Original Article

p53 Protein Evaluation on Spermatozoa DNA in Fertile and Infertile Males

Salvatore Raimondo, Tommaso Gentile, Mariacira Gentile, Alessia Morelli, Francesca Donnarumma, Felice Cuomo, Stefania De Filippo, Luigi Montano[1]

Laboratory Research "Gentile s.a.s." 80054 Gragnano, Naples, [1]Andrology Unit, "Saint Francis of Assisi" Hospital, 84121 Salerno, Italy

Abstract

Introduction and Objectives: Protein p53 role in the spermatogenesis is demonstrated, it guarantees both the appropriate quality and quantity of mature spermatozoa. In this observational study we evaluate the eventual correlation between "corrected" p53 concentration on human spermatozoa DNA and male fertility potential. **Materials and Methods:** Our work is based on an observational study made of 169 male in a period between March 2012 and February 2017. The entire study group is composed by 208 male partners aged between 26-38 years with ejaculate volume from 0.6 to 5.8 mL and heterogeneous seminal valuation: 86/208 (41,3%) normospermic; 19/208 (9,1%) mild oligospermic; 51/208 (24,5%) moderate oligospermic to; 52/208 (25,1%) with severe oligospermic. The "control" group A includes 39 male partners considered "fertile", because we did the p53 "corrected" test on their spermatozoa after 28 ± 3,5 days from the positives of their partners pregnancy test (betaHCG> 400 m U/m L). The group B, subdivided in B1, B2 and B3, includes 169 male partners for a observational period of 60 months. This partners don't report previous conceptions, they aren't smokers, don't make use neither of alcohol nor drugs and don't present pathologic varicocele studied with ecoColorDoppler. They are all married or stable cohabitants from a period of 27-39 months and report to have frequent sex without protection with their partners. Determination of p53 procedure: To separate the spermatozoa from seminal fluid we utilized the Differex™ kit System and the DNA IQ™ kit (Promega). For the p53 test we used the direct DuoSet IC kit and quantitative (R&D System). The p53 values were corrected in respect to the spermatozoa concentration expressed in ng/millions of spermatozoa. **Results:** Group A (39 male) presents "correct" p53 values that vary from 0.35 to 3.20 ng/millions of spermatozoa and group B presents values that vary from 0.68 to 14.53. From group B (48 male) in the observational period we have recorded 21 pregnancies with initial "correct" p53 values that vary from a minimum of 0.84 to a maximum of 3.29. In the subgroup B1 we obtained 8 pregnancies from male partners with a "correct" p53 concentration included between 0.84 to 1.34. In the subgroup B2 we obtained 13 pregnancies from male partners with a "correct" p53 concentration included between 1.66 and 3.29. In the subgroup B3 (121 male) there weren't neither pregnancies nor miscarriages and "correct" p53 values were included between 3.58 and 14.53. **Conclusion:** The results show that the member of the group A with values of 'corrected' p53 between 0.35 and 3.20 were considered

Address for correspondence: Dr. Salvatore Raimondo, Laboratory Research Gentile s.a.s.; 80054 Gragnano, Napoli, Italy.
E-mail: salvatoreraimondo57@gmail.com

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"Fertile", although in the observational period 3 miscarriages happened for 3 partners. 36 partners on 39 (92,3%) had a p53 concentration inferior to 1.65, this value were considered as the extreme to identify this group. The member of the group B1 had "corrected" p53 concentration that were included in the group. In the group B2 were observe 13 pregnancies, so its member were considered "potentially fertile" In the group B3 (121 male) weren't observe neither pregnancies nor miscarriages, so its member were considered "potentially infertile". If further studies confirm these data, we will consider the p53 test ELISA inspected in "correct" p53 as a new and accurate marker of the potential of male fertility.

Keywords: *Bromo domain testis-specific, p53 method ELISA assay quantitative, Sperm DNA fragmentation index*

Introduction

In the industrialized countries birth rate reduction is more common, in Italy is drastically reduced under level of substitution (Eurocast Regional Yearbook 2015).[1]

The male factor is involved in a large percentage of sterility cases because there are many factors that are involved such as genetic abnormalities, acute and chronic diseases, certain medical procedures, environmental and occupational impact, lifestyle, and infective agents. All these factors play a negative role on male fertility.

Even now, about the infertility etiology, lots of questions remain unanswered. The idiopathic sterility is the major male infertility type.[2,3]

Spermatozoa's genetic[4] and molecular biology were neglected over the last decade for an indiscriminate use of assisted reproductive technology that bypassed the need of a "normal" sperm sample.

