Cissampelos capensis Rhizome Extract Induces Intracellular ROS Production, Capacitation and DNA Fragmentation in Human Spermatozoa

S. Shalaweh, P. Bouic, F. Weitz, R. Henkel

Abstract—More than 3000 plants of notable phyto-therapeutic value grow in South Africa; these include *Cissampelos capensis*, commonly known in Afrikaans as dawidjie or dawidjiewortel. *C. capensis* is the most significant and popular medicinal plant used by the Khoisan as well as other rural groups in the Western region of South Africa. Its rhizomes are traditionally used to treat male fertility problems. Yet, no studies have investigated the effects of this plant or its extracts on human spermatozoa. Therefore, this study aimed at investigating the effects of *C. capensis* rhizome extract (CRE) fractions on ejaculated human spermatozoa *in vitro*. Spermatozoa from a total of 77 semen samples were washed with human tubular fluid medium supplemented with bovine serum albumin (HTF-BSA) and incubated for 2 hours with 20 µg/ml progesterone (P4) followed by incubation with different concentrations (0, 0.05, 0.5, 5, 50, 200 µg/ml) of fractionated CRE (F1=0% MeOH, F2=30% MeOH, F3=60% MeOH and F4=100% MeOH) for 1.5 hours at 37°C. A sample without addition of CRE fractions served as control. Samples were analyzed for sperm motility, reactive oxygen species (ROS), DNA-fragmentation, acrosome reaction and capacitation. Results showed that F1 resulted in significantly higher values for ROS, capacitation and hyper-activation compared to F2, F3, and F4 with P4-stimulated samples generally having higher values. No significant effect was found for the other parameters. In conclusion, alkaloids present in F1 of CRE appear to have triggered sperm intrinsic ROS production leading to sperm capacitation and acrosome reaction induced by P4.

Keywords—Capacitation, acrosome reaction, *Cissampelos capensis,* DNA fragmentation, ROS.

I. INTRODUCTION

REPRODUCTIVE problems such as poor quality or quantity of sperm, sexual dysfunction, and hormone disorders are of great concern and cause considerable distress, anxiety and a decrease in sexual confidence especially in males [1]. Studies have shown that the male factor contributes approximately 30-50% to fertility-related cases and that almost 50% of male infertility cases are classified as idiopathic [2]. Most sexual problems tend to increase with age, affecting more men than women [3]. Environmental and

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biological factors may have contributed to the male factor and could possibly have resulted in defects during spermatogenesis [4]. Herbal medicines are being used by many people as a form of therapy worldwide [5]. There is a variety of herbal practices that are dominant today such as Chinese herbs, Ayurvedic medicine [6]. About 70% of traditional Chinese medicine consists of natural herbs which are used to treat illnesses such as bronchial asthma, irritable bowel syndrome, arthritis [6]. Due to the fact that modern medicines are expensive, most people living in Third World countries, especially in Asia and Africa, are forced to use herbal medicines due to their affordability and accessibility. However, only a small number of plant extracts have been investigated in relation to male reproductive health [7]. Different parts of the world incorporate a variety of mechanisms to combat issues pertaining to male reproductive health. Each method entails its own specific or particular herb of choice; the basis of which depending on socio-economic factors as most underdeveloped countries cannot afford to use western medicine. In Asia and Eastern Europe *Tribulus terrestris* is a medicinal plant that has been used for centuries to increase sexual desire and enhance erection [8]. People have also been using plants such as *Panax ginseng* for the treatment of impotency and improving sexual stamina [9]. However, Tongkat Ali (*Euryco malongifolia*) is a native plant located throughout South East Asia which has been used as aphrodisiac to enhance testosterone levels and to treat erectile dysfunction [10]. In Central Andes, South America *Lepidum meyenii* (Maca) is used to improve male fertility [11]. In Africa, the alkaloid yohimbine derived from the yombi tree (*Pausinystalia yohimbe*) is used to increase sexual arousal and sexual dysfunction [7]. In West Africa, a study on plant extracts of *Hibiscus macranthus* and *Basella alba* revealed effects to enhance testosterone production [12]. *In vitro* studies on *Mondia whitei*, which is used as an aphrodisiac in Ghana, have shown that an aqueous administration enhanced total motility and progressive motility [13]. The African medicinal plant *Securidacalongi pedunculata* (Polygalaceae) and *Fadogia agrestis* (*Rubiaceae*) is used to treat erectile dysfunction [14].

