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Effect of *Eucalyptus globulus* essential oil supplementation on advanced motility parameters and DNA integrity of human sperm

Modou M. Mbaye,[1,2,3] Bouchra El Khalfi,[1] Achraf Zakaria,[2] Mustafa Zakaria[4], Noureddine Louanjli [2,3] and Abdelaziz Soukri[1]

[1]*Laboratory of Physiopathology, Genetics Molecular and Biotechnology (PGMB), Faculty of Sciences Ain Chock, Research Center, Health and Biotechnology, University Hassan II of Casablanca, Morocco*
[2]*In Vitro Fertilization Center IRIFIV, IRIS Clinic, Casablanca, Morocco*
[3]*Laboratory of Medical Analyses, Reproductive Biology, Labomac, Casablanca, Morocco*
[4]*Department of Reproductive Biology, and Assisted Reproductive Technology, Northwestern University, Evanston, Illinois, USA; 2IRIFIV, Fertility Center - ART IRIFIV Scientific Research Group (AISRG), Casablanca, Morocco*

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ABSTRACT

Reduced sperm motility remains one of the most important causes of male infertility. Many reports have indicated that treatment of subnormal semen samples with specific agents (pentoxifylline, relaxin, prostaglandin E, diltiazem, etc.) before artificial insemination significantly improves potential fertilization of spermatozoa. In our previous work, it has been shown that the essential oil of Eucalyptus globulus has interesting biological properties on the motility and vitality of human spermatozoa. Therefore, in this study, we aimed to verify the effect of Eucalyptus globulus essential oil supplementation on advanced motility parameters and DNA integrity of 25 semen samples from male volunteers diagnosed as asthenozoospermic. Our results showed that eucalyptus globulus essential oil, over an incubation period of 5 to 10 minutes of exposure at 37°C under 5% CO_2, immensely significantly improved advanced motility parameters: curvilinear velocity (VLC), linear velocity (VSL), average path velocity (VAP), and amplitude of movement (ALH), with the effect of increasing VCL decreasing linearity (LIN), mean line (STR), and mean oscillation (WOB). Eucalyptus globulus essential oil at 5 minutes had no significant effect on DNA fragmentation index (DFI) and chromatin decondensation index (SDI).

1. Introduction

In 20% of cases of couple infertility, the male factor is responsible and is almost always marked by spermogram abnormalities. Among them, asthenozoospermia is characterized by a decrease in sperm motility Liu et al., 2004. This decrease in motility is due to a reduction in energy level caused by a failure of the leading ATP hydrolysis pathway, structural damage to the spermatozoa (flagella), or infections (Jodar *et al*., 2007). To compensate for this energy deficit, reproductive biology experts resort to numerous chemical solutions, most of which are toxic. Thus, finding natural biomolecules capable of improving the mobility and vitality of spermatozoa remains a promising and very attractive way to significantly improve the poor results of in vitro fertilization, which stagnate at less than 20%. The results of our previous work had shown that in vitro supplementation of *Eucalyptus globulus* essential oil during 5 and 10 minutes of incubation at 37° C under 5% CO_2 significantly improved sperm motility and vitality (Mar *et al*., 2020). These results pushed us to see the effect of the supplementation of this essential oil (*Eucalyptus globulus*) on the advanced parameters of the mobility of the spermatozoids, namely: Curvilinear velocity (VCL), linear velocity (VSL), average path velocity (VAP), the amplitude of movement (ALH), linearity (LIN), average straight line (STR) and average oscillation (WOB) and its impact on the integrity of sperm DNA through the evaluation of the DNA fragmentation index (DFI) and sperm decondensation index (SDI).

**Corresponding Author: Mbaye Modou Mamoune*
E-mail Address: mbayeass87@gmail.com
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2. Materials and Methods

2.1. Chemicals

All chemicals used in this study were obtained from Sigma-

Aldrich (St. Louis, MO, USA).

2.2. Plant material

The essential oil (EO) was obtained directly from the leaves of *Eucalyptus globulus* (Fliyou) plants collected in the region of Fez (central Morocco). The plant material was collected randomly, washed and dried in a well-ventilated area at room temperature for ten days before being used according to the method of Sabir *et al* 2017. The sample was then isolated and stored for extraction.

2.3. Essential oil

The essential oil was obtained by hydro distillation for 3.5 hours using the Clevenger apparatus according to the method of Sabir et al. The oil was extracted from the distillate with hexane and dehydrated through anhydrous sodium sulfate. After filtration, the solvent was removed by distillation under reduced pressure in a rotary evaporator at 35° C, and the pure oil was stored in an amber bottle in a refrigerator (4°C), until use Sabir *et al.,* 2017. The non-lethal concentrations of the essential oil were determined by the serial dilution technique according to the method of Mar *et al*., 2020.

