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RESEARCH ARTICLE

Activation of the mobility of human spermatozoa with the use of pentoxifylline: effects on spermal DNA

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Abstract

Introduction: 50% of pregnancy failure is due male factors. One of these factors is the absence of spermatozoa in the ejaculate; in these cases the patient is submitted to epididymal or testicular sperm aspiration. The spermatozoa acquired from these technics usually are immobile, what makes necessary the use of pentoxifylline, which activates sperm movement, to distinction of viable spermatozoa to ICSI. **Objectives:** To analyze if a low concentration of pentoxifylline causes spermatic chromatin damages. **Materials and Methods:** Sperm motility activation was tested in different concentrations of pentoxifylline. After the screening of doses, the lowest effective concentration was choose to analyze the chromatin damage rate. 15 samples of fresh sperm were tested for DNA damage with HALOSPERM® Kit. Blades' analyses were made in bright field microscopy. The fragmented DNA was settle by the absence of chromatin dispersion halo or small halo. **Results:** The lowest and effective pentoxifylline dose was 1.5 mM. There was no significant difference on chromatin damage rate between study and control groups (p = 0.55). **Conclusion:** The use of pentoxifylline at 1.5 mM does not affect the sperm DNA.

Keywords: pentoxifylline; sperm motility; DNA fragmentation.

Introduction

Infertility is a disease of the reproductive system defined by the failure or impossibility to achieve a clinical pregnancy. According to Inhorn[1] it is estimated that 8 to 12% of world population is infertile, which represents about 60 to 80 million people across the world. Infertility is the inability of a sexually active, non-contracepting couple to achieve pregnancy in one year.[2]

The causes of infertility may derive equally from men or women, and sometimes both present this condition simultaneously.[3]

Up to 50% of the infertility causes may be due male factors.[4] The main reasons are: low sperm count (age related or not), low sperm motility, abnormal morphology, absence of spermatozoa in ejaculate, vasectomy, varicocele, high DNA fragmentation, inflammation, anatomic issues, endocrine disorders and sexually transmitted diseases.[5]

The diagnosis starts with an anamnesis, which consists of assessing the couple's clinical history, followed by physical examination in order to determine possible abnormalities. Moreover, exams such as sperm count test, sperm DNA fragmentation, hormonal dosage, urine test and testicular biopsy could be required if necessary.

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The treatments to overcome the difficulty of conception include treatments for reversible causes or solutions through assisted human reproduction techniques.

In cases of azoospermia (absence of spermatozoa in ejaculate), percutaneous epididymal and/or testicular aspiration, or open surgery of testis (including microsurgery) may be considered. These techniques are: PESA (percutaneous epididymal sperm aspiration), MESA (microsurgical epididymal sperm aspiration), TESA (testicular sperm aspiration), TESE (testicular sperm extraction) and micro TESE (microdissection testicular sperm extraction). For such techniques, local sedation or general anesthesia are required.[6]

In assisted human reproduction, the use of high-complexity artificial fertilization techniques such as IVF (in vitro fertilization), and more precisely ICSI (intracytoplasmic sperm injection), has become increasingly common in laboratory routines. The ICSI has had excellent results in couples with male infertility, increasing the chance of pregnancy. The spermatozoa used in ICSI could be obtained from ejaculate, frozen samples, PESA, MESA, TESA, TESE or Micro-TESE. The spermatozoa achieved through surgical sperm retrieval techniques usually have little or no motility.

In order to use those spermatozoa with little or no motility for ICSI it is necessary perform a technique of spermatozoa selection. There are three types of selection, the sperm tail flexibility test, the hypo-osmotic test, and more recently the use of pentoxifylline. The sperm tail flexibility test consists in gently touch the tail of the spermatozoa with the end of the micropipette and analyze its movement. The tail may present an independently movement or appear to be rigid, moving jointly with the head; from this movements, the spermatozoa may be considered alive (viable) or dead (unviable), respectively. The hypo-osmotic test analyses the integrity of the spermatozoa plasma membrane, the viable spermatozoa present an increase in the tail size, which causes the tail to coil in different shapes, in case this do not occur, the spermatozoa is considered unviable. Some laboratories have used the pentoxifylline, substance that active the sperm motility, making the detection of alive spermatozoa visually detectable.[7]

The motile tail of a sperm is a long flagellum, whose central axoneme is formed by microtubules and diplomicrotubules, which are connected by protein portions known as dynein arms. When the dynein arms undergo a dephosphorylation, they become active making one portion of the diplomicrotubule glide over the other, resulting in movement.[8]

The dephosphorylation results from two signaling pathways, one is the link between the AMPc (cyclic adenosine monophosphate) a PK-A (protein kinase A). The link between these two molecules allows the catalysis of the phosphorylation of the dynein arms, and for the reverse process (the protein phosphorylation), it is necessary a reduction on the levels of AMPc through the action of the phosphodiesterase enzyme.

