Universidade de Aveiro Departamento de Ciências Médicas
2018

João Cláudio Fonseca Nunes

Síntese proteica em espermatozoides de mamífero

Protein translation in mammalian spermatozoa

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Mestrado Biomedicina Molecular, realizada sob a orientação científica da Professora Auxiliar com Agregação Margarida Fardilha do Departamento de Ciências Médicas, co-orientação do Professor Rui Vitorino do Departamento de Ciências Médicas da Universidade de Aveiro e co-orientação da Doutora Joana Vieira Silva, Investigadora de Pós-Doutoramento do Instituto de Biomedicina da Universidade de Aveiro; do Instituto de Investigação e Inovação em Saúde (I3S) da Universidade do Porto; e da Unidade para a Investigação Multidisciplinar em Biomedicina (UMIB) da Universidade do Porto.

Este trabalho é financiado por Fundos FEDER através do Programa Operacional Fatores de Competitividade -COMPETE e por Fundos Nacionais através da FCT – Fundação para a Ciência e a Tecnologia no âmbito do projeto «PTDC/BBB-BQB/3804/2014»; e do Instituto de Biomedicina -iBiMED «UID/BIM/04501/2013».

Dedico esta tese aos meus pais.

o júri

presidente

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agradecimentos

Agradeço à minha mãe e ao meu pai por apoiarem sempre todas as minhas decisões.
À professora Margarida e a toda a equipa do Signal Transduction/Fardilha's Lab, em especial à Joana e à Santiago por toda a orientação.
À professora Catarina e à Carolina por todas as dicas relativas à citometria de fluxo.
Ao Manuel pela ajuda com as imagens.
A todos os meus amigos e família por estarem sempre presentes.

palavras-chave tradução, espermatozoides, inibição da tradução, *surface sensing of translation*

resumo

A fosforilação proteica reversível é o principal mecanismo que regula as funções vitais dos espermatozoides, uma vez que, os espermatozoides são altamente compartimentados e praticamente desprovidos de transcrição e tradução. No entanto, existe uma pequena área de cromatina descondensada, na qual a transcrição e tradução ainda são possíveis. Existe apenas um estudo na literatura a demonstrar que ocorre síntese proteica nos espermatozoides durante a capacitação.

O principal objetivo deste trabalho foi avaliar se a síntese proteica ocorre em espermatozoides de bovino e de humano. Deste modo, monitorizámos a síntese proteica, utilizando a técnica *Surface Sensing of Translation* (SUnSET), seguida de citometria de fluxo. Os resultados revelaram tradução de mRNA nos espermatozoides. Para além disso, avaliamos o impacto da inibição da tradução pelos ribossomas mitocondriais e citoplasmáticos nos níveis de expressão de diversas proteínas do espermatozoide. Os resultados demonstraram que, enquanto algumas proteinas são afetadas pelos inibidores da tradução, outras mantêm-se estáveis.

Concluindo, os nossos resultados suportam que ocorre síntese proteica nos espermatozoides e demonstram, pela primeira vez, que a técnica SUnSET permite monitorizar e quantificar a síntese global de proteínas em espermatozoides de mamíferos.

keywords translation, spermatozoa, translation inhibition, surface sensing of translation

abstract

Reversible protein phosphorylation is the key general mechanism for regulating vital sperm cells functions since sperm cells are highly compartmentalized and almost devoid of transcription and translation. However, there is a small area of uncondensed chromatin where transcription and translation are still possible. There is only one study in the literature showing protein synthesis occurs in spermatozoa during capacitation.

The main goal of this work was to study if protein translation occurs, both in human and bovine spermatozoa. In order to achieve that, we monitored protein synthesis using the *Surface Sensing of Translation* (SUnSET) technique, followed by flow cytometry. The results revealed mRNA translation in spermatozoa. Furthermore, we analysed the impact of translation inhibition by mitochondrial and cytoplasmic ribosomes in the expression levels of diverse spermatozoa proteins. The results show that, while some proteins are affected by translation inhibitors, others remain stable.

To conclude, our results support protein synthesis occurs in spermatozoa and show, for the first time, SUnSET technique allows to monitor and quantify the global protein synthesis in mammalian spermatozoa.

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List of Abbreviations, symbols and acronyms

AKAP	A-kinase anchor protein
ALCs	Adult Leydig Cells
AR	Acrosome reaction
AT1-R	Angiotensin II Type I Receptor
CaMKIV	Ca2+/calmodulin- dependent protein kinase IV
CH	Cycloheximide
D-CP	D-Chloramphenicol
FLCs	Fetal Leydig Cells
FSC	Forward Scatter
FSH	Follicle Stimulating Hormone
GSK-3	Glycogen synthase kinase 3
hCG	Placental human Chorionic Gonadotropin
HSP27	Heat shock protein 27
iBiMED	Institute for Biomedicine
IMM	Inner Mitochondrial Membrane
LH	Luteinizing Hormone
MFI	Median fluorescence intensity
miRNAs	microRNAs
mitoribosomes	mitochondrial ribosomes
mRNA	messenger RNA
MS	Mitochondrial Sheath
mtDNA	mitochondrial DNA
NC	Negative control
ODFs	Outer Dense Fibers
OMM	Outer Mitochondrial Membrane
OXPHOS	Oxidative Phosphorylation
p70 S6 kα	p70 S6 kinase α
PBS	Phosphate-buffered saline

PC	Positive control
PKA-Cs	Protein Kinase A Catalytic Subunit
PKC	Protein Kinase C
PPP1CC2	Phosphoprotein phosphatase 1 subunit gamma 2
PR	Progesterone Receptor
puro	Puromycin
RT	Room Temperature
SAGE	Serial analysis of gene expression
SGEs	Selfish Genetic Elements
SSC	Side Scatter
SUnSET	Surface sensing of translation

1. Introduction

1.1. Male Reproductive System

The male reproductive system is involved in spermatogenesis and steroidogenesis, production of spermatozoa and male sex hormones, respectively; in addition to the delivery of sperm to the female reproductive tract. This system is composed by two testes; a system of genital ducts formed by the epididymis, vas deferens and ejaculatory duct; accessory sex glands as the seminal vesicles, prostate, and bulbourethral glands; and the penis [1].

1.2. Testis

Testis are oval shaped reproductive organs, located inside detached compartments within the scrotum, whose main roles are testosterone secretion, along with sperm production [1–3]. The testicular parenchyma consists in highly-coiled seminiferous tubules, the testis' functional unit , where spermatogenesis occurs [1,4]; surrounded by interstitial tissue, made of Leydig cells, responsible for testosterone secretion [1].

The seminiferous epithelium is formed by a stratified layer of developing male germ cells (spermatogonia, spermatocytes and spermatids) and Sertoli cells, its only somatic cells, which keep germ cells nourished and organized [2].

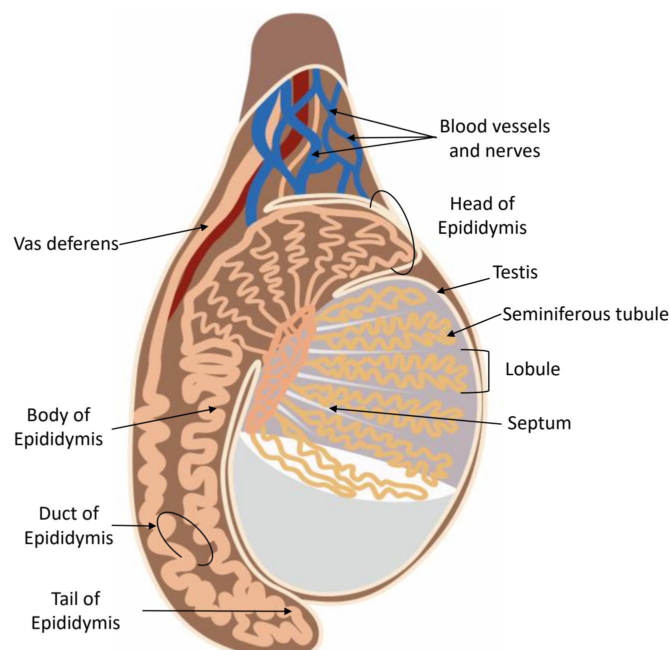


Figure 1- Testis and epididymis structure. The testicular parenchyma contains highly-coiled seminiferous tubules. The accessory ducts shown are the epididymis and vas deferens. Adapted from [5,6].

1.2.1. Sertoli Cells

Sertoli cells are heavily involved in testis' development and maturation [7]. At the onset of puberty, these cells cease mitosis and their number is indispensable in regulation of spermatogenesis and testis size [8,9].

Sertoli cells are nurse-like somatic cells, essential for spermatogenesis, since they extend from the base to the lumen of the seminiferous tubule, allowing them to support and nurture germ cells survival and maturation, as they pass through the seminiferous epithelium. These cells secrete glycoproteins and other important biomolecules such as transport or bioprotective proteins as transferrin; protease inhibitors, vital in tissue remodeling processes during spermiation; glycoproteins that form the basement membrane between the Sertoli cells and the peritubular cells; a class of regulatory glycoproteins that function as growth factors or paracrine factors; bioactive peptides, nutrients or metabolic intermediates [4,8,10].

Furthermore, Sertoli cells aid spermatozoa detachment at spermiation and establish the blood–testis barrier, a physical barrier that isolates meiotic and post-meiotic germ cells from immune and lymphatic systems [4].

1.2.2. Leydig Cells

In humans, Leydig cells are split in two distinctive populations: fetal (FLCs) and adult Leydig cells (ALCs) [11]. FLCs develop under the stimulation of placental human chorionic gonadotropin (hCG), responsible for testosterone production and testis descent [11,12]. ALCs derive from Leydig stem cells and produce testosterone, in the presence of Luteinizing hormone (LH), which starts at puberty, fundamental in the establishment and maintenance of secondary sex characteristics and spermatogenesis continuity [10,13].

These cells exist in the interstitial space, adjacent to blood vessels. Its main function is testosterone production, which diffuses into the seminiferous tubules [10].

1.2.3. Spermatogenesis

Spermatogenesis is the complex process of spermatozoa production, that occurs in the seminiferous tubules. It starts in puberty and lasts the rest of the male's lifetime [1,2,10,14,15]. Spermatogenesis is essential to preserve the number of chromosomes in the offspring, as it is responsible for the cell division of diploid spermatogonia into haploid gametes.

It requires hormones as follicle stimulating hormone (FSH), LH and testosterone, essential to maintain spermatogenesis. Furthermore, Sertoli cells, which modify rates of spermatozoal production and produce key factors to gamete development [1,4,10].

The entire process of spermatogenesis can be divided into three phases [1]. The first phase, spermatocytogenesis is the process that starts with spermatogonium and finishes with spermatids. Firstly, diploid spermatogonia divides by mitoses, producing primary spermatocytes and other spermatogonia. After puberty, primary spermatocytes through meiosis originate haploid secondary spermatocytes, that later suffer meiosis, dividing into haploid spermatids [1].

The second phase, spermiogenesis is the maturation of spermatids into haploid spermatozoa, regulated by testosterone that helps remove the residual bodies (excess organelles and cytoplasm) via phagocytosis, by adjacent Sertoli cells. The spermatozoon maturation involves the development of the axoneme, flagellum formation, nuclear maturation that includes condensation and packaging of DNA, initially with basic proteins, afterwards with protamines. The resultant chromatin is then enveloped by a Golgi apparatus creating the acrosome [1,15].

The third phase, spermiation refers to the release of mature spermatozoa from the protective Sertoli cells into the lumen of the seminiferous tubule, prior to their transition into the epididymis. It can last several days to complete. The mature motile spermatozoa is still unable to penetrate the oocyte [1,15].

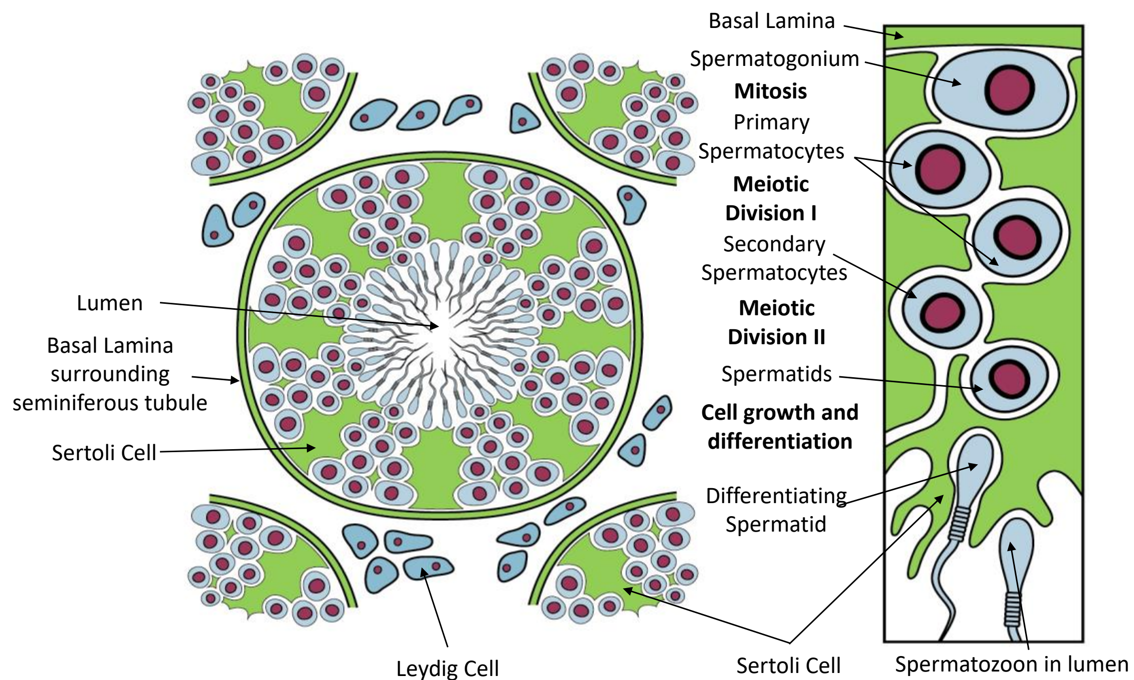


Figure 2- Spermatogenesis. The testicular parenchyma contains highly-coiled seminiferous tubules; surrounded by interstitial tissue, made of Leydig cells. The seminiferous epithelium is formed by a stratified layer of developing male germ cells (spermatogonia, spermatocytes and spermatids) and Sertoli cells. Adapted from [16,17].

1.3. Spermatozoa

Sperm is a small, condensed cell, extremely specialized in fertilizing an egg [16]. The mammalian spermatozoa is composed by a head and a flagellum [18], surrounded by the plasma membrane, and has a specie-specific length [19]. These cells are absent of cytoplasmic organelles such as endoplasmic reticulum and Golgi apparatus, which are unnecessary for DNA delivering to the egg [16]. However, it contains mitochondrial and cytoplasmic ribosomes [20].

1.3.1. Head

The sperm head contains the acrosome, a condensed nucleus and a compartmentalized perinuclear cytoskeleton [18].

The acrosome is a cap-like form, Golgi-derived, highly diverse structure that covers the first two thirds of the sperm head [19,21]. It contains several hydrolytic enzymes that are key for the acrosome reaction (AR), which enables spermatozoa to penetrate the zona pellucida

and fuse with the oocyte membrane [19,21,22].

