

## A New Oral Supplementation Based on the Ataxia-Telangiectasia-Mutated Repair Pathway Enhancement Reduces Sperm DNA Damage and Improves Semen Parameters

Sandra Lara-Cerrillo<sup>1</sup>, Josu Franco Iriarte<sup>2</sup>, María Velasco Álvarez<sup>3</sup>, Estefanía Rodríguez Ferradas<sup>3</sup>, Álvaro Vives Suñé<sup>4</sup>, Marta Ballester Ferrer<sup>4</sup>, Tania Lacruz-Ruiz<sup>1</sup>, Candela Rosado-Iglesias<sup>1</sup>, Ana Raquel Jiménez Macedo<sup>5</sup>, Joan Carrera Rotllan<sup>5</sup> and Agustín García-Peiró<sup>1\*</sup>

<sup>1</sup>CIMAB, Barcelona Male Infertility Centre, Research and Development Department, Sant Quirze del Vallès, Barcelona, Spain

<sup>2</sup>Hospital Ruber Internacional, Assisted Reproduction Department, Madrid, Spain

<sup>3</sup>Instituto Vasco de Fertilidad, Grupo IVF Life San Sebastián, Assisted Reproduction Department, Donostia, Guipuzkoa, Spain

<sup>4</sup>Hospital Universitario Dexeus, Department of Obstetrics, Gynaecology and Reproduction, Reproductive Medicine Unit, Barcelona, Spain

<sup>5</sup>Unitat d'Endocrinologia Ginecològica, Assisted Reproduction Department, Vic, Barcelona, Spain

**\*Corresponding Author:** Agustín García Peiró, CIMAB, Barcelona Male Infertility Centre, Research and Development Department, Sant Quirze del Vallès, Barcelona, Spain.

**Received:** April 30, 2021; **Published:** May 08, 2021

### Abstract

**Background:** A male factor is present in half of infertile couples and the presence of sperm DNA damage has been shown to be a key factor in these cases. The presence of Double-Strand Breaks (DSB) could cause alternations in embryos' development and recurrent miscarriage. DSB are naturally produced during male meiosis and the Ataxia-Telangiectasia-Mutated (ATM) repair pathway plays an essential role in its repair. However, defects in DNA repair or an excessive production of DNA breaks may lead to high DSB values in the ejaculate. Single-Strand Breaks (SSB) have an oxidative origin, therefore, antioxidant supplements showed to reduce its values. Unfortunately, there are no specific approaches to reduce DSB. Here it is proposed that the enhancement of DSB-repair-pathways could be an efficient strategy to improve sperm quality. For this purpose, new oral supplements containing Curcumin and Gingko Biloba extract have been tested based on their antioxidant properties and their ability to enhance the ATM repair pathway.

**Objective:** To test three different oral supplements regarding their capacity to reduce DSB values in affected males. SSB and semen parameters were also studied.

**Methodology:** This prospective and multicentric study tested two formulas containing different doses of Curcumin and Gingko Biloba extract that were administered to infertile males presenting high DSB values. A commercialized antioxidant not containing these substances was also tested. Sperm DNA damage and semen parameters were evaluated in 71 patients at baseline and after 13 weeks. DSB and SSB were measured using the CometAssay.

**Results:** The greatest reduction of DSB values was observed in Formula 1 containing the highest dose of Curcumin and Gingko Biloba extract (27.1%). Formula 2 and the Control group reduced 19.9% and 15.8%, respectively. Formula 1 also reduced SSB (19.5%). Regarding semen parameters, Formulas 1 and 2 increased sperm concentration, sperm count and progressive motility. The control group reduced these values.

**Conclusion:** Formula 1 is a new oral supplement designed to enhance the ATM repair pathway which significantly reduced DSB and SSB, and improved semen parameters. The prevention of sperm DNA damage through effective oral supplementation focused on improving DNA damage repair could be of great interest in patients affected by DSB.

**Keywords:** Double-Strand Breaks; Curcumin; Gingko Biloba Extract; Antioxidants; Sperm DNA Damage; Male Fertility

## Abbreviations

8OHdG: 8-Hydroxy-29-Deoxyguanosine; ATM: Ataxia-Telangiectasia-Mutated; CUR: Curcumin; DSB: Double-Strand Breaks; GBe: Ginkgo Biloba Extract; ICSI: Intracytoplasmic Sperm Injection; ROS: Reactive Oxygen Species; SCD: Sperm Chromatin Dispersion Assay; SCSA: Sperm Chromatin Structure Assay; SDF: Sperm DNA Fragmentation; SSB: Single-Strand Breaks; TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling

## Introduction

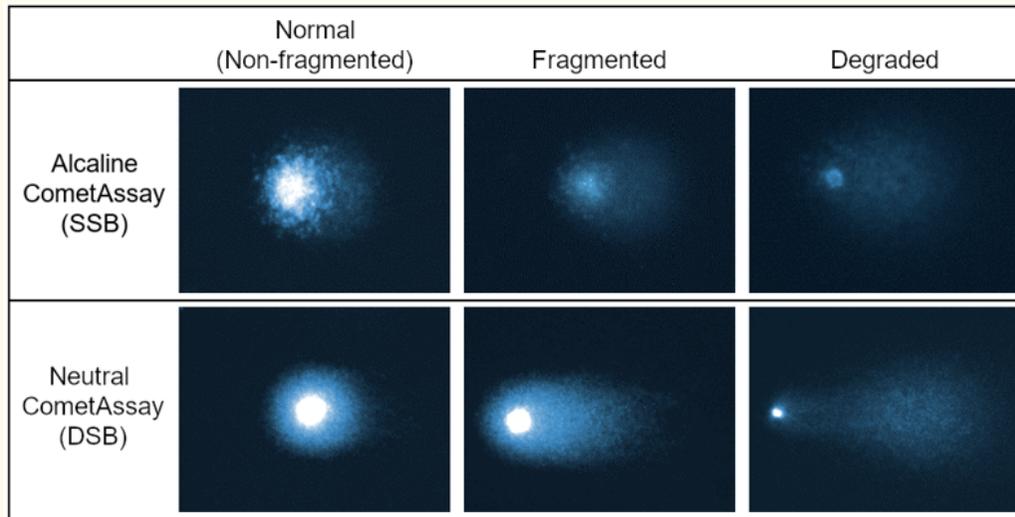
Infertility is defined as the absence of conception after one year of regular and unprotected sexual intercourse [1]. It is estimated that about 15% of couples in reproductive age suffer this problem, being 20 - 30% of cases only related to the male and around 15% of idiopathic origin [2].

The traditional microscopic analysis of the semen measures concentration, motility and morphology of sperm. However, these parameters present a high variability between different ejaculations and might not represent the global fertility potential of a male [3]. Therefore, the identification of new diagnostic and treatment biomarkers is a topic of high interest. In the last decade, multiple studies have been focused on the detrimental effects of Sperm DNA Fragmentation (SDF) on fertility [3]. SDF can be divided in Single-Strand Breaks (SSB) and Double-Strand Breaks (DSB), which have different origins and different effects on male fertility [4].

The SSB damage has been extensively studied and showed to be one of the main causes of male infertility (30 - 80% of cases) [5]. These breaks are mainly produced by Reactive Oxygen Species (ROS) through the formation of 8-hydroxy-29-deoxyguanosine (8OHdG) all over the genome in an extensive manner [6]. ROS can also induce sperm motility loss through the alteration of the normal function of mitochondria to produce ATP and/or through alterations of the cell membrane related to lipid peroxidation [4]. Even reactive oxygen metabolites are necessary for common physiological processes, such as sperm capacitation or apoptosis, the balanced redox equilibrium can be disrupted leading to oxidative stress as a consequence of different phenomena, such as leucocytospermia or dysfunctions in sperm mitochondria [7]. Fortunately, substances with antioxidant properties can delay/inhibit cellular oxidation, can have radical scavenging capacity or can be able to modify the expression of genes coding for some antioxidant enzymes [8]. Multiple antioxidant oral supplements have been described and are increasingly used for fertility enhancement and targeted to reduce oxidative damage (SSB) [9], for example: Vitamin C, Vitamin E, L-carnitine, L-arginine, Lycopene, Coenzyme Q10, Selenium, Inositol and Zinc [10-13]. However, when endogen or exogen antioxidant agents are insufficient to reduce pathological ROS values, spermatozoa with a high number of SSB through all its genome could be present in the ejaculate. The presence of this damage has been shown to reduce the chances of a natural pregnancy since these embryos may contain a high number of SSB in the paternal genome overcoming the repair capacity of the oocyte [14]. Different methods are available for the detection of this damage in the semen sample, such as: the Sperm Chromatin Structure Assay (SCSA); the Sperm Chromatin Dispersion test (SCD); the Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and the Alkaline Comet assay (Figure 1) [6].

On the other side, DSB have an enzymatic origin. These breaks are naturally produced in a controlled manner to enable DNA recombination of homologous chromosomes during the Prophase I stage of meiosis [4]. After recombination, the presence of DSB induce DNA repair machinery activation, being the ATM protein kinase the cornerstone of this process [15-17]. Even these breaks are naturally produced and repaired during male meiosis, unrepaired DSB can be present in sperms' DNA if the repair response is not properly activated or is insufficient. After fertilization, the repair machinery from the oocyte could be able to repair paternal DNA damage at some extend [18]. However, this repair process could be inefficient for DSB since maternal and paternal pronuclei are separated during the first stage of the embryo, and no complementary chains are available as a template. Consequently, the effect of DSB might not appear until the zygote

is formed or later [19]. If present, DSB may cause a delay in embryo's development and could impair its implantation [20]. Interestingly, high levels of SSB in semen samples did not show this relationship [20]. High levels of DSB have also been associated with repeated early pregnancy loss in couples with unknown female factor [21]. In recent years, techniques such as the Neutral Comet assay have been developed to specifically detect this damage (Figure 1) [6].



**Figure 1:** Sperm nuclei classification using the Alkaline Comet Assay (for SSB detection) and the Neutral Comet Assay (for DSB detection).

About options to reduce DNA damage, antioxidants have consistently shown to be useful reducing oxidative damage (SSB) as mentioned before [9]. Moreover, a recent study have shown that sperm selection of a morphologically normal and motile spermatozoa during ICSI treatments is highly efficient reducing SSB values [22]. However, when a semen sample contains a high number of spermatozoa with DSB, conventional semen preparation methods (such as Swim-up or Density gradients) or even the sperm selection process in ICSI treatments performed by embryologists would not reduce this damage and could be finally transmitted to the embryo [22]. Consequently, specific actuations to reduce physiological DSB values in the semen sample are of special interest, especially those focused on the enhancement of ATM-mediated DNA repair. In this regard, Curcumin (CUR) is one of the most important natural compounds with the ability to activate ATM [23,24], but also with antioxidant, anti-cancer, anti-tumour and anti-inflammatory properties and the ability to induce apoptosis and cell cycle arrest [25,26]. Additionally, the BRCA1 protein also plays a critical role in male meiosis participating in the recruitment of RAD51 and promoting homologous recombination and DSB repair [27]. It has also been described that BRCA1 activity can also be encouraged by CUR and other natural compounds such as Ginkgo Biloba extract (GBe).