South Africa has an exceptional variety of more than 30,000 higher plants, of which about 3,000 are used as traditional remedies [15]. However, only very little is known about the action and use of these plants, their extracts and their phytochemicals, particularly for male reproductive health problems. One of the plants that are traditionally used, but

have not been scientifically investigated on its effects on male fertility/infertility is *Cissampelos capensis*. This plant is endemic to the Western Cape, South Africa, is also known by the Afrikaans name "dawidjiewortel". It is the best known and most frequently used species in its family (*Menispermacea*). In Khoisan ethnomedicine, *Cissampelos capensis* is of special significance because of its variety in treatment applications [16]-[18]. The rhizomes are widely used as a blood purifier and a diuretic medicine; it is also applied to treat ailments such as fever, diabetes, stomach and skin cancer, cholera, syphilis, colic, bladder problems, snakebite, tuberculosis, menstrual problems, prevention of miscarriage and expelling the placenta [19]-[21]. Amongst these different usages, it is also said that the rhizome extract can be used to treat male fertility problems. In general, it is assumed that the medicinal therapeutic activity of the rhizomes is due to alkaloids such as the alkaloid bisbenzyltetrahydroisoquinoline which is known to have anti-inflammatory effects, muscle relaxant and anti-carcinogenic activity [16]-[18]. However, only one study [22] reporting the effect of *C. capensis* rhizome crude extract on male reproduction functions is available. Therefore, this study aimed at obtaining a more detailed insight in the nature of the bioactive compounds after fractionation of *C. capensis* crude extract and the effects of these fractions on human sperm functions.

II. MATERIALS AND METHODS

A. Chemicals

Unless otherwise mentioned, all chemicals were obtained from Sigma, St. Louis, MO, USA.

B. Preparation of Aqueous Cissampelos capensis Rhizomes Extract Fractions

Cissampelos capensis rhizomes were collected in the Cape Nature Reserve, Bellville, South Africa, cleaned and cut into 1-2 cm pieces, which were dried in a drying oven at 25°C and finally milled to a powder. This powder was extracted for 20 minutes with hot distilled water, filtered (Whatman 1 filter; Whatman, Madestone, UK), frozen at -20°C and finally freeze-dried using a Virtis freeze-dryer (Virtis, Warminster, PA, USA) to obtain *C. capensis* crude extract (CRE).CRE was then fractionated on a discontinuous methanol (MeOH) gradient (0%, 30%, 60% and 100%) using an Oasis® Hydrophilic-Liphophilic Balance (HLB) 6cc cartridge (Waters Corporation, Milford, MA, USA) connected to the Supelco-Visiprep™ manifold under vacuum at a constant pressure of 7 kPa. This technique separated the extract into hydrophobic or hydrophilic compounds. Accordingly, fraction 1 (F1) is most hydrophilic (0% MeOH) and F4 most hydrophobic (100% MeOH).Following the activation of the gel with 4 ml MeOH and calibration with 4 ml purified water, freeze-dried CRE was dissolved in double-distilled water (ddH$_2$O) at a concentration of 0.1 g/ml and 1 ml of this sample was loaded. The initial test tube was then replaced with one for F1 (0% MeOH) collection. After absorption of the extract into the bed, 4 ml of purified water were added to clean out the remaining debris. This process was continued for the F2 to F4 fractions.

For the F2, the cartridge bed was loaded with 1 ml 30% MeOH, the F3 with 1 ml 60% MeOH and for the F4 fraction with 1 ml 100% MeOH. After collection, fractions F1, F2 and F3 were frozen at -20 C° and subsequently freeze-dried, whereas the F4 fraction, due to the 100% MeOH content, had to undergo an evaporation step before freeze-drying. The extracts (F1, F2, F3 and F4) were then used in the bioassays at concentrations of 0.05, 0.5, 5, 50, 200 µg/ml. The extraction process of CRE led to a yield of 9.47% freeze-dried yellowish powder, of which the distribution amongst the 4 fractions was quite equal. F1, F2, F3 and F4 fractions contributed to CRE of 25.7%, 21.6%, 24.3% and 28.4%, respectively. After freeze-drying, all these fractions were of whitish color.

