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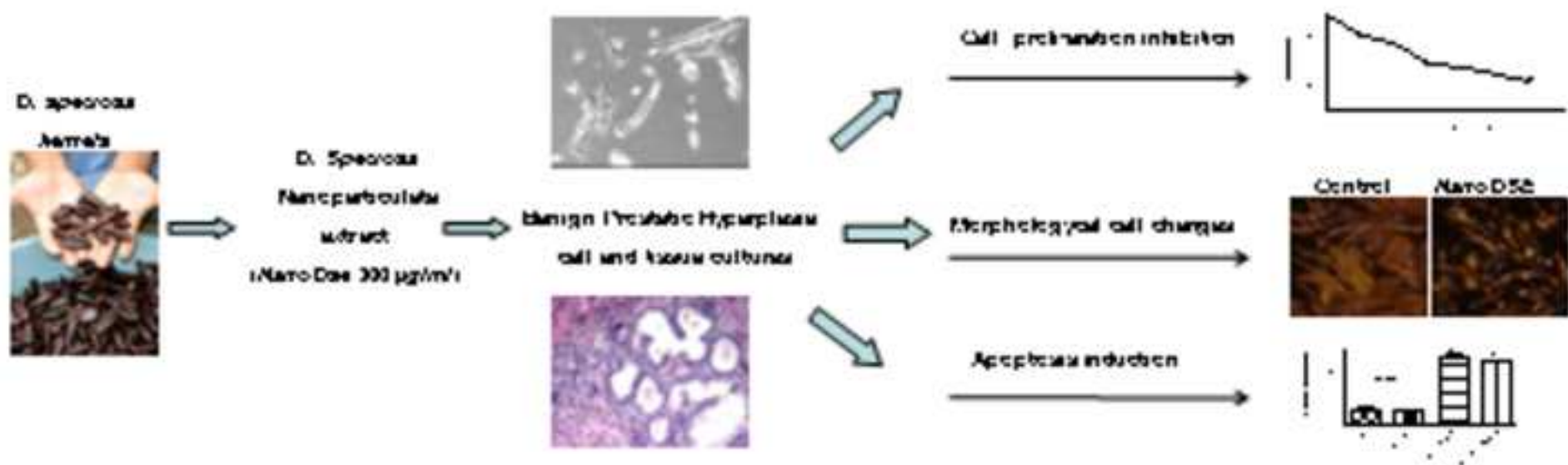
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Abstract

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Materials and Methods: *Orbignya speciosa* extract was obtained from the kernels, a nanoparticulate system was developed and acute toxicity test was performed. BPH primary stromal cell and tissue cultures were established and treated with 300 $\mu\text{g/mL}$ *Orbignya speciosa* nanoparticle (*NanoOse*) extract in order to evaluate its effects on apoptosis induction, cytotoxicity, cell morphology and proliferation.

Results: Our results indicated that *NanoOSE* shows no toxicity in animals and acts incisively by promoting morphological cell changes, reducing cell proliferation as well as inducing necrosis/apoptosis on BPH cells and tissues.

Conclusions: This study provided the first report of the successful use of *NanoOSE* on BPH treatment which corroborates with the popular use of the kernels of this plant. The results also suggest the potential of *NanoOSE* as a candidate new phytotherapeutic agent on the management of BPH.

Effects of a nanocomposite containing *Orbignya speciosa* lipophilic extract on Benign Prostatic Hyperplasia

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Conclusions: This study provided the first report of the successful use of *NanoOSE* on BPH treatment which corroborates with **the popular use of the kernels of this plant**. The results also suggest the potential of *NanoOSE* as a candidate new phytotherapeutic agent on the management of BPH.

Keywords: Benign Prostatic Hyperplasia, Phytotherapy, *Orbignya speciosa* extract, cell proliferation, apoptosis

1. Introduction

Symptomatic BPH is a common condition in men intimately related to ageing, and to bladder outlet obstruction associated with lower urinary tract symptoms (Berry et al., 1984; McVary, 2006). It is mainly caused by fibrotic changes and a non-proportional hyperproliferation of prostate stromal cells (McNeal, 1990; Chagas et al., 2002). Epithelial–mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation are the main source of BPH reactive stroma, contributing to alterations in cytokines, growth factors, and extracellular matrix components (Schauer et al., 2008, 2009; Alonso-Magdalena et al., 2009). Treatment options include pharmaceutical and phytotherapeutic preparations (Barry, 1997; Oesterling, 1995). The main pharmacological agents used for this approach are 5 α -reductase inhibitors, which regulate the levels of 5 α -DHT and α -adrenergic blockers (Tiwari, 2005). The extract of the dried ripe fruit from the American dwarf saw palmetto plant *Serenoa repens* (SPE) is routinely prescribed in Europe and in USA to relieve the symptoms of BPH and promote prostate health. Multiple mechanisms of action have been proposed, including inhibition of 5 α -reductase, interfering with the synthesis and metabolism of prostaglandins, inhibition of binding of DHT to the androgen receptor and inhibition of prostate cell proliferation mediated by growth factors (Tacklind et al., 2009; Gerber, 2000; Koch, 2001; Goldmann et al., 2001).

Babassu is the popular name of *Orbignya speciosa* or *Orbignya phalerata* Mart. (syn. *Attalea glazmanii* Zona Palms) [Arecaceae (Palmae)]. This palm has been used by Apinaye and Guajajara Indians from northeastern Brazil, yielding a variety of important products. Babassu palms provide food, fuel, shelter, fiber, construction materials, medicine, magic and other basic necessities of life for these people (Balick, 1988). The oral consumption of the aqueous extract of the babassu mesocarp is a common ethno botanical practice in the treatment of inflammatory diseases as it is the use of the kernel of this fruit to treat lower urinary tract symptoms, particularly in the surroundings of the Brazilian Amazon Rain

Forest. Regarding the kernels, unlike the preparation of an infusion for drinking, people eat them entirely or freshly grounded.

Some compounds have been isolated from *O. phalerata* such as tannins, sugars, saponins, steroid compounds (Bandeira et al., 1986) and triterpenes (Garcia et al., 1995). The anti-inflammatory and analgesic properties of babassu mesocarp were confirmed experimentally using a chloroform extract of the dried fruits **as shown by the inhibition of carrageenan-induced acute inflammation, cotton pellet granuloma and formalin-induced arthritis in rats (Maia and Rao, 1989; Sila and Parente, 2001).** The use of babassu epicarp /mesocarp ethanol extract in the *in vitro* treatment of three strains of human leukaemia (HL 60, K562 and K562 LUCENA 1) has been successfully shown a dose-dependent induction of apoptosis and cell necrosis, a decrease of cell proliferation, and profound morphological changes. Furthermore, significant anti-inflammatory effects were also observed (Rennó et al., 2008). Insofar, as BPH involves a tumor disease associated with a significant inflammatory component, these apoptotic, anti-inflammatory and antiproliferative effects indicate that the use of babassu could be effective in treating this disease. In addition, previous studies performed by Menezes F.S. showed that the lipophilic content of *O. speciosa* **kernel** extract is quite similar to the commercial extract of *S. repens*, raising the hypothesis that this species might also have pharmacological activity on BPH.