The pentoxifylline is a methylxanthine, which plays an inhibitory action of phosphodiesterase. With the inhibition of the phosphodiesterase, there is an increase in the available AMPc levels, resulting in larger amount of active PK-A, which leads to a gain of sperm motility.[9]

However, a question raised by Unsal et al.[10] is the chance of DNA damage induced by pentoxifylline. The presence of fragmentation in the chromatin may have a negative impact on fertility rates, particularly in ICSI.

Infertile men have a major tendency to sperm chromatin fragmentation. Therefore, it is important to determine a safe threshold for use of pentoxifylline, and then to avoid the increase of sperm DNA damage. In this regard, this study proposes to find a minimum concentration of pentoxifylline which could satisfactory activate sperm motility, in order to select viable sperm for ICSI, and concomitantly avoid chromatin damage.

Materials and methods

This study was performed after the approval by the Ethics Committee of the School of Medicine from Federal University of Rio Grande do Sul – COMPESQ-FAMED / UFRGS project nº 31892, according to ethical principles of research with human beings (Appendix B). The experiments were conducted during October and November of 2016 in the Insemine Human Reproduction Center, situated in 2825 Nilo Peçanha Avenue, Porto Alegre, RS.

Pentoxifylline concentration

A pilot test with different concentrations of pentoxifylline was performed during analysis of TESA materials performed in Insemine Human Reproduction Center. These samples have been placed in 3 drops of culture media with the addition of pentoxifylline in a final concentration of 1.5mM, 1.25mM and 1mM. After pentoxifylline the samples were analyzed for 20 minutes. In these tests the minimum concentration for satisfactory sperm motility activation for ICSI was found.

Sample selection for sperm DNA fragmentation test

Following the definition of the concentration to be used, 15 fresh sperm samples were selected from patients undergoing assisted reproduction treatment, who were properly clarified regarding the content of the research and agreed to participate signing the free and informed consent form (Appendix A).

Sample analysis

The sample analysis was performed after liquefaction. A simple sperm count test was performed before the sperm DNA fragmentation test. This sperm count test assessed the sperm motility and concentration.

Sperm DNA fragmentation test

Each sample was split in control group (without pentoxifylline) and pentoxifylline group (with addiction of pentoxifylline), and incubated for 20 minutes in incubator with 37 °C and 6% CO_2. After incubation the DNA fragmentation test was performed, based on the SCD test (sperm chromatin dispersion), using the Halosperm G2 kit®.

The slides were assessed under bright field microscopy. The spermatozoa that showed intact DNA possess a pink halo around the head. In contrast when there was DNA fragmentation the spermatozoa showed a stained head with coloration ranging from pink to purple, with a small or absent halo.

Statistic analysis

The samples were grouped in control and pentoxifylline groups, and the groups were compared using the Wilcoxon signed-rank test for paired samples. In addition, the correlation between DNA fragmentation and sperm motility was tested using the Spearman's rank correlation coefficient.

Results

The aim of this study was to compare control and pentoxifylline groups in order to evaluate the potential implications of pentoxifylline in sperm DNA damages. The pilot test has identified the concentration of 1.5mM as the minimum for satisfactory increase in sperm motility for ICSI purposes.

The group pairing was effective (85.8%, p≤ 0.0001), and therefore the groups were evaluated through Wilcoxon signed-rank test. There are no statistical difference between the groups (p=0.55) in regard to sperm DNA fragmentation (Figure 1). The mean, median and standard deviation were respectively 18.51, 15.70 and 3.04 for control group and 17.66, 13 and 2.71 for pentoxifylline group.

The correlation test showed no association between DNA fragmentation and sperm motility (r=-0.41 and p=0.27), the same occur when DNA fragmentation and sperm concentration were compared (r=-0.08 and p=0.76).

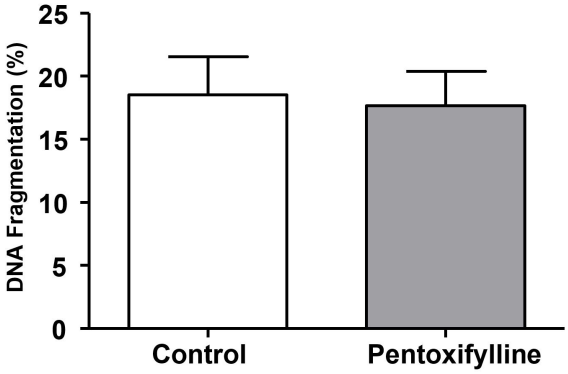

Figure 1. Analysis of the sperm DNA fragmentation rate through Wilcoxon signed-rank test for paired samples showing no differences between the control and pentoxifylline groups (p=0.55).