### **Aim of the Study**

The aim of this proof-of-concept study was to evaluate the safety and effectiveness of three different oral formulas addressed to reduce DSB values in patients with high values of this damage: a commercialized antioxidant and two new oral supplements containing CUR and GBe, natural components with antioxidant, anti-inflammatory and radical scavenging properties, in addition to a remarkable capacity to enhance the DNA repair systems of the cell.

## Materials and Methods

### Participants

This multicentric, randomized and prospective study included 80 infertile men attending the CIMAB centre (Barcelona Male Infertility Centre), the IVF centre (Instituto Vasco de Fertilidad), the Hospital Universitario Dexeus and the UEG centre (Unitat d'Endocrinologia Ginecològica) in Spain. The study was carried out in strict compliance with international recommendations on ethics in research and clinical trials on human subjects, set out in the latest version of the Helsinki Declaration, recommendations on Good Clinical Practice laid down at the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH-E6), and applicable regulatory requirements. The study was approved by the Hospital Universitari Parc Taulí Ethics committee (REF: 2017/901) and an informed consent was signed by all participants.

Candidates for the study were evaluated for DSB, SSB and semen parameters (volume, total sperm count, concentration and motility). Only adult patients presenting idiopathic infertility for more than 12 months who showed altered DSB values ( $\geq 60\%$ ; internal cut-off values for normality) were included in the study. Exclusion criteria included allergies to any of the components of the studied supplements and the intake of any other treatment for fertility improvement or any other treatment affecting sperm quality such as antibiotics, antidepressants, analgesics, antihistamines, cardiovascular drugs, anticoagulants and chemotherapeutic drugs during, at least, three months before the inclusion date. Moreover, patients were excluded from the study when presenting any adverse effect or if the compliance with protocol was not guaranteed.

### Study design

Patients who met the requirements to be included in the study received a randomized oral supplement (T0). A control visit (T1) was programmed after six weeks to evaluate the antioxidant and anti-inflammatory effects on sperm during their transit through the epididymis and during spermiogenesis. A final visit (T2) was programmed after 13 weeks, which corresponds to the period to complete a spermatogenesis cycle by human males. DSB, SSB and semen parameters were evaluated at T0, T1 and T2.

### Studied products

Three oral supplements were assigned to patients following a randomization process: a) antioxidant commercial formula (Androferti; Q Pharma laboratories, Alicante, 03008 Alicante, Spain); b) Formula 1, containing a high concentration of CUR and GBe and c) Formula 2, containing a low concentration of CUR and GBe. Formulas 1 and 2 were developed by Laboratorio Reig Jofre, S.A. (Sant Joan Despí, 08970 Barcelona, Spain) based on the proposal given by the CIMAB centre to improve DNA repair systems and reduce inflammation and oxidation in male patients. These formulas contained CUR and GBe due to their ATM-activation (with the objective of reducing DSB values) and antioxidant (with the objective of reducing SSB and increase sperm motility) properties. New formulas also contained Inositol (as an energy activator which favours ATP synthesis to increase sperm motility), Selenium, Zinc, Vitamin E, Vitamin C, Piperine, Coenzyme Q10 and folic acid. Only the manufacturer knew the exact quantitative composition of Formulas 1 and 2 during the study conduction and its analysis phase.

According to manufacturer instructions, Androferti was administered twice per day (morning and night), while Formulas 1 and 2 were administered once per day (morning) for 13 weeks.

### DSB, SSB and semen parameters evaluation

The Neutral and Alkaline Comet assays (CometFertility; CIMAB S.L., Spain) were performed simultaneously on two different slides to assess DSB and SSB, respectively. Semen samples were mixed with low-melting-point agarose, jellified at 4°C and were immersed in

two consecutive lysis solutions. The Neutral Comet electrophoresis was performed at 20V for 12.5 minutes in TBE buffer (pH 8.5) and the Alkaline Comet electrophoresis was performed at 20V for 4 minutes in 0.03M NaOH buffer (pH 12.2) after a denaturing treatment. Slides were washed in neutralization solution, dehydrated with increasing concentrations of ethanol and horizontally dried. Samples were stained with DAPI SlowFade Gold anti-fade (Invitrogen; OR, USA) and 400 sperm were classified as fragmented or non-fragmented following the criteria reported before (Figure 1) [28]. Laboratory internal cut-off values for DSB and SSB were 60% and 45%, respectively.

Semen parameters (volume, concentration, total number of sperm and motility) were assessed after liquefaction using the Sperm Class Analyser (SCA) CASA software (Microptic S.L., Spain) following the latest WHO recommendations (WHO, 2010).

**Statistical analysis**

Variables were studied during 13 weeks. The T-student test was performed when variables presented a normal distribution. Otherwise, a Wilcoxon test was performed. Differences between products were studied using the ANOVA test when variables presented Normal distributions and the Kruskal-Wallis test when not. The Wilcoxon test for related samples was used to compare DSB between T0, T1 and T2. The level of statistical signification was 95%.

**Results**

**Patients’ withdrawal and dropout from the study**

A total of 80 patients (Androferti: n = 25; Formula 1: n = 27 and Formula 2: n = 28) were included in the study and were administered, at least, one dose of the product. From these, a total of 71 patients (Androferti: n = 21; Formula 1: n = 25 and Formula 2: n = 25) completed the protocol and were evaluated for SDF and semen parameters at T0, T1 and T2. Nine patients were excluded from the study due to different reasons (Table 1).