Herein, we analysed the *in vitro* effect of nanoparticles containing *O. speciosa* kernels extract on BPH stromal cell and tissue cultures, concerning to morphological changes, proliferation, viability, necrosis and apoptosis induction. Our results showed that this extract can be a valuable alternative approach to treat BPH.

2. Material and Methods

2.1 *Orbignya speciosa* nanocomposites extract (NanoOSE) obtention

O. speciosa was collected in the state of Piauí, northeastern Brazil. A herbarium sample has been deposited in the Herbarium Gabriella Barroso at UFPI under the voucher number TEPB 18985. Its fruits were dried and separated in its parts: mesocarp, endocarp, pericarp and kernel, being the kernel of interest in this study. *O. speciosa* kernels were then submitted to solvent extraction with ethanol, using cold dynamic maceration in order to avoid potential degradation of the active constituents. The resulting extract had the solvent fully evaporated under reduced pressure using a rotatory evaporator, thereby assuring the presence only of its chemical constituents. The ethanol extract has been analyzed by gas chromatography-mass spectroscopy (GC-MS) and had the identity of all its constituents determined. It is a lipophilic extract composed **almost exclusively** of fatty acids, and its main constituent is lauric acid. Other constituents found were capric acid, myristic acid, palmitic acid, stearic acid, oleic acid and linoleic acid. A single batch of the extract was used for the treatment of all cultures. It was obtained as an ethanol *O. speciosa* oily extract, redissolved in ethanol, to produce a concentrated stock solution of 10 mg/mL. In initial experiments, it was observed a problem with the solubilisation of the natural extract (oily) in culture media (aqueous), with the obvious implication that the extract could not possibly come into contact with cells and tissues plated at the bottom of culture wells and bottles. To try to overcome this problem, a nanoparticulate system was developed by intercalation of *O. speciosa* kernels extract in a Viscogel B8. This is an organically modified bentonite (a natural clay) derived form, using polyvinylpyrrolidone (PVP) as plasticizer at 1:1:2 (w/w) ratio. The intercalation process was carried out using dichloromethane as diluent during 72 h under stir. The solvent was removed by rotaevaporation under vacuum at 45 °C. Interesting to mention that PVP (**Hanka, 1971; Angervall and Berntsson, 1961**) and bentonite is perfectly inert and has no effect alone in the models used in this study. Each gram of the resultant nanocomposite (*NanoOSE*) contains exactly 159.05 mg $\pm$ 4.21% of *O. speciosa* extract (average $\pm$ RSD), as determined by several GC-MS analyses. The *O. speciosa* extract release from the *NanoOSE* was evaluated using the apparatus 1 of United

States Pharmacopeia (Hanson research, CA, USA), with simulated gastric fluid (HCl 0.1 M, pH 1.2) and 1% sodium lauryl sulphate (LSS) as dissolution medium, at 75 RPM and 37 °C. All cultures were treated with the *NanoOSE*. A stock solution containing 30 mg/mL of *NanoOSE* was prepared in absolute ethanol. The *NanoOSE* stock solution was further diluted in DMEM supplemented with 10% fetal bovine serum (FBS), 0.5% ethanol and 20 nM 5 α -dihydrotestosterone (DHT-Sigma Chemical Company, St Louis, MO, USA) to provide a 300 μ g/mL of *NanoOSE* working solution.

2.2 Source of prostate tissue

These studies were approved by the Ethics Committee from Federal University of Rio de Janeiro, protocol n° CAAE-0029.0.197.000-05, and informed consent was obtained from the donors. Prostate tissues were obtained under sterile conditions from patients submitted to transurethral resection of the prostate (TURP) or open prostatectomy for Benign Prostatic Hyperplasia (BPH) at the Andaraí General Hospital, Rio de Janeiro. All of the patients had moderate to severe lower urinary tract symptoms and were diagnosed by having BPH based on the combination of their clinical symptoms, the digital rectal examination, PSA determinations and prostate ultrasonography. An independent and experienced pathologist made the histopathological analysis. Tissue samples of patients with BPH with some of the following conditions were excluded: prior prostate surgery, prostate cancer, treatment with α -blockers, 5 α -reductase inhibitors, LH-RH analogues, anti-androgens and herbal extracts. Following removal, these specimens were immediately placed in ice-cold Dulbecco's Modified Eagle's Medium (DMEM-Gibco by Invitrogen, Carlsbad, Calif) and transported to the laboratory.

2.3 BPH stromal cell and tissue cultures establishment and characterization

BPH stromal cells were isolated according to previously described methods (Kassen et al., 1996). Briefly, prostate tissue was washed with phosphate-buffered saline (PBS) to remove

all traces of blood, before being diced into approximately 1-mm³ pieces using forceps and scissors. The fragments were transferred to 10 mL dissociation flasks containing a solution of DMEM supplemented with 10% FBS and 1mg/mL of type I collagenase (Sigma, St Louis, MO). Tissue specimens were dissociated by magnetic bar constant stirring for 2-4h at 37°C. The supernatant was frozen at 4°C and the remaining tissues were submitted to a new cycle of dissociation likewise above described. The supernatant of the first and the second cycle were centrifuged and washed with balanced saline solution-calcium magnesium free (BSS-CMF) at 1200 RPM three times. The resulting cells were seeded in 25 mm³ flasks and left to allow attachment in a defined medium composed of supplemented DMEM (10% FBS, antibiotic/antimycotic mixture (Gibco): Penicillin 100 U/mL, Streptomycin 100 µg/mL and Fungizone 25 µg/mL) and placed in a tissue culture incubator at 37°C in humidified air containing 5% CO₂. Cells were fed 3 times a week and at sub-confluence (approximately 90% occupancy in each bottle), they were harvested using 0.05% trypsin/EDTA (both from Sigma), and replated. Cell cultures were initially analyzed by phase contrast microscopy in order to observe cell confluence and morphology. Immunocytochemistry characterization of the culture was carried out between first and sixth passages with 20 X 10⁵ cells fixed with 3.7% paraformaldehyde (PFA) for 20 min. After three washes with PBS, cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS. Cells were then incubated for 18 h at room temperature with the following primary antibodies: anti-laminin (Sigma – LAM-89; dilution 1:100); anti-fibronectin (Sigma-FN-15; dilution 1:200); anti-type IV collagen (Sigma-COL-94; dilution 1:100); anti-chondroitin sulfate (Sigma-CS-56; dilution 1:200); anti-vimentin (Sigma-LN-6; dilution 1:100); anti-α smooth muscle actin (Sigma-1A4; dilution 1:400) and anti-cytokeratin peptide 18 (Sigma-CY-90; dilution 1:400). The abelin proteins were detected using anti-monoclonal secondary antibody (Alexa 546; dilution 1:200, Invitrogen). The cells were then incubated with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, 5-µ g/mL, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS and 5% bovine serum albumin (BSA). For each