The sperm nucleus contains highly condensed chromatin with the genetic information [19], involved by a redundant nuclear envelope and protected by the perinuclear theca, a condensed cytosolic protein layer [23,24].

During the last phases of spermatogenesis, a structural chromatin change occurs. It is characterized by a partial replacement of histones for protamines, the main nuclear proteins of mature spermatozoa, increasing nuclear DNA condensation. Therefore, sperm's genome remains inactive until it is deposited inside an egg, becoming reactivated [25,26]. However, in the neck region of spermatozoa, a short linking segment between the sperm head and the flagellum, there is a small area of uncondensed chromatin where transcription, translation and protein-biosynthesis are still possible [19,27,28].

1.3.2. Flagellum

The flagellum, the longest sperm's fraction [15,19], consists in connecting piece, midpiece, principal piece, and end piece [19,29].

The connecting piece is the small linkage, between the sperm head and the midpiece of the flagellum, which looks vital in stabilizing the proximal part of the sperm tail [29].

The axoneme is a 9+2 (nine doublet microtubules surrounding a central pair) structure, required for male fertility and sperm motility. This structure extends all throughout flagellum's length. The nine doublet microtubules are linked around the axoneme by nexin links [30–32]. Inner and outer dynein arms, indispensable for flagellum movement, project from each of the nine outer doublets [31,32]. Furthermore, nine radial spokes project inwards towards the central pair [30,31].

The midpiece is characterized by a mitochondrial sheath (MS) that envelops nine morphologically different outer dense fibers (ODFs). Each ODFs is linked to its own axonemal doublet microtubule [31]. Mitochondria is exclusive to the midpiece and produces ATP, via oxidative phosphorylation (OXPHOS) [33,34]. It is split in four defined inter-connected compartments: the outer mitochondrial membrane (OMM), the inner mitochondrial membrane (IMM), the intermembrane space and the mitochondrial matrix [33]. Mature mammalian sperm include a small number of mitochondria, between 22 to 75, which form

a tight helix around the flagellar basis of the midpiece, strategically placed in order to provide the ATP necessary for flagellar propulsion [16,35–37]. Mammalian mitochondrial ribosomes (mitoribosomes) have a 55 sedimentation coefficient (55S), which splits into a 28S small subunit, which contains 12S rRNA; and a 39S large subunit, which has 16S rRNA [38]. In eukaryotic cells, mitochondria-type ribosomes are present outside mitochondria [39]. A unique mitochondria feature is its own circular genome, mitochondrial DNA (mtDNA) and specific ribosomes, therefore enabling local protein synthesis [33,40]. Each mitochondria has one copy of mtDNA [38,41].

Furthermore, sperm mitochondria possess numerous exclusive proteins or protein isoforms [34]. Even though, mtDNA simply codes for 13 polypeptides, producing five proteins overall, tens of proteins are synthesized during sperm capacitation [38].

The principal piece , the longest flagellum's fraction, is constituted by fibrous sheath (FS) ribs which enclose the dense fibers, along with two longitudinal FS columns that replace two ODFs [31,42].

The endpiece only includes the axoneme, surrounded by the plasma membrane [31,34,43].

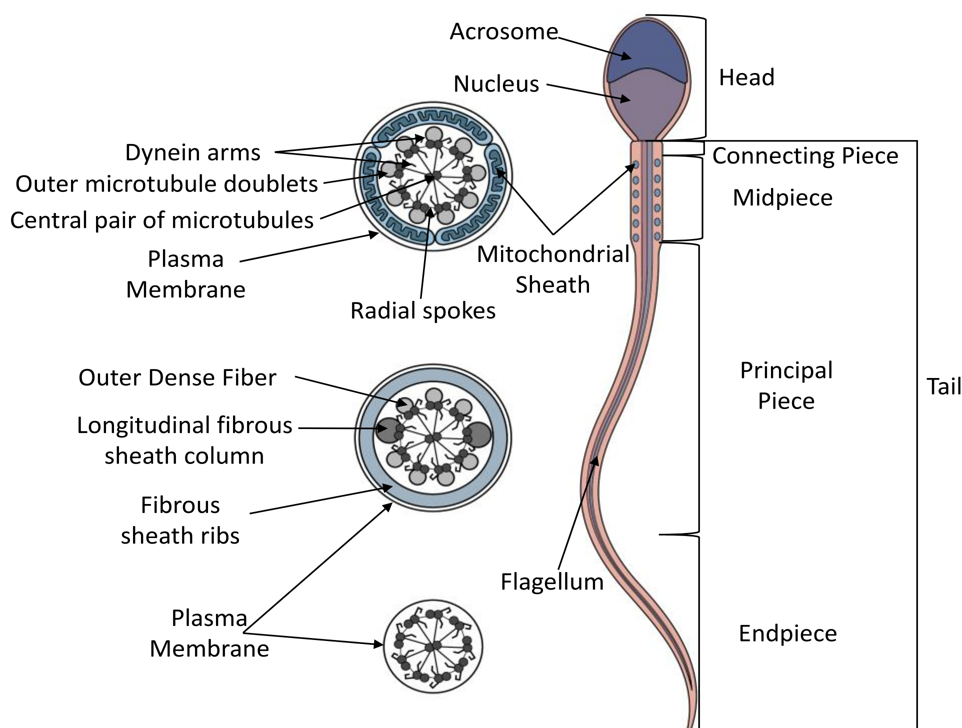


Figure 3- Spermatozoon structure and flagellum ultrastructure. The flagellum consists in connecting piece, midpiece, principal piece, and end piece. The connecting piece is the smallest flagellum's fraction. The midpiece is characterized by a MS that envelops nine morphologically different ODFs and the axoneme. The principal piece is constituted by FS ribs which enclose the dense fibers, along with 2 longitudinal FS columns that replace two ODFs. The endpiece includes the axoneme, enclosed by the plasma membrane. Adapted from [5,17].

1.4. Protein translation in spermatozoa

Reversible protein phosphorylation is the key general mechanism for regulating vital sperm cells functions since sperm cells are highly compartmentalized and almost devoid of transcription and translation [44]. However, in the neck region of spermatozoa, there is a small area of uncondensed chromatin where transcription, translation and protein-biosynthesis are still possible [19,27,28].

A new insight was given by Gur & Breitbart, which proved protein synthesis occurs in spermatozoa during capacitation, which is the sequence of biochemical and physiological changes, that occur in the female reproductive tract prior to the AR [20,38,45].

They concluded sperm's translation is time-dependent, capacitation-dependent and sensitive to mitochondrial translation inhibitors and insensitive to cytoplasmic translation inhibitor via inhibition of the incorporation of labeled [35S] amino acids into polypeptides during sperm capacitation [20,38].

Furthermore, as expected by sperm's morphology, BODIPY-lysine-tRNALys incorporation happens, apparently, in the sperm midpiece [20,38]. In capacitating conditions, labeled amino acids incorporation started almost immediately, refuting previous thoughts [20,38,46]. In fact, Gur & Breitbart believe, this newly made proteins, are indispensable to start and even continue sperm capacitation.

Gur & Breitbart proposed mitoribosomes are responsible for sperm translation (Gur & Breitbart, 2006, 2008). Additionally, during capacitation, sperm-specific, nuclear-encoded, proteins were sensitive to the mitochondrial ribosome inhibitor [20,38].

Inhibition of protein translation greatly reduced sperm functions, essential to fertilization such as motility, actin polymerization, and the AR [20].

Translation was unaffected by transcription inhibitor, along with incorporation of labeled amino acids proving that the source for protein translation is long lasting mRNA [20,38].

In conclusion, since protein production in spermatozoa, revealed to be sensitive to several mitochondrial mRNA translation inhibitors, while insensitive to the cytoplasmic 80S ribosomal inhibitor, mitochondrial machinery is the responsible for sperm translation [20,38]. Additionally, spermatozoa protein synthesis is key in the last maturation phase leading to successful fertilisation [20].

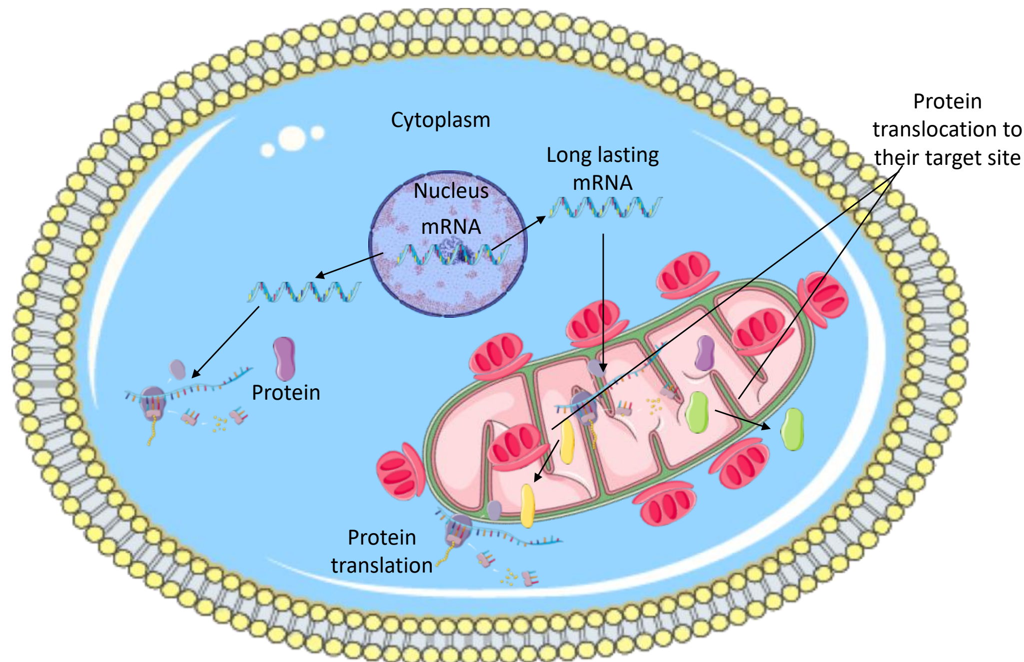


Figure 4- The sperm protein translation model proposed by Gur & Breitbart. It suggests nuclear-encoded long lasting mRNA are translated by mitochondrial-type ribosomes localised both in the cytoplasm, outside and inside the mitochondria. The translated proteins are then translocated to their active site [38]. Adapted from [38].

1.4.1. Sperm RNA potential functions

Although, it was originally believed sperm RNAs were merely spermatogenesis artefacts [28]. Recent findings suggest otherwise, Hosken & Hodgson propose sperm RNA could have four potential functions such as relatedness markers, helping sperm cooperation; paternally established suppressors of haploid interests; nuptial gift, delivering the female with resources that attract her to fertilise; Trojan horse, given by males to manipulate female reproduction [47].

They propose sperm RNA could act as relatedness markers that help sperm cooperation. Sperm cooperation, in other words, means mutual interaction between sperm of one male

to increase a male's fertilization success[48].

The changes with morphology and motility in sperm microRNAs (miRNAs), whose role remains undetermined, indicate a critical biological function. Possibly they are remnants of spermatogenesis, stored for a later role in fertilization, or are delivered to the oocyte to guide early embryonic development [49].

They also suggest sperm hypothetically could have some control over their own behavior [47]. Recent observations suggest that some transcription occurs in postmeiosis. For instance, in Drosophila melanogaster, it has been identified direct evidence for de novo transcription of RNA during the postmeiotic phases. Reinforcing the emerging notion that postmeiotic transcription is dynamic and integral to the overall process of spermatogenesis [50]. Even though, it is controversial and possibly reveals translation of male transcripts packed into the gametes [51].

It was also proposed that sperm acts as nuptial gifts, a method of male reproductive investment provided to females in return for mating [52]. They are found across a large number of taxa, and males have been found to tailor these gifts in relation to the likelihood of siring success [52]. However, females can accept gifts and use them in ways that do not benefit the gift provider [52].

Furthermore, sperm could also act as Trojan horses delivering manipulative RNAs to influence female reproduction [47] since there are a lot of male-derived ejaculatory substances that alter female reproduction [53].

These two last suggestions are the least likely, because the cost of packaging gifts into each spermatozoon will be wasted if only a few sperm fertilize ova [47].

Additionally, Holman & Price suggested sperm RNA could intermediate an anticipatory paternal effect that encodes environmental information [54]. Males possibly adjust the RNA content of their sperm under different conditions to prime the embryo to develop appropriately for the environment [55].

Additionally, they proposed sperm RNA could originate from selfish genetic elements (SGEs), which promote their own transmission in ways that damage the fitness of the rest of the genome [56], or they probably represent a defense against SGEs [55].

Lastly, they suggest sperm RNA may also trigger a male equivalent of Maternal-effect

dominant embryonic arrest (Medea) in *Tribolium* beetles [55]. Medea is a maternal offspring killer that poisons all offspring, excluding those that inherit it and, therefore, have the antidote [56].

All this lead us to think RNA sperm could be more important than originally believe (Table 1).

Table 1- Sperm RNA potential functions and matching descriptions [47,55].

Sperm RNA potential functions	Description

Relatedness markers	• Help sperm evolution and maintenance of cooperation
	• Results benefit group members
	• Via:
	  ○ direct recognition
	  ○ behavioral algorithm

Paternally imposed suppressors of haploid interests	• Suppress the selfish interests of the haploid cell • Keep sperm under paternal control
Nuptial gift	• Deliver resources to the female that attract her to fertilise
Trojan horse	• Manipulation of the female fertilisation machinery • Benefits the paternal haplotype
Anticipatory paternal effect	• Sperm RNA content varies under different conditions • Prime the embryo to develop properly for the environment
SGEs	• Promote their own transmission • Harm the fitness of the rest of the genome [56]
Defense against SGEs	• Represent a protection against SGEs
Male equivalent of Medea in *Tribolium* beetles [55]	• Medea is a maternal offspring killer • Poisons all offspring, excluding those that inherit it ○ have the antidote [56]

1.5. Aims

The main aim of this study was to evaluate protein synthesis in mammalian spermatozoa. In order to achieve that, we stablished a series of steps:

- Monitoring and quantifying the global protein synthesis in human and bovine spermatozoa using Surface Sensing of Translation (SUnSET):
  - Preliminary assay in bovine and human spermatozoa
  - Positive control establishment
  - Protocol optimization in bovine spermatozoa
  - Flow Cytometry analysis
- Assessing the expression levels of signalling proteins after treatment with translation inhibitors:
  - Bovine spermatozoa incubation, under capacitation conditions, with or without translation inhibitors (D-chloramphenicol or cycloheximide)
  - Immunoblots with antibodies for GSK-3∝/β, PPP1CC2, CaMKIV and HSP27

2. Material and methods

Experimental procedures were performed in Signal Transduction Laboratory, Institute for Research in Biomedicine (iBiMED), University of Aveiro (Aveiro, Portugal). The details of the solutions used in this thesis are stated in the Supplementary Table 1.

2.1. Sperm Sample Processing

This study was approved by the Ethics and Internal Review Board of the Hospital Infante D. Pedro E.P.E. (Aveiro, Portugal) and was conducted in accordance with the ethical standard of the Helsinki Declaration. Ejaculated human semen samples from volunteer donors were collected by masturbation into a sterile container. All donor signed an informed consent allowing the use of the samples for scientific proposes. Basic semen analysis was conducted in accordance with World Health Organization (WHO) guidelines and only normal sperm samples were used. Briefly, after complete liquefaction of the semen samples at 37ºC, during approximately 30 minutes, a macroscopic examination was performed. The microscopic examination included the analysis of spermatozoa motility, concentration and morphology. All microscopy analyses were performed using a Zeiss Primo Star microscope (Carl Zeiss AG, Germany). The results of basic sperm analysis were detailed in Supplementary Table 2.