	<b>N. of patients</b>	<b>Reason for withdrawal</b>
No compliance with protocol	02	Mix the study oral supplement with other alimentary supplements for fertility enhancement.
	04	Not take the oral supplement according to the established protocol and not attend to the control (T1) and the final visit (T2).
	01	Not attend the final visit (T2).
Adverse effects	01	Infection by <i>Helicobacter pylori</i> detected between T0 and T1.
	01	Nephritic colic detected between T0 and T1.
Total	09	

**Table 1:** Excluded patients and reasons for withdrawal.

**Sperm DNA fragmentation**

The analysis of DSB and SSB showed reductions between T0 and T2 after the administration of all products. Formula 1 was the most effective in these reductions (Table 2).

Variable	Androferti				Formula 1				Formula 2			
	Initial (T0)	Final (T2)	Difference	p value	Initial (T0)	Final (T2)	Difference	p value	Initial (T0)	Final (T2)	Difference	p value
Double-strand SDF (DSB %)	72,71	61,19	-15.8%	0.033*	72,08	52,56	-27.1%	0.002*	71,00	56,88	-19.9%	0.200
Single-strand SDF (SSB %)	46,48	42,48	-8.6%	0.200	36,92	29,72	-19.5%	0.040*	39,96	37,24	-6.8%	0.200
Volume (mL)	3,16	2,87	-9.2%	0.004*	3,11	3,47	+11.6%	0.200	2,78	3,17	+14.0%	0.156
Concentration (M/mL)	51,94	44,48	-20.1%	0.167	65,01	66,38	+2.1%	0.046*	48,63	74,67	+53.6%	0.200
Total sperm count (M)	143,56	109,62	-23.6%	0.001*	197,87	215,28	+8.8%	0.135	125,39	238,52	+90.2%	0.012*
Progressive motility (A+B %)	36,77	31,57	-14.1%	0.200	41,74	45,54	+9.1%	0.072	37,85	38,26	+1.1%	0.067

**Table 2:** Mean values of DSB, SSB and semen parameters at baseline (T0) and at the end of study (T2). Positive differences indicate an increase, while negative differences indicate a reduction. \*: Indicates statistical differences.

All patients presented altered values of DSB at T0. After 13 weeks (T2), 90.5% of patients taking Androferti; 100.0% of patients taking Formula 1 and 92.0% of patients taking Formula 2, reduced DSB values at some extent. Moreover, 38.1%, 64.0% and 56.0% of patients reduced DSB up to normal values (< 60%), respectively (Table 3 and figure 2).

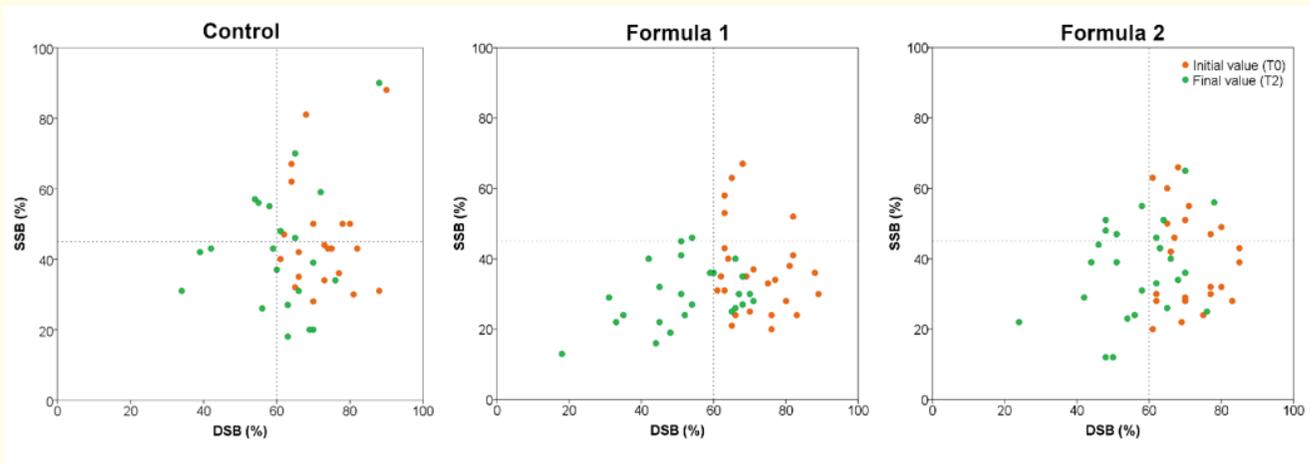
	N. of patients	No reduction	Reduction	Reduction below 60% at T2
Androferti	21	2 (9.5%)	19 (90.5%)	8 (38.1%)
Formula 1	25	0 (0.0%)	25 (100.0%)	16 (64.0%)
Formula 2	25	2 (8.0%)	23 (92.0%)	14 (56.0%)
Total	71	4	67	38

**Table 3:** Reduction of DSB values after the administration of each oral supplement during 13 weeks. All patients presented DSB ≥ 60% at T0.

On the other hand, 57.1% of patients taking Androferti; 72.0% of patient taking Formula 1 and 60.0% of patients taking Formula 2 reduced SSB values after 13 weeks (T2). From all patients, 22 presented altered values at T0. Results show that 25.0%; 80.0% and 55.5% of them reduced SSB values up to normal values (< 45%), respectively (Table 4 and figure 2).

