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Chapter

Presence of p53 Protein on Spermatozoa DNA: A Novel Environmental Bio-Marker and Implications for Male Fertility

Salvatore Raimondo, Mariacira Gentile, Tommaso Gentile and Luigi Montano

Abstract

Many studies suggest a direct relationship between toxic effects and an increase in the p53 protein on cellular DNA. For our studies, we used sperm DNA as an indicator of environmental toxic effects, dosing p53 quantitatively. To assess possible variations, we used semen samples from two homogeneous male groups living permanently in areas with different environmental impact. The toxic effects of the selected high environmental impact area are caused by both soil and air pollution, while the selected low environmental impact area is a nature reserve where there are no landfills, but only rural factories. As we work with reproductive cells, our interest was inevitably focused on sperm DNA damage and whether this damage could affect their fertilizing capacity. The length of telomeres and the quantification of protamines are being studied to better define the possible damage.

Keywords: p53, DFI, spermatogenesis, infertility, sperm DNA damage

1. Introduction

The combination of health and environment is now a major issue on the political agenda of many governments because of its social and cultural relevance to both individual and collective health.

The World Health Organization (WHO) has set – as one of its main priorities – the understanding of the relationship between sources of pollution and the effects on health, the development of indicators and the prevention of diseases linked to an unhealthy environment, which are a major cause of mortality and morbidity [1]. Sitography (https://apps.who.int/iris/handle/10665/204585).

Therefore, the 'eco-epidemiological' study of the determinants of health and their spatial and temporal distribution is of great interest, as these are strongly linked to social, cultural and environmental factors that mutually interact and affect the genetic heritage of individuals.

To understand which elements should be taken into account, from an epidemiological point of view, in order to assess the impact of different factors on health status is a very complex task.

The combination of environmental, territorial and epidemiological data, as well as other health, demographic, cultural and social indicators, allows us to draw up risk thresholds or possible risk scenarios for a specific population (www. epicentro.iss.it).

It's now well acknowledged that pollution plays a major role in determining an adverse health effect, and that the health condition of the population varies according to whether environmental pressure is greater or lower in an area compared to another, varying not only over countries, but also within the same country or even the same region.

Human semen is an early sensor of the environmental contamination status and therefore the first to be affected [2, 3]; Kimberley [4–6].

Chemical substances found in the environment (such as heavy metals and dioxins) in food (such as agro-pharmaceuticals or insecticides), as well as unhealthy life styles or electromagnetic pollution are the main cause of alterations of semen parameters [7, 8]. The well-known mechanisms whereby chemical and physical environmental factors, whether combined or not, interfere with reproductive function are: induction of oxidative stress, hormonal imbalance, genetic and epigenetic alterations [9, 10].

Concerning the sperm decline of the last few decades, there is much concern among researchers dealing with human reproduction. More specifically, a major meta-analysis study on data collected from 1973 to 2011 among the male population in Western countries suggest that the concentration of spermatozoa drastically decreased by more than 50%, from 99 million per milliliter to 47 million per milliliter [11] and the situation is certainly no better in some countries such as Africa, India, Brazil and China [12–15]. The decline in semen quality seems to mirror the impact that pollution and bad lifestyles have had and are still having on human health.

Usually, all forms of stress, whether endogenous or exogenous, affecting the organism lead to a response from the latter, primarily from the basic morpho-functional unit, i.e. the cell.

The cell fate decision machinery is composed of multiple complex signaling pathways, in which p53 plays a central role in coordinating the multiple cellular signaling pathways as well as determining cell fate [16, 17].

When this factor is diverted from its normal control and repair functions, the regulation of cell growth may be blocked and the cell rapidly multiplies abnormally [18, 19].

The first evidence that p53 could control cell fate was gathered from studies using a myeloid leukemia cell line [20]. The finding that p53 can lead to apoptosis was confirmed by analogous experiments in which a temperature-sensitive p53 or WT p53 was also forcibly expressed in erythroleukemia cells [21], in a colon cancer cell line [22] and in a Burkitt's lymphoma cell line [23]. The p53 protein is not essential for our survival, but its role in protecting our organism from modified cells is crucial, hence the definition of '*Guardian of the Genome*', referring to its role in preserving stability by preventing mutations [24]. Since the biological role of p53 is to ensure the integrity of the genome in cells, it can stimulate repair processes and protective mechanisms, or stop cell division and stimulate induction of cell death (apoptosis) [25]. Primarily through its transcription factor function, p53 has the ability to induce cell-cycle arrest and apoptosis, both of which protect the cell and the organism from DNA damage that leads to genome instability [26]. The activity of the p53 protein is stimulated in response to DNA damage and various genotoxic insults that ultimately compromise genome integrity [27]. Following genotoxic stress, p53 decides cell fate: it may induce growth arrest, DNA repair or, in case of exposure to severe DNA damage, even induce cell death by apoptosis. The loss of

p53 regulatory functions and activities are involved not only in the development of malignant diseases, but also in cardiovascular, neuro-degenerative, infectious and metabolic diseases, as well as participating in the aging process of the body. p53 is capable of binding specific reactive DNA elements, and the specificity of transcriptional activation depends on the ability of the DNA-binding domain and p53 protein to interact with the regulatory regions of certain genes. Transcriptional activation is determined by the N-terminus of p53, this contains several regions which interact with the transcriptional mechanism and recruiting factors that modify the local chromatin structure [28]. The p53 protein is mainly regulated by post-translational modifications, primarily phosphorylation, and the accumulation of p53 is the first step in response to cellular stress [29].

The N-terminus is strongly phosphorylated while the C-terminus contains phosphorylated, acetylated and sumoylated residues. N-terminal phosphorylations are important for stabilizing p53 and are crucial for acetylation of C-terminal sites, which in combination lead to the p53-mediated response to genotoxic stresses [30].

The degradation of p53 depends on the interaction between two proteins and is mediated by the proteasome. The link between N-terminal Mdm2 and C-terminal p53 leads to the degradation of p53 by Mdm2. Any alterations in the central DNA binding domain of p53 do not cancel the sensitivity of the protein to degradation mediated by Mdm2 [29, 31, 32].

In response to DNA damage, ATM kinase rapidly phosphorylates p53 at Ser15. The serine/threonine kinase Chk2 acts downstream of ATM by phosphorylating p53 at Ser20. These phosphorylated sites in the N-terminus of p53 are in proximity to the Mdm2 binding region of the protein, thus blocking the interaction with Mdm2, leading to stabilization of p53, which eludes proteosomal degradation [30]. Recent studies suggest that constitutive phosphorylation of p53 by protein kinase inhibits the regulation of sequence-specific DNA binding, oligomerisation status, nuclear import/export and ubiquitination [30]. Furthermore, constitutive phosphorylation of p53 by protein kinase C (PKC) at the C-terminal domain contributes to its degradation through the ubiquitin-proteasome pathway [33].