C. Semen Sample Collection and Preparation

This study was ethically approved by the local Institutional Review Board and patients and sperm donors gave informed consent. A total of 45 semen samples were collected after 3-5 days abstinence from 25 patients attending the infertility clinics of Tygerberg Hospital, Tygerberg, South Africa, and Vincent Palotti Hospital, Pinelands, South Africa, respectively, as well as 20 fertile semen donors. After liquefaction at room temperature, semen samples were diluted 1:5 with human tubular fluid medium, supplemented with 1% bovine serum albumin (HTF-BSA) (280 mOsmol/kg) and centrifuged for 10 minutes at 500xg. The supernatant was discarded and the pellet resuspended in fresh HTF-BSA. After subsequent washing, sperm were incubated for 2 hours in HTF-BSA allowing for capacitation. Thereafter, sperm suspensions were incubated with fractions F1, F2, F3 and F4, respectively, at different concentrations (0.05, 0.5, 5, 50, 200 µg/ml) with (20 µg/ml) or without progesterone (P4) for 1.5 hours at 37°C. P4 was prepared as a stock solution in dimethylsulphoxide (DMSO).

D. Determination of Sperm Motility

Sperm motility was measured with the Motility Concentration module of the Sperm Class Analyzer version 4.1.0.1 (Microptics, Barcelona, Spain). An aliquot of 10 µl of the sperm suspension was put on a glass slide covered with a cover slip and the motility of at least 100 sperm was analyzed. The following kinematic parameters were analyzed: total motility, progressive motility, beat cross frequency (BCF), linearity (LIN), straightness (STR), average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL) and the percentage of hyper-activated sperm.

E. Determination of Reactive Oxygen Species (ROS) Producing Sperm

Sperm ROS production was determined using dihydroethidine (DHE) (Molecular Probes, Eugene, OR, USA) as fluorescing probe according to [23]. In brief, a stock solution was prepared by using 20 µM DHE in PBS at pH 7.4. After incubation of sperm samples with F1-F4 fractions for 1.5 hr at 37°C, an aliquot of 100 µl of spermatozoa was centrifuged for 10 min at 500xg. Subsequently, the supernatant was discarded and samples were re-suspended in 100 µl PBS and 20 µl DHE stock and then incubated for 15

min at 37ºC. Afterwards, 10 µl of each sample was viewed on a slide covered by a cover slip under oil immersion using an epifluorescence microscope with 488 nm excitation and 590 emission filters (Zeiss, Oberkochen, Germany). Red fluorescing sperm indicate excessive ROS production. The percentage of ROS-producing sperm was calculated from at least 200 spermatozoa.

F. Determination of Sperm DNA-Fragmentation

The DNA fragmentation of spermatozoa, according to [24] was determined by using the TUNEL (Terminal deoxynucleotidetransferase-mediated dUTP nick-end labelling) assay. The suitability and reproducibility of the assay is accompanied by the adequate detection of single and double DNA strand breaks [25]. In brief, spermatozoa were incubated with different concentration of CRE fractions for 1.5 hour at 37°C. Samples of 100 µl aliquots were centrifuged for 10 min at 300xg, the supernatant discarded and the pellet was resuspended with 100 µl PBS. A smear was made on Superfrost® slides and air dried. After air drying, slides were fixed in freshly prepared 4% methanol-free formaldehyde in PBS for 25 min at 4°C. Slides were then washed in PBS for 5 min at room temperature, permeabilized with 0.2% triton X-100 in PBS for 5 min and washed with fresh PBS twice for 5 min. After removing excess liquid samples were equilibrated for 5-10 minutes in 100 µl equilibration buffer. Thereafter, 20 µl TdT buffer was added, the sample covered with a cover slip and incubated for 1 hour at 37°C. Eventually, slides were immersed in 2X SSC for 15 minutes, washed thrice in distilled water and evaluated in an epifluorescence microscope at 630-times magnification. The percentage of green-fluorescing spermatozoa (TUNEL-positive) was calculated.