Frozen semen from bulls was obtained from LusoGenes, LDA (Aveiro, Portugal). Bovine semen was thawed in a 37ºC water bath for 1 minute.

Human and bovine spermatozoa were isolated and washed three times from seminal plasma by centrifugation (600g for 10 minutes at room temperature (RT)) using 1x Phosphate Buffered Saline (PBS). Pellet was re-suspended in Sperm Preparation Medium (SPM) (Origio, Denmark) to a final concentration desired and incubated at 37ºC with 5% CO_2 until the appropriated treatments were added. The concentration of sperm cells after the washing procedures was assessed using the Sperm Class Analyzer CASA System (Microptic S L, Barcelona, Spain) with SCA® v5.4 software. Samples and controls (2 µl) were loaded into individual chambers of Leja Standard Count 8 chamber slide 20 µm depth (Leja Products B. V., The Netherlands) which were pre-heated at 37ºC.

2.2. Cell Culture

CAL-1 and PNT2 cells were maintained in RPMI 1640 (GIBCO BRL Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum (FCS, HyClone, PERBIO, Aalst, Belgium) and 1% penincillin/streptomycin mixture, maintained in a humidified atmosphere at 37°C and 5% CO2.

CAL-1 (suspension) and PNT2 (adherent) cells were teased apart and washed by repeating pipetting in PBS, additionally PNT2 were harvested with trypsin (500 µl).

2.3. Surface Sensing of Translation (SUnSET)

2.3.1. Preliminary assay in bovine and human spermatozoa

Bovine spermatozoa (5 x 10^6 cells or 20 x 10^6 cells) were incubated with puromycin (1 µg/mL for 15 minutes or 10 µg/mL for 1 h) in a humidified atmosphere at 37°C and 5% CO_2.

Human spermatozoa (20 x 10^6 cells) were incubated with 10 µg/mL of puromycin for 1 h in a humidified atmosphere at 37°C and 5% CO_2.

Negative controls were performed in the absence of puromycin.

The sperm cells were centrifuged 5 minutes at 500g and the supernatant was discarded. Cells were washed with PBS (5 minutes, 500g) and subsequently treated for Flow Cytometry analysis (see section 2.4.).

2.3.2. Positive control establishment

CAL-1 (suspension) and PNT2 (adherent) cells were used since translation is highly described in these cell types and they would, certainly, incorporate puromycin. 1 x 10^6 of each cell type was incubated for 15 minutes with 1 µg/mL of puromycin in a humidified atmosphere at 37°C and 5% CO_2.

For CAL-1 cells, the medium containing the cells was resuspended, transferred to a falcon and centrifuged at 1000 rpm for 3 minutes.

For PNT2 cells, the medium was removed, and cells were washed with 2 mL of PBS. 500 µl of trypsin was added and incubated for 2 minutes in a humidified atmosphere at 37°C and 5% CO_2. Cells were transferred to a falcon and centrifuged at 1000 rpm for 3 minutes.

In both cells types, the supernatant was removed after centrifugation and the cells were subsequently used for Flow Cytometry analysis (see section 2.4.).

2.3.3. Protocol optimization in bovine spermatozoa

After the preliminary assays, a wide range of puromycin concentrations and incubation times were tested.

Bovine sperm cells (20 x 10^6 cells) without treatment (negative control) or treated with five different puromycin concentrations (1; 5; 7,5; 10; 15 µg/mL) incubated for 15 or 30 minutes at 37°C and 5% CO_2.

A control condition was added: 20 x 10^6 cells of bovine sperm cells were incubated with 7,5 µg /mL of puromycin and 100 µg /mL of D-CP, a specific inhibitor of mitochondrial translation [57].

PNT2 cells were treated in parallel in 3 conditions: (1) negative control (incubated with RPMI 1640 (GIBCO BRL Life Technologies, Rockville, MD) supplemented with 10% foetal calf serum (FCS, HyClone, PERBIO, Aalst, Belgium) and 1% penincillin/streptomycin mixture); (2) positive control incubated with 1 µg /mL of puromycin concentration for 15 minutes at 37°C and 5% CO_2; (3) control incubated with 1 µg /mL of puromycin and 100 µg/mL of D-CP, a specific inhibitor of mitochondrial translation [57], for 15 minutes at 37°C and 5% CO_2.

The sperm cells were centrifuged 5 minutes at 500g and the supernatant was discarded. Cells were washed with PBS (5 minutes, 500g) and subsequently treated for Flow Cytometry analysis (see section 2.4.).

2.4. Flow Cytometry analysis

Cells were fixed in 400 µl of 4% paraformaldehyde (PFA). Resuspended and incubated 15 minutes at 37°C. Cells were harvested by centrifugation at 4 °C 300 g for 6 minutes. Discarded the supernatant, and washed three times with 400 µl of cold PBS. Cells were resuspended in 50 µl of Fluorescence activated cell sorting (FACS) solution including 0,1% saponin and transferred to a 96 round bottom well plates (Thermo Fisher Scientific). The plate was centrifuged at 4ºC 400 g for 3 minutes, discarded the supernatant and added 50 µl of anti-puromycin Alexa488 (Thermo Fisher Scientific) diluted 1:100 in FACS solution

including 0,1% saponin. The negative control was incubated with only 50 µl of FACS solution including 0,1% saponin. Cells were resuspended and incubated 30 minutes on ice in the dark. Added 150 µl of FACS solution including 0,1% saponin. Resuspended and centrifuged 4ºC 300g for 3 minutes and discarded the supernatant. Washed twice with 200 µl of FACS solution including 0,1% saponin. Washed once with 200 µl of cold PBS, resuspended the pellet in 200 µl of cold PBS. Filtered the samples and carried out a FACS analysis.
Events were collected on a BD Accuri™ C6 Cytometer and the data was acquired and analysed using BD Accuri™ C6 software (BD Biosciences).

2.5. Sperm treatment with translation inhibitors

Bovine spermatozoa (20 x 10^6 cells) were incubated with D-CP, a specific inhibitor of mitochondrial translation [57] (100 µg/mL shortly (time 0) or for 4 h) or CH, a specific cytoplasmic ribosomal inhibitor [20] (1 mg/mL shortly (time 0) or for 4 h) in a humidified atmosphere at 37°C and 5% CO_2.
Negative controls were performed in the absence of D-CP, nor CH.
The sperm cells were centrifuged 5 minutes at 500g and the supernatant was discarded. Cells were washed twice with PBS (5 minutes, 500g) and subsequently lysed with 60 µl of Sodium Dodecyl Sulfate (SDS) 1X on ice for 15 minutes.
The sperm cells were centrifuged 4ºC 12000g for 10 minutes and the supernatant was kept.

2.6. Western Blotting

Sperm lysates were resolved by 10 % SDS - Polyacrylamide gel electrophoresis (PAGE) run at 200 V. Followed by electrotransference onto nitrocellulose membranes at 200 mA for 2 hours. 8,75 x 10^6 sperm cells were loaded per condition/well.
Membranes were later blocked with 5% Milk or 5% Bovine Serum Albumin (BSA) in Tris-buffered saline containing 0,1% Tween 20 (TBST) 1X at RT for 1 h. The blots were washed with TBST and incubated with the following primary antibodies: mouse anti-Glycogen synthase kinase 3 (GSK-3α/β (0011-A): sc-7291 obtained from Santa Cruz Biotechnology, Inc.), mouse anti-protein 70 S6 kinase α (p70 S6 kα (B-5): sc-393967 obtained from Santa Cruz Biotechnology, Inc.), mouse anti- heat shock protein 27 (HSP27 (F-4): sc-13132)

overnight at 4ºC; mouse anti-Protein kinase C (PKC βI (E-3): sc-8049 obtained from Santa Cruz Biotechnology, Inc.), mouse anti- Ca^{2+}/calmodulin- dependent protein kinase IV (CaMKIV (A-3): sc-166156 obtained from Santa Cruz Biotechnology, Inc.), at RT for 2h; rabbit anti- Phosphoprotein phosphatase 1 subunit gamma 2 (PPP1CC2) (homemade), at RT for 1h.

Afterwards, the blots were washed three times with TBST for 10 minutes, followed by incubation with the proper secondary antibody (anti-mouse) for 1 hour at RT (IRDye®800CW anti-mouse (926-32210) secondary antibody (1:5000) obtained from LI-COR Biosciences (Lincon, NE, USA)).

Blots were washed three times for 10 min with TBST and once with TBS and immunodetected using the Odyssey Infrared Imaging System (LI-COR® Biosciences, US).

Table 2- Primary antibodies used for Immunoblotting

Antibody	**Host**	**Dilution**	**Molecular Weight (kDa)**	**Reference**	**Supplier**
Anti-GSK-3α/β	Mouse	1:1000	~51/47	sc-7291	Santa Cruz Biotechnology, Inc.
Anti-PKC βI	Mouse	1:800	~79	sc-8049	Santa Cruz Biotechnology, Inc.
Anti-CaMKIV	Mouse	1:800	~60	sc-166156	Santa Cruz Biotechnology, Inc.
Anti-p70 S6 kinase α	Mouse	1:1000	~70	sc-393967	Santa Cruz Biotechnology, Inc.
Anti-PPP1CC2	Rabbit	1:2000	~37	G502	Homemade
Anti-HSP 27	Mouse	1:1000	~27	sc-13132	Santa Cruz Biotechnology, Inc.

3. Results

3.1. Strategy for protein synthesis analysis in human and bovine spermatozoa

In order to explore the existence of protein synthesis in spermatozoa, a nonradioactive fluorescence-activated cell sorting–based alternative assay - Surface Sensing of Translation (SUnSET) - was used due to its useful ability of direct translation activity monitoring, in heterogenous cells populations, by flow cytometry [58].

SUnSET takes advantage of the use of puromycin (puro), an aminonucleoside antibiotic produced by *Streptomyces alboniger*, structural analog of aminoacyl tRNAs, whose incorporation into the nascent polypeptide chain prevents elongation [58,59]. Puromycin incorporation, when used in minimal quantities (< 10 µg/mL), directly echoes the rate of mRNA translation in vitro [58,60–62], detected and monitored by an anti-Puromycin staining.

Our first challenge was to establish a suitable gate for bovine and human spermatozoa. As a result of the small dimension of this type of cells (Figure 5A and C), both axis (forward scatter (FSC) and side scatter (SSC)) demanded the use of a logarithmic scale, which aid the definition of both gates (Figure 5B and D). FSC reveals the cellular size, while SSC corresponds to cellular granularity or density [63]. As demonstrated in Figure 5A and C, bovine spermatozoa samples are usually cleaner than human samples, which might contain other types of cells and cellular debris. In both populations (bovine and human) (Figure 5B and D), there is only one distinct population of cells. The gates were established where the population was denser, since the other smaller populations could represent cellular debris (Figure 5B and D).

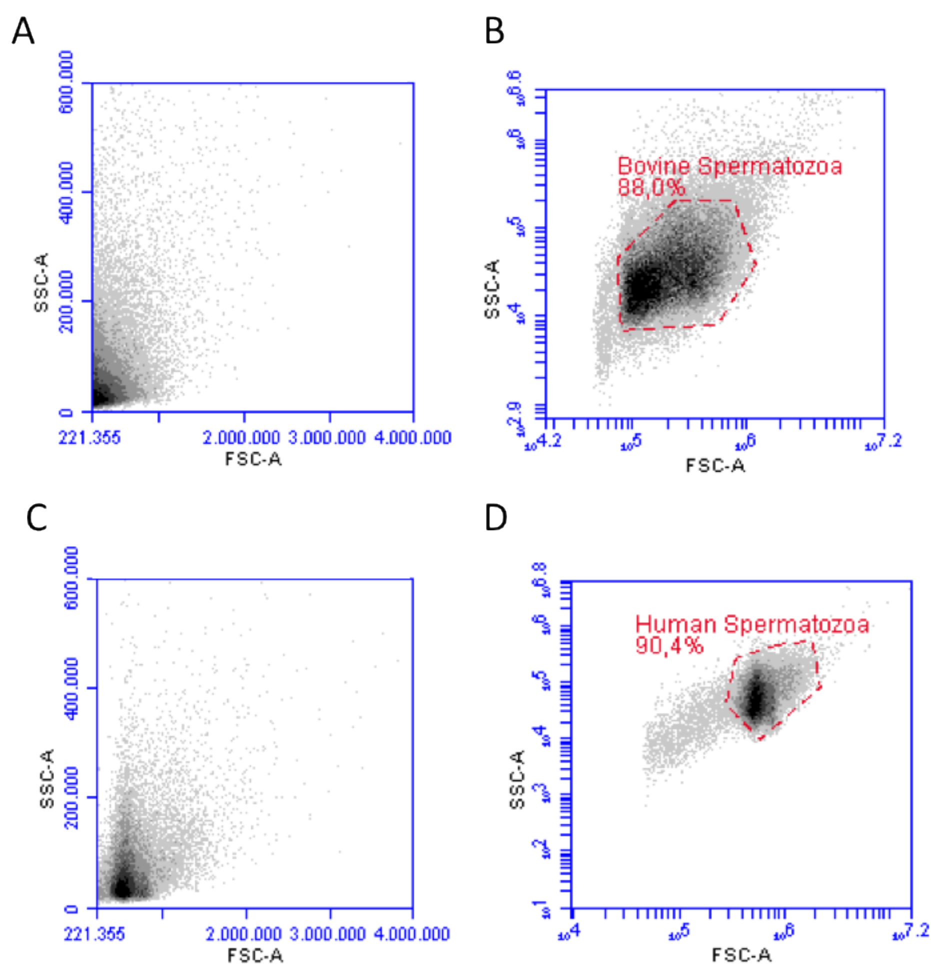


Figure 5- Bovine and Human spermatozoa density plots and gate establishment. Bovine (A) and Human (C) spermatozoa density plots (FSC vs. SSC in linear scale). Bovine and Human spermatozoa gate establishment (FSC and SSC in logarithmic scale) (B and D). FSC, forward scatter; SSC, side scatter.

3.1.1. Positive control establishment

Afterwards, two types of cell lines, suspension (CAL-1) and adherent cells (PNT2), were used to establish a positive control (PC) for this assay. Both cells lines (CAL-1 and PNT2) performed in a similar way, showing an identical shift, in the Puro-Alexa488 axis, in the overlay histograms, between the NC and puromycin 1 µg/mL condition (Figure 6A and B). Therefore, the PNT2 cell line was established as the PC, as it's regularly used by our group.