In spite of different improvements in this area, we had no significant increase of birthrate,[5] so it is necessary to study more the spermatozoa.

We focused on DNA present in the spermatozoa nucleus which is obviously the most important cellular component to complete the reproductive process.

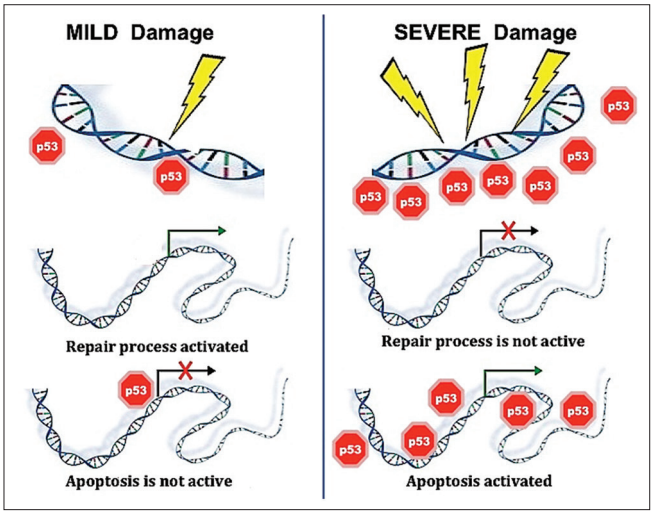

Figure 1: Cellular control mechanism when p53 presence vary

The spermatic chromatin is organized in a specific way to be compact and stable in the nucleus.

The spermatozoa nuclear condensation is due to the substitution of about 85% of hystones (rich in lysine) associated with DNA, with transition protein and protein rich in arginine: protammine.[6-8]

In contrast to the hystones that form a ring structure with DNA, the so-called nucleosome, the protamine are bound to the DNA helix, by strictly flinging around filaments (about 50 kb of DNA per protamine). They form tight loops and are highly organized.

Furthermore, the crossed intra- and inter-molecular disulfide bonds between protamine rich in cysteine are responsible for the compaction and stabilization of spermatozoa nucleus.[6,9]

The result of these specific associations is an extreme nuclear condensation with the reduction of about 10% of the nuclear volume.[8]

The key protein which mediates the chromatin compactation is the protein Bromo domain testis-specific that can promote the nuclear modeling that guarantees the transition between an hystonic chromatinic organization, that is somatic, to protaminic organization which is typical of mature spermatozoa.[8,10]

The characteristic nuclear compactation is important to protect the spermatozoa genome from extern stresses, in fact, physiologic and environmental factors of stress, so genetic mutation and chromosomal abnormalities can possibly interfere the different spermatogenesis phases.

These disorders can cause an anomalous structure of chromatin that is incompatible with fertility.

The genetic disorders that shall arise and are in mature spermatozoa could be packing defects (defective substitution hystone-protamine), nucleus maturation defects, DNA fragmentation (that is single- or double-strand breaks), integrity DNA defects, or chromosome spermatic aneuploidies.[6,7]

One of the factors that contribute to male infertility is the oxidative stress that can alter the different spermatogenesis phases.

In fact, in the testicle exposed to oxidative stress, the p53 expression is increased and spermatic cells proliferation is inhibited.[11]

P53 protein is so called for its molecular mass, of 53 kD,[10] it is involved in the cellular response to the DNA damage.[12,13]

It is known as "the genome guardian",[9,10,14,15] it is a transcription factor specific-sequence that respond to a lots of stress signals and cellular functions.[9,14]

It regulates, deactivates, and represses gene and protein expression responsible for the cellular cycle break and of the apoptosis process [Figure 1].[16,17]

It was assumed a p53 ancestral gene role to ensure the integrity of genomic germline and a correct reproductive process.[18,13]

In fact, p53 protein is highly expressed in the testis, spermatogonia,[19] and primary spermatocytes during the pachitene[20] or pre-leptotene, in the initial phases of pachytene,[20-22] during chromosomal pairing and DNA recombination and repair.[23]

The p53 expression in these phases of spermatogenesis suggests that this protein has a role during meiosis.[14,24,25]

It is possible that p53 protein plays different roles in the DNA reparation, depending on the type of damage[15,26] on the stadium in which the damage happened, and on the available reparation systems.[25,27]

If the damage is serious, p53 has a role to activate the apoptosis process in the germinal cells. But it exist also 53-independent way to activate the apoptosis.[19]

In normal condition, in the majority of body cells, p53 protein is present in low concentration; however, when normal cells are deprived of oxygen or exposed to treatments that could damage the DNA, such as ultraviolet or gamma rays, there is an increased p53 protein expression by the reduction of the molecular rapid degradation process.[10]

Apoptosis is a critical process for the DNA integrity in the germinal cells and to regulate their number.