We studied the p53 protein by using it as a direct indicator of cellular DNA damage caused by environmental toxic factors, comparing levels in male gametes (spermatozoa) and associating them with the fertilizing capacity of the spermatozoa themselves. On average, it takes 64 days to complete spermatogenesis, but this varies from individual to individual. Spermatozoa are produced non-stop every day from puberty onwards over a lifetime [34]. This feature could be used to monitor changes in environmental impact, drug response (antioxidants) and/or lifestyle. Sperm chromatin is very compact and stable in the nucleus, unlike the structure of somatic cells. Nuclear condensation in spermatozoa is due to the replacement of about 85% of the DNA-associated lysine-rich histones with protamines, arginine-rich transition proteins [35, 36].

While histones form a ring with DNA (nucleosomes), protamines are bound to the grooves of the DNA helix, wrapping tightly around the strands of DNA (approximately 50 kb of DNA per protamine) to form tight and highly organized rings. Moreover, inter- and intramolecular disulphide bonds between cysteine-rich protamines are also responsible for the compaction and stabilization of the sperm nucleus [36, 37]. This leads to an extreme nuclear condensation and a reduction of about 10% in the size of the nucleus [35]. The BRDT protein (Bromodomain Testis specific) is the key protein that mediates chromatin compaction and can facilitate nuclear remodeling, thus ensuring the transition between the histone organization of the chromatin, or somatic, and the protamine nucleus, typical of the mature spermatozoon [38]. Specific nuclear compaction is relevant to protect the sperm genome from stressogenic insults. Indeed, both physiological and environmental

stress, as well as genetic mutations and chromosomal abnormalities, can interfere with the processes of spermatogenesis. These changes can lead to an abnormal chromatin structure incompatible with the reproductive plan. The faults of genomic material found in mature spermatozoa can impair nucleus formation (defective histone and protamine substitution) and maturation, leading to DNA fragmentation (i.e. single- or double-strand breaks) and DNA integrity defects or chromosomal aneuploidy in the spermatozoa [36]. In atypical and immature spermatozoa, DNA may fragment, lose its functional integrity and thus result in functional defects in the spermatozoa. As a matter of fact, DNA fragmentation is particularly common in sub-fertile human spermatozoa [36].

p53 is one of the most investigated tumor suppressor proteins and is involved in cell cycle regulation, through its effects on transcription regulation in response to DNA damage and cell stress, resulting in DNA repair, cellular senescence, growth suppression, or apoptosis. Studies also indicate the involvement of p53 in spermatogenesis [39]. During normal spermatogenesis, p53 is expressed in the intermediate layer of the seminiferous tubules, in spermatocytes and round spermatids, suggesting that it might play a role in spermatogenesis [40, 41].

It has actually been suggested that the role of the ancestral p53 gene is to ensure the integrity of the genomic germ line and the accuracy of developmental processes [42]. The p53 protein fulfills several functions in the meiotic and premeiotic stages of spermatogenesis [43]. Possibly, p53 plays different roles in DNA repair, depending on the type of damage [44], the stage at which the cell was damaged and the possible repair pathways available [43]; in short: p53 helps the spermatozoon to deal safely with DNA damage [45].

DNA damage, resulting from normal metabolic processes in the cell, occurs at a rate of 1000 to one million molecular lesions per cell per day. Nevertheless, several causes of damage can increase this rate. Causes of alterations in sperm DNA include both extrinsic (environmental and lifestyle factors) and intrinsic causes. Apoptosis, or programmed cell death, is a natural process of cells whereby an aged or damaged cell dies without damaging its neighbors [46].

As for sperm cells, apoptosis mainly occurs to spermatogonia during spermiohistogenesis, a significant factor in blocking the complete development of a damaged cell. Apoptosis also occurs in mature spermatozoa when they manifest alterations that could be passed on to their offspring or that hinder the normal functions of the cell itself [47].

Many studies have been carried out over the years to assess the harmful effects of environmental factors on sperm DNA. The first studies were carried out on the effects of cigarette smoking and new techniques were developed to highlight the damage [48]. When comparing the DNA fragmentation index of spermatozoa from smoking and non-smoking patients, researchers were able to determine that the DNA damage detected in smokers was greater [49]. DNA breaks can be caused by the presence of carcinogens and mutagens in cigarette smoke [50]. Harmful substances, including alkaloids, nitrosamines, nicotine, cotinine and hydroxycotinine are found in cigarettes and produce free radicals [51]. Kunzle et al. [52], an association between cigarette smoking and sperm quality was found among extrinsic causes, i.e. due to environmental factors. Rodgman and Perfetti [53] and Alchinbayev et al. [54] highlight mutagenic properties of cigarette constituents and altered sperm quality.

Oxidative stress (OS) is the focus of in-depth studies, due to the potential harmful effects of high levels of reactive oxygen species (ROS) [55]. An increase in leukocytes is supposed to determine an increase in ROS production in semen but the process is still not very clear [56].

Environmental toxic effects damage sperm nuclear and mitochondrial DNA. The assessment of damage related to non-functional spermatozoa is extremely

significant for male fertility [57]. Sperm DNA damage reaches higher levels in infertile men than in fertile men and, as a matter of fact, more and more studies prove that sperm DNA damage negatively affects reproductive outcomes [58]. These damages may not only impair fertility, but also increase the transmission of genetic diseases during ART procedures [59]. Spermatozoa produce small amounts of ROS and these play a significant role in many sperm physiological processes, such as capacitation, hyperactivation and sperm-oocyte fusion [60, 61]. However, ROS must be inactivated continuously to keep only a small amount necessary to preserve normal cell function. Overproduction of ROS in semen can result in sperm DNA damage. An overproduction of ROS in semen can result in sperm DNA.

During their maturation process, spermatozoa extrude their cytoplasm, the main source of antioxidants. Once this process is slowed down, the residual cytoplasm forms a cytoplasmic droplet in the sperm mid region. These spermatozoa carrying cytoplasmic droplets are immature and functionally defective [62]. The residual cytoplasm contains a high concentration of certain cytoplasmic enzymes (G6PDH=Glucose-6-Phosphate DeHydrogenase, SOD= SuperOxide Dismutase), which are also a source of ROS [62]. The lack of cytoplasm leads to a decrease in antioxidant defense. This process is the link between poor sperm quality and high levels of ROS [56, 63].

Human ejaculate consists of different cell types: mature and immature spermatozoa, round cells from different stages of the spermatogenic process, leukocytes and epithelial cells. Peroxidase-positive leukocytes and abnormal spermatozoa continuously produce free radicals. Spermatozoa are extremely sensitive to damage caused by excessive ROS because their cytoplasmic membranes contain large amounts of polyunsaturated fatty acids (PUFAs), which intensify lipid peroxidation by ROS, resulting in a loss of membrane integrity [55, 64, 65]. There is a strong positive correlation between immature spermatozoa and ROS production, which in turn is negatively connected to semen quality [66]. Moreover, the concentration of mature spermatozoa with damaged DNA was found to increase along with immature spermatozoa in the human ejaculate [47].

Over the last few decades, scientific evidence of the harmful effects on spermatogenesis of occupational exposure chemicals known as endocrine disruptors (EDCs) on the reproductive system has been progressively accumulating [67, 68]. Environmental pollution is one of the main sources of ROS production and has been involved in the pathogenesis of poor semen quality [69]. A study carried out on workers at motorway toll booths, who are constantly exposed to environmental pollutants, correlated blood methaemoglobin and lead levels in semen were inversely correlated, compared to local male inhabitants not exposed to heavy traffic pollution levels. These results suggest that nitrogen oxide and lead, both found in the composition of car exhaust, negatively affect semen quality [70]. Furthermore, increased industrialization has led to a high deposition of highly toxic heavy metals in the atmosphere. Paternal exposure to heavy metals such as lead, arsenic and mercury is associated with a decrease in semen parameters, resulting in a reduced fertility capacity [71, 72].