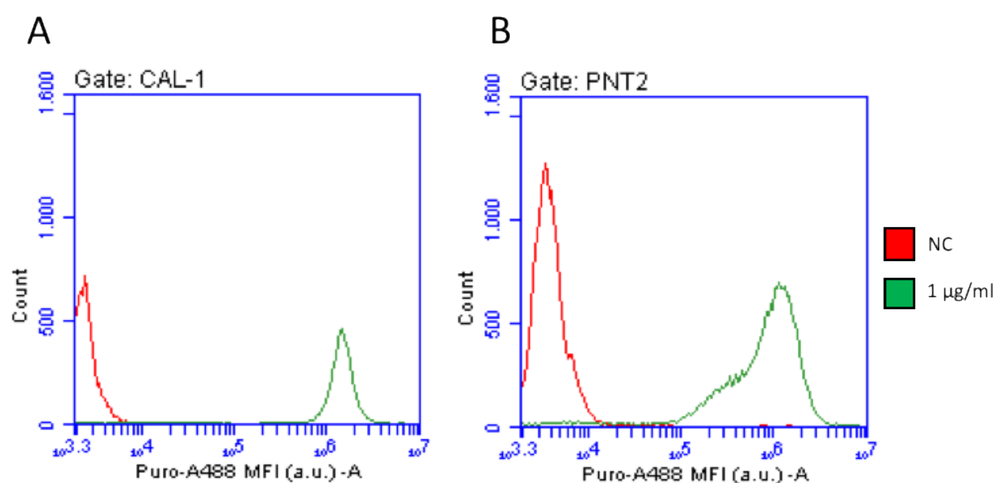


Figure 6- Overlay histograms of CAL-1 and PNT2 cells. Two cell lines (CAL-1 and PNT2 cells) were treated with puromycin at a concentration of 1 µg/mL, for 15 min. The samples were left unstained (NC) or stained for anti-puromycin and analysed by flow cytometry. The plots represent the overlay histograms of CAL-1 (A) and PNT2 cells (B) between NC and puromycin samples. Puro, puromycin; NC, negative control.

3.1.2. Protein synthesis in human spermatozoa

To exploit if protein synthesis occurs in human spermatozoa, a concentration of 10 µg/mL of puromycin during 15 min was used. The results showed a shift between the NC and puromycin 10 µg/mL condition (Figure 7B).

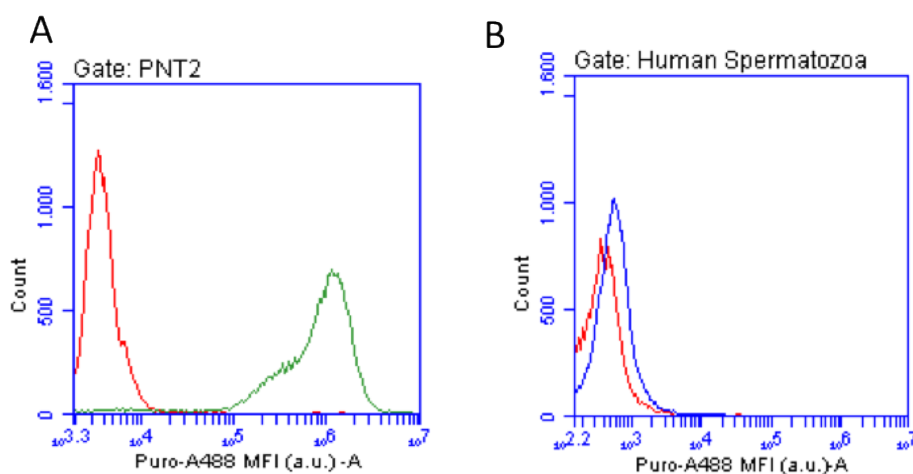


Figure 7- Overlay histograms of PNT2 cells and human spermatozoa. PNT2 cells (PC) and human spermatozoa and were treated with puromycin at a concentration of 1 µg/mL and 10 µg/mL, respectively, for 15 min. The samples were left unstained (NC) or stained for anti-puromycin and analysed by flow cytometry. The plots represent the overlay

histograms of PNT2 cells (A) and human spermatozoa (B) between NC and puromycin samples. PC, positive control; NC, negative control; puro, puromycin.

3.1.3. Protein synthesis in bovine spermatozoa

To optimize the puromycin concentration, bovine spermatozoa were treated with two different concentrations of puromycin (1 µg/mL and 10 µg/mL) (Figure 8B).

The shift in the Puro-Alexa488 axis, in the overlay histograms (Figure 8A and B) represent the increase of median fluorescence intensity (MFI) of puromycin showed in Figure 8C.

Both concentrations of puromycin (1 µg/mL and 10 µg/mL) revealed a shift, between NC and puromycin-incubated samples (Figure 8B). Nevertheless, puromycin 10 µg/mL condition showed a greater increase in MFI (Figure 8C).

As observed in Figure 8A, PNT2 cells (PC) MFI increase is much superior than bovine spermatozoa (Figure 8C).

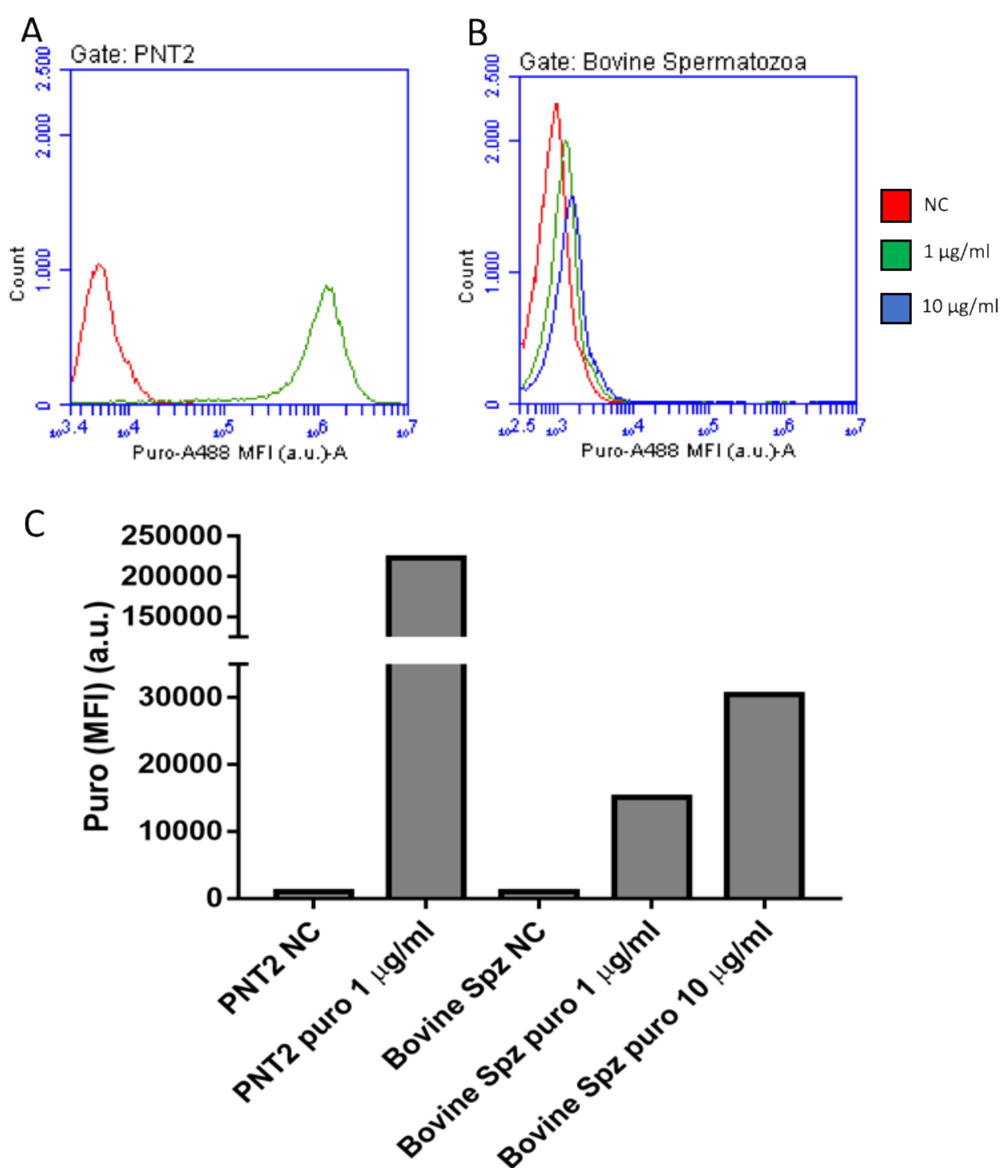


Figure 8- Overlay and Puro (MFI) histograms of PNT2 cells and bovine spermatozoa. PNT2 cells (PC) and bovine spermatozoa were treated with puromycin at a concentration of 1 µg/mL; 1 and 10 µg/mL, respectively, for 15 min. The samples were left unstained (NC) or stained for anti-puromycin and analysed by flow cytometry. The plots represent the overlay histograms of PNT2 (A) and bovine spermatozoa (B) between NC and puromycin samples, represented in the Puro (MFI) histogram (C). MFI were normalised to the NC. Spz, spermatozoa; MFI, median fluorescence intensity; PC, positive control; puro, puromycin; NC, negative control.

Subsequently, 5 different puromycin concentrations (1; 5; 7,5; 10; 15 µg/mL) were used, along with two incubation time (15 minutes or 30 minutes), in independent assays.

In both incubation times, all different puromycin concentrations (1; 5; 7,5; 10; 15 µg/mL) revealed a shift, comparing to the NC (Figure 9B and Figure 10 A).

At 15 min, the biggest MFI increase was the puromycin 7,5 µg/mL condition (Figure 9C); while in the incubation of 30 min, it was the puromycin 5 µg/mL condition (Figure 10B).

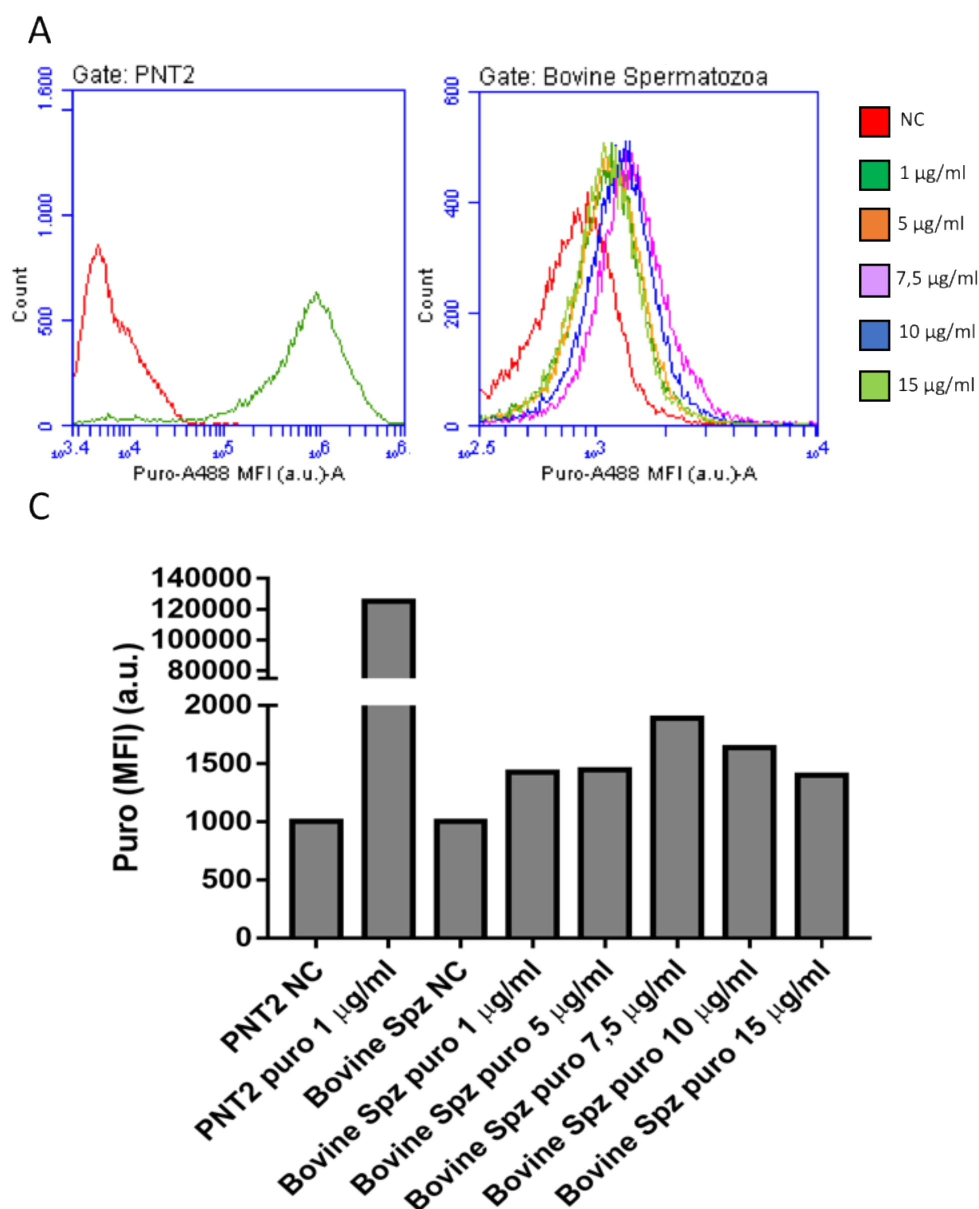


Figure 9- Overlay and Puro (MFI) histograms of PNT2 cells and bovine spermatozoa (15 min). PNT2 cells (PC) and bovine spermatozoa were treated with puromycin at a concentration of 1 µg/mL and 1; 5; 7,5; 10; 15 µg/mL, respectively, for 15 min. The samples were left unstained (NC) or stained for anti-puromycin and analysed by flow cytometry. The plots represent the overlay histograms of PNT2 (A) and bovine spermatozoa (B)

between NC and puromycin samples, represented in the Puro (MFI) histogram (C). MFI were normalised to the NC. Spz, spermatozoa; MFI, median fluorescence intensity; PC, positive control; puro, puromycin; NC, negative control.