In fact, p53 is an important apoptosis regulator and for spontaneous mutation in the different spermatogenesis phases.[28]

It was assumed that the absence of p53 protein adapt concentration could involve aberrant spermatogenesis or seminal fluids containing DNA damaged.

Although, from a study of Sue Marty *et al*., 1999[29] qualitative differences are not observed in the anomalies incidence in spermatozoa form and number related to the p53 concentration; these data contrast with Yin *et al*., 1998[30] data which report p53 protein take the control of germinal cells quality inducing spontaneous apoptosis. In fact, the lack of this control comport the accumulation of defeated cells that produce an increased concentration of anomalous spermatozoa and later it compromises the male fertility; these data are supported by more recent studies based on a negative correlation with nemaspermica motility.[31]

Spermatozoa vitality correlates strongly with DNA fragmentation index.[32]

The aim of this work is to analyze and to compare the p53 concentrations obtained by ELISA assay, repetitive and objective method, in the evaluation of spermatic DNA damage. The assay is accomplished on human spermatozoa DNA came from individuals with normal and pathologic seminal parameters.

Materials and Methods

Our work is based on an observational study on 169 male in a period included between March 2012 and February 2017.

The whole study group is made up from 208 male partners aged between 26 and 38 years with volume ejaculate from 0.6 to 5.8 mL and with heterogeneous seminal valuation: 86/208 (41.3%) normospermic; 19/208 (9.1%) mild oligospermic; 51/208 (24.5%) moderate oligospermic; and 52/208 (25.1%) severe oligospermic.

Individuals spermatic evaluation in exam have been detected with seminal fluid text,[33] adopting standard analysis criteria according to the WHO manual fifth edition – 2010 (WHO laboratory manual for the examination and processing of human semen).

Makler counting (Makler Counting Chamber, Sefi-Medical Instruments Ltd.) has been utilized for the evaluation of nemaspermica concentration, expressed in millions in the number of spermatozoa other than in the study of cellular component non nemaspermica (leukocytes, red blood cells, germ line cells).[12]

Processing procedures on samples were made after 30 min from ejaculation.

Samples were divided in two aliquots, one of which has immediately been analyzed with ELISA assay for p53 and the other frozen to −20°C for future investigations.

The subjects signed the informed consent form for the processing of personal and sensitive data, as well as of genetic and biological samples collection in compliance with the applicable laws.[34,35]

The signed informed consent was obtained from the human participants of this study. The procedures

followed were in accordance with the ethical standards of experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 2000.

Our work includes a control group (Group A) made up from 39 male partners called "fertile", because p53 concentration "correct" text is has been carried out on spermatozoa DNA after 28 ± 3,5 days later their partners positive pregnancy test (betaHCG >400 m U/m L).

The team noted (Group B), total observational period 60 months, includes 169 male partners that don't report previous spontaneous conceptions; they aren't ordinary consumers of cigarettes, alcohol or drugs and they don't present pathologic varicocele studied with ecoColorDoppler.[36-39]

They are married or stable cohabitant (for a period of 27–39 months) and they report to have frequent and free sex with their partners. The observational period for each male partners was 24 months.

All samples were analyzed with p53 quantitative dosage "correct" in comparison to spermatozoa number.

The method was presented in an our precedent work[40] and it is reported below.

Separation of sperm from seminal fluid

To perform the isolation of sperm from semen, the Differex System™ – for use with the Differex Magnet™, and the DNA IQ™ SystemSmall Sample Casework Protocol kits were used (Promega Corporation, Madison WI, USA). In 1985 Gill *et al*.,[41] developed a method to separate spermatozoa from epithelial cells in a sample of human semen. The separation protocol reported below has been developed for this project. It requires about 150 min to obtain the complete separation and purification of the sperm DNA.

The number of sample and reagents quoted has been calculated for a single sample and a single experiment in the following protocol.

