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Research

International Journal of Gynecology and Reproductive Sciences

ProAKAP4 polypeptide as a biomarker of sperm functionality and male fertility disorders

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Received: October 10, 2019 | **Published:** January 10, 2020

Abstract

Infertility is nowadays a raising societal problem. An increasing prevalence of male reproductive disorders, such as testicular cancers, and a global decline in sperm counts have been reported worldwide. Over the last decade, there have been accumulating evidences that proAKAP4 protein may represent a new molecular sperm parameter that could be measured routinely, to evaluate the quality of spermatogenesis and the sperm quality. Structurally proAKAP4 polypeptide must be converted by motile and alive spermatozoa in mature AKAP4 that consequently regulate sperm functionality, i.e motility, capacitating and fertility. In testis, the precursor proAKAP4 exists from spermatide stage up to the mature ejaculated spermatozoa as a storage form of the AKAP4 active molecule. With the use of a protein marker such as proAKAP4, researchers and clinicians have now a useful functional assay to assess semen quality in preclinical, toxicological and clinical studies. ProAKAP4 concentrations can be easily measured with the 4MID® Kits, that are robust sandwich ELISA. These functional assays give a number that allow to qualify proAKAP4 directly in the ejaculated spermatozoa. Being correlated to sperm motility, and therefore to living spermatozoa, proAKAP4 could also be useful at terms to assess semen functionality before selecting the more appropriate artificial insemination settings. In the present review, we will first focus about what is the proAKAP4 biomarker and how this molecule organizes flagella structure and regulate motility. Then we will present the data about proAKAP4 and AKAP4 under expression and or metabolism impairment in male fertility disorders, and how proAKAP4 parameter can bring functional indications for a better understanding of the molecular effects of any pharmaceuticals products (including vaccines, biopharmaceuticals, contraceptives and veterinary drugs), industrial chemicals such as endocrine disruptors and food additives on male reproduction. Further in–depth clinical investigations should then be performed to evaluate the proAKAP4 variations in antioxidative therapeutic approaches of male infertility and more generally to evaluate any therapeutic compounds that may impair, preserve or restore spermatogenesis as well as sperm functionality.

Keywords: proAKAP4, biomarker, spermatozoa, male fertility, motility

Abbreviations: AKAP4: A–kinase anchor protein 4; PKA: protein kinase A

Introduction

ProAKAP4 (A–kinase anchor protein 4) has been described over the last decade as a functional marker of spermatozoa.[1,2] This key protein is largely conserved between species and its specific regulation during spermatogenesis seems essential to increase success rate in artificial insemination settings.[2] Recent cross species analyses have identified the potential use of proAKAP4 as a pertinent biomarker of overall semen quality in clinical settings.[2–4] In terms of structure, proAKAP4 is a precursor polypeptide that must be converted by motile and alive spermatozoa in mature AKAP4 that consequently will dock and coordinate the core transduction signals regulating sperm functionality, i.e motility, capacitating and fertility.[1,2]

There have been accumulating evidence that modern lifestyle exposure can cause adverse reproductive effects on spermatozoa.[5] Infertility is nowadays a raising societal problem with more than 80 million couples worldwide suffering of fertility problems and with semen quality declining in many species.[5] To evaluate developmental or environmental impacts on male fertility, the sperm quality assessments were used to be mainly morphological and dynamic assessments using computer–assisted sperm analysis (CASA) without taking into account how spermatozoa have to be motile up to the fecundation site.[2] Recently functional assays have raised the market. They detect and quantify specifically the proAKAP4 biomarker in spermatozoa and are called 4MID® Kits.[2,4] Evaluating the semen functionality with this pertinent functional sperm parameter should improve at terms the birth success rates in assisted reproductive technologies setting that are still largely unsatisfactory.

This review will then focus on proAKAP4 biomarker that appears today as a useful tool to evaluate impact of unhealthy behaviours, endocrine disruptors or therapeutic drugs on global sperm quality and functionality.

What is proAKAP4?