Global pollution was negatively associated with sperm count in a group of Californian sperm donors. This study shows a significantly negative relationship between sperm concentration and ozone levels measured 0–9, 10–14 and 70–90 days prior to semen collection. Since ozone appears not to be involved in oxygen transport mechanisms, the mechanism of action remains to be clarified, although the observed effect reinforces the evidence on the relationship between spermatogenesis and traffic-related pollution [73].

As for pesticides to which the population is exposed or has been exposed in the past, the available results of specific studies on their effects on spermatogenesis are still inconsistent. This also applies to the well-known DDT, which is now banned in Western countries: the effect of this pesticide on spermatogenesis is low [74].

Reproductive capacity, on the other hand, does not seem to be adversely affected other than marginally [75, 76].

Similar considerations apply to other persistent contaminants in the environment. Contrary to this general consideration, an American study reported a highly significant association between urinary levels of the metabolites of three pesticides and a reduced number of spermatozoa in the ejaculate. However, this study also found a decrease in the number of spermatozoa, albeit insignificant [77].

However, Marty et al. [78] found no qualitative differences in the incidence of abnormalities in spermatozoa form and number related to p53 concentration, in contrast to the data reported by Yin et al. [79]. The latter reported that the p53 protein controls germ cell quality by inducing spontaneous apoptosis, failure to do so results in the accumulation of defective cells, which increases the concentration of abnormal spermatozoa and subsequently compromises male fertility. These data are supported by more recent studies reporting a negative correlation with nemaspermic motility [80]. Sperm vitality correlates strongly with the DNA fragmentation index [81] and oxidative stress, caused by harmful environmental exposure, is believed to have a significant role in the development and progression of diseases [82].

The function of p53 to govern the fate of cellular life, when it is damaged, is now well known. p53 monitoring is useful for assessing the effects of pollutants on DNA. Considering the changes of p53 in relation to the degree of the DNA damage, quantitative measurement of the p53 protein on sperm DNA was performed to evaluate:

a. possible negative effects of pollutants on male fertility in subjects living in high environmental impact area;

b. possible sperm DNA damage following manipulation of spermatozoa during the separation procedures for ART techniques, evaluating the quality of the embryos too.

For this aim, the method proposed by Raimondo et al. [83] consists of 3 steps:

1. separation of spermatozoa from seminal fluid using a forensic method [84].

2. isolation of nuclear DNA from spermatozoa.

3. quantitative evaluation of the p53 protein by ELISA.

The concentration of the spermatozoa is reported in Mil/ML, 100 micronliters of seminal fluid are used for the p53 protein assay, therefore the p53 protein concentration is correlated to 1/10 of the sperm count per ML. The correlation existing between p53 concentration and number of spermatozoa per ML, allows us to report the p53 values in "p53 ng/million spermatozoa" [83].

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

p53 protein values are expressed in ng/million spermatozoa.

2. p53 concentration on sperm DNA and environmental impact

The ancestral p53 gene is involved in ensuring the integrity of the genomic germline and the replication of developmental processes. The p53 protein is highly

expressed in testicles, spermatogonia and primary spermatocytes during pachytene or pre-leptotene, when chromosome pairing, recombination and DNA repair occur. The expression of p53 at these stages of spermatogenesis suggests that it plays a role in meiosis. Apoptosis is a critical process for the integrity of germ cell DNA and in regulating their quantity.

If p53 concentrations are not adequate, this would lead to aberrant spermatogenesis or sperm containing damaged DNA. Failure to control p53 leads to the accumulation of defective cells, which increases the concentration of abnormal spermatozoa [85] and subsequently impair male fertility. These data are supported by more recent studies reporting a negative correlation with nemaspermic motility [81, 86]; additionally, sperm vitality is strongly correlated with DNA fragmentation index [87, 88].

We carried out an observational study on 117 male subjects, aged 18–38 years (28.02 + 4.99), permanently living in low and high environmental impact areas from July 2015 to June 2020.

Our purpose is to assess the concentration of the p53 protein on spermatozoa DNA using an immunoenzymatic assay (ELISA) as a marker of possible damage. The whole group consisted of 117 males divided as follows: 49 of them permanently living in low environmental impact areas (southern area of Salerno; Campania, Italy), aged 18–38 (28.04 + 4.84) years identified as Group A; 68 of them permanently living in high environmental impact areas (northern area of Naples 'terra dei fuochi'; Campania, Italy), aged 18–37 (28.01 + 5.13) years identified as Group B. The observation lasted 60 months, among the requirements: homogeneous behavior and lifestyle, no habitual smokers, no alcohol abusers and except for some of them who has used cannabis in the past (whose suspension is reported from 6 to 36 months before the collection of semen), they do not perform activities considered to be an environmental occupational risk and did not suffer from pathological varicocele at preliminary examination with Color Doppler [50, 55, 59, 89, 90].

The examination of the human semen was evaluated using the standardized analysis criteria according to the WHO Laboratory Manual for the examination and processing of human semen, fifth edition – 2010. In Group A, the ejaculate volume ranged from 1.1 to 4.9 mL, and the seminal evaluations were as follows: 24 samples (48.9%) normospermic; 14 (28.6%) mild oligospermic; 7 (14.3%) medium oligospermic; 4 (8.2%) severe oligospermic. In group B, the ejaculate volume varies from 0.6 to 7.1 ml, the seminal evaluations were as follows: 13 (19.1%) normospermic; 20 (29.4%) mild oligospermic; 27 (39.7%) medium oligospermic; 8 (11.8%) severe oligospermic. The Makler Counting Chamber (Sef-Medical Instrumens ltd.) was used to evaluate the nemasperm concentration expressed per ml, the number of spermatozoa as well as the study of the non-nemaspermic or immature nemaspermic cellular component (leukocytes, red blood cells, germ line cells) (**Table 1**) [49, 53, 91].

Sample processing procedures were carried out 30 minutes after ejaculation. Samples were divided into two aliquots, one of which was processed immediately for the p53 ELISA assay and the other frozen at −20° for later examination. A quantitative assessment of p53 corrected according to the number of spermatozoa was performed on all samples and values are expressed in ng/MLN spermatozoa. The method employed was that suggested by Raimondo et al. [83].

Data suggest that there are significant differences in seminal parameters from groups A and B.

These variations are probably due to the effects of environmental factors on the organism, and on semen in particular (**Figure 1**). This finding is further supported by the fact that the examined groups are homogeneous, as previously reported.