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2.5. Sample collection

This study was performed at the Laboratory of Medical Analysis and Reproductive Biology, "Labomac", Casablanca, Morocco. We formed a study group of 25 samples from male volunteers diagnosed as asthenozoospermic (≥ 20 x concentrations x 106 ml /, progressive motility is < 32%). Informed consent was obtained from all included patients before their semen was used in this study. Then, samples were collected after 3 to 4 days of sexual abstinence in sterile, labeled containers (Mar et al., 2020). For liquefaction, samples were stored at 37°C under 5% CO_2 until use. We checked at an interval of 10 minutes until liquefaction. Microscopic analysis was performed according to World Health Organization (WHO) standards and guidelines (Mar et al., 2020).

2.6. Semen processing

After one hour of semen production, a routine analysis was performed to determine sperm count, motility, and vitality. For this purpose, a 20µl sample of semen was deposited in a Makler room. Pretreatment of the semen was performed using the density gradient optimization technique. Thus, 1 ml of PureSperm® 70%, 1 ml of PureSperm® 40% and 1 ml of semen sample were added respectively to a 10 ml Falcon tube and centrifuged at 500 rpm for 20 minutes. The pellet (approximately 0.5 ml) was enriched with 10-20 µl of BM1 and then divided into two equal aliquots in 10 ml Falcon tubes, the first tube containing the pellet and BM1 was incubated as a control, the second was also incubated with 1.5 µl of *Eucalyptus globulus* oil at a final concentration of (Barlow *et al*., 1991; Mar *et al*., 2020). The incubations were performed at 37°C under 5% CO2 (Figure 1) (Mar *et al*., 2020).

2.7. Motility analysis

The effect of *Eucalyptus globulus* oil on advanced parameters of human sperm motility was evaluated at different incubation times: 0, 5, and 10 min at 37°C under 5% CO_2. The protocol consists of depositing a 20 µl aliquot of the mixture (semen/oil, control) in a preheated Makler chamber and analyzed by CASA (Hamilton-Thorne Semen Computer Assisted Analysis version 10 HTM IVOS Analyzer (Hamilton-Thorne Biosciences, Beverly, MA, USA). The parameters used for the analysis were: number of images, 8; frame rate, 20 Hz; layer thickness 10µl; temperature, 37°C; minimum contrast, 6; minimum size, 6; small grid size, 0.4; large grid size, 2.0; low intensity grid, 2.5; high intensity grid, 4.3 (Liu et al., 2004). Eight representative fields containing at least 100 motile spermatozoa were examined. Parameters recorded for each sample were: percent viability, percent progressive motility, and movement characteristics such as curvilinear velocity (VCL), linear velocity (VSL), mean path velocity (VAP), lateral displacement amplitude (ALH), mean linearity (LIN: VSL/VCL), mean straight line (STR: VSL/VAP), mean oscillation (WOB: VAP/VCL) (Liu et al., 2004).

2.8. Vitality analysis

Sperm viability was assessed with a 2% eosin stain (Mar et al., 2020). For this purpose, a semen sample was mixed with an equal volume of eosin solution. A smear on a glass slide was made from this mixture and allowed to air dry. Immediately after drying, each slide was examined under a light microscope. The test is performed immediately after contact between spermatozoa and different concentrations of EO (t = 0 min), at exposure times ranging from 5 to 10 min and the preparation was incubated at 37°C under 5% CO_2 (Mar *et al*., 2020).

2.9. Measurement of DNA fragmentation index by the TUNEL assay

Semen DNA integrity was assessed by the TUNEL assay using a commercial kit (Roche Diagnostic, Lewes, UK) according to the manufacturer's recommendations. The principle of the TUNEL technique is to use an enzyme, terminal deoxynucleotransferase (TdT), capable of adding nucleotides to the 3'-OH ends of free DNA.

The semen sample was washed twice in phosphate-buffered saline (PBS, Sigma-Aldrich, Gillingham, UK) and adjusted to a concentration of 2 x 107 cells/ml in PBS. The cell suspension was then fixed in PBS containing 2% formaldehyde (Sigma-Aldrich) for 60 minutes at room temperature. After a double wash with PBS, the sample was centrifuged at 1200 rpm.