As largely described in the literature, proAKAP4 and the mature AKAP4 are specific structural and functional proteins of the spermatozoa.[1,2,6]

The full–length AKAP4, the so-called proAKAP4, is encoded by a single AKAP4 gene located at Xp 11.22 locus of the short arm of the human X chromosome.[7] The AKAP4 gene belongs to the large family of the A–kinase anchor proteins (AKAPs) that includes around 50 of structurally diverse members all sharing in common at least one anchoring domain to the regulatory subunits of the protein kinase A (PKA).[1,6,8] The AKAPs have unique cell localizations and have the ability to form complexes with other signaling molecules thus enabling a precise and spatial signaling cascade to discrete sub–cellular compartments.[1,9,10] However in contrast to other AKAPs, proAKAP4 and AKAP4 have a strict localization in the flagellum of male sperm cells.[1,2,6]

Structurally proAKAP4 is the polypeptide precursor form of AKAP4. In humans, the proAKAP4 is expressed as a 854 amino-acid protein.[1,2] This precursor protein is then processed to a "mature" AKAP4 protein that are lacking the 188 first amino acids.[1,2,8] The excised 188 first amino acids formed the so-called prodomain. Up to now, the regulatory mechanisms involved in the synthesis, the metabolism and the endoproteolytic processing of proAKAP4 are still unknown. AKAP4 polypeptides are expressed in a variety of mammalian species and are highly conserved (with an overall 70% of homology) in animal kingdom highlighting their essential roles in gene transmission.[3,11,12]

ProAKAP4 is a spermatozoa specific protein

Among biomarkers, the specificity of proAKAP4 polypeptide is to be inside the spermatozoa, more precisely in the column and ribbons of the fibrous sheath of the principle piece of the flagellum.[1,6–9] The proAKAP4 is then an intracellular protein, only found in spermatozoa and never found in seminal plasma.[13–16] This is neither a transmembrane protein nor a membrane–associated protein or a secreted protein, conversely to inhibit B or the anti–Mullerian hormone (AMH) that were previously described as potential markers of idiopathic infertility.[17,18]

Both proAKAP4 and AKAP4 were specifically localized to the fibrous sheath of the principal piece[8,9,14,19] and not found in the intermediate or in the terminal piece of the flagellum.[13–16] AKAP4 is one of the major components (~50%) of the sperm fibrous sheath and play a major role in completing fibrous sheath assembly.[8,9] As a scaffolding and functional protein, AKAP4 has been shown to interact with many protein partners including the Fibrous Sheath Interacting Protein 1 and 2 (FSIP1 and FSIP2), AKAP3, glycolytic enzymes such as enolase 4, protein phosphatase PP1CC2 and the PKA regulating subunit 1 and 2 (PRKAR1A and PRKAR2A).[8,20–22] The latter studies demonstrated the central contribution of AKAP4 to anchor the glycolytic enzymes that are regulating sperm motility and hyper motility in human and mouse spermatozoa. Thus the fibrous sheath with proAKAP4 and AKAP4 proteins constituted a unique cytoskeleton structure that is surrounding the axon me and with the outer dense fibres that extends throughout the principal piece of the sperm flagellum.[9] This particular structure is only observed in the highly differentiated spermatozoa and is never encountered in other flagellated cells (Figure 1).

As described, AKAP4 anchors cAMP dependent protein kinase A (PKA) to the fibrous sheath of the flagella where the kinase is required for the regulation of spermatozoa motility.[6–9] AKAP4 has then been implicated as a sub cellular scaffold responsible for compartmentalizing PKA within the immediate proximity of its enzymatic substrates.[6,9–11] Consequently, AKAP4 exerts impact over the specificity of the signal transduction and metabolic processes that support sperm motility, hyper motility and capacitation.[2,3]

By gene ablation studies, the importance of AKAP4 in the organization and integrity of the fibrous sheath was described with male mice lacking AKAP4 expression.[23–25] In these mice, the interaction between outer dense fibers and microtubules were maintained in the sperm of mutant mice, and middle–piece dimensions of mutant sperm were similar to those of wild type sperm, however the sperm flagellum was generally shorter and immotile.[24]