	Low environmental impact Group A	High environmental impact Group B
MLN spermatozoa/mL	41.26 ± 14.6	27.12 ± 9.8
Motility type (a)	33.7 ± 11.5	28.1 ± 9.6
Morfology	15.6 ± 2.8	13.8 ± 3.8
Vitality	61.2 ± 6.3	57.4 ± 8.1

Table 1.
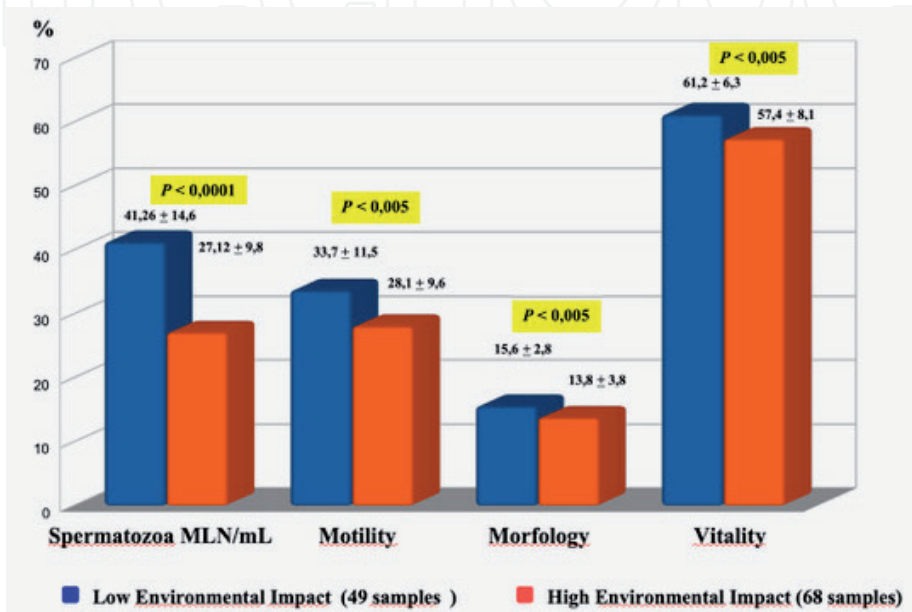*Description of seminal parameters of the two groups.*

Figure 1.
Significant variations in the main seminal parameters in groups A and B.
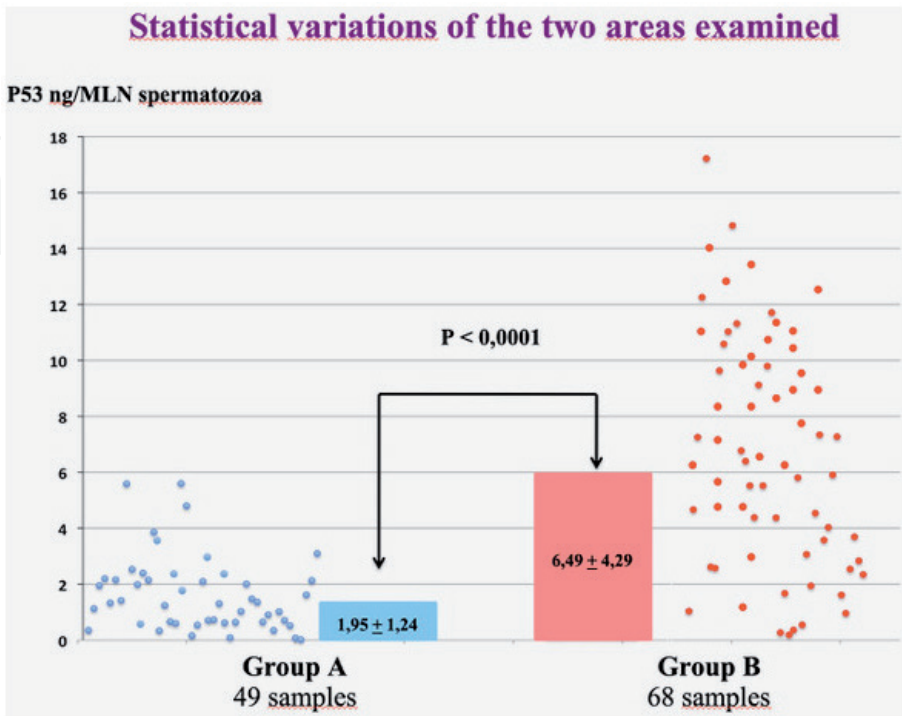

Figure 2.
Statistical changes in p53 values in the two areas under examination.

In order to assess the possible damage to the spermatozoa DNA, we used the quantitative analysis of the p53 protein and results show a significant variation (p < 0.0001) between the two groups: group A; p53= 1.95+1.24: group B; p53= 6.49+4.29 (**Figure 2**).

These data highlight that environmental factors are strongly associated with seminal parameters alteration and with sperm DNA damage in subjects living in high environmental impact areas and, inevitably, these alterations may interfere with the reproductive plans of couples living in these areas.

3. p53 concentration on sperm DNA and male fertility

Spermatogenesis is male gametogenesis, i.e. the maturation process of male germ cells that takes place in testicles under the stimulus of the hormones FSH and testosterone when the individual has reached puberty. Although it's the equivalent of oogenesis in women, it differs from the latter mainly in terms of timing: sperm production begins at puberty and lasts a lifetime, oogenesis begins before birth and then stops and resumes when the woman reaches sexual maturity, ending at menopause. Spermatogenesis is not to be confused with spermiogenesis, which is the third and final stage of spermatogenesis, during which the final differentiation takes place, leading to the development of mature spermatozoa [92, 93].

At the end of spermatogenesis, only 15–20% of spermatozoa are normal, the residual being functionally or morphologically abnormal spermatozoa.

Spermatogenesis takes place inside the testes and more precisely in the seminiferous tubules, which are blind-ending tubules that converge in the recti seminiferous tubules. The tubules recti then converge to form the *rete testis*, from which 15–20 efferent ducts drain into the epididymis and then continues into the vas deferens. The wall of these seminiferous tubules consists of supporting cells, called Sertoli cells, and various germ cells that make up the various stages of spermatogenesis. The duration of spermatogenesis can take 70 to 90 days and begins with the division of undifferentiated cells located near the basal lamina of the seminiferous tubule (spermatogonia). These cells undergo mitosis and meiosis, resulting in the production of mature cells (spermatozoa) which detach from the most luminal part of the tubular wall. Germ cells then undergo a process that brings them from the most marginal regions of the wall towards the most apical regions, until they are released into the lumen of the tubule. The cells involved in spermatogenesis are divided into two large groups: germ cells, consisting of spermatozoa and their precursors, and non-germ cells, cells that are not precursors and never become gametes, but have trophic and regulatory functions [92, 93]. The primordial germ cells settling in male gonads form hollow structures called seminiferous tubules, whose wall consists of somatic cells called Sertoli cells. Outside the seminiferous tubule, within the connective tissue that surrounds it, there are the Leydig cells, responsible for the production of testosterone. In this situation, the germ cells, represented by the A1 type spermatogonia, which have already undergone a cellular multiplication during organogenesis, remain dormant until sexual maturity. The Sertoli cells are tightly connected to each other in the basal compartment by occluding junctions that together form the blood-testicular barrier. This barrier means that the seminiferous tubule is structured into two compartments: the basal compartment (housing the spermatogonia and the leptotene spermatocytes) and the adluminal compartment (housing the more mature spermatocytes, spermatids and spermatozoa) [94, 95].