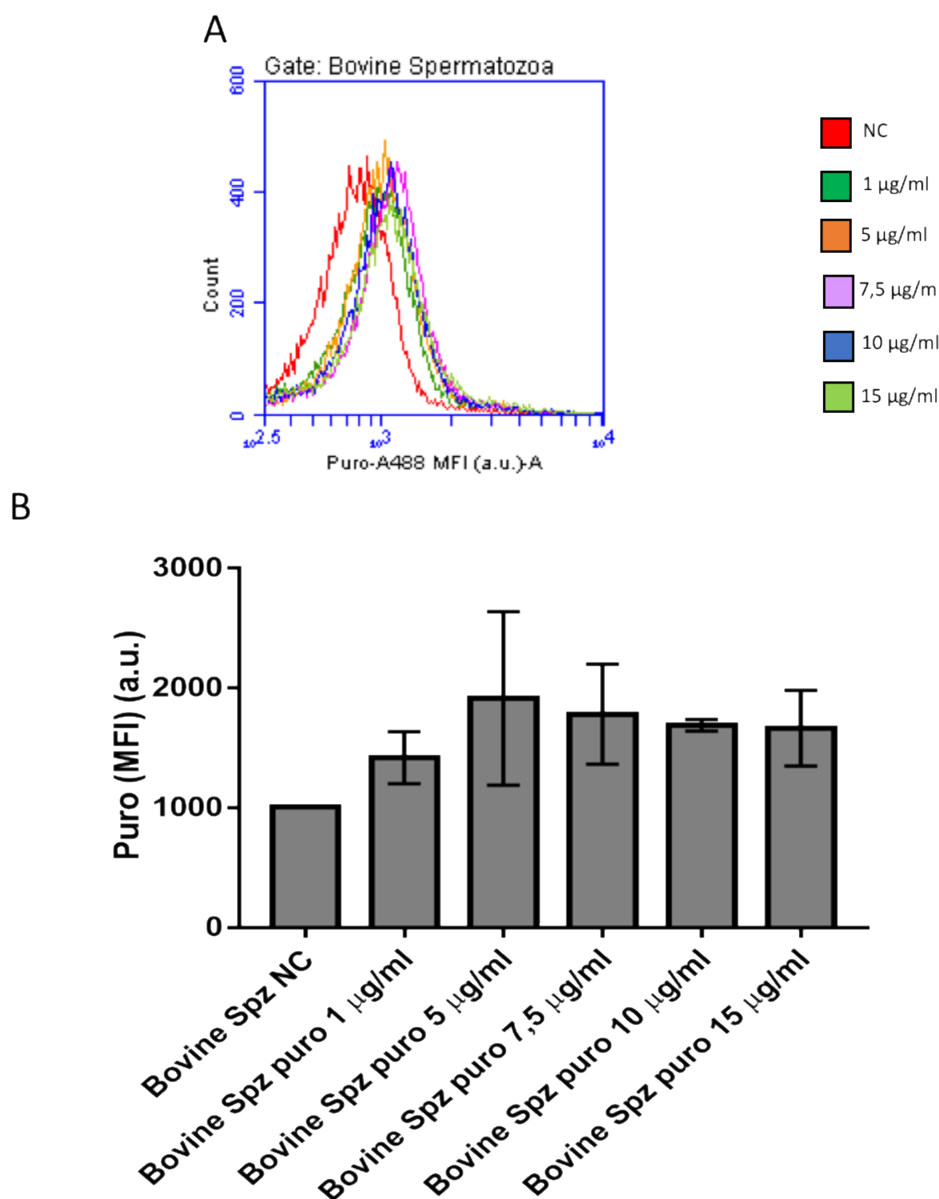


Figure 10- Overlay and Puro (MFI) histograms of bovine spermatozoa (30 min). Bovine spermatozoa were treated with puromycin at a concentration of 1; 5; 7,5; 10; 15 µg/mL for 30 min. The samples were left unstained (NC) or stained for anti-puromycin and analysed by flow cytometry. The plots represent the overlay histograms of bovine spermatozoa (A), represented in the Puro (MFI) histogram (B). The Puro (MFI) histogram (B) represents two independent experiments. MFI were normalised to the NC. Spz, spermatozoa; MFI, median fluorescence intensity; puro, puromycin; NC, negative control.

3.2. Impact of translation inhibition in bovine spermatozoa

In order to test if D-CP, a specific inhibitor of mitochondrial translation [57] , reduces PNT2 cells' translation, 2 different time points were used (15 and 30 min). In both situations, MFI was similarly reduced (Figure 11A).

Therefore, in order to test the translation inhibition in bovine spermatozoa, two incubation times (30 min and 4 h) were tested (Figure 11B). In both inhibition conditions (30 min and 4 h), a puromycin concentration of 7,5 µg/mL was used, since it previously revealed to have the biggest protein translation rate (Figure 9C). Both D-CP conditions (30 min and 4 h) showed lower MFI levels, supporting D-CP as a translation inhibitor (Figure 11D) [20]. D-CP treatment (4h) did not have a significative impact on sperm motility (Supplementary Table 3).

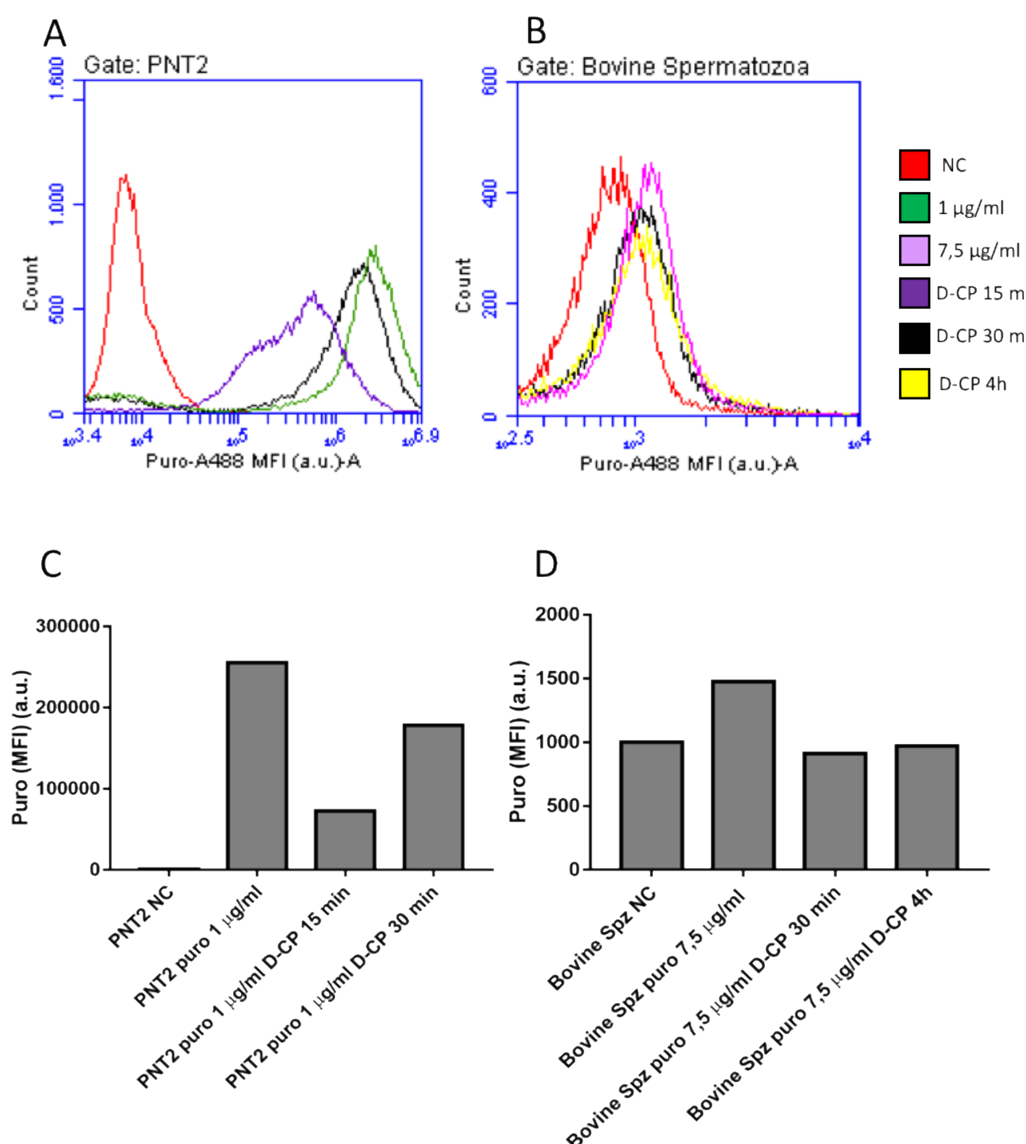


Figure 11- Overlay and Puro (MFI) histograms of PNT2 cells (D-CP 15 and 30 min) and bovine spermatozoa (D-CP 30 min and 4 h). PNT2 cells were treated with puromycin at a concentration of 1 µg/mL, and with D-CP for 15 min and 30 min (A); bovine spermatozoa were treated with 7,5 µg/mL puromycin and with or without D-chloramphenicol for 30 min or 4 h. The samples were left unstained (NC) or stained for anti-puromycin and analysed by flow cytometry. The plots represent the overlay histograms of PNT2 cells (A) and bovine spermatozoa (B), represented in the Puro (MFI) histogram (C and D). MFI were normalised to the NC; MFI levels with D-CP were normalised to the similar condition without the inhibitor. MFI, median fluorescence intensity; puro, puromycin; NC, negative control.

3.2.1. Impact of translation inhibition on the levels of several signaling proteins in bovine spermatozoa

In order to identify differentially expressed proteins after translation inhibition, bovine spermatozoa were exposed, in capacitating conditions, along with two translation inhibitors for 4h named: (i) D-CP, a specific inhibitor of mitochondrial translation [57] and (ii) CH, a specific cytoplasmic ribosomal inhibitor [20].

After exposure of bovine spermatozoa to D-CP and CH for 4h, a reduction in the levels of GSK-3α (Figure 12), PPP1CC2 (Figure 12) and HSP27 (Figure 13), was observed, compared with the negative control (NC) at 4h and the T0 conditions. Interestingly, the levels of GSK-3α, PPP1CC2 and HSP27 were lower with the translation inhibitor CH than with D-CP. These results suggested that both mitochondrial and cytoplasmic ribosomes might be involved in protein translation in bovine spermatozoa, and GSK-3α, PPP1CC2 and HSP27 could be translated during sperm capacitation.

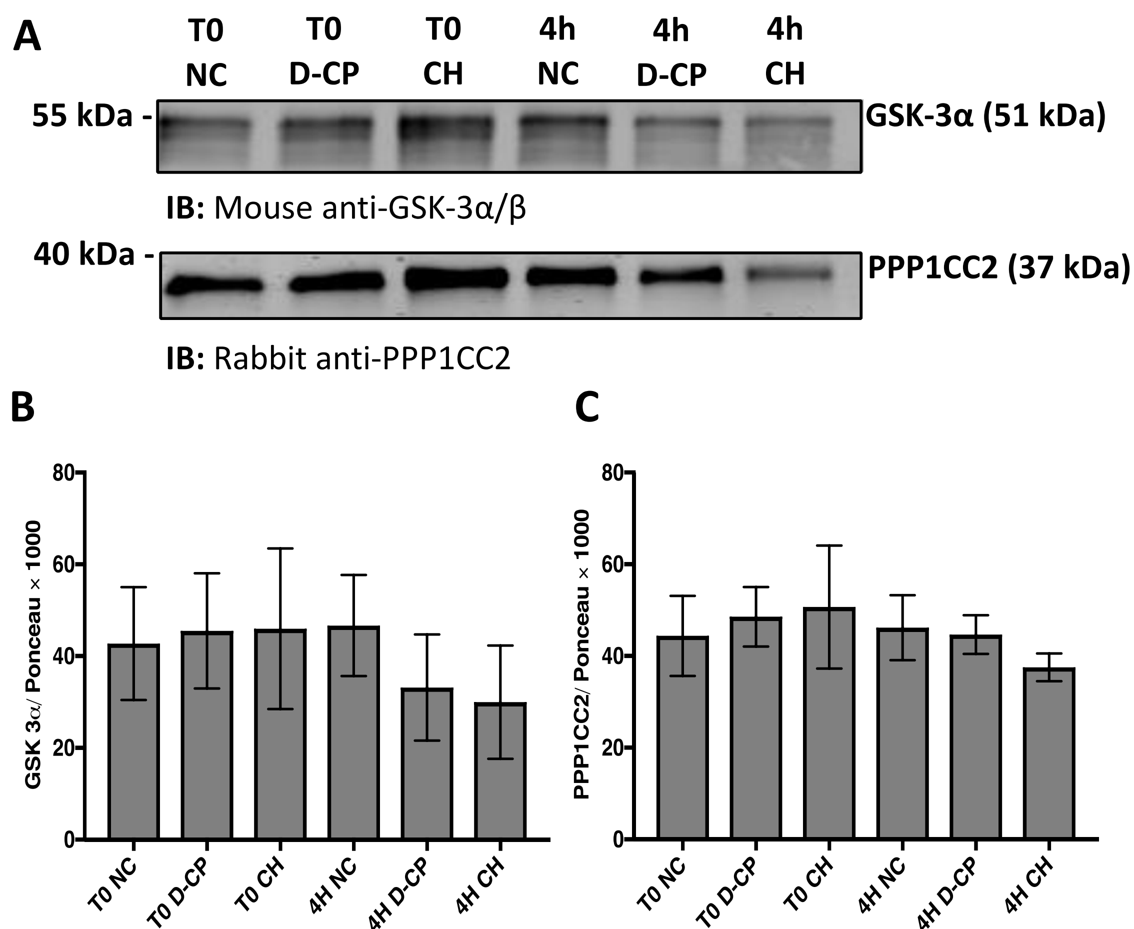


Figure 12- Immunoblot with mouse anti-GSK-3α/β and rabbit anti-PPP1CC2. Bovine sperm soluble extracts corresponding to 8,75 x 10^6 cells were loaded, followed by immunoblot with mouse anti-GSK-3α/β and rabbit anti-PPP1CC2 (A). Pixel intensity was quantified using Quantity One® Software (B and C) and Ponceau staining was used as loading control (Supplementary Figure 1A). GSK-3α graph bars represent the mean values of three independent experiments. PPP1CC2 graph bars represent the mean values of two independent experiments. NC, negative control; D-CP, D- Chloramphenicol; CH, cycloheximide; IB, immunoblot.

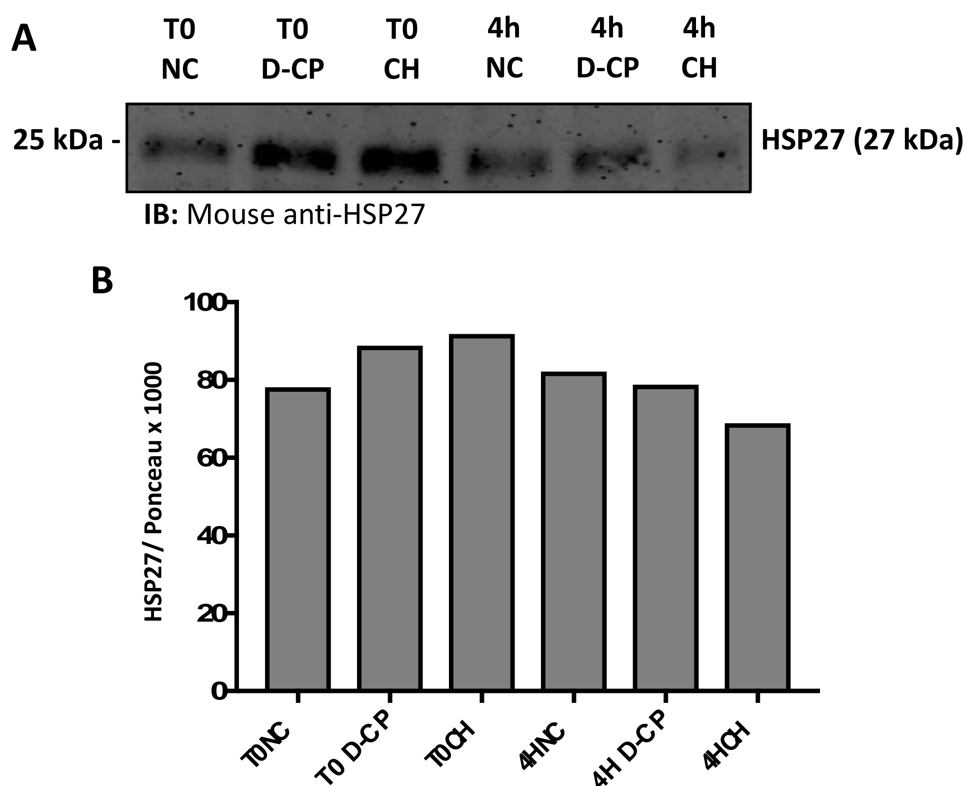


Figure 13- Immunoblot with mouse anti-HSP27. Bovine sperm soluble extracts corresponding to 8,75 x 10^6 cells were loaded, followed by immunoblot with mouse anti-HSP27 (A). Pixel intensity was quantified using Quantity One® Software (B) and Ponceau staining was used as loading control (Supplementary Figure 1B). NC, negative control; D-CP, D- Chloramphenicol; CH, cycloheximide; IB, immunoblot.