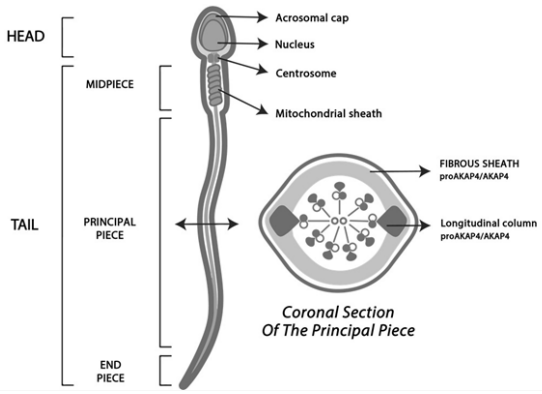

Figure 1 ProAKAP4/AKAP4 Expression in Spermatozoa.

As ejaculated spermatozoa are transcriptional inactive, the proAKAP4 has to be fully synthesized in testis during spermatogenesis. Both proAKAP4 and AKAP4 were shown to be expressed at the round spermatide stage.[2,26] As AKAP4 gene is on the X chromosome[7,27], AKAP4 mRNA and or polypeptides must be shared among conjoined X- and Y-bearing spermatids.[27] The precursor proAKAP4 exist then from spermatic stage up to the mature ejaculated spermatozoa as a storage form of the AKAP4 active molecule, that will then be playing a central role in flagella structure, chemotaxis, capacitating and sperm motility.[1,2,28] The functional protein proAKAP4 as a fully synthesized stock of AKAP4, orchestrates then carefully all the signaling messages involved in living, moving and functional spermatozoa.

ProAKAP4 as a functional protein

According to the literature, the proAKAP4 polypeptide is then clearly instrumental for sperm flagella morphology and consequently sperm motility. For ensure sperm functionality, both AKAP4 and proAKAP4 were found to be serine and tyrosine-phosphorylated. Tyrosine phosphorylation of AKAP4 have been well described in spermatozoa of various species from in mouse, hamster, pig, bull and human.[1,19,29–37] In mouse, AKAP4 was identified among the sperm proteins that were highly modified during epididymal maturation.[35] A structural study showed that proAKAP4/AKAP4 can experienced up to 13 phospho peptide modifications from the caput to the caudal location during epididymal maturation.[36] One of these peptides (LSSLVIQMARK) was found within a region of AKAP4 involved in binding the regulatory subunits RI and RII of PKA. Confining the holo enzyme PKA to the principal piece in the sperm cells was previously reported to lead to a 4.62–fold increase in Ser3 of the same LSSLVIQMARK peptide of proAKAP4 during spermatozoa capacitation.[37]

Tyrosine phosphorylation of AKAP4 clearly influence PKA recruitment to specific cell compartments.[32,34] Inhibition of PI 3-kinase results in an increased of intracellular cAMP levels, in tyrosine phosphorylation of AKAP4 and PKA recruitment to sperm tails.[34] Interestingly, ERK1/2 was shown to specially phosphorylate proAKAP4 on Threonine 265 in human spermatozoa both in vitro and in vivo.[10] Furthermore, ERK1/2 phosphorylation was shown to

Citation: Delehedde M, Carracedo S, Selleslagh M, et al. ProAKAP4 polypeptide as a biomarker of sperm functionality and male fertility disorders. *Int J Gynecol and Reprod Sci.* (2019);2(1):13-19.

be important to control the cellular distribution of proAKAP4 and AKAP4 protein.[10] Interestingly, *in vitro* experiments showed that the mature AKAP4 was localized to the principal piece of the flagellum under basal conditions but was translocated to the principal piece, in the mid–piece and in the post–acrosomal region under PMA stimulation in human spermatozoa.[10] The prodomain of AKAP4 seems necessary to delocalize AKAP4 in the fibrous sheath of the flagellum. After processing, the "mature" AKAP4 acquires the ability to bind to microtubules and mediates the transduction signals from kinase to the axoneme me to promote motility (Figure 1).