G. Determination of Capacitation and Acrosome Reaction

To determine capacitation and acrosome reaction, the chlorotetracyclin (CTC) fluorescence assay as described by [26] was employed. In brief, a 100 mg/ml stock solution of Hoechst 33258 was made up in distilled water and stored at 4ºC for up to 1 month. Before use, this stock solution was diluted 1:1000 in HTF and then further 1:100 with sperm suspension in HTF-BSA. Thereafter, the samples were incubated at room temperature for 2 minutes before being washed by centrifugation through 4 ml of 2% polyvinylpyrrolidone (PVP40) in HTF at 900xg for 5 minutes. The CTC staining solution was prepared on the day of use and contained 750 µM CTC in a buffer of 130 mMNaCl, 5 mM cysteine, 20 mMTris-HCl. The pH was adjusted to pH 7.8. This solution was kept wrapped in aluminum foil at 4ºC until use. Hoechst-treated spermatozoa (45 µl) were mixed with an equal volume of CTC solution and 8 µl of 12.5% w/v paraformaldehyde in 0.5 M Tris-HCl (pH 7.4) were added. Subsequently, 10 µl of the this suspension were placed on a slide and one drop of 0.22 M 1,4-diazabicyclo(2.2.2)octane (DABCO) dissolved in glycerol:PBS (9:1) was mixed in carefully. Slides were viewed with a 100x oil immersion objective using a fluorescence microscope. In each sample, 200 live (Hoechst-negative) cells were assessed for CTC staining patterns as follows: uniform fluorescence over the entire head (non-capacitated, acrosome-intact cells); fluorescence-free band in the post-acrosomal region (capacitated, acrosome-intact cells); and dull or absent fluorescence over the sperm head (capacitated, acrosome-reacted cells).

III. STATISTICAL ANALYSIS

All statistical calculations were performed using the MedCalc Statistical Software (Version 14.12.0; Medcalc Software bvba, Ostend, Belgium).After testing for normal distribution by means of the Kolmogorov-Smirnov test; appropriate tests (ANOVA, repeated measures analysis and independent t-test) were performed for further analysis. A P-value $P < 0.05$ was regarded as significant

IV. RESULTS

A. Sperm Motility

Although total and progressive motility, velocity curvilinear and straightness were not very much affected by increasing concentration of all fractions without P4, significant, dose-dependent effects on various motion parameters were observed. While values for VAP, VSL and linearity decreased the percentage of hyper-activated sperm and beat cross frequency increased significantly ($P < 0.001$) ANOVA: $P < 0.001$, with F1 yielding a higher significant effect compared to F2, F3 and F4. In addition, motility parameters analyzed for fractions with P4 in also depicted F1 to produce significant effects ($P < 0.001$) compared to F2, F3 and F4. While progressive motility and STR were not affected by increasing concentrations of F1, the values for VSL and linearity decreased with increasing concentrations of the F1 extract ($P < 0.001$) with increasing concentration of the fractions as well as a significant positive trend (ANOVA: $P < 0.001$). On the other hand, the percentage of hyper-activated sperm and the beat cross frequency increased significantly ($P < 0.001$).