One hundred microliters of sample were placed in a 1.5 ml tube with 400 µl of Digestion Solution containing 6 µl of diluted Proteinase K and 364 µl of Digestion Buffer. The tube was vortexed for 30 s at 14,000 rpm, incubated for 90 min at 56°C and then centrifuged for 10 min at 14,000 rpm in a microcentrifuge at room temperature, not before having marked the position where the pellet would form.

Then 3.5 µl of DNA IQTM Resin were added in the opposite position of the pellet and the tube was placed on Differex Magnet™ so that the resin, attracted by the magnet, would coat the pellets. The yellow liquid layer, containing epithelial cells, was then removed.

The sample was then washed three times with 500 µl of nuclease-free water and the last washing volume was not removed.

The tube was centrifuged again at 14,000 rpm for 10 min and 3.5 µl of DNA IQTM Resin were added in a position opposite the pellet and positioned in Differex Magnet™ so that the resin would coat the pellets. After three washes, a further 500 µl of nuclease-free water together with 100 µl of Separation Solution were added, so that the resin would coat the pellets. The washing and separation solutions were then removed and the pellet was resuspended by adding 400 µl of 0.9% sodium chloride solution.

To extract the sperm DNA, 250 µl of Lysis Solution (containing 2.5 µl of DTT and 252.5 µl of Lysis Buffer) were added to the tube, which was then vortexed for 3 s at high speed and incubated for 5 min at room temperature. After having vortexed again for 3 s, the tube was positioned in the Differex Magnet TM so that the separation would occur instantaneously and the supernatant was then removed and stored in another tube ("lysed sample").

Then 100 µl of Lysis Solution were added and the tube was removed from the Differex Magnet™. After vortexing for 2 s, the tube was put back in Differex Magnet™ and the entire Lysis Solution eliminated.

To perform the washing 100 µl of Wash Buffer were added after removing the tube from the Differex Magnet™ and the sample was vortexed for 2 s at high speed.

Once the tube was repositioned in Differex Magnet™, the entire wash solution (containing 500 µl of Wash Buffer, 250 µl of Isopropyl Alcohol and 250 µl of ethanol) was eliminated.

The washing procedure was repeated three times.

The resin was allowed to air dry for 5 min, leaving the tube with the cap open.

Subsequently, 100 µl of elution buffer were added (10 mM Tris pH 8.0 and 0.1 mM ethylenediaminetetraacetic acid [EDTA]), and the tube was vortexed for 2 s. incubated for 5 min at 65°C vortexed again and placed immediately on Differex MagnetTM. The solution containing the DNA was carefully transferred in a new tube ("lysed sample and refined DNA").

Quantitative p53 dosage with ELISA assay

A direct and quantitative ELISA assay was used to measure p53 (DuoSet IC, Human Total p53 R and D Systems Inc. Minneapolis, USA).

Briefly, 100 µl of the capture antibody, appropriately diluted, were pipetted into each well of a 96 well microplate, which was then sealed and incubated