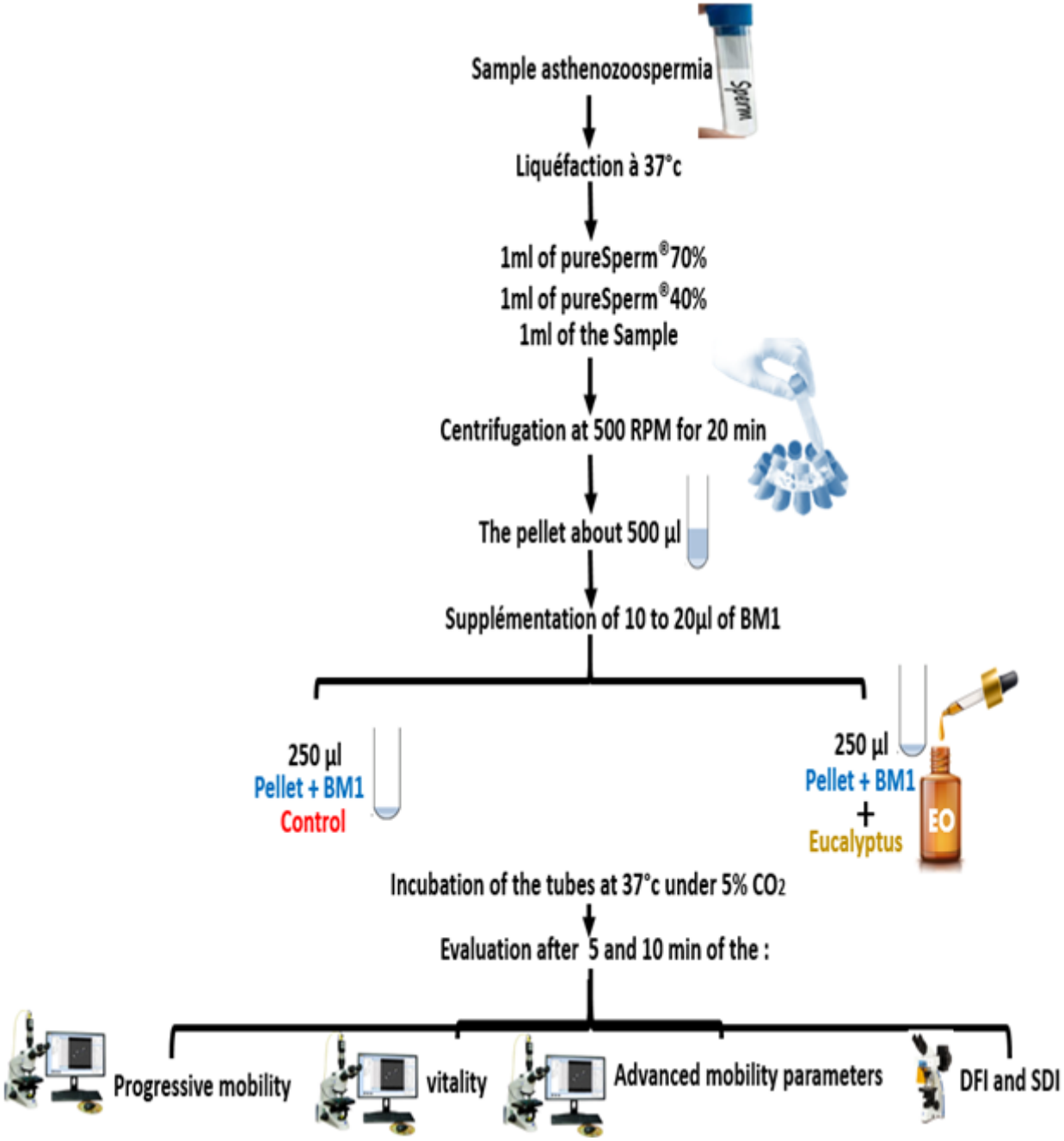

Figure 1: Sample processing method

This step was repeated twice. The prepared slides were immediately analyzed using a fluorescence microscope (Nikon Eclipse 80i) equipped with appropriate filters. Images were captured using a CCD camera and XytoGen software (Excilone, version 3.8.46, France) (Figure 2) (Kaarouch et al., 2018).

2.10. Measurement of sperm decondensation index with aniline blue

Slides of the prepared sperm samples were rinsed twice with distilled water and then stained with a 5% aniline blue bath at pH 3.5 for five minutes. They were then quickly rinsed with distilled water, then dehydrated in alcohol baths (70, 90 and 100°, one minute each). Reading is done under white light at × 1000 magnification and by immersion (Figure 2). A total of 500 sperm were counted on the slides made from the WHO sample.