	N. of patients	No reduction	Reduction	N. of patients with SSB ≥ 45% at T0	Reduction below 45% at T2
Androferti	21	9 (42.9%)	12 (57.1%)	8	2 (25.0%)
Formula 1	25	7 (28.0%)	18 (72.0%)	5	4 (80.0%)
Formula 2	25	10 (40.0%)	15 (60.0%)	9	5 (55.5%)
Total	71	26	45	22	11

**Table 4:** Reduction of SSB values after the administration of each oral supplement during 13 weeks. From all patients, 22 presented SSB ≥ 45% at T0.



**Figure 2:** Sperm DNA fragmentation (SDF) values at baseline (T0) and at the end of study (T2) on each study group. Discontinuous lines indicate cut-off values for normality.

### Semen parameters

Formula 1 was the most effective supplement increasing sperm progressive motility. Formula 2 was the most effective supplement increasing the ejaculate volume, sperm concentration and total sperm count. On the contrary, Androferti reduced these parameters (Table 2).

### Discussion

The objective of the present study was to evaluate the safety and effectiveness of three supplementary oral formulas at reducing DSB in affected infertile males. For this purpose, sperm DNA damage (both DSB and SSB) and semen parameters were measured. All supplements contained antioxidants with the objective to reduce oxidative damage (SSB), but only Formulas 1 and 2 contained CUR and GBe at different doses with the objective to reduce DSB. To our knowledge, this is the first study evaluating the effects of oral supplements containing CUR, GBe and antioxidants in relation to DSB and semen parameters.

The fertility potential of a spermatozoa depends, not only on the ability to fertilize the oocyte, but also on the capacity to transfer an intact paternal genome to generate a healthy embryo. Paternal DNA integrity plays a critical role in embryogenesis and sperm DNA damage may lead to lower embryo kinetics, embryo pre- and post-implantation losses, early miscarriage and congenital malformations [20]. According to internal data from our centre, about 50 - 70% of males from infertile couples attending reproductive centres present high DSB values. Moreover, about 25 - 35% of semen donors (apparently non-infertile males) could also present high DSB values [28].

Prevention of SDF through effective oral supplements focused on improving DNA repair and/or apoptosis in spermatozoa could be of great interest for both natural conception and before *in vitro* fertility treatments. All three oral products tested in the present work showed an important reduction of DSB and SSB values after 13 weeks (Table 2). Overall, the greatest reduction was observed for Formula 1 (higher dose of CUR a GBe), which resulted in a reduction in DSB values of 27.1% and a reduction in SSB values of 19.5% after 13 weeks

(Table 2). Regarding Formula 1 results, all patients reduced DSB values and 72.0% of patients reduced SSB values. Moreover, in this group, 64.0% of patients reduced DSB and 80.0% reduced SSB up to normal values (< 60% and < 45% respectively). Lower reductions were observed in the control group (Androferti), showing that 90.5% of patients reduced DSB and 57.1% reduced SSB values at some extent. In this group, only 38.1% and 25.0% of patients showed a reduction of DSB and SSB up to normal values, respectively (Table 3 and 4; Figure 2).

Our results suggest that oral administration of Formulas 1 and 2 reduces the incidence of SDF in a dose-dependent manner. Regarding DSB, CUR is known to be an important natural compound with ability to activate ATM [23,24]. In response to DNA damage, cells activate the sensor kinases ATM, ATR and DNA-PK that, in turn, phosphorylate several downstream substrates, resulting in cell-cycle checkpoint initiation and/or apoptosis [29,30]. The effectiveness of Formulas 1 and 2 at reducing DSB could also be due to CUR's ability to activate BRCA1. *BRCA1* gene is related to DNA repair, cell cycle checkpoints and transcription. During male meiosis, BRCA1 plays a critical role in homologous recombination by promoting the recruitment of RAD51 to DNA damage sites allowing DSB repair [27,31]. Recently, it has been described that CUR can restore *BRCA1* gene expression in HCC-38 and UACC-3199 cells [32]. In addition, CUR has shown to activate the Nucleotide Excision Repair pathway and homologous recombination through the up-regulation of BRCA1, BRCA2 and ERCC1 expression enhancing the DNA repair pathways in bone marrow cells [33]. Formulas 1 and 2 also contained GBe, compound with well-known anticancer properties. Recent *in vitro* studies on GBe also showed beneficial effects on cell proliferation inhibition, tumour suppression and DNA repair [34,35].

The effectiveness of CUR and GBe in Formulas 1 and 2 to reduce SSB could be related to its antioxidant, anti-cancer, anti-tumour and anti-inflammatory properties through the promotion of antioxidant gene expression [26,36-39]. The Nrf2 protein is known to bind promoters of antioxidant genes to induce their expression [39]. Interestingly, the level of Nrf2 mRNA expression is significantly lower in human males with low sperm motility [40]. It has been described that CUR can activate the Nrf2 signals and, as a result, it can improve the antioxidant levels in the kidneys of rats with type 2 diabetes [41]. CUR also significantly increases the expression levels of Nrf2,  $\gamma$ -GCS and GSH-Px antioxidant enzymes in the mouse testes [38]. In this sense, a study performed in asthenozoospermic men concluded that the up-regulation of Nrf2 induced by CUR might protect spermatozoa through decreasing ROS production and apoptosis. GBe has been observed to display protective effects against oxidative stress through the activation of Nrf2 and its downstream antioxidative genes such as *NQO-1*, *SOD2* and *HO-1* [38].

Electrolytes and metals are essential for the viability and function of spermatozoa and were included in Formulas 1 and 2 [42]. It is well known that the semen of infertile males is characterized by reduced levels of some micronutrients such as Calcium and Magnesium, and trace metals like Zinc and Selenium [43]. In this regard, several studies demonstrated that semen parameters can be improved by oral supplementations including antioxidants and micronutrients [44]. Different studies measuring the effect of antioxidants on sperm DNA damage also indicate a significant improvement [45].