case, negative control slides consisted of sections incubated with the antibody vehicle or non immune rabbit serum or mouse serum. BPH tissue was dissected into 3 mm³ fragments, and two explants were transferred onto a well of a 24-well tissue culture plate, prepared in advance with 500 µL Agar gel previously heated to make it liquid. Cells were homogenized in 500 µL culture media + 10% FBS supplemented with antibiotics and subsequently cooled. Afterwards, 1 mL of medium composed by DMEM supplemented with antibiotics, 10% FBS and 20 nM DHT were added to each well and the plate was kept in a humidified atmosphere containing 5% CO₂ at 37°C. With this technique, we prevented cell release and adherence to the bottom of the wells, allowing the realization of tissue cultures. BPH tissue culture was maintained 72 h in culture before fixed with 3.7% PFA. Immunohistochemical staining (IHC) was performed as follows. Paraffin-embedded tissue sections (5mm thick) were cut, placed on silane-treated slides, and maintained at room temperature. After dewaxing, sections were treated with a solution of 3% H₂O₂ in 0.01 mol/L PBS, pH 7.5, in order to inhibit endogenous peroxidase activity. The slides were then immersed in 10 mM/L citrate buffer (pH 6.0) and heated in a microwave oven for 5 min to recover masked antigens; to reduce nonspecific antibody binding the sections were then incubated with PBS containing a 10% solution of normal goat serum and 5% BSA for 30 min. Sections were incubated with the following antibodies: the rabbit anti-PSA polyclonal antibody (Dako, Carpinteria, CA, A0562, dilution 1/400) and the mouse anti-p63 (clone 4A4, Santa-Cruz Biotechnology, Santa Cruz, CA; dilution 1/200). Incubations were carried out overnight and then revealed using Strept AB Complex/HRP Duet Kit (Dako, Carpinteria, CA), with 3, 3'-diaminobenzidine (Liquid DAB-Plus Substrate Kit –Zymed, South San Francisco, CA) as the chromogen and counterstained with haematoxylin. For each case, negative control slides consisted of sections incubated with the antibody vehicle or non immune rabbit serum or mouse serum.

2.4 BPH stromal cell and tissue cultures treatment with *NanoOSE*

It was used a stock solution of 10mL in a concentration of 30mg/mL of *NanoOSE* dissolved in absolute ethanol. The *NanoOSE* stock solution was further diluted in DMEM supplemented with 10% FBS, 0.5% ethanol and 20 nM 5 α -dihydrotestosterone (DHT-Sigma Chemical Company, St Louis, MO, USA), to provide working solutions ranging between 100 μ g/mL and 300 μ g/mL of *NanoOSE*. Controls were untreated and only exposed to DMEM supplemented with 10% FBS, 0.5% ethanol and 20 nM DHT.

2.5 Acute toxicity test

All experiments were carried out with male and female Swiss mice (18-25 g). The animals were kept in a temperature-controlled room ($22 \pm 2^\circ \text{C}$) by cycles of light / dark for 12 h with free access to food and water. The care of animals and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation. The parameters of acute toxicity were determined as described by Lorke (1983), with some modifications. The daily oral dose of *NanoOSE* (100 mg/kg) was administered to a group of 20 mice (ten males and ten females) for 10 consecutive days. After 30 min, 1, 4, 6 and 24 hours and daily until the tenth day, the following parameters were observed: the state of attention care and welfare (general appearance, irritability), motor coordination (general activity, response to touch, constriction response of the tail, abdominal contraction, walking, reflex stiffness), muscle tone, central nervous system activity (tremors, convulsions, hyperactivity, sedation, hypnosis, anaesthesia), the autonomic nervous system activity (lacrimation, defecation, urination, pilo-erection, hypothermia, breathing), and water and food intake. After the tenth day, the animals were killed by cervical dislocation, the stomachs were removed, an incision along the greater curvature was held, and the number of ulcers (single or multiple erosions, ulcers or perforation) and hyperaemic areas were counted. Blood samples were collected on the tails on the tenth day. An aliquot was diluted in 1/20 in Türk fluid. The

total number of cells was determined with the aid of haematocytometre. The differential cell counts were made after centrifugation and staining with haematoxylin-eosin.

2.6 Cell viability assay

Cellular viability or survival was obtained using the MTT (3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyltertrazolium bromide) colorimetric assay. For this assay, 7.5×10^4 BPH stromal cells were seeded into 96-well cell culture plate and were exposed for 24 h to *NanoOSE* concentrations ranging from 100 to 300 $\mu\text{g/mL}$, for the initial kinetics assay. After incubation, the medium was removed, the cells were washed, and a solution containing 5 mg of MTT per mL of 0.15 M PBS was added to each well to reach a final concentration of 0.5 mg/mL. Following incubation for 4 h at 37° C, the medium containing MTT was partially removed, and dimethyl sulfoxide (100 μL) was added to solubilise the MTT formazan product. MTT formazan formation was measured at 570 nm by using a spectrophotometer. **Afterwards, cell viability assay was performed with both the crude, oily extract and *NanoOSE* at the same concentration, comparing their results in 24, 48 and 72 h. Then, cell viability assays were done solely with *NanoOSE* again in 24, 48 and 72 h, with the chosen -300 $\mu\text{g/mL}$ - extract concentration.** After subtracting background absorbance, results were expressed as percent of viable cells, in relation to extract-free control cells. In addition, cell viability assays were also done comparing BPH stromal cell cultures (maintained for 72 h) with the nanoparticle carrier material (PVP/Bentonite) or DMEM, in order to assess if PVP/Bentonite were inert. All assays were done in triplicate.

2.7 Actin staining

The changes in cell morphology caused by treatment with *NanoOSE* were evaluated indirectly, through the use of the fungal toxin Phalloidine coupled to fluorochrome Cy3. 7.5×10^4 BPH stromal cells were seeded on 12-mm round cover slips placed in 24-well medical-grade

polystyrene plates (BD Falcon) and were treated as described for 4, 6, and 12h. After treatment and supernatant discards, cells were washed in PBS and fixed in 4% PFA for 10 min at room temperature in Sorensen's phosphate buffer for 20 min and washed with PBS. Cells were then incubated in 50 nM NH₄Cl in PBS for 30min and permeabilized in 0.5% Triton X-100 (Sigma) before washed and incubated with rhodamine-phalloidin (1/100-Sigma) for 20 min at room temperature. Cells were washed again, the nucleus stained with DAPI, mounted and photographed with an immunofluorescence microscope (Nikon, Tokyo, Japan) connected to a digital camera (Coolpix 990; Nikon). The images shown are representative of at least three separate experiments.

2.8 Lactate dehydrogenase (LDH) cytotoxicity assay

LDH release was measured by a nonradioactive protocol using the LDH kit (Doles). The end-point of the LDH assay is an estimate of damage to the target after exposure to *NanoOSE* for defined period (4, 8, 12, 24 and 48 hours). HPB stromal cells were detached from 25 mm³ bottles, and 1 x 10⁴ cells were seeded into 96-well cell culture plates. After culture incubation for a period of 72 h at 37°C in a humidified atmosphere containing 5% CO₂, cells were exposed to *NanoOSE* 300 µg/mL for 4, 8, 12, 24 and 48 h. 50µL of supernatant was transferred in quadruplicates into wells in a 96-well microtiter plate (MTP), and 100 µL of LDH reaction mixture added to each well. After 15 min at 37° C, the absorbance generated was read on iMARK microplate reader (BIO-RAD) at 490 nm with a 96-well plate ELISA reader. The LDH percent was expressed using the formula (sample value/maximal release) x 100. Maximal release was obtained treating control cells with 0.5 % of Triton X-100 (Sigma) for 10 min at room temperature, and the spontaneous release of LDH was obtained by control cells incubated with DMEM for the same period than *NanoOSE* treated cells.