B. Reactive Oxygen Species Production

The treatment of human spermatozoa with increasing concentrations of fractions of CRE in the presence or absence of P4, respectively, caused a significant (ANOVA: $P < 0.0001$), dose-dependent increase in the percentage of ROS-positive spermatozoa (Figs. 1 A and B). This significant dose-dependent increase was evident in fraction 1 (ANOVA: $P < 0.0001$), but not in F2, F3 and F4. The percentage of ROS-positive spermatozoa was significantly ($P < 0.0001$) after exposure to various concentrations of the F1 fraction compared to the ones of the other fractions. In addition, progesterone stimulation caused a significant dose-dependent increase in the intracellular ROS production as expressed as the difference between the two values (ΔROS). This effect was even more pronounced after exposure of the spermatozoa to increasing concentrations of the F1 fraction (Fig. 2).
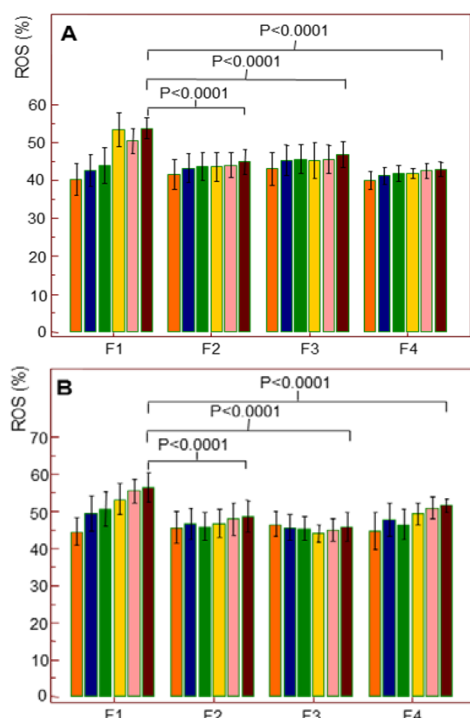
Fig. 1 The effect of *C. capensis* methanolic fractions on reactive oxygen species (ROS) *in vitro*: The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml.A) without P4; ANOVA for F1 $P<0.001$, F2 $P=0.028$, F3 $P=0.011$ and F4 $P<0.001$. B) with P4 ANOVA for F1 $P<0.001$, F2 $P=0.331$, F3 $P=0.105$ and F4 $P<0.001$
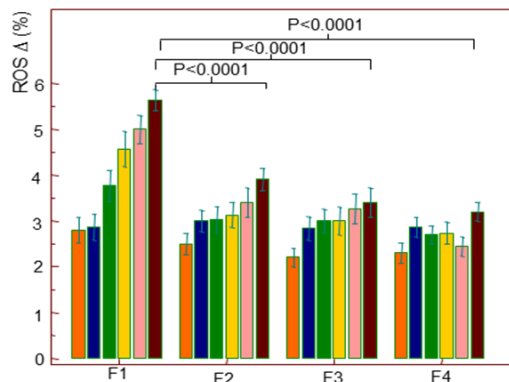

Fig. 2 The effect of *C. capensis* methanolic fractions on the difference in increase in intracellular production of reactive oxygen species (ROS) in the absence and presence of progesterone (ΔROS) *in vitro*: The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml. For ANOVA F1 $P<0.001$, F2 $P<0.001$, F3 $P<0.001$ and F4 $P<0.001$

C. Capacitation and Acrosome Reaction

Figs. 3 A and B demonstrate the percentages of capacitated spermatozoa for the different fractions in the presence or absence of progesterone. Results reveal that the F1 fraction led to higher numbers of capacitated spermatozoa compared to fractions F2-F4. Once again, the effect is dose-dependent

(ANOVA: $P<0.0001$). Furthermore, a significant positive trend (ANOVA: $P<0.001$) towards higher percentages of capacitated, acrosome-intact and capacitated, acrosome-reacted spermatozoa was found at higher concentrations from fraction F1 (Figs. 4 A and B). Moreover, progesterone stimulation caused a dose-dependent increase in the inducibility (induced AR - spontaneous AR) of acrosome reaction as shown for the percentage of capacitated, acrosome-reacted spermatozoa (ΔAR) (Fig. 5). While all concentrations for fractions F2, F3 and F4 similarly caused an initial increase and then a decrease, fraction F1 resulted in a constant dose-dependent increase, which was much bigger than for the other fractions. While comparing fractions on capacitated delta acrosome-reacted, fraction F1 showed a highly significant increase in a dose-dependent manner.
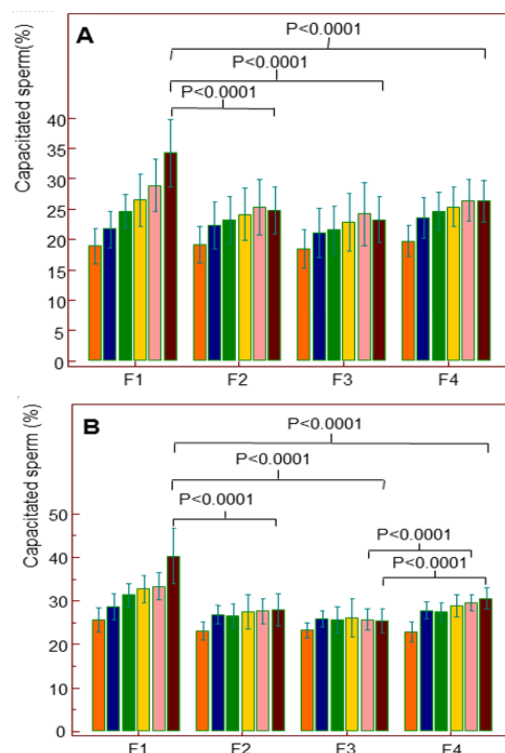