2.11. Statistical test

The data obtained in our experiments were statistically studied. The results of *Eucalyptus globulus essential* oil supplementation on the characteristic parameters of mobility and nuclear quality were obtained by Student's t-test (t-test). All graphs and histograms represented in this article were created with GraphPadPrism7 software.

3. Results

3.1. Effects of *Eucalyptus globulus* oil on sperm motility

In vitro supplementation with *Eucalyptus globulus* essential oil significantly improved the percentage of progressive motility as well as the vitality of human spermatozoa (Figure 3) (P<0.001). (Figure 3) (P<0,001).

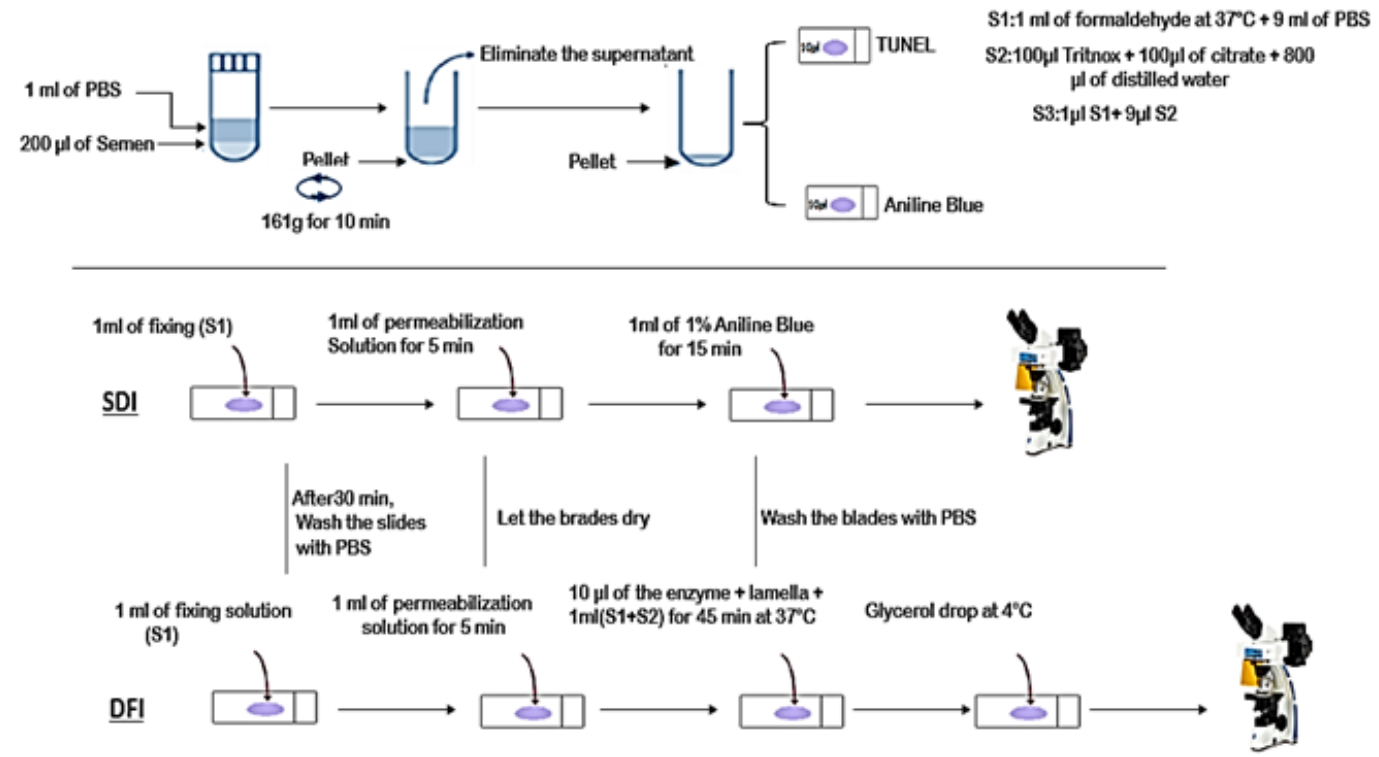

Figure 2: Processing method used for evaluating the sperm decondensation index (SDI) and the DNA fragmentation index (DFI).