In the literature, the proAKAP4 concentrations have been largely reported to be correlated with total and progressive motility in humans as in main mammals such as stallions, pigs, dogs and bulls.[2–4,13–16] The proAKAP4 concentrations as a reflect of the long–lasting motility give a more objective figure compared to microscopic observations of spermatozoa that are motile at the time of analysis.[2] Indeed, proAKAP4 amount reflect the ability of spermatozoa to keep active and functional in time, up to the site of fertilization. In this context, spermatozoa from mice lacking AKAP4 failed to show progressive motility and homozygous male mice were unfertile.[24,25] Independent studies of a targeted disruption of the AKAP4 gene show that the number of spermatozoa produced by AKAP4 knockout animals were unchanged but they failed to be functional.[24,25] These KO cells exhibited defects in sperm flagellum, were failing to acquire progressive motility and exhibited a reduced abundance in transduction signals and in glycolytic enzymes associated to the fibrous sheath.[24,25] In a male patient, an absence of proAKAP4 and of AKAP4 expression have been also associated with sperm fibrous sheath dysplasia.[38–40]

Taken together, these data highlighted that any modification of proAKAP4 expression during spermatogenesis or modification in its metabolism will have consequences on sperm motility, hyper motility, sperm capacitation and then fertility.

ProAKAP4 as a biomarker of male fertility dysfunctions

Recently, positive correlations between the amounts of AKAP4, and and/or of the proAKAP4 precursor molecule, with key sperm quality and fertility indicators were found in humans, large mammals and small mammals.[2–4,13–15,41,42]

The proAKAP4/AKAP4 biomarker has been described for years in male fertility dysfunctions studies.[14,43–58] The decrease in expression of proAKAP4/AKAP4 have been described in infertile patients with normal sperm parameters, gathered as patients with idiopathic infertility or unexplained infertility.[14,43–47] Results from Jumeau and collaborators have recently showed that human sperm motility was positively correlated with proAKAP4 amounts in a series of 77 normozoospermic men consulting for unexplained fertility.[14] Both AKAP4 and proAKAP4 proteins were down regulated in the infertile group of normozoospermic men compared to fertile group.[46] Interestingly AKAP4 peptides were more highly represented in the spermatozoa of fertile men compared with spermatozoa of the infertile men that had lost their capacity to bind *in vitro* to the zone pellucida.[43] Furthermore, a preliminary retrospective analysis of a series of patients followed for unexplained infertility, high level proAKAP4 proteins were shown to be significantly correlated with a lower proportion of abortions in intrauterine insemination settings.[4] AKAP4 was then recently suggested as a marker to select rare and best quality sperm in assisted reproduction technologies.[48]

Consequently, measuring expression and levels of concentrations of proAKAP4 in semen appears today as an interesting approach to evaluate semen quality in male infertility disorders (Table 1). There have been lines of evidences of the striking underrepresentation of AKAP4 in the spermatozoa of infertile human patients.[43,45–47] The AKAP4 gene was also described to be the most significantly down–regulated transcripts in infertile patients with various types of non-obstructive azoospermia (n=18) versus patients with normal spermatogenesis (n= 4).[49]

Table 1 Main AKAP4/ProAKAP4 Variations in Male Fertility Disorders

Male Fertility Disorders	ProAKAP4 /AKAP4 Expression	References
Idiopathic infertility	Downregulation of AKAP4	47
	Downregulation of AKAP4	44
	Downregulation of both proAKAP4 and AKAP4	46
	Downregulation of AKAP4	43
	Downregulation of AKAP4	45
	Downregulation of AKAP4 proteins	14
Asthenozoospermia	Decrease expression	50
	Downregulation of AKAP4 protein	51
	Downregulation of AKAP4 protein	52
	Coding mutation : G>A in one patient	53
Azoospermia	Downregulation of AKAP4	49
	Downregulation of AKAP4 gene	58
Fibrous Sheath Dysplasia (DFS)	Absence or decrease expression	54
		55
		38
		39
		47
		40
	Deletion mutation observed in one patient	39
Multiple Morphological Abnormalities of sperm Flagella (MMAF)	Lack of AKAP4 expression in FSIP2 mutants	56
		57

In asthenozoospermia, the down regulation of AKAP4 expression was reported in different studies.[50–53] AKAP4 protein expressions were markedly down regulated in asthenozoospermic patients compared to donors.[51] Both the AKAP4 expression and phosphorylation level were significantly altered in asthenozoospermic patients compared to normo zoospermic men.[52]