2.9 PCNA immunohistochemical staining

BPH tissue cultures were treated with 300 µg/mL *NanoOSE* for 24 h, immunolabelled for proliferation cell nuclear antigen (PCNA) (PC-10, Dako Cytomation, Carpinteria, CA; dilution 1/100) and compared to non treated tissue cultures (controls). Briefly, immunohistochemical staining was performed as follows. Paraffin-embedded tissue sections (5 mm thick) were cut, placed on silane-treated slides, and maintained at room temperature. After dewaxing, sections were treated with a solution of 3% H₂O₂ in 0.01 mol/L phosphate-buffer saline (PBS), pH 7.5, in order to inhibit endogenous peroxidase activity. The slides were then immersed in 10 mmol/L citrate buffer (pH 6.0) and heated in a microwave oven for 5 min to recover masked antigens; to reduce nonspecific antibody binding the sections were then incubated with PBS containing a 10% solution of normal goat serum and 5% BSA for 30 min. Sections were incubated with PCNA. Incubations were carried out overnight and then revealed using Strept AB Complex/HRP Duet Kit (Dako, Carpinteria, CA), with 3, 3-diaminobenzidine (Liquid DAB-Plus Substrate Kit – Zymed, South San Francisco, CA) as the chromogen and counterstained with haematoxylin. For each case, negative control slides consisted of sections incubated with the antibody vehicle or non immune rabbit or mouse serum. The cells with brownish yellow granules in nuclei were regarded as PCNA positive cells.

2.10 Identification of apoptotic nuclei by TUNEL assay

Apoptotic nuclei were identified using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit S7100 (Chemicon, Temecula CA, USA) according to the manufacturer's protocol. Paraffin-embedded tissue sections (5mm thick) were cut, placed on silane-treated slides, and maintained at room temperature. After dewaxing, endogenous peroxidase was blocked by immersing slides in 3% H₂O₂. Negative controls were treated in the same manner except that the TdT labelling enzyme was omitted (Gavrieli et al., 1992). Apoptotic nuclei were visualized with diaminobenzidine (DAB-Biogenex, San Ramon - CA) as the chromogen substrate, and

counterstained with haematoxylin. Cells showing dark brown staining from the colorimetric reaction were considered positive for DNA fragmentation (Huppertz et al., 1999).

2.11 Histomorphometry

All tissue sections were examined by two blinded observers using an X 40 objective lens of a light microscope (Nikon, Tokyo, Japan) connected to a digital camera (Cool pix 990; Nikon). Ten fields of immunostained section (PCNA or TUNEL) were chosen at random and captured from each specimen. Quantification was assessed on captured high-quality images (2048 X 1536 pixels buffer) using the Image Pro Plus 4.5.1 (Media Cybernetics, Silver Spring, MD). Data was stored in Adobe Photoshop, version 3.0, to enable uneven illumination and background colour to be corrected. The number of PCNA or TUNEL stained nucleus in the BPH tissue cultures was counted. A semi quantitative evaluation of immunohistochemical staining for PCNA and TUNEL was performed according to the method described by Donnez et al. (1998). This method involves the analysis of the distribution and the intensity of staining within the glandular epithelium or stroma. The staining result was expressed as mean $\pm$ standard deviations.

2.12 Statistical analysis

For viability and LDH release assay results, the data represent the mean $\pm$ standard deviation from values of the number of independent experiments indicated. The differences between groups were analyzed by ANOVA one-way followed by Newman-Keuls multiple comparison test. The $p < 0.05$ (*) value was considered statistic significant. For TUNEL and PCNA assay, all statistical calculations were carried out using the Stat- Xact-5 software program (CYTEL Software Corporation, Cambridge, MA). The differences between groups were calculated using nonparametric analyses (Mann-Whitney U test). A p value of < 0.5 was established as statistically significant.

3. RESULTS

3.1 Physical-chemical features of *NanoOSE*

O. speciosa oil extracted from almonds has a yellow tinge and smell like coconut oil, having a melting point of 24th C, a saponification number of 225.6, density 0.924; 16-16,6 Bellier index, viscosity (0E, 500) = 10. It was found in *O. speciosa* oil a percentage of fatty acids such as caprylic 6.5%, capric 5.5%, lauric 44.5%, myristic 15.5%, palmitic 8.5%, stearic 3%, oleic 10%, linoleic 1%, arachidonic 0.7% and traces of caproic acid. As mentioned before, there is a considerable similarity between *S. repens* and *O. speciosa* extracts (data not shown).

3.2 *NanoOSE* does not show toxicity in animals

In order to evaluate potential toxic effects in animals, 100 mg/kg of *NanoOSE* was administered to mice. After 10 days of continuous administration, the animals showed no behavioural changes and or no bleeding gastric lesions were observed. Moreover, there were no signs of intoxication such as seizures, death and gastric ulcer during the observation period. These findings suggest that *NanoOSE* was practically nontoxic in mice up to an oral dose of 100 mg/kg body weight. There were also no significant changes in the estimate of white blood cells of treated animals compared to controls (Table 1).

Table 1: Hematological evaluation of *NanoOse* toxicity assay in mice
Total count of leukocytes ($\times 10^6/\text{mL}$)

Group	30 min	1h	4h	6h	24h	10 days
Vehicle	7.8 $\pm$ 1.4	7.1 $\pm$ 1.1	6.7 $\pm$ 0.5	6.9 $\pm$ 1.0	6.6 $\pm$ 0.7	6.3 $\pm$ 3.8
<i>NanoOs</i>	8.1 $\pm$ 0.9	7.4 $\pm$ 0.9	7.6 $\pm$ 0.8	7.3 $\pm$ 1.2	7.0 $\pm$ 1.0	7.3 $\pm$ 2.1

There were no significant changes in the assessment of white blood cells of treated animals compared to controls. Results are presented as mean $\pm$ SD. Statistical differences (* $p < 0.05$) between mean values were determined by one-way ANOVA followed by Bonferroni post-test.

3.3 NanoOSE decreases cell viability

From the third passage, we established a homogeneous BPH stromal cell cultures (Fig. 1A-B) with a diffuse immunolabelling of vimentin microfilaments in all cells (Fig. 1C); in some cells, it was also observed α -smooth muscle actin immunostaining (Fig. 1D). These stromal cell cultures had the expression of several important extracellular matrix components, including chondroitin sulfate, typical feature in BPH specimens, showing that these cultures have similarity in their behaviour, with the *in vivo* situation (Fig. 1E-H). As expected for such proliferating cells, we detected an increase in anti-vimentin labelling and a decrease in the number of cells labelled with anti- α -smooth muscle actin along the passages. This marker, however, never disappeared, suggesting the coexistence of fibroblasts and myofibroblasts.