The blood-testicular barrier has several functions: it ensures the preservation of distinct microenvironments between the two compartments so as to help meiosis and spermiohistogenesis in the adluminal compartment and prevent possible

immunological responses following exposure to germ antigens or the transit of macromolecules from the adluminal compartment into the bloodstream.

The spermatogenesis is a complex process in which differentiation and mitosis of a group of starting stem cells take place. The germ cell is called a spermatogonium and divides by mitosis into two cells. The first is a differentiated spermatocyte while the second maintains the features of spermatogonium, to ensure the turnover of the germ cell base [96]. The primary spermatocyte is different from the spermatogonium and takes part in the meiosis process. During the first stage, the primary spermatocyte (a diploid) divides into two secondary spermatocytes (haploids) containing half the genetic patrimony of the primary spermatocyte. The newly formed secondary spermatocyte is still in the meiosis stage and with the second reduction, not reducing its genetic patrimony, it divides into two spermatids. Each spermatid is then 'refined' inside the gonad because it is not yet capable of undergoing fertilization. The 'refining' is to be understood as a variable length process, aimed at creating and reinforcing the structure of the future spermatozoon, which requires particular elements that are not present in the spermatids in order to fulfill its task [97]. At the final stage, the spermatozoon has a typical structure: mature spermatozoon [98].

The p53 protein was found to have several functions in the meiotic and premeiotic stages of spermatogenesis [99]. Possibly, p53 plays different roles in DNA repair, depending on the type of damage, or on the stage at which the cell was damaged, and on the possible repair pathways available [42]. The p53 protein helps sperm to deal safely with DNA damage [100]. A study by Lane shows that p53 plays a role in spermatogenesis: as a matter of fact, mRNA and p53 protein are highly expressed during mouse and rat spermatogenesis and we deal with primary premyiotic spermatocytes at the zygotene-pachytene stages, before the beginning of meiotic division [101]. In addition, p53-knockout mice and mice with reduced levels of p53 show germ cell degeneration during the meiotic prophase, which occurs with the appearance of multinucleated giant cells [102]. p53 knockout mice show a higher incidence of testicular cancer, suggesting that p53 plays a role in the prevention of carcinogenesis during the mitotic stages of spermatogenesis [102–104]. p53 is also capable of mediating stress-induced apoptosis of spermatogonia after DNA damage and after overheating of testicular tissue [105]. The role of p53 in the stress response of spermatogonia is also supported by the extreme reactivity to chemo- and radiotherapy of testicular cancer cells expressing wild-type p53 [106–108]. This has been proven to be the result of the activation of 'normally latent' wild-type p53, which in turn induces a wide apoptotic response [109]. Several studies report the role of the p53 protein in the pre-meiotic and meiotic stages of spermatogenesis [110]. Recently, it has been shown that the accuracy of meiotic crossing over at different genomic locus does not cause severe difficulties in p53 knockout mice [111], moreover, the DNA damage in spermatogonia that induces apoptosis is p53 dependent, the meiotic quality control of chromosomes at meiotic metaphase I is p53 independent. On the other hand, it has been observed that knockout mice for both p53 and ATM genes proceed to later stages of meiosis than those knockout mice with only the ATM gene. Yin et al. [79] reported that p53 mice had impaired apoptosis especially in the tetraploid DNA state. These results suggest that DNA damage at the meiotic stage is p53 dependent.

The proper presence of the p53 protein in spermatogenesis ensures both the quality and the right amount of mature spermatozoa necessary for successful conception. In this observational study, we evaluate the possible correlation between p53 concentration on human sperm DNA and male fertility potential.

Our report is based on an observational study involving 169 males over a period from March 2014 to February 2019. The whole group consists of 208 male partners aged 26–38 years with ejaculate volume from 0.6 to 5.8 ml and heterogeneous

seminal evaluation: 86/208 (41.3%) normospermic; 19/208 (9.1%) mild oligosper-mic; 51/208 (24.5%) moderate to oligospermic; 52/208 (25.1%) with severe oligo-spermic. The 'control A' group includes 39 male partners considered 'fertile' because we performed the p53 test on their sperm DNA 28 ± 3.5 days after the positive pregnancy test results of their partners (betaHCG> 400 m U/mL). Group B, divided into B1, B2 and B3, includes 169 male partners and was observed over a period of 60 months. These partners do not report previous conceptions, do not smoke, do not abuse alcohol, do not use drugs and do not suffer from pathological varicoceles examined with Color Doppler. The whole group includes married and stable cohab-iting partners over a period of 27–39 months, reporting frequent unprotected sex. The p53 values were corrected with respect to spermatozoa concentration, therefore, expressed in ng/million spermatozoa, hence called 'corrected' p53 values.

3.1 Results

Group A (39 males) shows 'corrected' p53 values ranging between 0.35 and 3.20 ng/million spermatozoa and group B (169 males) shows values ranging between 0.68 and 14.53. In group B over the observation period we recorded 21 pregnancies with initial 'corrected' p53 values ranging from a minimum of 0.84 to a maximum of 3.29. In subgroup B1, 8 spontaneous pregnancies were obtained from male partners with a 'corrected' p53 concentration ranging between 0.84 and 1.34. In subgroup B2, 13 pregnancies were obtained from male partners with a 'corrected' p53 concentration ranging between 1.66 and 3.29. In subgroup B3 (121 males) there were neither pregnancies nor miscarriages and the 'corrected' p53 values ranged between 3.58 and 14.53.

3.2 Conclusion

The results show that participants in group A had 'corrected' p53 values between 0.35 and 3.20 and are considered 'fertile', although 3 miscarriages occurred over the
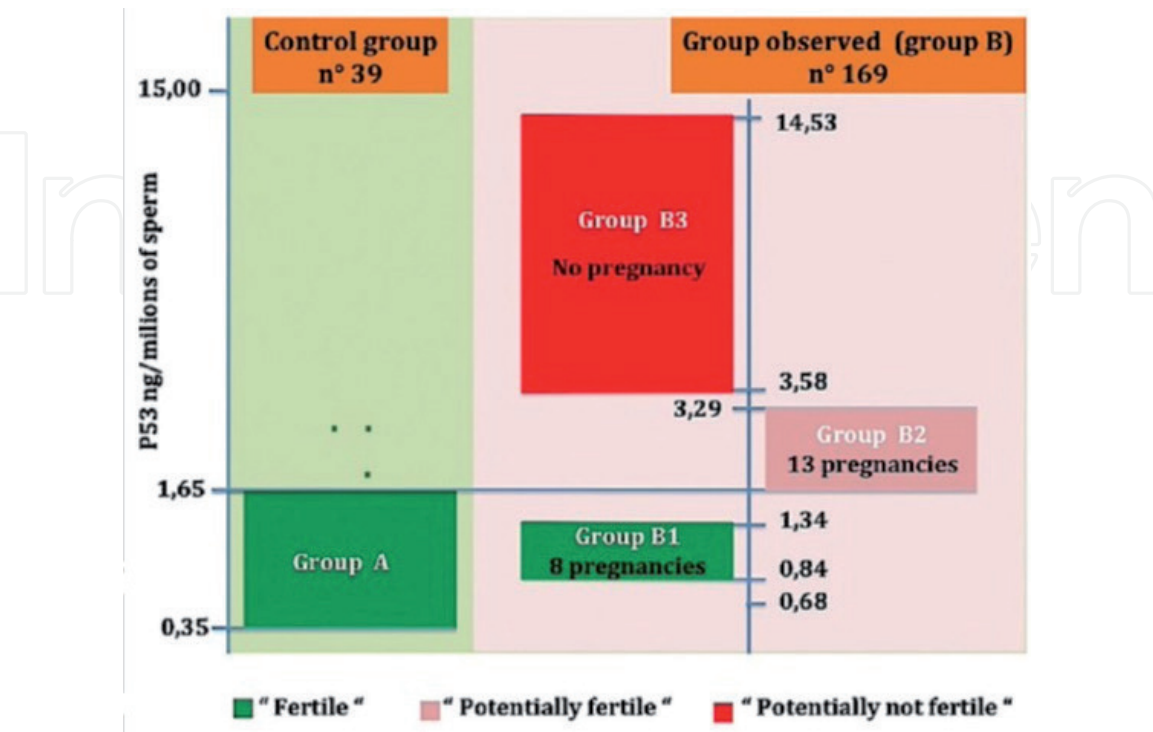