On the other hand, following 4 hours incubation, neither D-CP, nor CH affected CaMKIV expression (Figure 14).

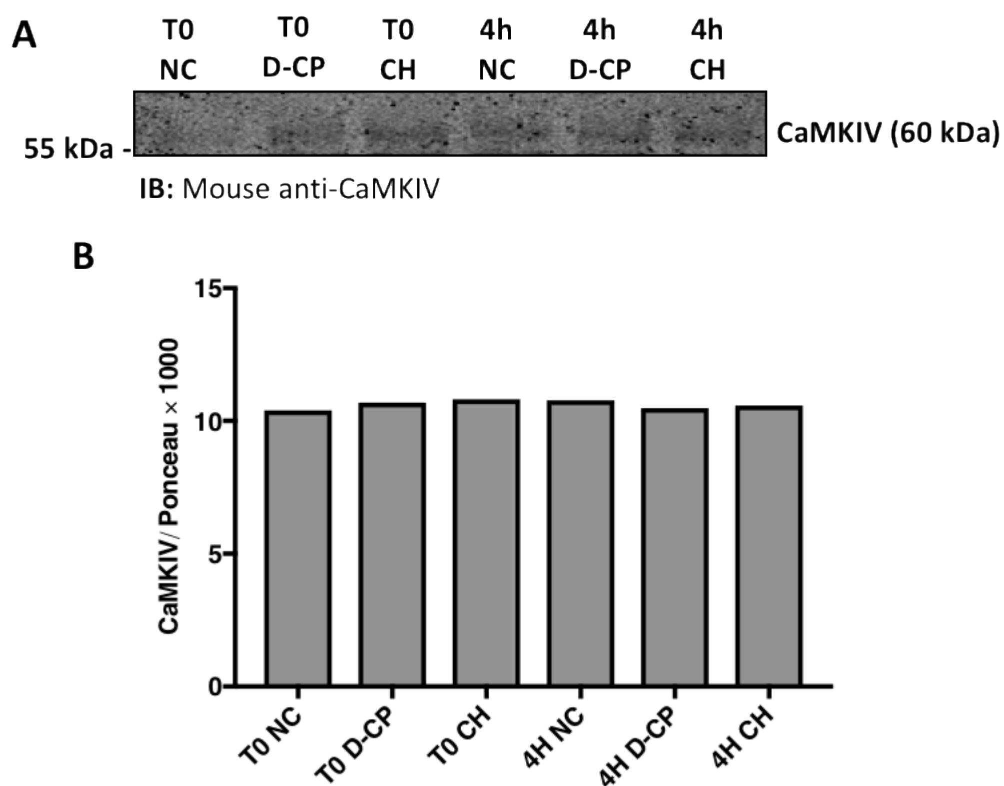


Figure 14- Immunoblot with mouse anti-CaMKIV. Bovine sperm soluble extracts corresponding to 8,75 x 10^6 cells were loaded, followed by immunoblot with mouse anti-CaMKIV (A). Pixel intensity was quantified using Quantity One® Software (B) and Ponceau staining was used as loading control (Supplementary Figure 1C). NC, negative control; D-CP, D- Chloramphenicol; CH, cycloheximide; IB, immunoblot.

4. Discussion

Protein translation in spermatozoa remains a controversial subject, mainly due to sperm cell's high compartmentalization [44], along with its lack of cytoplasmic organelles such as endoplasmic reticulum and Golgi apparatus [16]. However, the presence of an area of uncondensed chromatin, in the neck region [19,27,28] , as well as mitochondrial and cytoplasmic ribosomes [20] increases the likelihood of protein translation in spermatozoa.
A new insight was given by Gur & Breitbart, which proved protein synthesis occurs in spermatozoa during capacitation [20,38] through labeling of sperm's nascent proteins via [35S] amino acid, which was completely inhibited by D-chloramphenicol (D-CP), while unaffected by Cycloheximide (CH). Furthermore, BODIPY-lysine-tRNALys incorporation allowed visualization of the translation's progress [20,38], which, as expected by sperm's morphology, occurs, apparently, in the sperm midpiece [20,38]. Thereby, concluding sperm's translation is time-dependent, capacitation-dependent and sensitive to mitochondrial translation inhibitors.
To test that hypothesis, in this study we implemented, for the first time in bovine and human spermatozoa, a nonradioactive method to monitor protein synthesis named surface sensing of translation (SUnSET) [58].
Our approach using the SUnSET technique revealed to be extremely enlightening, since it allowed to monitor the rate of mRNA translation in spermatozoa (Fig. 5-10) [58], by anti-puromycin staining detection of puromycin incorporation in neosynthesized proteins, supporting that protein translation occurs in both bovine and human spermatozoa. Nevertheless, spermatozoa protein translation levels were significantly inferior when compared with somatic cells (PNT2) levels (Fig. 8).
Bovine spermatozoa treated for 15 min with puromycin, presented the highest mRNA translation levels with a puromycin concentration of 7,5 µg/mL (Fig. 9); while, for a puromycin incubation of 30 min, the optimal puromycin concentration was 5 µg/mL (Fig. 10). The 30 min incubation exhibited, in all conditions, higher protein translation levels than the 15 min incubation suggesting it is a better puromycin incubation time to measure

spermatozoa translation levels, since as described by Gur & Breitbart, translation levels increase until it reaches a plateau after 1 h of incubation under capacitation conditions [20]. Therefore, further studies are indispensable to establish the absolute optimal puromycin concentration and incubation time to monitor protein synthesis in mammalian spermatozoa.

Additionally, in order to corroborate Gur & Breitbart's view that spermatozoa translation occurs in mitochondrial-type ribosomes [20], bovine spermatozoa was incubated with D-CP, which revealed, as expected, a decrease in protein translation (Fig. 11). However, the similar results obtained with D-CP incubated for 30 min and 4 h, display that short incubation times are enough for D-CP necessary inhibitor effect (Fig. 11D) [20].

Furthermore, to determine if, not only mitochondrial-type ribosomes, but also cytoplasmic ribosomes are involved in spermatozoa translation, bovine spermatozoa were incubated under capacitation conditions with or without D-CP and CH. Sperm cells lysates were electrophoresed and transferred into nitrocellulose membranes and membranes were incubated with antibodies for GSK-3α, PPP1CC2, PKC βI (Supplementary Figure 2), CaMKIV, p70 S6 kinase α (Supplementary Figure 3) and HSP27. PPP1CC2 and GSK-3 are crucial for motility initiation in the epididymis, along with regulation of mature sperm functions [64,65]; PKC is involved in flagellar motility and in the AR [66–68]; CaMKIV was also previously described in the human sperm flagellum [69]; p70 S6 kinase was reported to be a multifunctional protein that regulates spermatogenesis [70,71]; HSP27 was reported in human spermatozoa and its activation is negatively correlated with motility [70]. To our knowledge, it was the first time, HSP27 is identified in bovine spermatozoa.

As expected, GSK-3α (Fig. 12) levels decline with D-CP treatment. However, they all exhibited, surprisingly, a greater decline with CH than D-CP. Therefore, these results defy Gur & Breitbart's vision [20], suggesting that both mitochondrial and cytoplasmic ribosomes might be involved in spermatozoa protein translation. Our results support that GSK-3α is, as Gur & Breitbart previously suggested, translated during sperm capacitation [20]. It was the first demonstration, that PPP1CC2 (Fig. 12) and HSP27 (Fig. 13) levels decline, when translation is inhibited, indicating for the first time, both proteins are translated during sperm capacitation.

On the other hand, CaMKIV levels remained constant with D-CP (Fig. 14), as described by Gur & Breitbart , along with CH treatment, indicating CaMKIV remains relatively stable during sperm capacitation [20].

This study presented the first time the SUnSET technique was used to detect mRNA translation in spermatozoa. Additionally, PPP1CC2 and HSP27 were, for the first time, shown to be translated in spermatozoa during capacitation.

Our results corroborate, by other method, the only published paper about spermatozoa protein translation conclusions, except, we show for the first time, cytoplasmic ribosomes are also responsible for spermatozoa protein synthesis.

So, the initial key steps towards a change of believe about spermatozoa translation existence and importance were given.

5. Conclusions and future perspectives

5.1. Conclusions

The principal objective of this thesis was to demystify bovine and human spermatozoa protein translation using the SUnSET technique and Immunoblotting.

It was first time the SUnSET technique was used to detect mRNA translation in spermatozoa. The optimization of the SUnSET technique in bovine spermatozoa was unprecedent, which effectively demonstrated spermatozoa protein translation. The higher protein translation levels were obtained with an incubation time of 30 min and a 5 µg/mL puromycin concentration.

Immunoblotting results confirm, as previously described, GSK-3α is translated and CaMKIV is not. Furthermore, PPP1CC2 and HSP27 were, for the first time, shown to be translated in spermatozoa during capacitation.

Furthermore, translation inhibitors D-CP and CH were successfully used, showing surprisingly both, mitochondrial and cytoplasmic ribosomes, are involved in spermatozoa protein translation (Fig. 12 and 13).

However, due to spermatozoa anatomic features, spermatozoa protein translation levels are residual, when compared to somatic cell (PNT2) levels.

Our results validate, by other method, the only published data about spermatozoa protein translation. Nevertheless, we exhibit for the first time, cytoplasmic ribosomes are also accountable for spermatozoa protein synthesis.

In conclusion, this work proved protein translation occurs in bovine spermatozoa during capacitation.

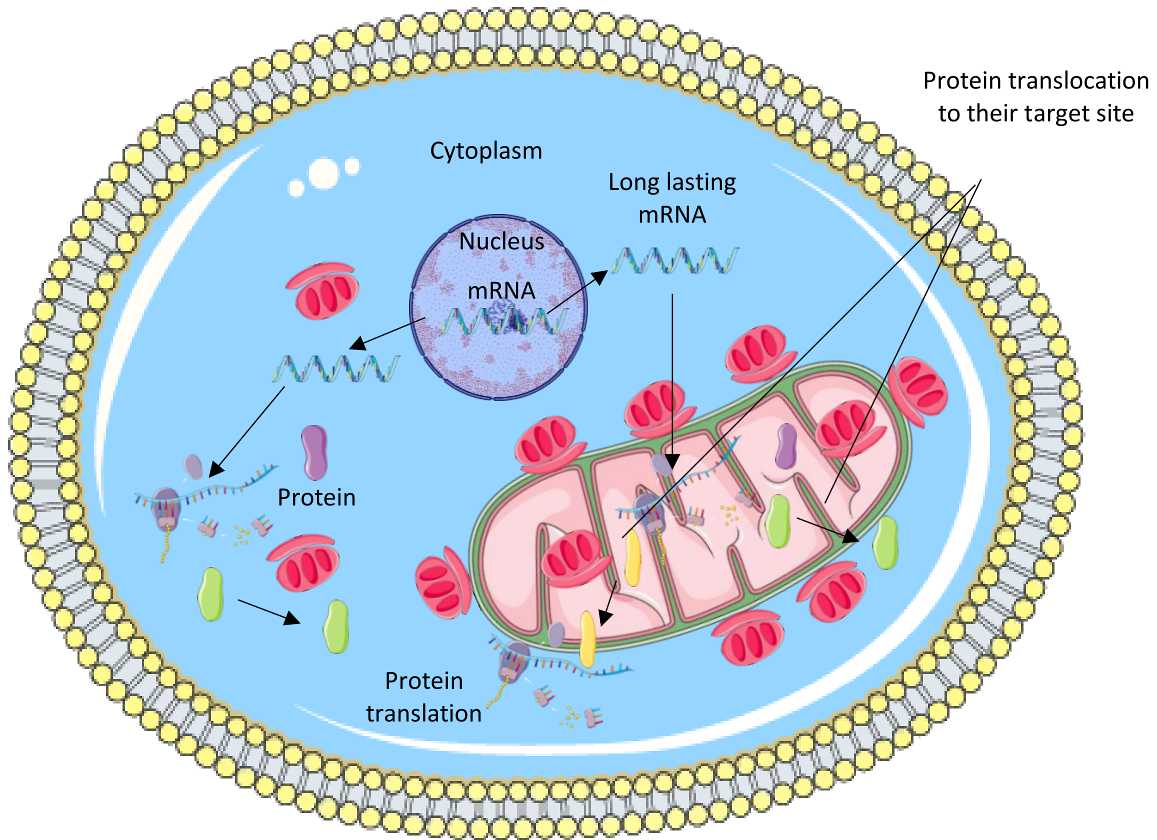


Figure 15- New sperm protein translation model proposed. It suggests nuclear-encoded long lasting mRNA are translated by cytoplasmic ribosomes, localised in the cytoplasm; along with mitochondrial-type ribosomes, localised outside and inside the mitochondria. The translated proteins are then translocated to their active site [38]. Adapted from [38].

5.2. Future perspectives

Upcoming work should focus upon the identification of the specific proteins translated in human spermatozoa during capacitation by mass spectrometry (MS), allowing also to validate if GSK-3α, PPP1CC2 and HSP27 are truly translated, while CaMKIV is not.

The SUnSET bovine spermatozoa results demand the use of other translation elongation inhibitors, namely emetine, in place of cycloheximide, a competitive inhibitor of the puromycin reaction, in order to validate if both, mitochondrial and cytoplasmic ribosomes, are involved in spermatozoa protein translation [72–74]. Furthermore, future replicas with human spermatozoa are essential to correlate if the results are similar to bovine spermatozoa.

References

1. Jiménez-Reina L, Maarten PJ, Jimena-Medina I, Agarwal A, Plessis SS du. Overview of the Male Reproductive System. In: *Exercise and Human Reproduction*. ; 2016:1-17. doi:10.1007/978-1-4939-3402-7.
2. Shalet SM. Normal Testicular Function and Spermatogenesis. *Pediatr Blood Cancer*. 2009;53:285-288. doi:10.1002/pbc.
3. Bhimji S, Leslei S. *Anatomy, Pelvis, Testicle*.; 2018. https://www.ncbi.nlm.nih.gov/books/NBK470201/.
4. Chojnacka K, Zarzycka M, Mruk DD. Molecular Mechanisms of Cell Differentiation in Gonad Development. 2016;58:225-251. doi:10.1007/978-3-319-31973-5.
5. Martini FH, Timmons MJ, Tallitsch RB. *Human Anatomy*. 7th ed. (Berriman L, ed.). Pearson Benjamin Cummings; 2012.
6. Marieb EN, Hoehn KN. *Human Anatomy & Physiology*. 9th ed. (Beauparlant S, ed.). Pearson Education, Inc; 2013. doi:10.1007/BF00845519.
7. Cortes D, Müller J, Skakkebaek NE. Proliferation of Sertoli cells during development of the human testis assessed by stereological methods. *Int J Androl*. 1987;10(4):589-596. doi:10.1111/j.1365-2605.1987.tb00358.x.
8. Griswold MD. The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol*. 1998;9(4):411-416. http://www.sciencedirect.com/science/article/B6WX0-45M31XN-H/2/c374f4f58035a68d8b2a948d4cc365f0 file://D:%5CPaper(pdf)%5CGriswold_1998.pdf.
9. Johnson L, Thompson DL, Varner DD. Role of Sertoli cell number and function on regulation of spermatogenesis. *Anim Reprod Sci*. 2008;105(1-2):23-51. doi:10.1016/j.anireprosci.2007.11.029.
10. Smith LB, Walker WH. The regulation of spermatogenesis by androgens. *Semin Cell Dev Biol*. 2014;30:2-13. doi:10.1016/j.semcdb.2014.02.012.
11. Tremblay JJ. Molecular regulation of steroidogenesis in endocrine Leydig cells. *Steroids*. 2015;103:3-10. doi:10.1016/j.steroids.2015.08.001.
12. Choi J, Smitz J. Luteinizing hormone and human chorionic gonadotropin: Distinguishing unique physiologic roles. *Gynecol Endocrinol*. 2014;30(3):174-181. doi:10.3109/09513590.2013.859670.
13. Walker WH. Testosterone signaling and the regulation of spermatogenesis. *Spermatogenesis*. 2011;1(2):116-120. doi:10.4161/spmg.1.2.16956.
14. Johnson L. Efficiency of spermatogenesis. *Microsc Res Tech*. 1995;32(5):385-422. doi:10.1002/jemt.1070320504.
15. Holstein A-F, Schulze W, Davidoff M. Understanding spermatogenesis is a prerequisite for treatment. *Reprod Biol Endocrinol*. 2003;16:1-16. doi:10.1186/1477-7827-1-107.
16. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Alberts - Molecular Biology Of The Cell 4th Ed. *J Chem Inf Model*. 1989;53:160. doi:10.1017/CBO9781107415324.004.
17. Fardilha M, Silva JV, Conde M. *Reprodução Humana Masculina*. 1st ed. (Fardilha M, Silva JV, Conde M, eds.). Aveiro: ARC Publishing e Editores; 2015.