Discussion

Fertile men have lower DNA fragmentation rates when compared with subfertile or infertile.[11] According to Irvine et al.,[12] fertile men presented about 10% of DNA fragmentation, while infertile men have rates above 20% of fragmentation.

The sperm chromatin is highly compacted, due to replacement of the histones by specific spermatozoa protamines during the spermatogenesis. However, chromatin damage may occur naturally due to several reasons such as apoptosis, oxidative stress or other reasons.[13] Since there is a chance of some degree of sperm DNA fragmentation, any method that may increase this fragmentation become unviable. According to Benchaib et al.,[14] a sperm DNA fragmentation higher than 10% may cause decrease in fertilization rates, embryo blockage, interfere with blastomere division, implantation impairment, and even be the cause of an eventual miscarriage.[15]

An important technique used to differentiate the live spermatozoa is the use of pentoxifylline. The concern about the chromatin damage caused by pentoxifylline use, as described by Unsal et al.[10] make this study very useful for assisted reproduction routines. Accordingly to our results, the use of pentoxifylline in 1.5mM concentration does not affect the genetic material of the patient, even when DNA fragmentation rates are higher. One of the samples that was included in the study presented high rates of DNA fragmentation (Table 1), but this patient underwent a chemotherapy treatment, which may have increase the DNA fragmentation levels. When the control and pentoxifylline samples of this patient was compared with the other samples there was no difference in their pattern, and we did not see statistical difference between the groups.

This study used fresh ejaculate samples to perform the sperm chromatin damage analysis, with sperm motility and concentration considered as normal. According to Benchaib et al.[14] the sperm DNA fragmentation does not have correlation with sperm concentration or motility, which was confirmed through our analysis of correlation that compared the sperm DNA fragmentation rates with the sperm concentration and motility of each sample. This allows us to extrapolate the findings of control and pentoxifylline groups with surgical sperm retrieval techniques from testis and epididymis, which possess low sperm concentration and motility rate. Therefore, we suggest that samples achieved from PESA, MESA, TESA, TESE and Micro-TESE could be safely submitted to pentoxifylline, in regard with DNA integrity, as long as the drug concentration is 1.5mM and the exposure do not exceed 20 minutes. However, we cannot claim that other pentoxifylline concentration or exposure for more than 20 minutes is safe, once these conditions were not tested in this study. More studies are needed to answer those questions.

Table 1. Data of DNA fragmentation rates relative to each sample

Sample	Volume (mL)	Concentrationmillion/mL	Motility (%)	Control group fragmentation (%)	Pentoxifylline groupfragmentation (%)	Obs.
1	2.7	150	90	4.66	5.66	
2	4	35	60	12	13	
3	3.2	20	70	17.66	13	
4	1	26	70	11.66	11	
5	1.9	18	60	27	22	
6	3.8	70	90	22	23	
7	8.7	38	60	18	18.67	
8	**3**	**100**	**50**	**56**	**50**	**Chemotherapy***
9	4.9	28	70	15.4	20	
10	2.3	70	75	8	8.3	
11	2	74	65	15.7	13	
12	4.7	25	65	14.7	11.4	
13	4.6	52	70	13.7	18.6	
14	1	62	60	21.4	24.3	
15	4.7	50	80	19.7	13	

*The sample number 8 presents highest DNA fragmentation rate in both group, probably due a previous chemotherapy, and do not present significant difference in the sperm DNA fragmentation between groups.

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Authors contribution

TCED - Development of the research project, reading related literature, performed the assays and wrote the results. DSS - Coordinator of the project, participated in the experimental design, selection and reading of reference articles, collaborated in the development of the experiments and written work. VML - Advisor by UniRitter, collaborated with the structure of written work and statistical development of the study. AS - Collaborated with collection of information regarding the use of pentoxifylline in the laboratory of andrology. JSCF - Responsible for the financial support that enabled the development of the research, besides helping in the development of the study and in the selection and clarification of the volunteers.

Appendix A. Informed consent form

INFORMED CONSENT FORM

You are been invited as volunteer to participate of the study:

THE USE OF PENTOXIFYLLINE ON ACTIVATION OF HUMAN SPERM MOTILITY: IDEAL CONCENTRATION TO AVOID DNA DAMAGES.

REASONS, OBJECTIVES AND PROCEDURES:

In the embryology laboratory many techniques are used in order to assist the fertilization process. One of the techniques is the ICSI (intracytoplasmic sperm injection) used mainly in cases where the number of spermatozoa are low and/or when the spermatozoa have low or none motility. The pentoxifylline is a drug that causes an activation of the sperm motility, being useful for identification and selection of viable spermatozoa for ICSI. However, there aren't many studies questioning your appropriate concentration or implication with sperm DNA damage. This study aims to find an ideal concentration where the risk of DNA damage could be avoided. Therefore, allows the use of pentoxifylline in laboratory routine for time and selection improvements. The sperm sample will be incubated with pentoxifylline and after the incubation period, a DNA fragmentation test will be performed.