Figure 3.
Group A and B with relative "corrected" p53 concentrations. Spontaneous pregnancies with relative p53 values are reported.

observation period, 36 out of 39 males (92.3%) had a p53 concentration of less than 1.65. Participants in group B1 had a 'corrected' p53 concentration ranging between 0.84 and 1.34, with 8 pregnancies. In group B2 the 'corrected' p53 concentration ranged between 1.65 and 3.29 and 13 pregnancies were observed, so this group can be considered 'potentially fertile'. In group B3 (121 males) with 'corrected' p53 values ranging between 3.58 and 14.53, neither pregnancies nor miscarriages were observed, so it was considered 'potentially infertile' (**Figure 3**).

4. p53 concentration on sperm DNA and sperm separation techniques (ART)

Many factors damage sperm DNA. Considering an increase in the use of assisted reproduction techniques, we would like to assess whether separation techniques can be counted among the probable causes of sperm DNA damage. Spermatozoa can be isolated for several reasons: for medically assisted procreation (MAP) or diagnostic tests [112]. In MAP, the techniques for separating spermatozoa are different and all of them aim to improve the pregnancy rate (PR). The need to select/separate spermatozoa is necessary in cases of infertility due to reduced seminal parameters or to avoid the transmission of sex chromosome diseases. The ideal technique for separating spermatozoa should be easy, fast and affordable, should allow the highest number of motile spermatozoa to be isolated, should not damage or physiologically alter the spermatozoa, should eliminate non-viable spermatozoa, leukocytes and bacteria and should allow selection in the event of hyperspermia (increased ejaculate volume). Currently, no technique meets all these requirements, so the choice of sperm preparation technique is dictated solely by the embryologist's ability and the quality of the semen [113, 114].

The three spermatozoa separation techniques considered in our work are some of those reported in the 5th edition of the WHO Laboratory Manual for the examination of human semen and are also the most frequently used in MAP (Medically Assisted Procreation) centres:

4.1 Direct swim-up

This requires semen with parameters at the lower standard limits for sperm number and motility and is often used for sperm preparation for intrauterine insemination (IUI).

4.2 Pellet swim-up

Exploits the natural ability of spermatozoa to migrate from the seminal plasma to the culture medium. This technique is less effective than direct swim-up, but is useful when the percentage of motile sperm in the semen is low. Pellet swim-up is often used for in vitro fertilization (IVF).

4.3 Density gradient centrifugation

By centrifuging seminal plasma, cells are separated according to their density. Moreover, motile spermatozoa actively migrate through the gradient forming a pellet at the bottom of the test tube. Usually, a two-layer discontinuous gradient with 40% density in the upper layer and 80% density in the lower layer is used. This technique is mostly used for sperm-deficient ejaculates and for ICSI (IntraCytoplasmic Sperm Injection).

In order to assess whether separation techniques can lead to spermatozoa DNA damage, we analyzed samples before and after selection procedures (DGC, pellet swim-up and direct swim-up), comparing data with pre-treatment values (control). To this end, we used an innovative technique able to quantify spermatozoa DNA damage. The reference technique is the one proposed by Raimondo et al. [83], the quantitative assessment of p53 protein on spermatozoa DNA corrected for sperm concentration. We used an Enzyme-Linked Immunoassay (ELISA), a technique that best meets laboratory requirements for accuracy, reliability and repeatability.

4.4 Population enrolled

For this study, we enrolled 63 males in the period from January 2016 to December 2019, aged 24–31 years, the volume of their ejaculates varies from 2.6 to 4.6 mL and have various patterns of dispermia. The sperm evaluations of the subjects were carried out by examining their semen using the standardized analysis criteria according to the WHO laboratory manual for the examination and processing of human semen, 5th edition, 2010.

The Makler Counting Chamber (Sef-Medical Instrumens Ltd.) was used for the assessment of nemaspermic concentration, expressed per mL, as well as for the study of the non-nemaspermic cellular component (leukocytes, red blood cells, germ line cells) [49, 53, 91].

Enrolled subjects do not suffer from chronic diseases, have not used drugs and medications during the 6 months prior to semen collection, are not exposed to environmental stress at work [115–117], did not suffer from pathological varicocele at preliminary examination with Color Doppler [118–120].

Semen samples were processed when liquefied within 30 to 45 minutes after ejaculation.

The samples were then aliquoted into four 0.5 mL aliquots and immediately processed.

The four aliquots were processed as follows:

4.5 Group (a): control

Control samples were quantitatively assessed for p53 protein at both 0 and 60 minutes. During this period of time, semen is not treated, incubated at 37°C at 5% CO2, in a 15 mL Falcon tube.

4.6 Group (b): direct swim-up

An aliquot of semen is placed under the 300 µL layer of culture medium (Quinn's, SAGE, USA). The test tube is placed at a 45° angle to increase the contact surface of semen and medium for 30 minutes at 37° C in a 5% CO_2 incubator. The supernatant fraction is removed and sent for further assessment [121, 122].

4.7 Group (c): pellet swim-up

A 0.5 mL aliquot of the whole sperm is gently mixed with 1.0 mL of sperm culture medium supplemented with 0.1% human serum albumin (Sigma-Aldrich. St. Louis, Catalog – A1653), heated to 37° C, in a 15 mL Falcon tube and centrifuged at 200 g for 8 minutes. The supernatant is discarded and the precipitate (pellet) is mixed with 1.0 mL of culture medium and centrifuged at 100 g for 45 minutes, the supernatant discarded, 300 micronL of culture medium is gently layered onto the final pellet. The test tube is placed at a 45° angle for 30 minutes

at 37° C in a 5% CO2 incubator. The supernatant fraction is removed and sent for further assessment [123, 124].

4.8 Group (d): density gradient centrifugation (DGC)

80/40 gradients (Pureception, SAGE, USA) were placed in 15 mL Falcon tubes, followed by layering of 0.5 mL of whole ejaculate and then centrifuged at 200 g for 20 minutes. The gradient is removed and the pellet is washed twice (200 g x 5 minutes) with 1.0 mL of culture medium. The final pellet is layered on the surface with 300 µL of culture medium and placed at 37°C in a 5% CO2 incubator for 30 minutes. The supernatant fraction is removed and sent for further assessment [125, 126].