18. Fouquet JP, Kann ML. The cytoskeleton of mammalian spermatozoa. *Biol Cell*. 1994;81(2):89-93. doi:10.1016/S0248-4900(94)80001-4.
19. Pesch S, Bergmann M. Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. *Micron*. 2006;37(7):597-612. doi:10.1016/j.micron.2006.02.006.
20. Gur Y, Breitbart H. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. *Genes Dev*. 2006;20(4):411-416. doi:10.1101/gad.367606.
21. Berruti G, Paiardi C. Acrosome biogenesis. *Spermatogenesis*. 2011;1(2):95-98. doi:10.4161/spmg.1.2.16820.
22. Brucker C, Lipford GB. The human sperm acrosome reaction: physiology and regulatory mechanisms. An update. *Hum Reprod*. 1995;1(1):51-62.
23. Ho H-C, Suarez SS. Characterization of the Intracellular Calcium Store at the Base of the Sperm Flagellum That Regulates Hyperactivated Motility1. *Biol Reprod*. 2003;68(5):1590-1596. doi:10.1095/biolreprod.102.011320.
24. Oko R, Sutovsky P. Biogenesis of sperm perinuclear theca and its role in sperm functional competence and fertilization. *J Reprod Immunol*. 2009;83(1-2):2-7. doi:10.1016/j.jri.2009.05.008.
25. Steger K. Transcriptional and translational regulation of gene expression in haploid spermatids. *Anat Embryol (Berl)*. 1999;199(6):471-487.
26. Brewer L, Corzett M, Balhorn R. Condensation of DNA by spermatid basic nuclear proteins. *J Biol Chem*. 2002;277(41):38895-38900. doi:10.1074/jbc.M204755200.
27. Dadoune JP. Expression of mammalian spermatozoal nucleoproteins. *Microsc Res Tech*. 2003;61(1):56-75. doi:10.1002/jemt.10317.
28. Miller D, Ostermeier GC, Krawetz SA. The controversy, potential and roles of spermatozoal RNA. *Trends Mol Med*. 2005;11(4):156-163. doi:10.1016/j.molmed.2005.02.006.
29. Ounjai P, Kim KD, Lishko P V., Downing KH. Three-Dimensional Structure of the Bovine Sperm Connecting Piece Revealed by Electron Cryotomography. *Biol Reprod*. 2012;87(3):1-9. doi:10.1095/biolreprod.112.101980.
30. Stephens RE, Oleszko-Szuts S, Linck RW. Retention of ciliary ninefold structure after removal of microtubules. *J Cell Sci*. 1989;92(3):391-402. http://jcs.biologists.org/content/92/3/391.abstract%5Cnpapers3://publication/uuid/0E03A8B0-2464-40D7-91AC-1941F8F0FD3E.
31. Linck RW, Chemes H, Albertini DF. The axoneme: the propulsive engine of spermatozoa and cilia and associated ciliopathies leading to infertility. *J Assist Reprod Genet*. 2016;33(2):141-156. doi:10.1007/s10815-016-0652-1.
32. Inaba K. Molecular Architecture of the Sperm Flagella: Molecules for Motility and Signaling. *Zoolog Sci*. 2003;20(9):1043-1056. doi:10.2108/zsj.20.1043.
33. Amaral A, Lourenço B, Marques M, Ramalho-Santos J. Mitochondria functionality and sperm quality. *Reproduction*. 2013;146(5). doi:10.1530/REP-13-0178.
34. Turner RM. Moving to the beat: A review of mammalian sperm motility regulation. *Reprod Fertil Dev*. 2006;18(1-2):25-38. doi:10.1071/RD05120.
35. Bahr GF, Engler WF. Considerations of volume, mass, DNA, and arrangement of mitochondria in the midpiece of bull spermatozoa. *Exp Cell Res*. 1970;60(3):338-340.

doi:10.1016/0014-4827(70)90526-4.
36. St John JC, Sakkas D, Barratt CLR. A role for mitochondrial DNA and sperm survival. *J Androl*. 2000;21(2):189-199.
37. May-Panloup P, Chrétien MF, Savagner F, et al. Increased sperm mitochondrial DNA content in male infertility. *Hum Reprod*. 2003;18(3):550-556. doi:10.1093/humrep/deg096.
38. Gur Y, Breitbart H. Protein synthesis in sperm: Dialog between mitochondria and cytoplasm. *Mol Cell Endocrinol*. 2008;282(1-2):45-55. doi:10.1016/j.mce.2007.11.015.
39. Amikura R, Kashikawa M, Nakamura A, Kobayashi S. Presence of mitochondria-type ribosomes outside mitochondria in germ plasm of Drosophila embryos. *Proc Natl Acad Sci*. 2001;98(16):9133-9138. doi:10.1073/pnas.171286998.
40. St John JC, Bowles EJ, Amaral A. Sperm mitochondria and fertilisation. *Soc Reprod Fertil Suppl*. 2007;65(May):399-416. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17644980.
41. Hecht NB, Liem H, Kleene KC, Distel RJ, Ho S mei. Maternal inheritance of the mouse mitochondrial genome is not mediated by a loss or gross alteration of the paternal mitochondrial DNA or by methylation of the oocyte mitochondrial DNA. *Dev Biol*. 1984;102(2):452-461. doi:10.1016/0012-1606(84)90210-0.
42. Cummins JM, Woodall PF. On mammalian sperm dimensions. *J Reprod Fertil*. 1985;75(1):153-175. doi:10.1530/jrf.0.0750153.
43. Rouge M. Sperm Morphology. 2004;17(1):http://www.vivo.colostate.edu/hbooks/pathphys/repr. http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/morph.htmL.
44. Fardilha M, Esteves SLC, Korrodi-Gregório L, Pelech S, da Cruz e Silva OAB, da Cruz e Silva E. Protein phosphatase 1 complexes modulate sperm motility and present novel targets for male infertility. *Mol Hum Reprod*. 2011;17(8):466-477. doi:10.1093/molehr/gar004.
45. Ickowicz D, Finkelstein M, Breitbart H. Mechanism of sperm capacitation and the acrosome reaction: Role of protein kinases. *Asian J Androl*. 2012;14(6):816-821. doi:10.1038/aja.2012.81.
46. Suarez SS. Formation of a reservoir of sperm in the oviduct. *Reprod Domest Anim*. 2002;37(3):140-143. doi:10.1046/j.1439-0531.2002.00346.x.
47. Hosken DJ, Hodgson DJ. Why do sperm carry RNA? Relatedness, conflict, and control. *Trends Ecol Evol*. 2014;29(8):451-455. doi:10.1016/j.tree.2014.05.006.
48. ImmLer S. Sperm competition and sperm cooperation: The potential role of diploid and haploid expression. *Reproduction*. 2008;135(3):275-283. doi:10.1530/REP-07-0482.
49. Curry E, Safranski TJ, Pratt SL. Differential expression of porcine sperm microRNAs and their association with sperm morphology and motility. *Theriogenology*. 2011;76(8):1532-1539. doi:10.1016/j.theriogenology.2011.06.025.
50. Vibranovski MD, Chalopin DS, Lopes HF, Long M, Karr TL. Direct evidence for postmeiotic transcription during Drosophila melanogaster spermatogenesis. *Genetics*. 2010;186(1):431-433. doi:10.1534/genetics.110.118919.

51. Fischer BE, Wasbrough E, Meadows LA, et al. Conserved properties of Drosophila and human spermatozoal mRNA repertoires. *Proc R Soc B Biol Sci*. 2012;279(1738):2636-2644. doi:10.1098/rspb.2012.0153.
52. Lewis S, South A. *The Evolution of Animal Nuptial Gifts*. Vol 44.; 2012. doi:10.1016/B978-0-12-394288-3.00002-2.
53. Birkhead TR, Hosken DJ, Pitnick S. *Sperm Biology - An Evolutionary Perspective*.; 2009.
54. Crean AJ, Bonduriansky R. What is a paternal effect? *Trends Ecol Evol*. 2014;29(10):554-559. doi:10.1016/j.tree.2014.07.009.
55. Holman L, Price TAR. Even more functions of sperm RNA: A response to Hosken and Hodgson. *Trends Ecol Evol*. 2014;29(12):648-649. doi:10.1016/j.tree.2014.09.014.
56. Burt A, Trivers RL. *Genes in Conflict: The Biology of Selfish Genetic Elements.* Vol 82. Paperback. Cambridge; 2006. doi:10.1086/523135.
57. Zhao C, Guo XJ, Shi ZH, et al. Role of translation by mitochondrial-type ribosomes during sperm capacitation: An analysis based on a proteomic approach. *Proteomics*. 2009;9(5):1385-1399. doi:10.1002/pmic.200800353.
58. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. *Nat Methods*. 2009;6(4):275-277. doi:10.1038/nmeth.1314.
59. Nathans D. Puromycin inhibition of protein synthesis: incorporation of puromycin into peptide chains. *Proc Natl Acad Sci*. 1964;51(1963):585-592.
60. Nemoto N, Miyamoto-Sato E, Husimi Y, Yanagawa H. In vitro virus: Bonding of mRNA bearing puromycin at the 3'-terminal end to the C-terminal end of its encoded protein on the ribosome in vitro. *FEBS Lett*. 1997;414(2):405-408. doi:10.1016/S0014-5793(97)01026-0.
61. Starck SR, Roberts RW. Puromycin oligonucleotides reveal steric restrictions for ribosome entry and multiple modes of translation inhibition. *Rna*. 2002;8(7):890-903. doi:10.1017/S1355838202022069.
62. Lelouard H, Ferrand V, Marguet D, et al. Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins. *J Cell Biol*. 2004;164(5):667-675. doi:10.1083/jcb.200312073.
63. Betters, PhD, RN DM. Use of Flow Cytometry in Clinical Practice. *J Adv Pract Oncol*. 2015;6(5):435-440. doi:10.6004/jadpro.2015.6.5.4.
64. Smith GD, Wolf DP, Trautman KC, da Cruz e Silva EF, Greengard P, Vijayaraghavan S. Primate sperm contain protein phosphatase 1, a biochemical mediator of motility. *Biol Reprod*. 1996;54:719-727. doi:10.1095/biolreprod54.3.719.
65. Vijayaraghavan S, Mohan J, Gray H, Khatra B, Carr DW. A Role for Phosphorylation of Glycogen Synthase Kinase-3α in Bovine Sperm Motility Regulation. *Biol Reprod*. 2000;62:1647-1654. doi:10.1095/biolreprod62.6.1647.
66. Naor Z, Breitbart H. Protein kinase C and mammalian spermatozoa acrosome reaction. *Trends Endocrinol Metab*. 1997;8(9):337–42. doi:10.1016/S1043-2760(97)00134-3.
67. Breitbart H, Lax J, Rotem R, Naor Z. Role of protein kinase C in the acrosome reaction of mammalian spermatozoa. *Biochem J*. 1992;281 (Pt 2:473-476. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1130709&tool=pmce

ntrez&rendertype=abstract.
68. Rotem R, Paz GF, Homonnai ZT, Kalina M, Lax J, Breitbart H NZ. Ca2+-Independent Induction Protein Kinase C in Human sperm. *Endocrinology*. 1992;131(5):2235-2243.
69. Marin-Briggiler CI. Evidence of the presence of calcium/calmodulin-dependent protein kinase IV in human sperm and its involvement in motility regulation. *J Cell Sci*. 2005;118(9):2013-2022. doi:10.1242/jcs.02326.
70. Silva JV, Freitas MJ, Correia BR, et al. Profiling signaling proteins in human spermatozoa: Biomarker identification for sperm quality evaluation. *Fertil Steril*. 2015;104(4):845-856e8. doi:10.1016/j.fertnstert.2015.06.039.
71. Feng LX, Ravindranath N, Dym M. Stem cell factor/c-kit up-regulates cyclin D3 and promotes cell cycle progression via the phosphoinositide 3-kinase/p70 S6 kinase pathway in spermatogonia. *J Biol Chem*. 2000;275(33):25572-25576. doi:10.1074/jbc.M002218200.
72. David A, Dolan BP, Hickman HD, et al. Nuclear translation visualized by ribosome-bound nascent chain puromycylation. *J Cell Biol*. 2012;197(1):45-57. doi:10.1083/jcb.201112145.
73. Aviner R, Geiger T, Elroy-Stein O. Novel proteomic approach (PUNCH-P) reveals cell cycle-specific fluctuations in mRNA translation. *Genes Dev*. 2013;27(16):1834-1844. doi:10.1101/gad.219105.113.
74. Hobden AN, Cundliffe E. The mode of action of alpha sarcin and a novel assay of the puromycin reaction. *Biochem J*. 1978;170(1):57-61. doi:10.1042/bj1700057.

Supplementary data

Supplementary Table 1 – Solutions used in the experiments.