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Absence of proAKAP4 and AKAP4 expression have been frequently associated with sperm fibrous sheath dysplasia (DFS) in infertile patients.[38–40,47,51,54] Furthermore, AKAP4 protein was detected by immuno staining in the testicular tissue of another patient affected by fibrous sheath dysplasia, concentrated in the cytoplasm of spermatids and in residual bodies of fibrous sheath.[50] Lack of AKAP4 expression was also reported in spermatozoa of patients with multiple morphological abnormalities of the sperm flagella (MMAF) when linked to the mutation of FSIP2 protein.[56,57]

Interestingly, mutations on *AKAP4* gene was never found in DFS patients [40,47,54,55] with the unique exception of a patient that exhibit a deletion mutation.[38,39] These partial deletions of *AKAP4* gene sequences seems to be related to defective assembly of fibrous sheath components and failure of compartmentalization of AKAP4 proteins in the tail, causing the sperm immotility.[38,39] A coding mutation in *AKAP4* gene (located in *AKAP4* c.887G>A; p. Gly296Asn) was also reported in only one among a group of 10 asthenozoospermic patients but was not identified in the group of 90 control men of the clinical study.[53] Taken together, the proAKAP4 appears today as a key molecular parameter to be evaluated in male fertility outcomes.

ProAKAP4 expression as a read out of good spermatogenesis

The complete stock of proAKAP4 in each individual cell is constituted during spermatogenesis and before ejaculation as spermatozoa are highly differentiated cells that are characterized to be transcriptional inactive. In contrast, AKAP4 amount is a consequence of proAKAP4 processing and is modulated according to the initial stock of the proAKAP4 and subjected to the metabolism regulatory systems during sperm maturation.

Hence, proAKAP4 concentrations reflect the maturation levels of ejaculated spermatozoa.[2,28] By using different density gradients, spermatozoa in human ejaculate can be separated into different fractions of sperm undergoing varying levels of maturation and therefore spermatozoa expressing different levels of proAKAP4/AKAP4 can be encountered.[28] High amounts of proAKAP4/AKAP4 were found in the fraction of more mature spermatozoa with increased motility parameter, normal morphology and exhibiting the lower reactive oxygen species levels and DNA fragmentation rate.[28] The proAKAP4 is a unique molecular parameter that evaluate level of maturation and then of competence of the spermatozoa in the ejaculate. Any defective event during spermatogenesis causing by testicular diseases (varicocele, cancers, inflammation) or by health habits (smoking, dietary deficiencies, alcohol consumption), genitourinary tract infections, drugs and treatments will affect the expression of proAKAP4, modified its metabolism and, consequently impact sperm functionality and fecundation.[2,4,36,40,59–67]

Concerning testicular pathologies, under expressions of AKAP4 have been reported in testicular cancer[60] and in epididymitis patients.[61] As described above, there has been clear evidence of proAKAP4/AKAP4 down regulation and or metabolism impairment in male fertility disorders.[14,43–58] Oxidative stress has been often implicated as a key contributor to the loss of sperm functional competence and consequently in male infertility.[5,62] The male germ cell by its nature and though its well–organized architecture exhibiting different stages of differentiation, can be the subject of free radical attacks.[5] AKAP4 has been for instance identified among the main 4-hydroxynonenal (4HNE) targets in oxidative stressed human spermatozoa.[63] The 4HNE is an aldehyde that has been described as one of the most abundant and cytosolic secondary oxidation products[5,61,62] and any reactive oxygen species (ROS) may cause functional lesions, which in turn will compromise the fertilization potential of mature spermatozoa.[28,62–64] Hamada and collaborators showed in a clinical report that AKAP4 levels were decreased in ROS positive semen samples which exhibit consequently lower motility.[64] More recently, using 4MID® Kits approach and proteomic methods, proAKAP4 amounts were shown to be significantly correlated with sperm motility and inversely correlated with DNA fragmentation.[4,14] Environment or non healthy lifestyles behaviors such as cigarette smoking was shown to have a negative impact on proAKAP4 expression in both human and mice spermatozoa.[4] With the read out of the proAKAP4 concentration modulations, toxicologists have then now a pertinent tool to assess the possible molecular abnormalities in sperm functionalities (Figure 2). AKAP4 and proAKAP4 expression were also shown to be modulated in a rat model of vinclozolin exposure, a di carboximide fungicide extensively used on fruits and vegetables that is clearly an anti-androgenic compound.[65] Perfluorododecanoic acid (PFDoA) that is a common environmental polluant, was shown to induce a marked decrease of the levels of AKAP4 phosphorylation in rat testes, providing evidence that PFDoA may disrupt the structure of the sperm fibrous sheath, leading to abnormal sperm motility.[66]