Fig. 3 The effect of *C. capensis* methanolic fractions on capacitated spermatozoa *in vitro*: The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml. A) without P4 ANOVA for F1 $P<0.001$, F2 $P<0.001$, F3 $P<0.001$ and F4 $P<0.001$. B) with P4 ANOVA for F1 $P<0.001$, F2 $P<0.001$, F3 $P<0.001$ and F4 $P<0.001$
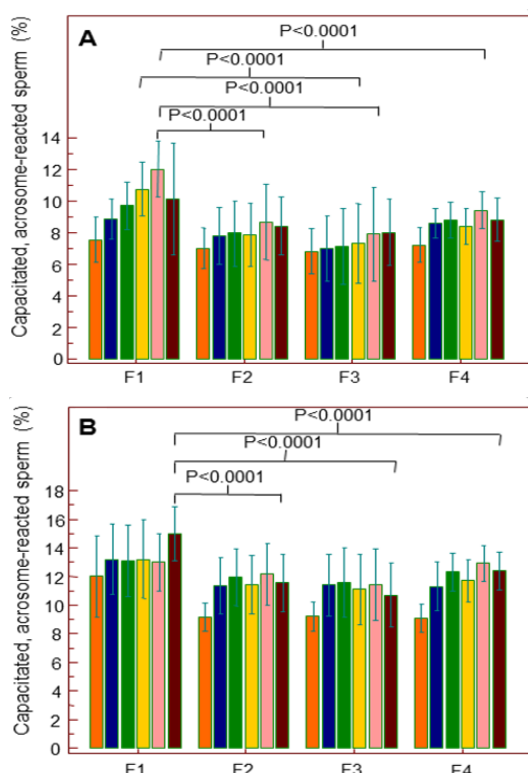
Fig. 4 The effect of *C. capensis* methanolic fractions on capacitated, acrosome-reacted spermatozoa *in vitro*: The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml. A) without P4 for ANOVA F1 P<0.001, F2 P=0.080, F3 P=0.222 and F4 P<0.001. B) with P4 for ANOVA F1 P=0.001, F2 P<0.001, F3 P<0.001 and F4 P<0.001
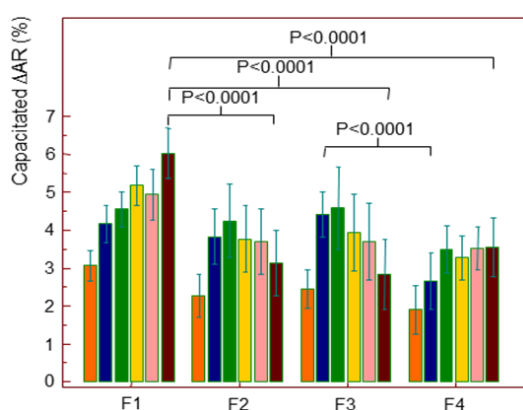

Fig. 5 The effect of *C. capensis* methanolic fractions on capacitated delta acrosome-reacted (ΔAR) spermatozoa *in vitro*: The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml. For ANOVA F1 P<0.001, F2 P<0.001, F3 P<0.001 and F4 P<0.001