Surface Sensing of Translation (SUnSET)		
Puromycin 100 ug/mL	For 10 mL, dissolve 1 mg of puromycin in 10 mL deionized water.	
Flow Cytometry analysis		
4% Paraformaldehyde (PFA)	For 15 mL, dissolve 1,5 mL of PFA stock 37% in 13,5 mL PBS.	
Fluorescence activated cell sorting (FACS) solution including 0,1% saponin	For 10 mL dissolve 0,025 g of BSA in 5 mL of PBS and add 2 mL saponin and 0,1 mL sodium azide and make up to 10 mL with PBS.	
Anti-puromycin Alexa488 diluted in 1:100 FACS solution	For 250 ul, add 2,5 ul Anti-Puromicina-Alexa488 to 250 ul FACS solution.	
Sperm treatment with translation inhibitors		
D-Chloramphenicol 100 µg/mL	For 1 mL, dissolve 1 mg d-chloramphenicol in 1 mL ethanol, add 9 mL MiliQ water.	
Cycloheximide 1 mg/mL	For 1 mL, dissolve 1 mg cycloheximide in 1 mL deionized water.	
Western Blotting		
Running gel 12% (2 gels, 1.5 mm thickness)	ddH2O Tris 1.5 M pH8.8 Acrylamide 40% Bisacrylamide 2% SDS 10% APS 10% TEMED	6.360 mL 5.000 mL 5.880 mL 2.360 mL 0.200 mL 0.100 mL 0.020 mL
Stacking gel 4% (2 gels, 1.5 mm thickness)	ddH2O Tris 0.5 M pH6.8 Acrylamide 40% Bisacrylamide 2% SDS 10% APS 10% TEMED	4.736 mL 2.000 mL 0.784 mL 0.320 mL 0.080 mL 0.040 mL 0.008 mL
Tris-HCl 1.5 M pH 8.8 buffer	For 1 L dissolve 181.5 g Tris in 800 mL deionized water. Adjust pH at 8.8 with HCl and make up to 1 L with deionized water.	

Tris-HCl 0.5 M pH 6.8 buffer	For 1 L dissolve 60 g Tris in 800 mL deionized water. Adjust pH at 6.8 with HCl and make up to 1 L with deionized water.
10% APS (ammonium persulfate)	For 10 mL of deionized water add 1 g of APS.
10% SDS (sodium dodecilsulfate)	For 500 mL of deionized water dissolve 50 g of SDS.
4X Loading gel buffer	For 10 mL add 44 mL glycerol, 250 µL Tris-HCl 0.5 M pH 6.8 buffer, 0.8 g SDS, 0.2 mL β-mercaptoethanol and 3.3 mL deionized water. Add bromophenol blue (a small amount). Keep it at RT for short periods or at 4ºC for longer periods.
Tris-Gly 10X Stock	For 1 L dissolve 30.30 g Tris (250 mM) and 144.10 g Gly (1.92 M) in 1 L of deionized water.
Running buffer 1X	For 1 L add 800 mL deionized water, 100 mL Tris-Gly 10X and 10 mL 10%SDS. Make up to 1 L with deionized water.
Transfer buffer 1X	For 1 L add 100 mL Tris-Gly 10X to 700 mL of deionized water and 200 mL methanol.
10X TBS Stock (Tris buffered saline)	For 0.5 L dissolve 6.055g Tris in deionized water and adjust pH at 8.0. Add 43.8325 g NaCl and make up to 500 mL with deionized water.
1X TBST (TBS + Tween 20)	For 1 L add 100 mL TBS 10X and 500 µL Tween-20 to 900 mL of deionized water.
5% BSA in TBST 1X	For 100 mL of solution dissolve 5 g of BSA in TBST 1X.

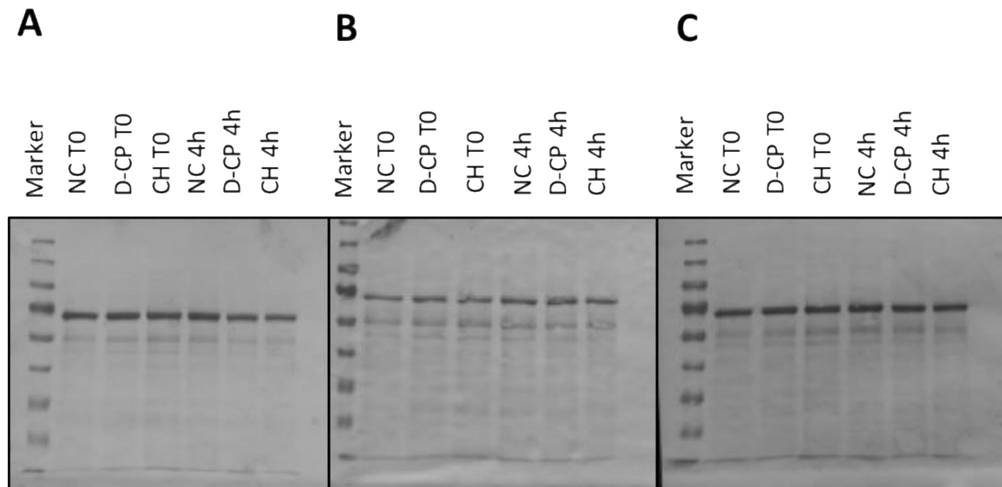
Supplementary Table 2 – Results of basic human spermatozoa analysis.

	Volume (mL)	Progressive motility (%)	Non-progressive motility (%)	Immotile (%)	Concentracion ($x10^6$ spz/mL)	Normal morphology (%)	Head defects (%)	Midpiece defects (%)	Tail defects (%)
Sample 1	5,5	43	23	34	22	5	90	62	22
Sample 2	4	63	16	21	108	8	85	55	8

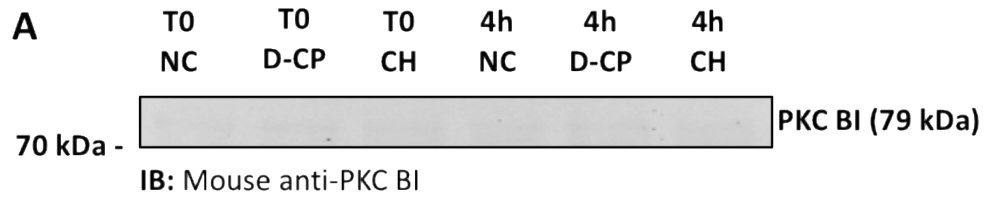
Supplementary Table 3 – Results of basic human spermatozoa analysis between time 0 (T0) and 4h incubation with D-CP.

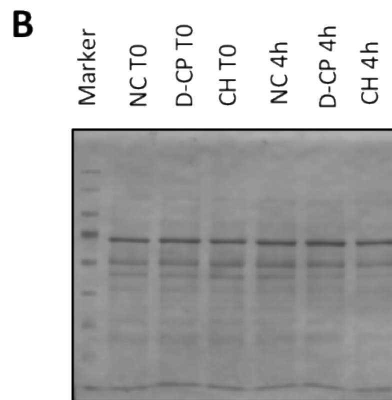
	T0	**D-CP 4h**
Progressive motility (%)	69,11	58,48
Non-progressive motility (%)	21,43	18,71
Immotile (%)	9,46	22,81

Supplementary Figure 1 - Ponceau S. staining used as loading control for Immunoblot with mouse anti-GSK-3α/β and rabbit anti-PPP1CC2 (**A**); mouse anti-HSP27 (**B**); mouse anti-CaMKIV (**C**).

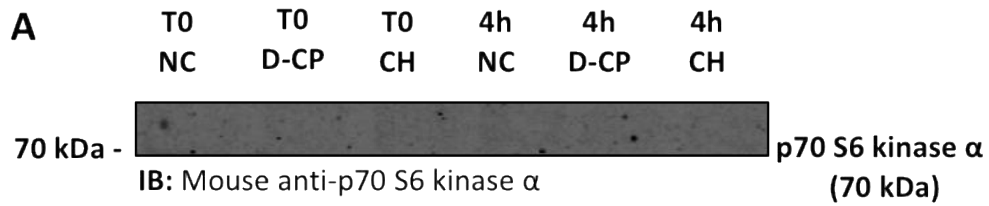


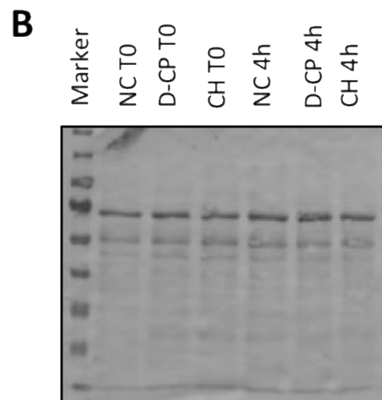
Supplementary Figure 2 - Immunoblot with mouse anti-PKC BI (**A**) revealed no staining in bovine spermatozoa; Ponceau S. staining used as loading control for Immunoblot with mouse anti-PKC BI (**B**).





Supplementary Figure 3 - Immunoblot with mouse anti-p70 S6 kinase α (**A**) revealed no staining in bovine spermatozoa; Ponceau S. staining used as loading control for Immunoblot with mouse anti-p70 S6 kinase α (**B**).





Supplementary Figure 4 - Protein translation in spermatozoa poster exhibited in I NoTeS Congress - Novel therapeutic strategies for noncommunicable diseases, 22-23 June 2018

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Protein translation in spermatozoa

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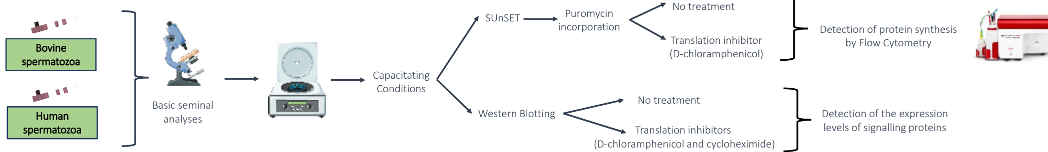
Goal Evaluate protein synthesis in mammalian spermatozoa

Background

Reversible protein phosphorylation is the key general mechanism for regulating vital sperm cells functions since sperm cells are highly compartmentalized and almost devoid of transcription and translation [1]. However, in the neck region of spermatozoa, there is a small area of uncondensed chromatin where transcription, translation and protein-biosynthesis are still possible [2,3,4]. A new insight was recently given showing protein synthesis in spermatozoa during capacitation [5]. Protein production in spermatozoa revealed to be sensitive to several mitochondrial mRNA translation inhibitors such as D-chloramphenicol, gentamycin and tetracycline, although insensitive to the cytoplasmic 80S ribosomal inhibitor cycloheximide. Furthermore, translation was unaffected by transcription inhibitor suggesting that the source of mRNA for protein translation is long lasting mRNA [5].

Experimental Design



Results

Human and bovine spermatozoa were treated, under capacitation conditions, with different puromycin concentrations with or without translation inhibitor (D-chloramphenicol). Samples were then analysed using the SUnSET technique, which allows to monitor the rate of mRNA translation by puromycin incorporation in neosynthesized proteins. Puromycin incorporation was detected by Flow Cytometry with an anti-puromycin antibody (Figure 1-4) [6].
To assess the impact of translation inhibition on the levels of signaling proteins, bovine spermatozoa was incubated under capacitation conditions with or without D-chloramphenicol or cycloheximide. Sperm cells lysates were then electrophoresed and membranes were incubated with antibodies for GSK-3α/β, PPP1CC2, CaMKIV and HSP27 (Figure 5).

SUnSET technique to detect mRNA translation in spermatozoa

Protein translation occurs in human spermatozoa | Protein translation occurs in bovine spermatozoa | Mitochondrial ribosomes are involved in spermatozoa protein translation

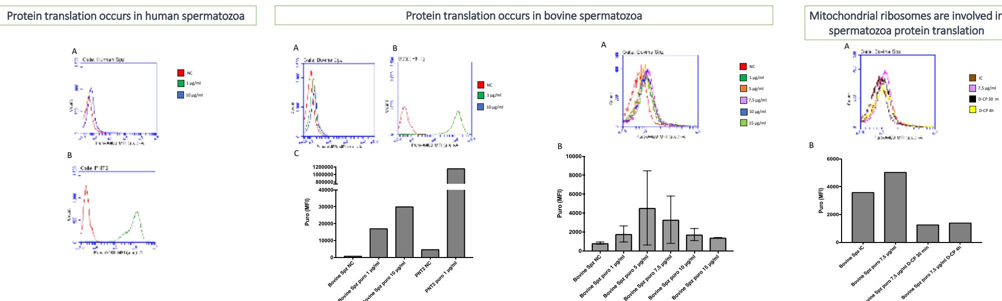


Figure 1 – Overlay histograms of human spermatozoa (A) and PNT2 cells (PC) (B). Human spermatozoa and PNT2 cells were treated with puromycin for 15 min. Spz, spermatozoa; PC, positive control; NC, negative control; puro, puromycin.

Figure 2 – Overlay and puromycin incorporation (MFI) (C) histograms of bovine spermatozoa (A) and PNT2 cells (PC) (B). Bovine spermatozoa and PNT2 cells were treated with different puromycin concentrations for 15 min. Spz, spermatozoa; PC, positive control; NC, negative control; puro, puromycin.

Figure 3 – Overlay and puromycin incorporation (MFI) (B) histograms of bovine spermatozoa (A). Bovine spermatozoa were treated with different puromycin concentrations for 30 min. Spz, spermatozoa; PC, positive control; NC, negative control; puro, puromycin.

Figure 4 – Overlay and puromycin incorporation (MFI) (B) histograms of bovine spermatozoa (A) between stained for anti-IgG or anti-puromycin samples. Bovine spermatozoa were treated with puromycin and with or without D-chloramphenicol for 30 min or 4 h. Spz, spermatozoa; IC, isotype control; PC, positive control; NC, negative control; puro, puromycin.

GSK-3α, PPP1CC2 and HSP27 are translated, while CaMKIV remains relatively stable during sperm capacitation

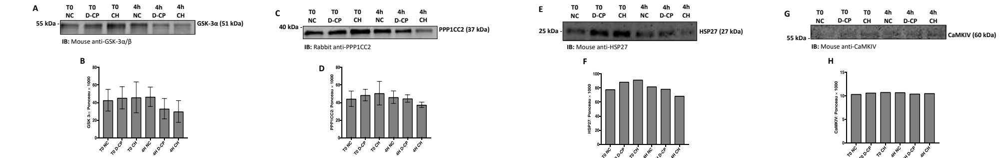


Figure 5 – Immunoblots with mouse anti-GSK-3α/β (A,B), anti-HSP27 (E,F), anti-CaMKIV (G,H) and rabbit anti-PPP1CC2 (C,D). Spz, spermatozoa; puro, puromycin; NC, negative control; D-CP, D-Chloramphenicol; CH, cycloheximide; IB, immunoblot.

Future work

- Identification of the proteins translated in human spermatozoa by mass spectrometry
- Validate mitochondrial and cytoplasmic ribosomes function in spermatozoa protein translation, with other translation elongation inhibitors

Conclusions

- Protein translation occurs in human and bovine spermatozoa during capacitation
- Mitochondrial and cytoplasmic ribosomes are involved in spermatozoa protein translation
- First time, PPP1CC2 and HSP27 were shown to be translated in spermatozoa during capacitation

References

1. Fardilha et al., Molecular Human Reproduction, 2011, 17, p.466–477
2. Dadoune, J. P., Microscopy Research and Technique, 2003, 61, p.56–75
3. Miller, D., Ostermeier, G. C., & Krawetz, S. A., Trends in Molecular Medicine, 2005, 11, p.156–163
4. Pesch, S., & Bergmann, M., Micron, 2006, 37, p.597–612
5. Gur, Y., & Breitbart, H., Genes & Development, 2006, 20, p.411–416
6. Pierre P. et al., Nature Methods, 2009, 6, p.275-277

Acknowledgements

This work was supported by FEDER funds through the "Programa Operacional Competitividade e Internacionalização- COMPETE 2020" and by National Funds through the FCT- Fundação para a Ciência e Tecnologia, Projects PTDB/BBB-BQB/3804/2014. We are thankful to iBiMED (UID/BIM/04501/2013 and POCI-01-0145-FEDER-007628) for supporting this project. This work was also supported by individual grant from FCT to JVS (SFRH/BPD/123155/2016).