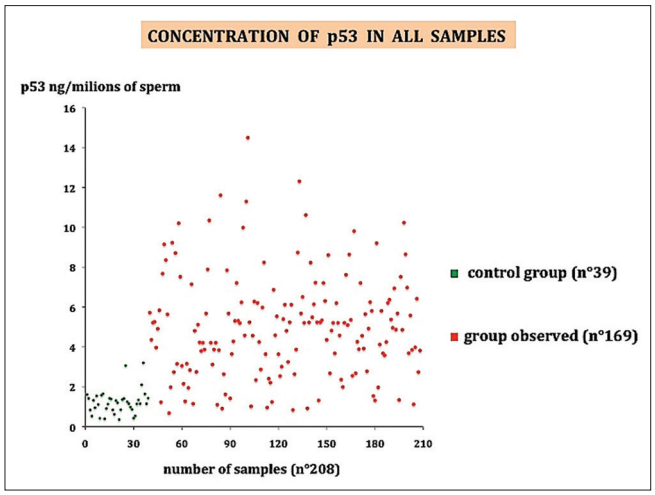

Figure 2: p53 diagram of concentrations into two groups in examination

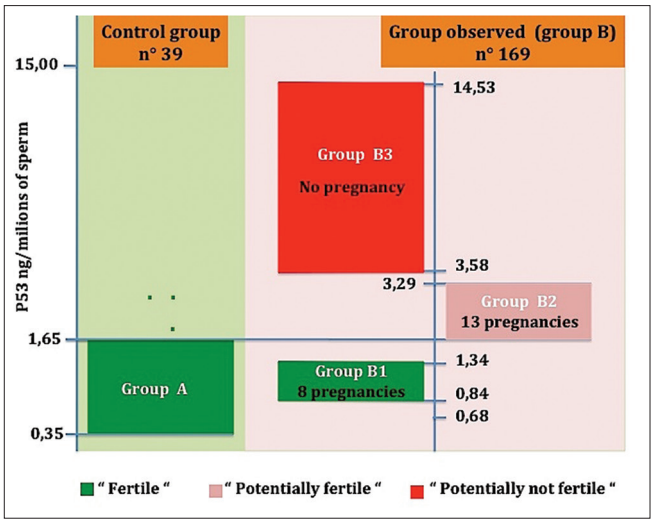

Figure 3: Summary of the obtained data and their grouping

overnight at room temperature. The next day the plate was washed three times with 400 µl Wash Buffer (0.05% Tween 20 in PBS, pH 7.27.4, filtered at 0.2 µl).

Each well was blocked with the addition of 300 µl of stop solution (Sample Diluent Concentrate: 5X PBS, 5 mM EDTA, and 2.5% Triton X100). The plate was then incubated at room temperature for 2 h. Extraction and washing steps were repeated. The standards were prepared by diluting in IC Diluent # 4 (1 mM EDTA, 0.5% Triton X100 in PBS, pH 7.2–7.4.) and using IC Diluent # 4 like standard zero.

Then 100 µl of sample or standard was added ("lysed and purified DNA" cell preparations), the plate was sealed and incubated for 2 h at room temperature. After the incubation, extraction and washing were repeated and 100 µl of detection antibody (total p53 detection antibody), appropriately diluted, were pipetted in each well.

The plate was sealed and incubated for 2 h at room temperature. After the incubation, extraction and washing were repeated.

One hundred microliters of StreptavidinHRP were then added and the plate was incubated for 20 min at room temperature. After the incubation, extraction and washing steps were repeated and 100 µl of Substrate Solution (1:1 mixture of reagent A and reagent B) were added to each well and the plate was incubated for 20 min at room temperature.

Finally, 50 µl of Stop Solution were added to each well.

The optical density was analyzed using a microplate reader set at 450 nm with a software that automatically calculated the concentrations expressed in pg/100 µl.

Results

The p53 values were also corrected ("corrected p53") because the dosage of p53 was performed on 100 µl of sample. The p53 values were therefore related to 1/10 of the value of sperm counts and the corrected p53 is a reliable estimate calculated for 1 ml of semen.

$$\text{Corrected p53} = \frac{\text{Value of p53 ng / 100 } \mu\text{l}}{\text{1/10 of the spermatic count/ml}} = \text{ng/million of spermatozoa}$$

The value of p53 was measured in three different cell preparations for each sample, there have been no significant changes.

P53 protein "corrected" concentration determinated in every subject that was included in this study are reported below in Figure 2 and subsequently they were subdivided into 3 groups with subgroups.

The Group A (39 male) presents p53 values "corrected" that vary from 0.35 to 3.20 ng/million of spermatozoa, the Group B presents values that vary from 0.68 to 14.53.

In accordance with the results obtained the Group B was divided, for conveniences interpretative, in B1, B2 e B3.

From group B (48 male) during the observational period, 21 pregnancies happened. The initial p53 protein "correct" values, for obtained conceptions, vary from a minimum of 0.84 to a maximum of 3.29.

In the subgroup B1 we recorded 8 pregnancies from male partners with a p53 concentration "corrected" included between 0.84 and 1.34.

In the subgroup B2 we recorded 13 pregnancies from male partners with a p53 concentration "corrected" included between 1.66 and 3.29.

In the subgroup B3 (121 male), there were neither pregnancies nor miscarriages, and p53 values were included between 3.58 and 14.53 [Figure 3].

The results show that the members of the group A with values of p53 between 0.35 and 3.20 can considered as "Fertile," although in the observational period three spontaneous miscarriages happened for three partners.

As 36 partners on 39 (92.31%) had a p53 concentration inferior to 1.65, we consider this value as the extreme to identify this group.

The members of the group B1 had p53 concentration "corrected" that were included in the Group A.

In the group B2 because of the 13 pregnancies are to be considered "potentially fertile."

In the group B3 (121 male) as we didn't observe pregnancies and miscarriages, we can consider its member "potentially infertile."