Apart from the marked positive effect on sperm DNA integrity, this study also shows a major improvement in all semen parameters after the administration of Formulas 1 and 2 in this patient's cohort. Previously published data on the effects of antioxidants on sperm count and motility are controversial: although some of the studies show an improvement on the semen parameters, other studies did not show any change [46-48] and in some cases, even a reduction of motile spermatozoa was observed [49]. It should be noted that differences observed between studies may be related to the composition, dose and time of administration of antioxidants, as well as the general baseline characteristics of the studied patients. To ensure effectiveness, in the present study, supplements were administered for 13 weeks, longer period than a complete cycle of human spermatogenesis. Formulas 1 and 2 containing CUR and GBe increased 8.80% and 90.22% the total sperm count compared to baseline values, respectively. These products also improved sperm concentration, especially in case of Formula 2, which showed an increase of 53.55%. Sperm motility was also increased, especially in patients taking Formula 1 (Table

2). In agreement with these results, CUR has also shown to increase sperm motility, rescuing mitochondrial function and reducing ROS production and apoptosis [39].

Surprisingly, a negative effect on semen parameters was observed in patients from the control group after the administration of Androfert. This food supplement is a widely used formula addressed to improve male infertility and previous studies support its effectiveness [50,51]. One must consider that our group of study represents a subpopulation of infertile patients characterized by elevated DSB values in semen and, some of them, with normal baseline semen parameters. Moreover, these patients could share a common aetiology. In this sense, results indicate that Androfert would not be the most appropriate approach to treat infertility in this specific patient's cohort.

Even both Formulas 1 and 2 reduced DNA damage and improved semen parameters, Formula 1 showed to be the most effective supplement reducing DSB values, while Formula 2 was the most effective supplement improving semen parameters (Table 2). This observation could be related to the anti-inflammatory properties of CUR and GBe. Recently, immunological changes has been described in blood and seminal plasma in subgroups of infertile patients with idiopathic infertility and male partners of couples with recurrent early pregnancy loss [52]. Oxidative stress leads to increased inflammatory cytokines, which inhibits apoptosis, triggering cell survival and proliferation of damaged cells [53]. This situation might explain the survival of sperm with damaged DNA that can be present in the ejaculate. Boyanapalli, *et al.* demonstrated that the administration of CUR reduces the expression of pro-inflammatory cytokines genes (iNOS, TNF- $\alpha$  and IL-6) and epigenetic modulators genes (DNMT 3A, HDAC2, 3 and 4) in rats [54]. CUR also increases non-enzymatic antioxidant levels, improves sperm quality and quantity and reduced both inflammatory biomarker levels and caspase-3 activities in rats treated with cyclophosphamide [55]. In the same way, GBe has also shown to reduce oxidative stress and inflammation through the activation of the AKT/Nrf2 pathway and increasing HO-1 expression [36]. Consequently, the resumption of apoptosis induced by CUR and GBe could explain the results observed in the present study: these substances would lead to an increase in the repair capacity of DSB in sperm cells through the activation of ATM repair pathways while, in addition, would promote apoptosis in extensively damaged spermatozoa in a dose dependent manner. In case of Formula 1, the highest dose of CUR and GBe, results in a smaller sperm count than in Formula 2, but with a substantial reduction of DSB. On the contrary, Formula 2 would be slightly less effective than Formula 1 at reducing DNA damage but its lower dose of CUR and GBe would not be enough to increase apoptosis and apparently better semen parameters are observed.

This proof-of concept study was performed with the objective of reducing DSB values in affected males. The results obtained from the 71 patients reveal the significant utility of those oral supplements containing CUR and GBe to reduce DSB. Added to the reduction of DSB values, SSB values were also reduced and semen parameters were increased. These data provide a first evidence on the clinical utility of these oral supplementation in this specific patients' cohort. Further studies including a major sample size or including male patients with different alterations in sperm may give some light on the potential global effect of oral supplementations containing CUR, GBe and antioxidants.

### Conclusion

Results show that the percentage of spermatozoa with DSB in infertile patients can be efficiently reduced by a relatively short oral supplementation (13 weeks) including a combination of CUR, GBe and antioxidants. SSB values can also be reduced and seminal parameters can also be improved by these formulas in this patients' cohort. Further studies including other alterations in sperm may provide evidence about the potential global effect of this oral supplementation. Moreover, further studies analysing natural and *in vitro* reproductive outcomes after the administration of these supplements would be of great interest.

### Conflict of Interest

None of the authors have a conflict of interest to declare.