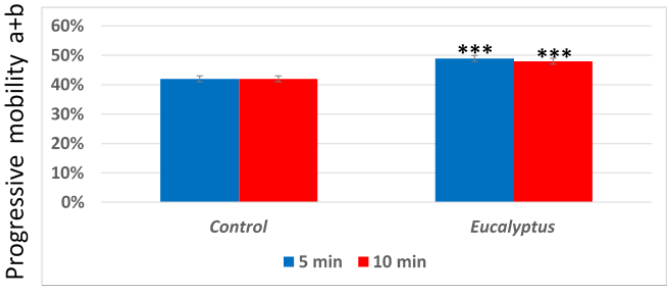

Figure 3: In vitro effect of Eucalyptus globulus oil on the proportion of motile spermatozoa. The essential oils were diluted in a sterile 0.2% (w/v) agar solution. The percentage motility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

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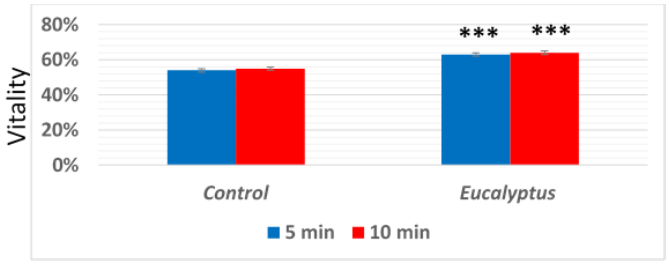

Figure 4: In vitro effect of Eucalyptus globulus oil on the proportion of live spermatozoa. The essential oils were diluted in a sterile 0.2% (w/v) agar solution. The percent motility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

3.3 Effects of *Eucalyptus globulus* on advanced mobility parameters

In vitro supplementation with *Eucalyptus globulus* essential oil showed a significant stimulating effect on advanced mobility parameters: curvilinear velocity (VCL) (P<0.01) (Figure 5), linear velocity (VSL) (P<0.01) (Figure 6), mean path velocity (MPV) (P<0.01) (Figure 7), amplitude of movement (ALH) (P<0.01) (Figure 8).

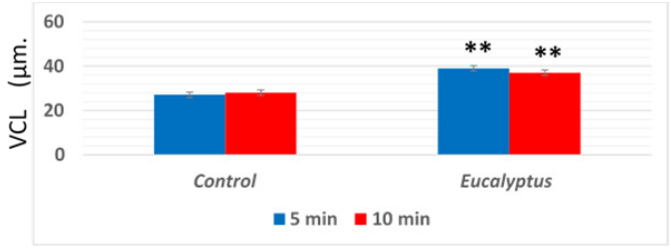

Figure 4: In vitro effect of Eucalyptus globulus oil on the proportion of live spermatozoa. The essential oils were diluted in a sterile 0.2% (w/v) agar solution. The percent motility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

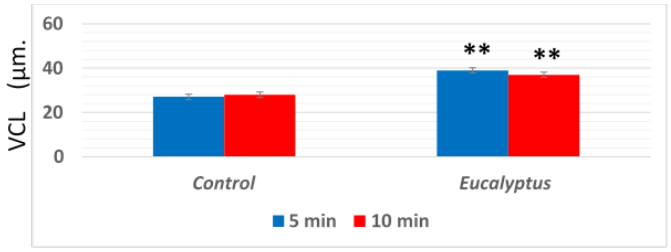

Figure 5: In vitro effects of Eucalyptus globulus oil on curvilinear velocity. Essential oils were diluted in sterile 0.2% (w/v) agar solution. The percent mobility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

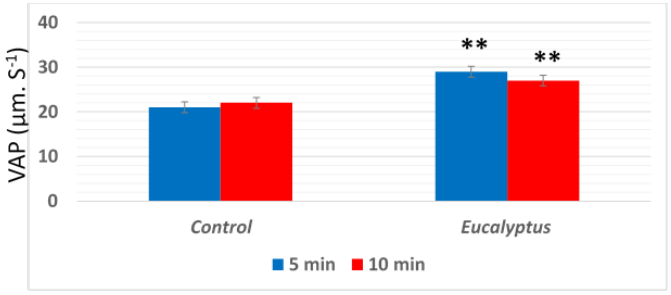

Figure 6: In vitro effect of Eucalyptus globulus oil on mean path velocity (MPV). Essential oils were diluted in sterile 0.2% (w/v) agar solution. The percent mobility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