ProAKAP4/AKAP4 may then also be a target for chemical contraception. Sperm stasis that are targeted with quinines was shown to be associated with the alkylation of AKAP4.[67] With these types of contraceptive compounds, a dramatic suppression of sperm motility was achieved by the alkylation of key thiols chemical moieties of AKAP4 in the human sperm flagellum. The alkylation of AKAP4 completely disrupted one of its main role in the coordination of the cAMP/PKA–dependent signaling pathways that are essential for motility.[67] Measuring proAKAP4 concentrations as a follow up of any types of molecules (drugs, cosmetics, food additive, vaccine, hormones, contraceptive agents, etc.) in a context of male infertility management should be then interesting to evaluate semen quality in preclinical, toxicological and clinical settings (Figure 2).

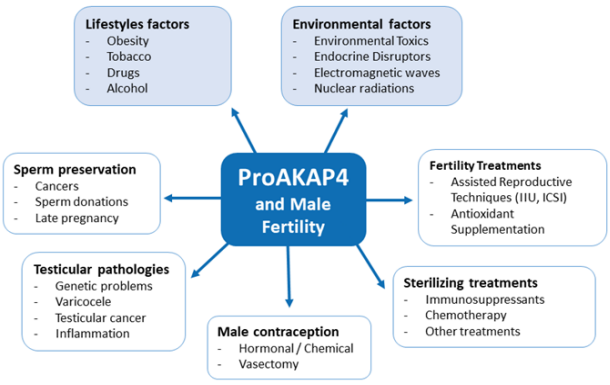

Figure 2 The proAKAP4 Parameter in Male Fertility Outcomes.

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Conclusion

During the last decades, an increasing prevalence of male reproductive disorders, such as cryptorchidism, testicular germ cell cancer and decline in sperm counts, has been reported worldwide. In this context, there have been clear evidence of proAKAP4/AKAP4 under expression and or metabolism impairment in male fertility disorders. As any modification of proAKAP4 expression during spermatogenesis or modification of its metabolism will have consequence on sperm motility, sperm capacitation and fertility, this new sperm parameter brings indications in the field of reproductive toxicology for a better understanding of the molecular effects of any toxic exposure that can be involved in male infertility. With the use of a protein marker such as proAKAP4, researchers and clinicians have now a useful tool and a functional assay to assess semen quality. ProAKAP4 concentrations can be easily measured with sandwich ELISA assays called the 4MID® Kits. These functional assays give a number that allow to qualify proAKAP4 directly in the ejaculated spermatozoa. Being correlated to sperm motility, and therefore to living spermatozoa, proAKAP4 could be useful at terms to assess semen functionality before selecting the more appropriate artificial insemination settings.

In conclusion, proAKAP4 represent today a pertinent new molecular sperm parameter that could be measured routinely, to evaluate the quality of spermatogenesis and consequently the sperm quality. Further clinical investigations should then be performed to evaluate the proAKAP4 variations in anti oxidative therapeutic approaches of male infertility and more generally to evaluate any therapeutic compounds that may impair, preserve or restore spermatogenesis as well as sperm functionality.

Acknowledgments

The authors are thankful to Dr. Mickael Perrais and Pr. Claude Alain Maurage (University of Lille) for their advices and critical reading of the manuscript; Paul Moreau and Ilaria Federici for graphism and figure designs, the Region Hauts–de–France and the BPI Innovation for their help and financial supports.

Conflicts of interest

MD and NS are co–founders of the company SPQI (Lille, France).

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