## **Bibliography**

1. Zegers-Hochschild F, *et al.* "The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology, 2009". *Human Reproduction* 24.11 (2009): 2683-2687.
2. Louis JF, *et al.* "The Prevalence of Couple Infertility in the United States from a Male Perspective: Evidence from a Nationally Representative Sample". *Andrology* 1.5 (2013): 741-748.
3. Agarwal Ashok, *et al.* "Should We Evaluate and Treat Sperm DNA Fragmentation?" *Current Opinion in Obstetrics and Gynecology* 28.3 (2016): 164-171.
4. Agarwal A, *et al.* "The Impact of Single- and Double-Strand DNA Breaks in Human Spermatozoa on Assisted Reproduction". *International Journal of Molecular Sciences* 21.11 (2020): 1-14.
5. Ashok Agarwal, *et al.* "What an Andrologist/Urologist Should Know about Free Radicals and Why". *Urology* 67.1 (2006): 2-8.
6. Ribas-Maynou J, *et al.* "Comprehensive Analysis of Sperm DNA Fragmentation by Five Different Assays: TUNEL Assay, SCSA, SCD Test and Alkaline and Neutral Comet Assay". *Andrology* 1.5 (2013): 715-722.
7. Aitken J and J Curry. "Redox Regulation of Human Sperm Function: From the Physiological Control of Sperm Capacitation to the Etiology of Infertility and DNA Damage in the Germ Line". *Antioxidants and Redox Signaling* 14.3 (2011): 367-381.
8. Smits RM, *et al.* "Antioxidants for Male Subfertility". *Cochrane Database of Systematic Reviews* 2019.3 (2019).
9. Tremellen K. "Oxidative Stress and Male Infertility: A Clinical Perspective". *Human Reproduction Update* 14.3 (2008): 243-258.
10. Festa R, *et al.* "Coenzyme Q 10 Supplementation in Infertile Men with Low-Grade Varicocele: An Open, Uncontrolled Pilot Study". *Andrologia* 46 (2013): 805-807.
11. Zareba P, *et al.* "Semen Quality in Relation to Antioxidant Intake in a Healthy Male Population". *Fertility and Sterility* 100 (2013): 1572-1579.
12. Ebisch IMW, *et al.* "Does Folic Acid and Zinc Sulphate Intervention Affect Endocrine Parameters and Sperm Characteristics in Men?" *International Journal of Andrology* 29 (2006): 339-345.
13. Appleton J. "Arginine: Clinical Potential of a Semi-Essential Amino Acid". *Alternative Medicine Review* 7.6 (2002): 512-522.
14. Ribas-Maynou J, *et al.* "Alkaline and Neutral Comet Assay Profiles of Sperm DNA Damage in Clinical Groups". *Human Reproduction* 27.3 (2012): 652-658.
15. Cooper TJ, *et al.* "Homeostatic Regulation of Meiotic DSB Formation by ATM/ATR". *Experimental Cell Research* 329 (2014): 124-131.
16. Lange J, *et al.* "ATM Controls Meiotic Double-Strand-Break Formation". *Nature* 479 (2011): 237-240.
17. Álvarez-Quilón A, *et al.* "ATM Specifically Mediates Repair of Double-Strand Breaks with Blocked DNA Ends". *Nature Communications* 5 (2014): 1-10.

18. Gunes S and S Sertyel. "Sperm DNA Damage and Oocyte Repair Capability". *A Clinician's Guide to Sperm DNA and Chromatin Damage* (2018): 321-346.
19. Gawecka JE, et al. "Mouse Zygotes Respond to Severe Sperm DNA Damage by Delaying Paternal DNA Replication and Embryonic Development". *PLoS ONE* 8.2 (2013).
20. Casanovas A, et al. "Double-Stranded Sperm DNA Damage Is a Cause of Delay in Embryo Development and Can Impair Implantation Rates". *Fertility and Sterility* 111.4 (2019): 699-707.
21. Ribas-Maynou J, et al. "Double Stranded Sperm DNA Breaks, Measured by Comet Assay, Are Associated with Unexplained Recurrent Miscarriage in Couples without a Female Factor". *PLOS ONE* 7.9 (2012): 1-9.
22. Lara-Cerrillo S, et al. "Sperm Selection during ICSI Treatments Reduces Single- but Not Double-Strand DNA Break Values Compared to the Semen Sample". *Journal of Assisted Reproduction and Genetics* 4 (2021).
23. Qiao Q, et al. "Curcumin Enhances the Response of Non-Hodgkin's Lymphoma Cells to Ionizing Radiation through Further Induction of Cell Cycle Arrest at the G2/M Phase and Inhibition of MTOR Phosphorylation". *Oncology Reports* 29.1 (2013): 380-386.
24. Hu A, et al. "Curcumin Induces G2/M Cell Cycle Arrest and Apoptosis of Head and Neck Squamous Cell Carcinoma in Vitro and in Vivo through ATM/Chk2/P53-Dependent Pathway". *Oncotarget* 8.31 (2017).
25. Salehi B, et al. "Plant-Derived Bioactives in Oral Mucosal Lesions: A Key Emphasis to Curcumin, Lycopene, Chamomile, Aloe Vera, Green Tea and Coffee Properties". *Biomolecules* 9 (2019).
26. Méndez-García LA, et al. "Curcumin Induces P53-Independent Inactivation of Nrf2 during Oxidative Stress-Induced Apoptosis". *Human and Experimental Toxicology* 38.8 (2019): 951-961.
27. Simhadri S, et al. "Male Fertility Defect Associated with Disrupted BRCA1-PALB2 Interaction in Mice". *The Journal of Biological Chemistry* 289.35 (2014): 24617-24629.
28. Ribas-Maynou J, et al. "Alkaline and Neutral Comet Assay Profiles of Sperm DNA Damage in Clinical Groups". *Human Reproduction* 27.3 (2012): 652-658.
29. Bartek J and J Lukas. "Chk1 and Chk2 Kinases in Checkpoint Control and Cancer". *Cancer Cell* 3 (2003): 421-429.
30. Squatrito M, et al. "Loss of ATM/Chk2/P53 Pathway Components Accelerates Tumor Development and Contributes to Radiation Resistance in Gliomas". *Cancer Cell* 18.6 (2010): 619-629.
31. Farmer H, et al. "Targeting the DNA Repair Defect in BRCA Mutant Cells as a Therapeutic Strategy". *Nature* 434 (2005): 917-921.
32. Al-Yousef N, et al. "Curcumin Induces Re-Expression of BRCA1 and Suppression of  $\gamma$  Synuclein by Modulating DNA Promoter Methylation in Breast Cancer Cell Lines". *Oncology Reports* 43 (2020): 827-838.
33. Chen X, et al. "Curcumin Activates DNA Repair Pathway in Bone Marrow to Improve Carboplatin-Induced Myelosuppression". *Scientific Reports* 7 (2017): 17724.