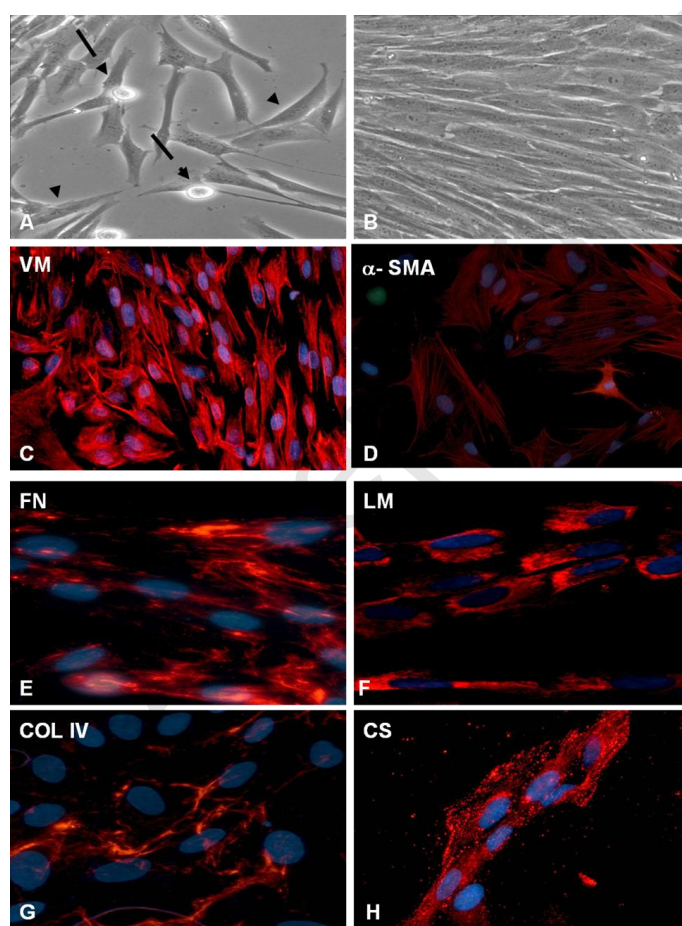


Figure 1. BPH stromal cell characterization.

(A) Phase contrast microscopy of BPH primary cell culture, 27 days after tissue dissociation, showing elongated fibroblastoid-like cells, and some round epithelial-like cells (dashed arrow). (B) Phase contrast microscopy of confluent established BPH stromal cell culture, fourth passage, with no epithelial-like cells. (C-H) Immunocytochemistry of BPH stromal cells, fifth passage, showing an immunolabeling of vimentin (C) and α -smooth muscle actin (α -SMA) (D) cytoskeleton microfilaments; and an immunodistribution of fibronectin (E), laminin (F), type IV collagen (G) and chondroitin sulfate (H) extracellular matrix components. A, B X250; C-H X400.

We treated these cells at 80% confluence for 24, 48 and 72 hour with doses ranging from 100 to 300 $\mu\text{g/mL}$. ***NanoOSE* at a concentration of 150 $\mu\text{g/mL}$ was able to inhibit cell viability by 50% (IC_{50}) in a 24 h exposure period. At 300 $\mu\text{g/mL}$ concentration, *NanoOSE* caused near 75% of cell viability inhibition in a 24 h exposure period and reached a plateau when these cells were exposed for longer periods such as 48 h and 72 h** (Fig. 2 A, B), even in the presence of FBS with its growth factors and hormones (Fig. 2 C). From this moment on, 300 $\mu\text{g/mL}$ was the *NanoOSE* **concentration chosen for the remaining experiments. Cell viability inhibition results were statistically significant higher with *NanoOse* than with *O. speciosa* crude, oily extract as shown in Fig. 2 D.** No significant difference was observed in cell viability, when stromal cell cultures were exposed to the nanoparticle carrier material compared to control **(data not shown).**

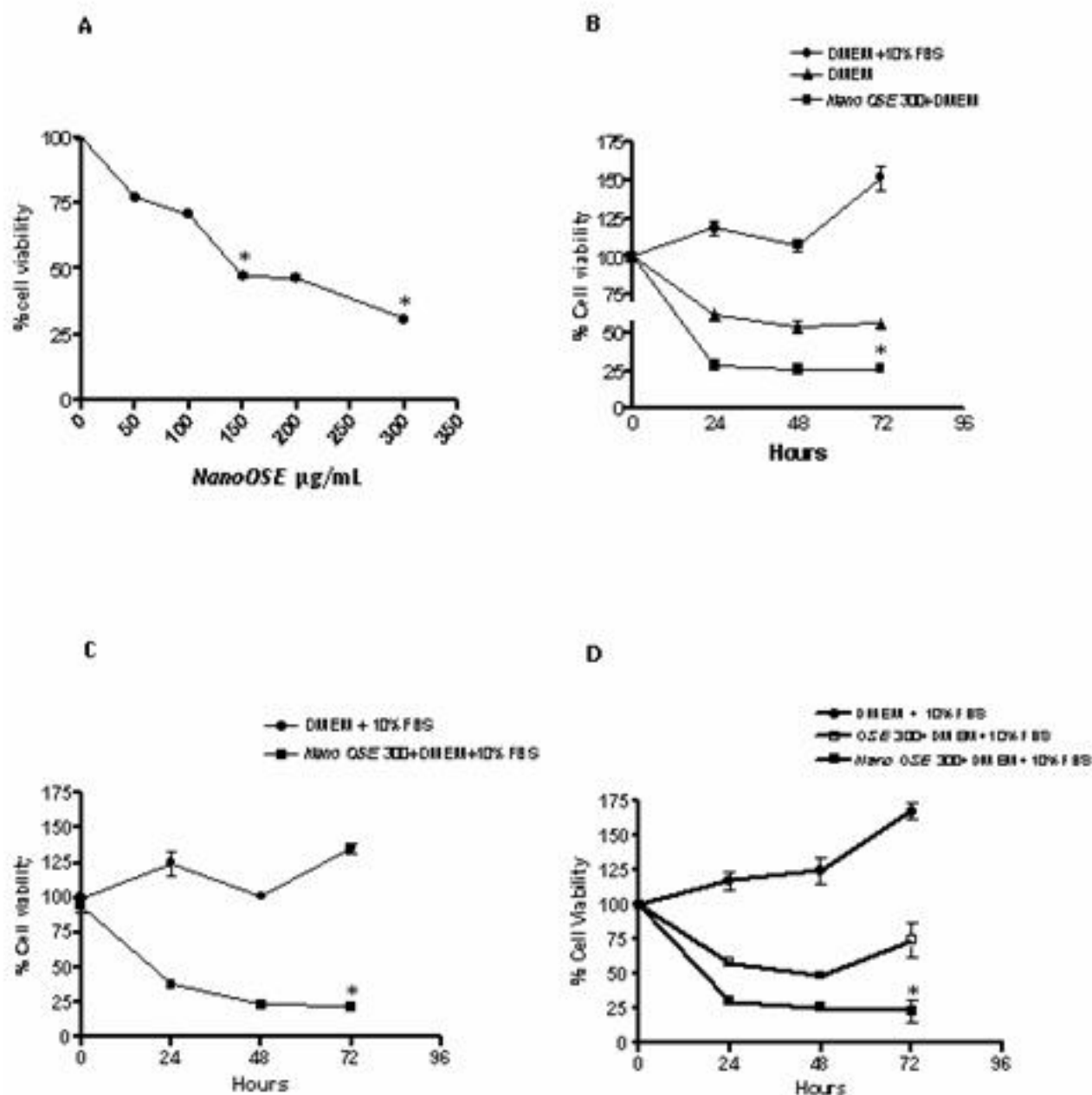


Figure 2. BPH stromal cell MTT viability assay.