All samples are subjected to a quantitative assay of p53 protein corrected in relation to the number of spermatozoa.

Separation of spermatozoa is an important step in ART techniques. Our data show that the Density Gradient Centrifugation (Group d) and Direct Swim-up (Group b) techniques provide superior quality in terms of motility, vitality and apoptosis indices compared to other conventional techniques. In Group (b), apoptosis is superimposable to that of Group (d), while motility and vitality are slightly lower. Group (c) has lower parameters than the other techniques. With regard to the assessment of the p53 protein, the results are in contrast with those of apoptosis: in Group (d), the values are significantly higher than the other techniques (**Table 2**).

The mean percentage of apoptotic spermatozoa in the processed samples was evaluated by the AO test [48] and samples processed by pellet Swim-up (Group c) were found to be significantly higher than those processed by density gradient (Group d) and direct Swim-up (Group b). The lower percentage of apoptotic spermatozoa found in Group (b) and Group (d) suggest that these techniques result in a supernatant with fewer spermatozoa with fragmented DNA. The use of apoptotic spermatozoa during ART may be one of the causes of failure of MAP cycles. The negative association between sperm apoptosis and fertilization rate has been documented with several studies [127, 128]. The selection of non-apoptotic spermatozoa should be one of the most important requirements for achieving optimal conception rates in ARTs [128]; it is beyond doubt that to achieve this important parameter, it is necessary to choose a separation technique that comes closest to natural selection.

This work suggests that the spermatozoa preparation techniques commonly used for assisted reproduction techniques result in different levels of apoptosis and spermatozoa DNA damage, which can be assessed by quantifying the p53 protein isolated from spermatozoa DNA. In the future, we plan to use p53 quantization to assess the damage already existing in spermatozoa DNA of potential

	P53 ng/Mln spermatozoa		
	Before	**After**	*P* value
Control	2,72 ± 0,0	3,17 ± 2,1	NS
Direct swum-up	2,72 ± 0,3	3,18 ± 2,9	NS
Pellet swim-up	2,72 ± 0,2	4,02 ± 3,2	P<0,001
Density gradient centrifugation	2,72 ± 0,3	7,87 ± 3,9	P< 0,0001

Table 2.
Variation in p53 protein concentrations, before and after the separation technique used, including statistical changes.

patients wishing to undergo assisted reproduction techniques, so as to prevent the final result from being further compromised. In case the p53 concentration in the untreated samples is already high, a possible therapy could be evaluated for such patients to improve the starting conditions of spermatozoa thus achieving a better result [5, 6]. This work fits well into a scenario of spermatozoa quality assessment and the importance of having an objective and repetitive data prior to conception both in vivo and in vitro [129].

5. p53, embryo quality and pregnancy rate

The p53 protein is thought to play an important role in oocyte maturation, blastocyst development and embryo implantation in human reproduction [130].

p53 protein expression is low in zygotes and at the cleavage stage, but then increases around the blastocyst stage. Blastocysts from in vivo fertilization have low concentrations of p53 protein, while p53 expression is higher in embryos produced by in vitro fertilization. These findings suggest that embryo culture leads to accumulation of p53 protein transcription activity in blastocysts and may be one of the reasons for the delayed growth of embryos. Human embryos generated by intracytoplasmic sperm injection (ICSI) have a high nuclear p53 expression, associated with delayed embryo development [131]. From these considerations, a more complex role for the p53 protein emerges, which is different from just controlling the integrity of sperm DNA; it is assumed to control the timing and mode of embryo development [132].

The p53 protein plays an important role in the cell and is normally found in all cell types in the human body. It plays a central role in an extensive control network of proteins that enable the 'healthy' condition of a cell and of cellular DNA. The p53 protein is the 'director' of a well-orchestrated cell damage detection and control system. When damage occurs, the activity of the p53 protein is crucial in deciding whether to repair it or induce cell death. The death of a cell that has suffered severe DNA damage is vital for the organism because it prevents the reproduction of cells with dangerous and harmful mutations and, in the event of conception, prevents abnormal embryonic development [133].

Its increase is proportional to cellular damage, so its quantitative assessment indicates DNA damage. Also interesting is its role in controlling and regulating the meiosis process of spermatogenesis and its function in monitoring embryonic development.

The idea that the p53 protein performs multiple tasks in systemic cellular control and development and in the control of human reproductive project is gaining momentum. Our work fits well with the knowledge of the presence of the p53 protein in differentiating male fertility.

For our study, we enrolled 117 partners of couples who had undergone medically-assisted procreation (MAP) for conception.

The seminal parameters were assessed according to the criteria of the WHO 2010 manual, shown in **Table 3**.

Participants were assessed for the concentration of the p53 protein on sperm DNA, first proceeding to a DNA extraction using a forensic method and then to a quantization of the p53 protein using ELISA-immunoassay technique, with another calculation of the results, and expressed in ng/MLN spermatozoa [83].

The embryologist chose the MAP technique to be performed, based on the seminal parameters obtained after the capacitation procedure and, in order to ensure consistency in comparison, it was the same for all samples (Percoll gradients): 90 couples (76.9%) using the IVF technique and 27 couples (23.1%) using the ICSI technique.

IVF stands for In Vitro Fertilization with Embryo Transfer and is the first arti-ficial insemination technique used. IVF is recommended for couples with proven fertility problems: for women, especially those suffering from tubal pathologies (obstruction of the fallopian tubes), and for men when there are minor problems with the semen. This technique can be used mainly in patients who have already conceived naturally, because the ability of the sperm to spontaneously penetrate the egg cell is more certain. With IVF (or in vitro fertilization), conception takes place outside the woman's body: the sperm spontaneously penetrate the egg cell, but everything takes place in a test tube.

ICSI stands for IntraCytoplasmic Sperm Injection and is used in patients of advanced maternal age (>36 years), in cases where oocyte production is low or,

Participants	MLN spermatozoa/mL	Type a motility %	Morfology % according to Kruger	Vitality %
117	20,14 ↔ 48,31	18,5 ↔ 51,6	8,5 ↔ 17,8	48,8 ↔ 76,5

Table 3.
Seminal parameters of the participants.