34. Marques F, *et al.* "Stimulation of DNA Repair in *Saccharomyces Cerevisiae* by Ginkgo Biloba Leaf Extract". *Food and Chemical Toxicology* 49 (2011): 1361-1366.
35. Jiang W, *et al.* "Ginkgo May Prevent Genetic-Associated Ovarian Cancer Risk". *European Journal of Cancer Prevention* 20.6 (2011): 508-517.
36. Chen XJ, *et al.* "Ginkgo Biloba Extract-761 Protects Myocardium by Regulating Akt/Nrf2 Signal Pathway". *Drug Design, Development and Therapy* 13 (2019): 647-655.
37. Daverey A and SK Agrawal. "Curcumin Protects against White Matter Injury through NF-KB and Nrf2 Cross Talk". *Journal of Neurotrauma* 37 (2020): 1255-1265.
38. Yang SH, *et al.* "Protective Role of Curcumin in Cadmium-Induced Testicular Injury in Mice by Attenuating Oxidative Stress via Nrf2/ARE Pathway". *Environmental Science and Pollution Research* 26.33 (2019): 34575-34583.
39. Zhou Q, *et al.* "Curcumin Improves Asthenozoospermia by Inhibiting Reactive Oxygen Species Reproduction through Nuclear Factor Erythroid 2-Related Factor 2 Activation". *Andrologia* 52.2 (2020): 1-11.
40. Chen K, *et al.* "Low NRF2 MRNA Expression in Spermatozoa from Men with Low Sperm Motility". *The Tohoku Journal of Experimental Medicine* 228 (2012): 259-266.
41. Kim BH, *et al.* "Protective Effects of Curcumin on Renal Oxidative Stress and Lipid Metabolism in a Rat Model of Type 2 Diabetic Nephropathy". *Yonsei Medical Journal* 57.3 (2016): 664-673.
42. Majzoub A and A Agarwal. "Systematic Review of Antioxidant Types and Doses in Male Infertility: Benefits on Semen Parameters, Advanced Sperm Function, Assisted Reproduction and Live-Birth Rate". *Arab Journal of Urology* 16 (2018): 113-124.
43. Chyra-Jach D, *et al.* "Levels of Macro- and Trace Elements and Select Cytokines in the Semen of Infertile Men". *Biological Trace Element Research* (2020): 1-9.
44. Agarwal A, *et al.* "Utility of Antioxidants during Assisted Reproductive Techniques: An Evidence Based Review". *Reproductive Biology and Endocrinology* 12 (2014): 112.
45. Gharagozloo P and J Aitken. "The Role of Sperm Oxidative Stress in Male Infertility and the Significance of Oral Antioxidant Therapy". *Human Reproduction* 26.7 (2011): 1628-1640.
46. Kessopoulou E, *et al.* "A Double-Blind Randomized Placebo Cross-over Controlled Trial Using the Antioxidant Vitamin E to Treat Reactive Oxygen Species Associated Male Infertility". *Fertility and Sterility* 64.4 (1995): 825-831.
47. Greco E, *et al.* "Reduction of the Incidence of Sperm DNA Fragmentation by Oral Antioxidant Treatment". *Journal of Andrology* 26.3 (2005): 349-353.
48. Majzoub Ahmad, *et al.* "Oxidation-Reduction Potential and Sperm DNA Fragmentation, and Their Associations with Sperm Morphological Anomalies amongst Fertile and Infertile Men". *Arab Journal of Urology* 16.1, *Arab Association of Urology* (2018): 87-95.
49. Hawkes WC and PJ Turek. "Effects of Dietary Selenium on Sperm Motility in Healthy Men". *Journal of Andrology* 22.5 (2001): 764-772.

50. López Granollers G., *et al.* "Effect of a Treatment with Vitamins, L-Carnitine and Coenzyme Q10 on Sperm Head Vacuolization and DNA Fragmentation on in Vitro Fertilization Patients". *Revista Internacional de Andrologia* 9.4 (2011): 154-159.
51. Balmori Boticario C., *et al.* "Impact of an Antioxidant Complex Supplementation on Sperm DNA Fragmentation in Infertile Men". *Revista Internacional de Andrologia* 8.3 (2010): 107-113.
52. Havrylyuk A., *et al.* "Cytokines in the Blood and Semen of Infertile Patients". *Central European Journal of Immunology* 40.3 (2015): 337-344.
53. Bisht S., *et al.* "Oxidative Stress and Male Infertility". *Nature Reviews Urology* 14.8 (2017): 470-485.
54. Boyanapalli SSS., *et al.* "Pharmacokinetics and Pharmacodynamics of Curcumin in Regulating Anti-Inflammatory and Epigenetic Gene Expression". *Biopharmaceutics and Drug Disposition* 39.6 (2018): 289-297.
55. Akomolafe SF and BT Aluko. "Protective Effect of Curcumin on Fertility in Cyclophosphamide Exposed Rats: Involvement of Multiple Pathways". *Journal of Food Biochemistry* 44.1 (2020): 1-15.

**Volume 10 Issue 6 June 2021**

**©All rights reserved by Agustín García-Peiró, *et al.***