BPH stromal cells treated with varying concentrations of *NanoOSE* for 24 h in DMEM, showing that 300 $\mu\text{g/mL}$ *NanoOSE* was able to induce about 75% reduction in cell viability (A); BPH stromal cells treated with 300 $\mu\text{g/mL}$ *NanoOSE* for 72 h in DMEM and compared with non treated cells in DMEM and DMEM + 10% FBS, showing that reduction in cell viability was maintained over 72 h (B), and even with the addition of 10% FBS, 300 $\mu\text{g/mL}$ *NanoOSE* was able to maintain its inhibition activity (C); BPH stromal cells treated with the crude, oily *O. speciosa* extract at 300 $\mu\text{g/mL}$ in comparison with 300 $\mu\text{g/mL}$ *NanoOSE* for 72 h. demonstrating superior inhibition of viability produced by *NanoOSE* over the crude extract (D). Data are expressed as the percentage of cells in the control treatment at 24 h. Bars $\pm$ SE, n = 3. Means with different letters are significantly different, $P < 0.05$.

3.4 *NanoOSE* promotes disorganization of the actin cytoskeleton

The results obtained demonstrated that *NanoOSE* induced disorganization of the structure of actin microfilaments. The actin microfilaments in cells treated with *NanoOSE* were disorganized, disassembled, or disrupted, on a time-progressive mode when compared to controls with no treatment, as shown on Figure 3.

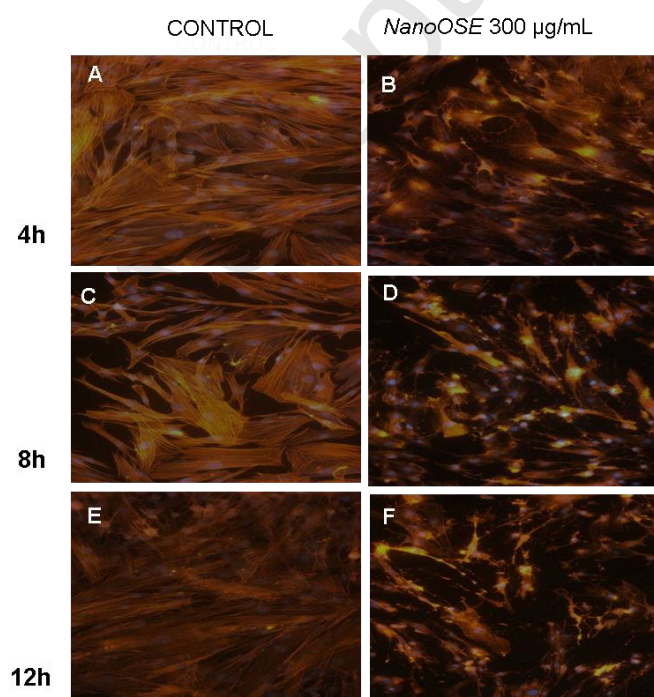


Figure 3. Phalloidin actin staining.

BPH stromal cells were stained with phalloidin after been maintained with complete medium for 4h (A), 8 h (C) and 12 h (E) or treated with 300 $\mu\text{g/mL}$ *NanoOSE* in complete medium for 4 h (B), 8h (D) and 12 h (F), showing an intense disorganization of the actin cytoskeleton in a time-progressive manner. A-F X200

3.5 *NanoOSE* increases LDH release in a time-progressive manner

Once determined that *NanoOSE* promotes decrease in cell viability and disorganized the cytoskeleton we were concerned about its cytotoxicity on human BPH stromal cells. Dose-response curve demonstrated that *NanoOSE* 300 $\mu\text{g/mL}$ induced a near 50% LDH release in 24 h (IC_{50}), rising up to 75% LDH release in 48 h (Fig. 4A). As shown in Figure 4B, *NanoOSE* 300 $\mu\text{g/mL}$ was able to increase LDH levels in periods of exposure as short as 4h. These findings point toward a significant *NanoOSE*-induced cytotoxicity on human BPH stromal cells.

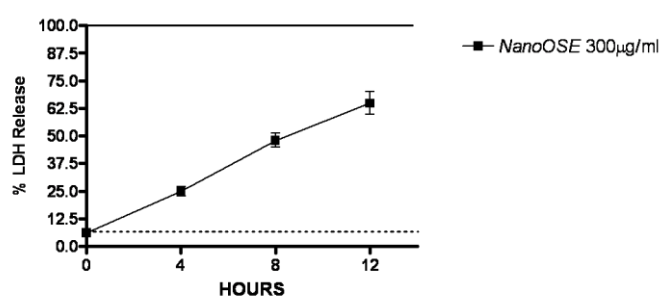
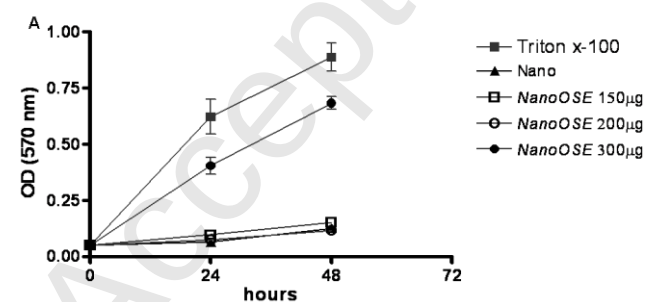


Figure 4. LDH cytotoxicity assay.

LDH release dose-response curve of BPH stromal cell culture treated with varying concentrations of *NanoOSE* in 24 and 48 h showing a progressive LDH release, reaching 70% as compared to cell lysis by 2% Triton (A), and treated with 300 $\mu\text{g/mL}$ *NanoOSE* for 4, 8 and 12 h (B), demonstrating an early effect on cell death. Dotted line represents normal spontaneous LDH release, while continuous line represents complete LDH release after Triton exposure.

3.6 *NanoOSE* reduces cell proliferation in BPH tissue cultures

BPH tissue cultures were established and maintained its morphological features, as observed in histological sections. Glandular architecture preservation was observed and reinforced by the expression of strong and focal immunolabelling for PSA and p63, pointing towards the presence PSA⁺ (epithelial) and p63⁺ (basal) cells. These cultures showed glandular and stromal immunoreactivity for PCNA (Fig. 5).