D. Sperm DNA Fragmentation

The percentage of sperm DNA fragmentation increased significantly, with F1 yielding a higher significant effect compared to F2, F3 and F4. Increasing concentrations of the extract resulted in a dose-dependent, highly significant (ANOVA: P<0.001) increase in the percentage of spermatozoa with DNA fragmentation (Fig. 6).
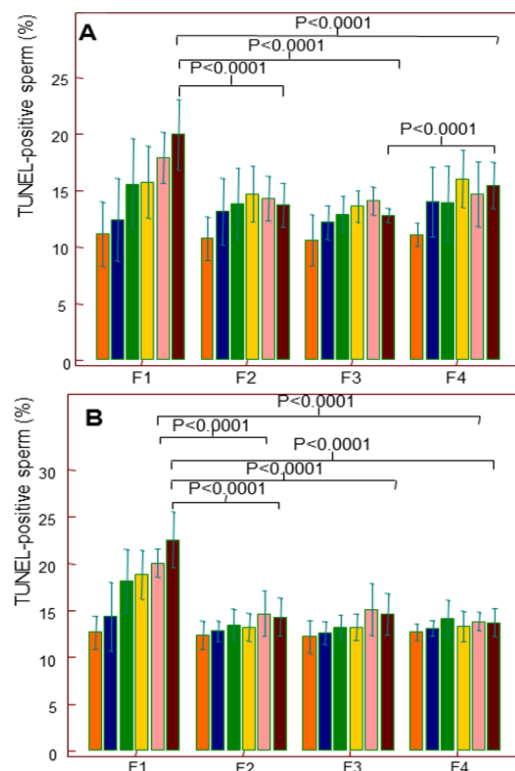

Fig. 6 The effect of *C. capensis* methanolic fractions on sperm DNA fragmentation *in vitro*: The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml. A) without P4; B) with P4

V. DISCUSSION

The use of plants as medicine dates back to 60,000 years ago. Today according to the World Health Organization [27], as many as 80% of the world's people depend on traditional medicine for their primary healthcare needs. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases [28], [39]. The Traditional Health Practitioners Act No.22 of 2007 is the regulatory framework and ensures the efficacy, safety and quality of the treatment in South Africa [29], [30], however traditional medicine is based on traditions and remedies passed down from generation to generation and is not always scientifically proven [31]. In Chinese medicine, more than 450 clinical trials are published investigating the efficacy of traditional medicines on ailments like asthma, cancer or diabetes [32], yet the amount of studies on investigating the effects of fertility is substantially lower [33]. Whilst investigating *Cissampelos capensis* it was found that the alkaloid content of the rhizomes is responsible for the medicinal value making it available throughout the year. While the leaves contain mainly three alkaloids namely bulbocapnine, dicentrine and salutaridine

with antimicrobial, antibacterial, antifungal and anti-inflammatory activity, respectively, the rhizomes contain mainly bisbenzyltetrahydroisoquinoline alkaloids, with cissacapine, 12-O-methylcurine and cycleanine as main alkaloids with average alkaloid yields of 15.3%, 35.9% and 46.3% respectively [34]. To date, only one report described the effects of *Cissampelos capensis* rhizome extract on human spermatozoa. In that report, an aqueous extract of *Cissampelos capansis* rhizomes causes a dose-dependent increase in sperm intrinsic superoxide production leading to sperm capacitation and DNA fragmentation, while acrosome reaction, total motility, sperm viability and mitochondrial membrane potential appear not to be affected. These processes might have been triggered by bisbenzyltetrahydroisoquinoline alkaloids, which represent the main compounds of the extract [22]. In current study, the major findings show that fraction F1 of CRE produced more significant, dose-dependent effects compared to fractions F2, F3 and F4. Increasing concentrations of F1 significantly decreased VAP, VSL and LIN and increased hyper-activation and BCF in human spermatozoa, but had no effect on sperm total motility, progressive motility, VCL and STR, whether in the presence or absence of progesterone. The F1 fraction also increased the percentage of ROS in the presence or absence of progesterone, which in turn caused the increased percentage of capacitated spermatozoa and inducibility of acrosome reaction. Freshly ejaculated spermatozoa must first undergo capacitation, a complex and timely series of changes, which is a prerequisite for acrosome reaction, before they can fertilize an oocyte in the female reproductive tract [35]-[37]. This process includes cellular changes that involve increased membrane fluidity, influx of calcium (Ca^{2+}), production of controlled amounts of ROS or phosphorylation of proteins on serine, threonine and tyrosine residues [35]-[38]. cAMP/PKA and P-PKA substrates, and the ERK pathway have been described as key in these phosphorylation processes during sperm capacitation [39]. In the present study, intercellular ROS concentrations increased after incubation of sperm with fractionated extract in a dose-dependent manner. Although, all fractions showed significantly increased values, the F1-fraction produced the most significant increase in ROS production. This effect may be attributed to the alkaloid content of the *C. capensis* rhizomes. Alkaloids can exhibit various effects on cells ranging from suppressing intracellular ROS-production [40], loss of mitochondrial membrane potential with intracellular ROS imbalance [41] to antioxidative [42] or antiproliferative effects [43]. For ergot alkaloids, a significant inhibitory effect on calcium ionophore-induced acrosome reaction was shown *in vivo* on stallion sperm [44]. In this study, seeing that superoxide ($\cdot O_2^-$) was detected and is triggered by CRE, one can deduce that CRE stimulates intracellular $\cdot O_2^-$ production, which subsequently triggered capacitation and caused sperm DNA fragmentation [45]. Therefore, even for the increase in sperm hyper-activation and all the relative changes in various motility parameters, this intercellular increase in $\cdot O_2^-$ might be responsible. Other factors might have either contributed to the induction of hyper-activation, or prevented a more distinct