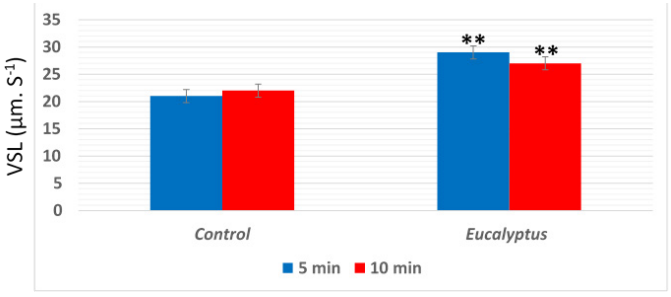

Figure 7: In vitro effect of Eucalyptus globulus oil on linear velocity (VSL). Essential oils were diluted in sterile 0.2% (w/v) agar solution. The percent mobility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

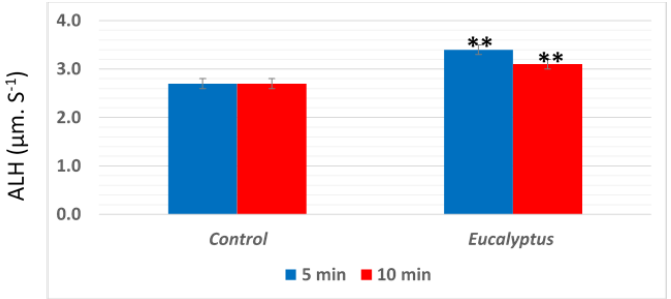

Figure 8: In vitro effect of Eucalyptus globulus oil on displacement amplitude (ALH). Essential oils were diluted in sterile 0.2% (w/v) agar solution. The percent mobility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

The effect of increasing VCL very significantly reduced linearity (LIN) (P<0.05) (Figure 9), mean line (STR) (Figure 10), and mean oscillation (WOB) (Figure 11)

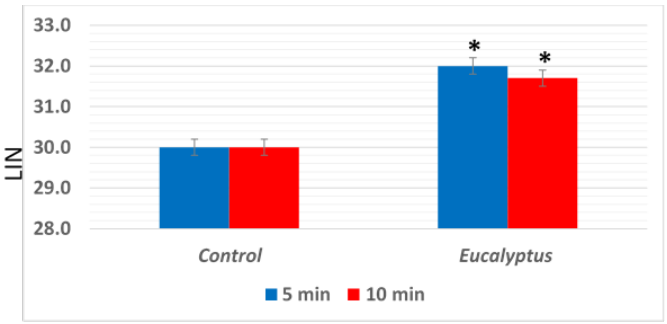

Figure 9: In vitro effect of Eucalyptus globulus on linearity (LIN). Essential oils were diluted in sterile 0.2% (w/v) agar solution. The percent mobility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

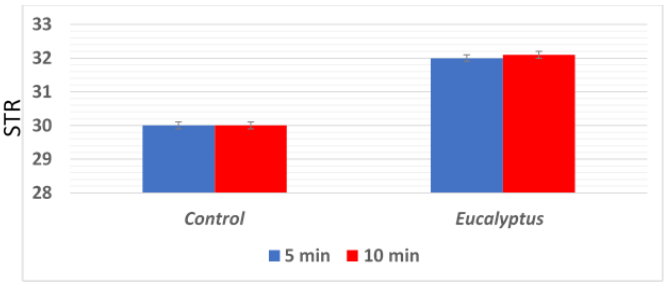

Figure 10: In vitro effect of Eucalyptus globulus oil on the midline (STR). Essential oils were diluted in sterile 0.2% (w/v) agar solution. The percent mobility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

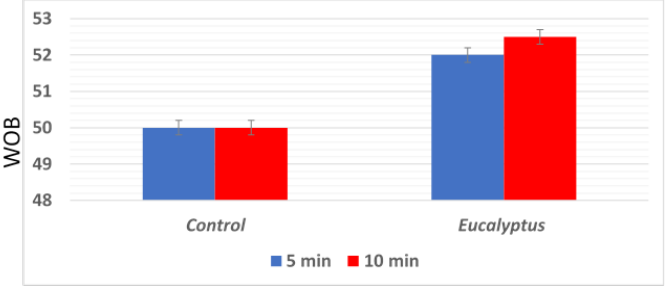

Figure 11 : In vitro effect of Eucalyptus globulus oil on the middle wobble (WOB). Essential oils were diluted in sterile 0.2% (w/v) agar solution. The percent mobility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard

deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

3.4. Effect of *Eucalyptus globulus* on DNA fragmentation index

Eucalyptus globulus supplementation in asthenozoospermic sperm after 5 and 10 minutes of incubation at 37°C under 5% CO_2 does not change the level of sperm with DNA fragmentation. Since the level of sperm with DNA fragmentation is as high in the fortified sample as in the control, the difference is not significant (p=0.49) at 5 min. In contrast, we found a significant difference at 10 min (p<0.05) (Figure 12).