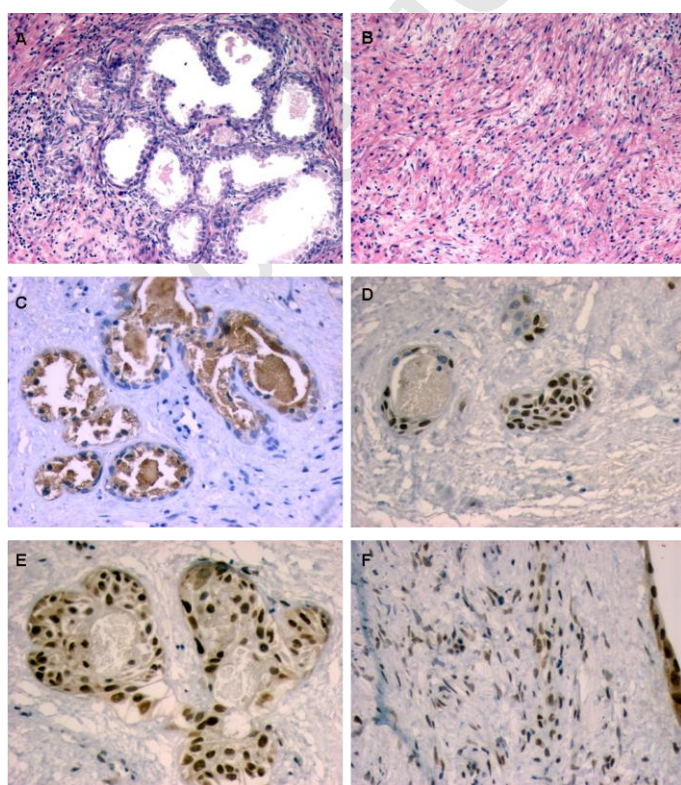


Figure 5. BPH tissue culture characterization.

A 72 h BPH tissue culture stained with hematoxylin and eosin demonstrating a typical BPH histological appearance, with enlarged prostatic glands and a folded epithelial line with eosinophilic luminal secretions and an intense stromal cellularity (A-B). Immunohistochemistry revealed PSA expression in the epithelium as well as in the glands lumen (C); p63 positive basal epithelial cells (D); and PCNA epithelium (E) and stroma (F) immunolabeling. A-F X200

BPH tissue cultures treated with a 24 h period of *NanoOSE* 300 $\mu\text{g/mL}$, demonstrated a remarkable reduction of PCNA immunostaining ($p < 0.001$) even in the presence of FBS, if compared to fresh BPH tissue and controls, indicating a significant reduction of cell proliferation as shown in Fig. 6. PCNA immunoreactivity within BPH tissue culture samples treated with *NanoOSE* was almost 50% lesser than that observed in fresh tissue and controls ($n=4$, $p < 0.05$ each) (Fig. 6).

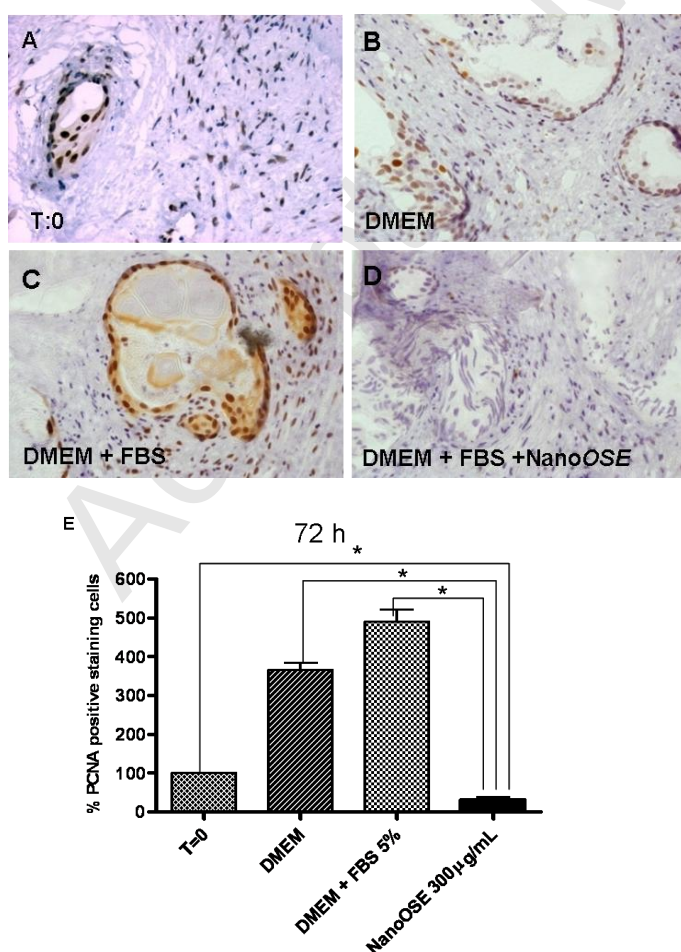
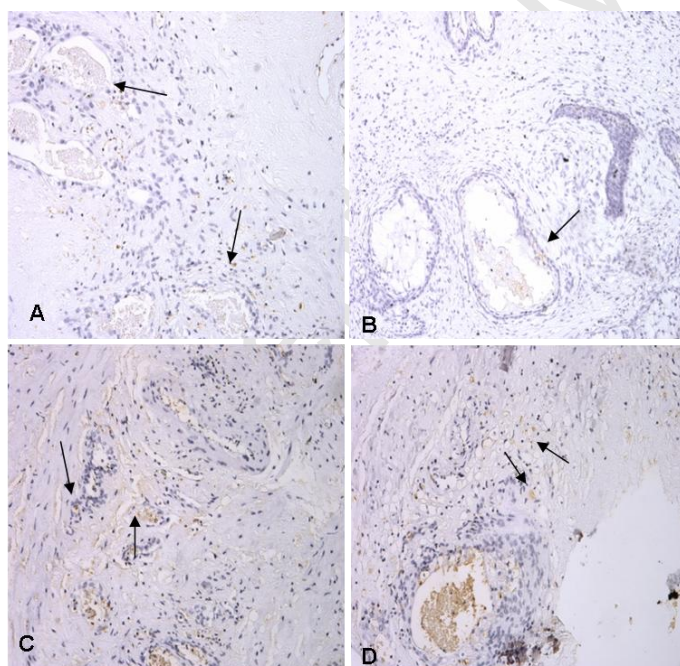


Figure 6. PCNA immunohistochemical staining.

PCNA immunolabelling of BPH tissue culture maintained 24 h in complete culture medium (A), 72 h in DMEM (B), 72 h in complete culture medium (C) and treated with 300 $\mu\text{g/mL}$ *NanoOSE* for 72 h in complete culture medium (D). Observe that the treatment produces a dramatic decrease in PCNA immunostaining, both in glandular epithelial and stromal cells. Histomorphometry analysis of PCNA immunolabelling (E). A-D X200.

3.7 *NanoOSE* promotes apoptosis in BPH tissue cultures

As shown in Figure 7, 24 h after exposure, there was a significant atrophy and the results of the TUNEL assay revealed diffuse apoptosis of prostate glands and a statistically significant increase in the apoptotic index observed in *NanoOSE* treated BPH tissue cultures compared with the untreated control groups.