The research material will not be used to fertilize an oocyte or for any reproductive ends.

DISCOMFORT AND RISKS: The collect does not present any kind of discomfort or risk. The material used for the research will bring no harm the IVF treatment. This material would be discarded!

WARRANTY OF CLARIFICATION, FREEDOM, REFUSAL AND CONFIDENTIALITY: You will be clarified about the study in any aspect of your desire. You are free to decline the participation, withdraw your consent or interrupt your participation at any time. Your participation is voluntary and the refusal in participate will not bring any penalty or injury in your IVF treatment.

Your identity will remain anonymous and the researchers will treat it with professional standards. Your name or material that indicate your participation will not be released without your permission. You will not be identified in any potential publication resulting of this study. The Research committee of the Federal University of Rio Grande do Sul will approve one copy of this consent, and you will have a copy of this document.

COSTS OF PARTICIPATION, COMPENSATION AND INDEMNITY FOR POSSIBLE DAMAGES: The participation in this study will not bring any costs to you and there will not be any kind of additional financial compensation.

I, _____ was clearly and detailed informed of the objectives of the above study, and clarified my doubts. I know that in any time I will be able to require new information and change my decision if desire. The Dr. João Sabino Cunha Filho and Alberto Stein have certified me that all the date from this study will remain confidential.

I also know that in case of any additional expenses, these will be absorved by the research budget. In case of questions I will be able to call the researchers Daniela Scherer da Silva at phone number (51) 33881212, Tamires Correia Evangelista Dutra at phone number (51) 84591363, or through the project number – 31892 at Ethics Committee of the School of Medicine from Federal University of Rio Grande do Sul, located at 110, Paulo Gama Avenue – Room 317, Building 1 of Rectory – Downtown Campus – Porto Alegre, RS.

I declare that I agree to participate of this study. I received a copy of this informed consent form, and the opportunity for read and clarify my doubts was provided.

Name	Participant signature	Date
Name	Participant signature	Date
Name	Participant signature	Date

Appendix B. COMPESQ/FAMED – UFRGS approval

UFRGS

Projetos de Pesquisa | Áreas de Atuação | Bolsas de Pesquisa | Programa de Iniciação Científica Voluntário | Programa de Fomento à Pesquisa(Fapergs) | Pós-doutorado | Docente em Tarefa Administrativa Convidado

Sistema Pesquisa - Pesquisador: Joao Sabino Lahorgue Da Cunha Filho

Retornar

Dados Gerais:

Projeto Nº:	31892	**Título:**	O USO DA PENTOXIFILINA NA ATIVACAO DA MOTILIDADE DE ESPERMATOZOIDES HUMANOS: CONCENTRACAO IDEAL PARA EVITAR DANOS AO DNA ESPERMÁTICO
Área de conhecimento:	Saúde Materno-Infantil	**Início:**	01/10/2016
Previsão de conclusão:	31/12/2017		
Situação:	Projeto Não Iniciado		
Origem:	Faculdade de Medicina	**Projeto Isolado**	
Local de Realização:	não informado		

Não apresenta relação com Patrimônio Genético ou Conhecimento Tradicional Associado.

Objetivo:

A pentoxifilina vem sendo usada como um meio mais seguro e objetivo para o embriologista na seleção de espermatozoides vivos nos procedimento de ICSI. Como existem poucuíssimos trabalhos relacionados ao uso desta substância em espermatozoides humanos, este projeto foi desenvolvido para trazer informações mais precisas no uso deste fármaco. Geral

O objetivo geral deste projeto é determinar a concentração da pentoxifilina para as rotinas de ICSI com espermatozoides de baixa ou nenhuma motilidade. Específico

Palavras Chave:

SEMEN, PENTOXIFILINA, ICSI, INFERTILIDADE

Equipe UFRGS:

Nome: JOAO SABINO LAHORGUE DA CUNHA FILHO
Coordenador - Início: 01/10/2016 Previsão de término: 31/12/2017

Equipe Externa:

Nome: Tamires Correia Evangelista Dutra
Instituição: Universidade Ritter dos Reis
Pesquisador desde 01/10/2016
Nome: Daniela Scherer da Silva
Instituição: Hospital de Clínicas de Porto Alegre
Pesquisador desde 01/10/2016
Nome: Virginia Meneghini Lazzari
Instituição: Universidade Ritter dos Reis
Pesquisador desde 01/10/2016

Avaliações:

Comissão de Pesquisa de Medicina - Aprovado em 24/09/2016 Clique aqui para visualizar o parecer

Anexos:

Projeto Completo

Data de Envio: 01/09/2016