levels of 3α-Diol and 5α-DHT were 1344 pg/mg DNA and 385 pg/mg DNA, respectively, or a total of 1729 pg/mg DNA [85].

In the formation of disulfide bonds, NAD(P)H has to be repeatedly oxidized. During fertilization and the dissolution of disulfide bonds, the reverse occurs, and $NAD(P)^{+}$ has to be repeatedly reduced. If chromatin decondensation utilizes the mechanism of chromatin condensation, but in reverse, then this will occur through the transfer of reducing equivalents from 3α-Diol to $NAD(P)^{+}$. We have found that the 3α-Diol bound to the head region is consistently 3 fold that of bound 5α-DHT [85], indicating that 3α-HSD is functioning as a reductase. For the enzyme to operate in the reverse direction, it is likely that activation by an external source is required. This activation could be in the form of a female sex hormone, such as es-

trogen or progesterone. Progesterone is known to act on the sperm to induce capacitation [86]. It could also cause 3α-HSD to function as an oxidase. There are approximately 10,000 molecules of 3α-Diol per spermatozoan [85]. This number would produce less than 1% of the reducing equivalents needed to break all disulfide bounds. Still, enough bonds could be broken to allow reduced glutathione, contained in the cytoplasm of the ovum [18], to penetrate the chromatin and affect the dissolution of the remainder of the disulfide bonds.

It has been suggested that glutathione is an intermediary in sperm condensation [29]. If this is indeed the case, reducing equivalents still need to be transferred, for which our proposed mechanism is applicable.

Testing the proposed mechanism for sperm chromatin condensation

The literature contains a number of reports that tentatively support our proposed mechanism. For example, a reduction in androgen levels in the lumen of the caput and cauda epididymides is correlated with decreased numbers of disulfide bonds in spermatozoa [87,88]. In addition, there is a significant correlation between ABP levels and sperm fertilizing ability in the pregnenolone-injected rat [60] and the restricted (H^{re}) rat [89]. In both experimental animals, decreased levels of ABP caused a defect in sperm quality, but had no affect on sperm quantity. While these studies provide supportive evidence, the best way to validate the proposed mechanism is to demonstrate that the formation of disulfide bonds in caput epididymal spermatozoa is completely dependent upon the conversion of 5α-DHT to 3α-Diol.

Significance of the proposed mechanism for sperm chromatin condensation

Knowledge of the actual mechanism of chromatin condensation/decondensation is important to the field of reproductive endocrinology. For example, if 5α-DHT is found to be the recipient of reducing equivalents during chromatin condensation, IVF clinics might consider preincubating spermatozoa with 5α-DHT in order to increase the efficiency of fertilization.

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