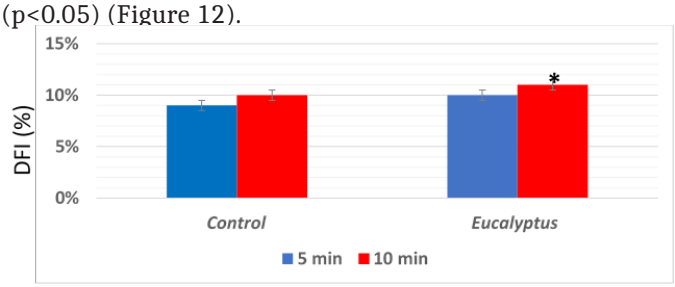

Figure 12: In vitro effect of Eucalyptus globulus oil on DNA fragmentation index. DFI was measured after the addition of EO and a 5 min and 10 min treatment. The essential oils were diluted in sterile 0.2% (w/v) agar solution. The percent mobility of each aliquot was calculated after EO addition (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

3.4. Effet d'*Eucalyptus globulus* sur l'indice de décondensation du sperme

The level of spermatozoa with a DNA condensation defect after 5 to 10 min of incubation at 37°C under 5% CO_2 shows that in vitro supplementation of Eucalyptus globulus oil in human sperm does not have a deleterious effect on normal sperm chromatin condensation since the mean values of the supplemented and control samples do not show a significant difference (p=0.41) at 5 min. However, we noted a significant difference at 10 min (p<0.43) (Figure 13).

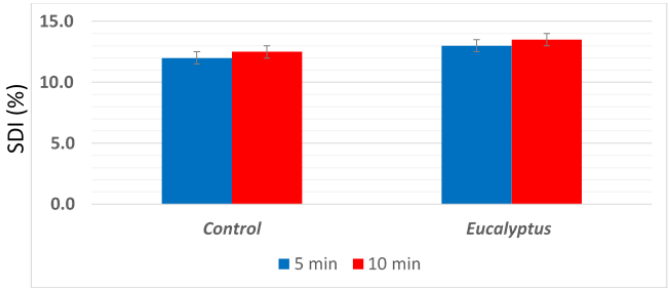

Figure 13: In vitro effect of Eucalyptus globulus oil on sperm decondensation index. SDI was measured immediately after addition of EO and after 5 min and 10 min of treatment. The essential oils were diluted in sterile 0.2% (w/v) agar solution. The percent mobility of each aliquot was calculated after the addition of EO (t = 5 min) and (t = 10 min). Data are expressed as mean ± standard deviation (n = 5). (*) Indicate "p" values: * significant (p<0.05); ** highly significant (p<0.01); *** highly significant (p<0.001); and **** highly highly significant (p<0.0001; n = 25).

4. Discussion

Sperm transferred during in vitro fertilisation procedures are treated with chemicals to increase their fertility (Liu *et al.*, 2004). These chemicals can sometimes be harmful to the sperm. This has an impact on the low chances of success of in vitro fertilization. Thus, finding natural, non-toxic molecules to boost sperm during IVF procedures would be a boon to reproductive biology professionals (Bączkowsk *et al.*, 2004).

Our previous work revealed that *Eucalyptus globulus* essential oil supplementation had beneficial effects on the characteristic parameters of human sperm quality namely human sperm motility and vitality (P<0.001) (Figure 3) (Mar *et al.*, 2020; Yang *et al.*, 1992).

Thus, we felt it necessary to test its effect on the advanced parameters of motility and sperm DNA integrity. In parallel, to see its effect with that of *Origanum vulgare* essential oil On the one hand, the results of *Eucalyptus globulus* essential oil supplementation on motility, vitality and advanced motility parameters showed that at 5 and 10 minutes of exposure at 37°C under 5 % CO_2, Eucalyptus essential oil significantly improved the curvilinear velocity (VCL) (Figure 4), linear velocity (VSL) (Figure 5), mean trajectory velocity (VAP) (Figure 6) and amplitude of movement (ALH) (Figure 7) of spermatozoa.

On the other hand, sperm linearity (LIN) (Figure 8) as well as mean line (STR) (Figure 9) and mean waviness (WOB) (Figure 10) decreased significantly due to the increase in VCL and VAP. Our results are in good agreement with those of the groups of Barlow and J. Liu (Barlow *et al.*, 1991; Liu *et al.*, 2004).