relationship. On the other hand, one could assume that the stimulus by the CRE fractions via an increased production of $\cdot O_2^-$ would not be sufficient to induce acrosome reaction and may need additional stimulation. This assumption could be supported by the fact that bisbenzyltetrahydroisoquinoline alkaloids including 12-O-methylcurine and cycleanine have been described as potent Ca-antagonists [46], [47], thus inhibiting a Ca^{2+}-influx, which has been described as a trigger of acrosome reaction [48]. Thus, the alkaloids might have stimulated capacitation but inhibited the initiation of acrosome reaction. The inhibition of calcium channels could be the plausible explanation as to why acrosome reaction was not or only marginally stimulated even though capacitation was significantly triggered in the present study. Yet, by the inclusion of P4 to induce acrosome reaction showed that this trigger is still necessary, and can then lead to acrosome reaction. Although an additional stimulation seems to be required when incubated with CRE fractions, other mechanisms of action by the alkaloids may also play a role in the induction of acrosome reaction. When spermatozoa progress from the seminal fluid to the female reproductive tract and come in contact with the oocyte, their movement change from a relatively linear fashion to hyper-activated motility. This is described as a non-progressive, frantic, whiplash type, high amplitude that is necessary to penetrate the viscous environment of the zonapellucida [35], [49]. Hyper-activation is one of the physiological changes that are associated with the process of capacitation, which can be regarded as a preparatory step of the male germ cell for acrosome reaction [50], [51]. In the current study, treatment of sperm with CRE fractions showed no effect on sperm total and progressive motility. However, a significant and dose-dependent increase in sperm hyper-activation was shown and indicated by all relevant parameters that characterize hyper-activation (decreased VAP, VSL and LIN; increased BCF). Furthermore and similar to this study [52] demonstrated a correlation between the fertilizing capacity of rat spermatozoa *in vitro* and a decline in VSL. Probably, CRE F1 led to a change in the motility pattern, at least in a small percentage of spermatozoa. Whether this stimulation of hyper-activation is a direct effect of the F1 or it is mediated perhaps by ROS, as ROS physiologically functions as a trigger for capacitation and hyper-activation [35], [53]-[55], needs to be clarified. However, CRE fractions when incubated with spermatozoa with or without the addition of progesterone to induce acrosome reaction, hyper-activation still showed an increase. Although the F2, F3 and F4-fractions revealed significant increases in these parameters, the F1-fraction produced even significantly higher levels.

In conclusion, the F1 fraction of a methanolic fractionation of aqueous *Cissampelos capensis* rhizome extract is much more potent in its effect on sperm intracellular ROS production, capacitation, DNA fragmentation and hyper-activation compared to the other fractions. These changes may be attributed to alkaloids that are present in CRE F1, whereby they are thought to trigger sperm intrinsic ROS production, which leads to sperm capacitation and acrosome reaction as

well as DNA fragmentation that is induced by progesterone. Further work needs to be done on F1. The HPLC and NMR require further work. In addition isolation, formulation and testing of some of the compounds herein reported will be important.

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