Discussion

We always try to find new potential male fertility markers that could help to predict the capacity of conceive and find deficits that could differentiate fertile and unfertile male.

The localization and the concentration of one particular protein in the spermatozoa could be useful to understand its function in the reproduction and be able to participate in case of deficit.

The glycolitic enzyme 6-phosfofruttokinase is localized in the acrosomial region and in the middle tract and regulate the semen principal functions as motility and fertilization.[42]

Proteolityc enzyme inside to acrosomial region were activated to be released during the acrosomial reaction before the fertilization.[43]

A complex protein contains arilsulfatasi A, binding molecule of semen 1 and Heat Shock Protein 2 (HSPA2), are localized in the human spermatozoa apical head region.[44]

The antioxidants secreted from male accessory sex glands are all important to support spermatozoa DNA integrity.

The semen DNA damage induced from oxidative stress is actually increased after removing accessory sex glands (ASG).[45]

The spermatozoa head morphology and the middle tract are critic to determinate the capacity of fertilize and spermatozoa motility; the DNA is accumulated in the spermatozoa head and mitochondria of the middle tract are as a power station that generates ATP for the spermatozoa movement.[46]

53BP-1 is a fixer protein of DNA damages during the mouse oocytes maturation.[47]

Even though 53BP-1 alone can't be the principle regulator for the initial embryos development but promotes p53 transcription activity, and so, it can play a role in the initial embryos development.

P53 nuclear expression is also in the bovine blastocystis after exposition to high temperature.[48]

An increased Heat HSPA2 production, caused from exposition to high temperature could provoke cellular apoptosis.[48]

Shimura *et al*.[49] studied the answer to the damage in mouse fertilized zygotes with spermatozoa irradiated and they noted a DNA damage in the S-phase p53-dependent.

When the p53 knockout oocytes were fertilized with irradiated spermatozoa, the resulting zygotes showed a chromosomes anomalous separation in the first phase of splitting, and an altered embryos morphology and they include a high number of micronucleus with embryos development block to 2 cells.[50]

The results showed that the control of S-phase p53-dependent is important in the chromosomes reparation in the initial embryos development phases with DNA damage.

It was assumed for the protein p53, an important role in the oocytes maturation, bastocystis development and embryos implant in the reproduction.[51]

The p53 expression is low in the mouse zygotes and in the scission phase, but it successively increases during the blastocystis stage.

Blastocystis with *in vivo* fertilization have p53 low concentration, on the contrary the protein p53 expression is higher in embryos produced from vitro fertilization.

These observations suggest that embryonic culture induces to accumulation of p53 protein transcriptional activity in blastocystis and it can be one of reasons for the late embryo growth.[52]

The human embryos produced from intracytoplasmatic sperm injection have a high p53 protein nuclear expression, associated to a late embryonic development.[53]

From these considerations emerge a more complex role for protein p53 that is different from the only spermatozoa DNA integrity control; it is also hypothesized a control on time and ways of embryonic development.

According to precedent works of others study groups, we understand better why in the "fertile" group subjects the p53 concentration is significantly lower respect to the "observed" group.

Our work integrates very well in this scenery and on the importance to have an objective and repetitive data, preliminary to conception both *in vivo* and *in vitro*.

Conclusion

The protein p53 plays an important role in the cell and it is normally presents in each type of cells, it has a central role in a extended protein net which allows the state of "good health" of a cell and cellular DNA.

The p53 protein is the "director" of "well-orchestrated" detection system and of control cellular damages.

When a damage happens, protein p53 activity is fundamental to decide if repair it or induce to death the cell.

The cell death that suffered severe DNA damages is providential for the organism because it impedes the cellular reproduction with dangerous and noxious mutations; moreover, in the case of conception, it impedes an anomalous embryonic development.

The p53 increase is proportionate to the cellular damage so its quantitative evaluation is index of DNA damage.

Its role is also interesting as controller and regulator of the spermatogenesis meiosis process and for its function to control embryonic development.

It just keeps getting always more incessant the idea that, the p53 protein plays different actions on control and systemic cellular development and on the human reproductive project control.

Our work integrates well on the knowledge presence of p53 protein to differentiate the different degrees of male infertility.

There is no doubt that further results will occur to compare this data but knowing the p53 concentration with ELISA method reported in relation to the spermatozoa number could have an important role in to the preconception screening identifying subjects with an "reduced fertility" and in the framework of PMA could be predictive on the *in vitro* fecundation result.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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