We believe that the major compounds in *Eucalyptus globulus* essential oil, the best known of which is 1,8-cineole or eucalyptol which is present in 50-80%, pinene, limonene, citronellal, cryptone and piperitone, are believed to be able to scavenge free radicals such as superoxide anion and hydroxyl (Torres *et al.*, 2016; Hollenbach *et al.*, 2015) which can become deleterious to many cells, including spermatozoa, if they increase in the cell environment, either due to increased production, or failure to degrade or eliminate them. Deleterious oxidative stress (OS) is therefore characterised by an imbalance between the production of ROS and the body's ability to detoxify them (antioxidant capacity) Agarwal and Said-2015; Armstrong *et al.*, 2002. Therefore, the improvement of motility, advanced parameters of mobility and vitality of spermatozoa by the essential oil of *Eucalyptus globulus* can be explained by its antioxidant activity against lipid peroxidation Hollenbach *et al.*, 2015. Recent epidemiological data conclude that about 15% of couples experience difficulties in having children and seek medical advice for this problem (Trussel-2013).

In addition, Sanocka et al, have shown that lipid peroxidation in sperm damages the lipid matrix structure of sperm membranes and is linked to a rapid loss of intracellular ATP that can lead to axonemic damage and reduced sperm viability (Williams-1992). In a similar vein, Aitken et al, determined that the peroxide radical (H2O2) could be

distributed across sperm membranes and thus inhibit the activity of key enzymes such as glucose-6-phosphate dehydrogenase (an enzyme that plays a major role in ATP production and sperm motility) (Aitken-1997).

Our ALH values found are consistent with the results of the work of Aitken et al, Bongo et al, and J. Liu et al, who showed that ALH was higher in the semen of fertile men than in that of infertile men. They also argue that the increase in displacement amplitude can be positively correlated with the ability or even efficiency of sperm to penetrate mucus (Aitken *et al*., 1987; Bongso *et al*., 1989; Liu *et al*., 2004).

Evaluation of the effect of eucalyptus oil supplementation on DNA integrity during a 5 min incubation at 37°C under 5% CO_2 gave no significant difference for DFI (Figure 11) and SDI (Figure 12) values. However, after 10 min of incubation, a significant difference was noted for both DFI ($p<0.05$) and SDI ($p<0.05$) compared to the controls (Figures 11 and 12). The lack of convention on the threshold values of the DNA fragmentation index and the chromatin decondensation index makes their interpretation difficult. We can say that *Eucalyptus globulus* essential oil has no impact on the DNA fragmentation index and the sperm decondensation index as we recorded values below the positive threshold value (4%) for DFI and (15%) for SDI (Roux *et al*., 2004 ; Mousa *et al*., 2020).

Sometimes the DNA is not compacted. This increases the risk of altering the genetic material. Indeed, the risk of DNA denaturation and fragmentation is higher and may compromise the fertilisation potential of the sperm. Among infertile men, 25% have a high level of DNA damage. Of these, only one in 10 has a normal sperm count.

Sperm abnormalities can lead not only to problems with embryo development (Jawad *et al*., 2014), but also to spontaneous miscarriage (Liu *et al*., 2004). The low DFI and SDI values may be the result of various factors in addition to Eucalyptus oil supplementation, as even in the control we noted low DFI and SDI values. Furthermore, many studies have shown that sperm DNA damage can result from various phenomena such as apoptosis, oxidative stress, production of endogenous reactive oxygen species (ROS), spontaneous and/or exogenous alterations (ionising radiation, toxic components of the environment), which act on DNA elements producing a very wide range of damage such as deletions or free nitrogen base breaks (Aitken and Krausz-2001; Kačániová *et al*., 2020).

The results of *Eucalyptus globulus* oil supplementation on the advanced parameters of sperm motility and DNA quality show that *Eucalyptus globulus* essential oil could be an alternative for the treatment of samples during in vitro fertilisation procedures. On the other hand, comparing the results of supplementation with *Origanum vulgare* essential oil, we clearly see that the results obtained with Origanum are better than those found with *Eucalyptus globulus* (Mousa *et al*., 2020). Thus, based on these results, we can conclude that oregano oil could be a safe therapeutic alternative for the management of motility dysfunction in asthenozoospermic patients compared to *Eucalyptus globulus* oil.

5. Conclusion

This study highlights the improving effects of in vitro supplementation with oregano essential oil on advanced sperm mobility parameters and its impact on sperm DNA quality. The results allowed us to observe an improvement in the advanced parameters of mobility, DFI and SDI after 5 min of incubation. Based on the results, we can conclude that oregano oil could be a safe therapeutic alternative for the management of motility dysfunction in Asthenozoospermic patients.

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