	1.145 < p53 > 2.45 ng/Mln spermatozoa	3.20 < p53 > 7.75 ng/Mln spermatozoa
Participants	51	66
No. MII oocytes	380	257
Embryos	248 (65.4%)	104 (40.5%)
Pregnancies	28 (PR=54.9%)	13 (PR=19.7%)

Table 4.
Number of participants, number of total (MII) oocytes, number of embryos that reached the 6–8 cell stage, pregnancies achieved (PR) for two groups of p53 values.
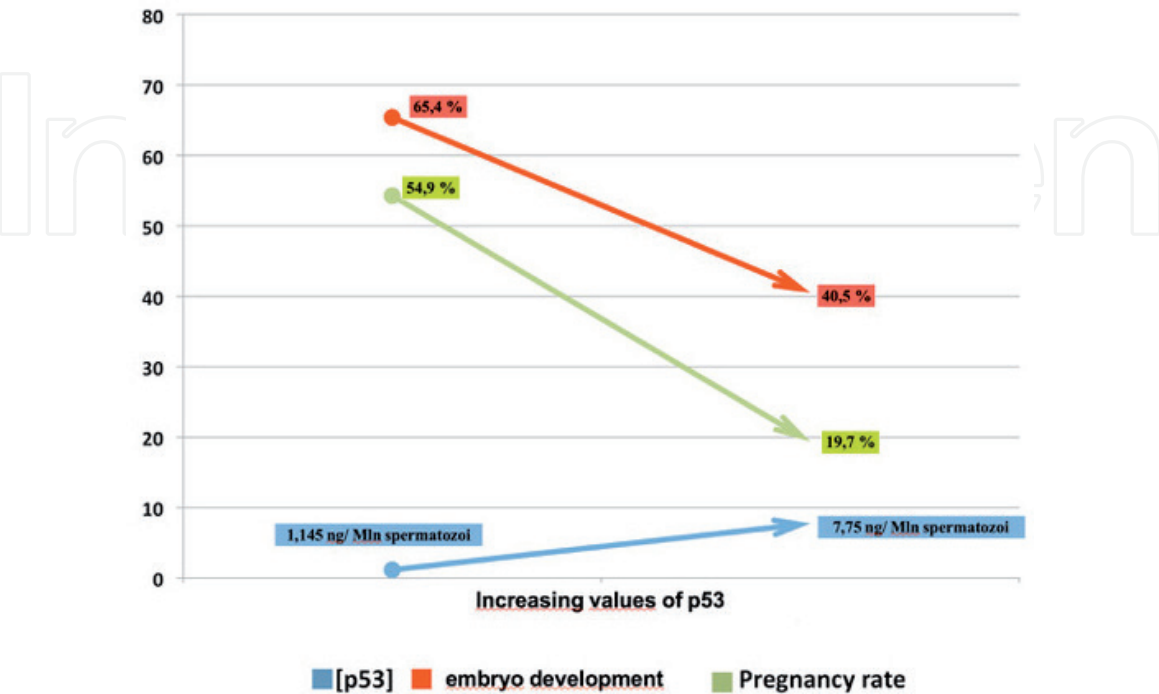

Figure 4.
Interrelation between p53 concentration, embryonic development and PR.

in the case of men, if there is severe seminal damage, such as the total absence of spermatozoa in the ejaculate fluid and it is necessary to proceed with the aspiration of sperm directly from the testicle.

The initial phase of ICSI is the same as that of IVF, starting with hormonal stimulation and then moving on to oocyte aspiration. The difference is that in ICSI a spermatozoon is selected by the biologist and injected into the cytoplasm of an oocyte using a micro needle to 'force' fertilization. This operation is repeated for all the oocytes to be inseminated. The following stages are exactly the same as IVF.

On the third day of embryo development, the number of embryos that reached the stage of 6–8 was assessed. Pick-up report (IVF + ICSI), fertilization and Pregnancy rate (PR) are shown in **Table 4**.

The results obtained support the theory that a high concentration of the p53 protein in spermatozoa DNA is associated with a low percentage of embryos able to reach the 6–8 cell stage on day three and a lower pregnancy rate (**Figure 4**).

Our work fits well with prediction models and the importance of having objective and repetitive data prior to conception, both in vivo and in vitro [134].

6. Conclusions

Cytochemistry, fluorescence and electrophoresis techniques have so far been used to assess DNA damage. For our studies, we employed an innovative method called 'quantitative proteomics', an analytical chemical technique for determining the amount of protein in a given sample. The methods for identifying proteins are identical to those used in general proteomics, but include quantification as an additional dimension. We used p53, a protein already known as the 'guardian of the genome', to assess the effect of environmental and/or dietary toxic factors on human bodies through DNA damage. From our studies, we have identified the spermatozoon as a sentinel cell of environmental impact, as its DNA damage is strongly correlated with pollution. Inevitably, the evolution of these preliminary
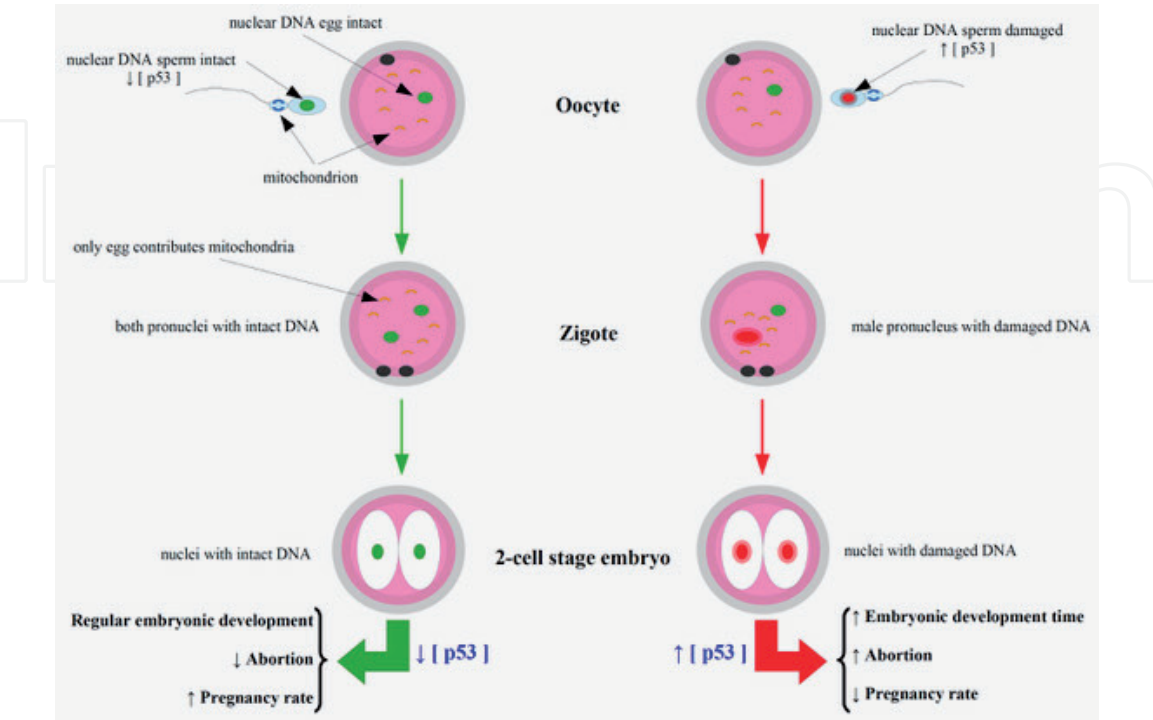

Figure 5.
Schematic representation of the effects of the different concentrations of the p53 protein on human reproduction.

studies turned to understanding whether DNA damage could influence the fertilizing capacity of males. We think that given our results, this protein can be used as an indicator of environmental impact, and given the renewal characteristics of spermatogenesis, it can also be used as a prevention and follow-up index for environmental remediation. A more extensive use would be to understand whether sperm DNA is compatible with the couple's optimal reproductive project both in vivo and in vivo (**Figure 5**).

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