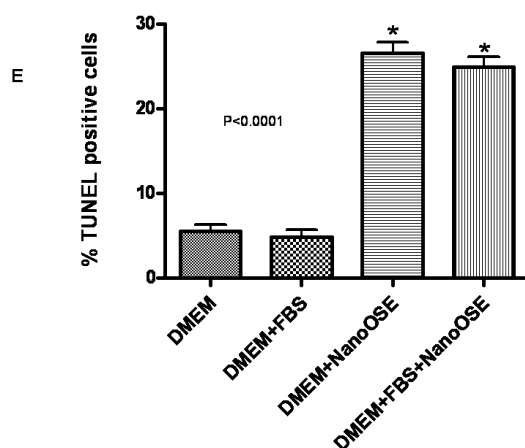


Figure 7. TUNEL assay.

TUNEL staining of BPH tissue culture maintained for 72 h in DMEM (A), in complete medium (B), treated with 300 $\mu\text{g/mL}$ *NanoOSE* for 72 h in DMEM (C) and treated with 300 $\mu\text{g/mL}$ *NanoOSE* in complete medium (D). Note that the treatment caused an intense and diffuse immunolabelling indicating increasing apoptosis induction. Arrows are indicating some apoptotic nucleus. Histomorphometry analysis of TUNEL assay (E). A-D X200.

4. DISCUSSION

BPH understanding and treatment have become really complex recently. At present, the treatment of BPH includes monitoring, pharmacological treatment and minimally invasive surgery, and operation. Most patients presenting initially with BPH are candidates for medical therapy, and during recent years, the number of surgical interventions in BPH has decreased in favour of medical treatments. Since the condition usually requires continuous medication, long-term tolerability is crucial issue. As some $\alpha 1$ -blockers may be associated with postural hypotension, and 5α -reductase inhibitors may cause sexual dysfunction (Clifford and Farmer, 2000), there is a considerable interest in well-tolerated and effective herbal remedies. Indeed, drugs from the latter group are still hugely popular in Europe and have gained widespread usage in the United States of America as well over the past 10 years (Lowe and Fagelman, 2002). Saw palmetto extract (*SPE*) in particular has been investigated extensively. It has been determined to

be superior to placebo and comparable to the 5 α -reductase inhibitor finasteride, in improving urinary tract symptoms and flow measures (Boyle et al., 2004; Gerber and Fitzpatrick, 2004; Gong and Gerber, 2004; Wilt et al., 1998). Currently, scientific evidence is suggestive, but not conclusive, of the beneficial effects of this agent for treatment of lower urinary tract symptoms. *SPE* selectively and specifically induces apoptosis in human primary epithelial and stromal cells of prostate origin, but not other target organs (Bayne et al., 2000). **In this study, ethanol has been used to extract the kernels of *O. speciosa* mainly due to the lack of toxicity of this solvent when compared with other solvents. Furthermore, after the evaporation of the solvent, the amount of possible contaminants left over is lower when ethanol is used, allowing the extract to be used in cell cultures. Knowing that the kernels are mainly composed of fatty acids, the decision to use ethanol instead of hexane was beneficial without concerns about the possibility of extracting anything more polar. After the evaporation of the solvent the *O. speciosa* kernel extract has been converted into a nanoformulation named *NanoOSE*.**

NanoOSE, a lipophilic **extract** composed by fatty acids has a significant chemical similarity to *SPE*, and we can infer, at least in part, that their mechanisms of action are quite similar. The recommended dose of *SPE* is 320 mg/d. Pharmacokinetic studies in humans show that a single 640 mg dose of *SPE* leads to peak plasma levels of 2.6 $\mu\text{g/mL}$ (De Bernardi et al., 1994). In addition, some reports indicate the specificity of *SPE* for prostate tissue. Oral administration of *SPE* supplemented with ^{14}C -labeled oleic and lauric acids to rats demonstrates that radioactivity is selectively concentrated in the prostate (Chevalier et al., 1997). Lauric acid, oleic acid, myristic acid and linoleic acid, major constituents of both *SPE* and *NanoOSE*, exerted binding activities of $\alpha 1$ -adrenergic, muscarinic and 1,4-DHP receptors and inhibited 5 α -reductase activity (Abe et al., 2009). Thus, although the concentrations of *NanoOSE* used to treat human BPH cultures *in vitro* were relatively high in comparison to the already known peak plasma levels of *SPE* after a single dose, they are relevant, given the possibilities for variability

in dose and for tissue-specific accumulation with prolonged treatment. In addition, the acute toxicity test showed that *NanoOSE* was practically nontoxic in mice up to an oral dose of 100 mg/kg body weight. This result was particularly important in that it demonstrated the safety of the use of *NanoOse* even with high concentrations.

In our initial experiments, it was observed a difficulty in solubility of the natural *O. speciosa* oily extract in the aqueous culture media, with the obvious implication that the extract could not possibly come into contact with cells and tissues **in culture. As expected, at the same concentrations, our results showed that NanoOse was able to induce a statistically significant higher cell viability inhibition when compared to *O. speciosa* crude, oily extract. In this way, we developed an unprecedented nanoparticle termed *NanoOse*, in order to investigate its action in the treatment of *in vitro* BPH. The concept of nanotechnology in pharmaceutical applications, specifically in drug delivery, is based on the production of nanoparticles containing the drug molecules which are exclusively deposited in the target organ. The product of this procedure, after proper removal of the solvent, is a calibrated size particle (Desphande et al., 1996). The development of a nanoparticle extract not only made the *in vitro* study possible for its better dissolution in culture aqueous medium, and also, the vectorial behaviour of nanoparticle extracts enhances the highest concentration in neoplastic or inflamed tissue. Cancer nanotherapeutics are rapidly progressing and are being implemented to solve several limitations of conventional drug delivery systems such as nonspecific biodistribution and targeting, lack of water solubility, poor oral bioavailability, and low therapeutic indices. To improve the biodistribution of cancer drugs, nanoparticles have been designed for optimal size and surface characteristics to increase their circulation time in the bloodstream. They are also able to carry their loaded active drugs to cancer cells by selectively using the unique pathophysiology of tumors, such as their enhanced permeability and retention effect and the tumor microenvironment (Cho et al., 2008).**

Although not malignant, BPH involves a tumor disease associated with an inflammation component and therefore, the use of nanoparticulate drugs seems to be a highly attractive option. **Furthermore, the future manufacture of *NanoOse* becomes more feasible in the form of pills that are more convenient** and less expensive than the traditional gelatinous tablets seen with saw palmetto formulations. **These findings however, do not exclude the effectiveness of *O. speciosa* crude extract, particularly in the *in vivo* situation, without the issue of dissolution observed in cell and tissue cultures.**

Although the causes of BPH remain poorly understood, overgrowth of stromal cells is an underlying mechanism for the development of symptomatic BPH. We established here a homogeneous stromal cell population constituted of fibroblasts (vimentin positive) and myofibroblasts (α -smooth muscle actin positive), which similarly to the *in vivo* situation, express many eminent extracellular matrix components, including chondroitin sulfate, a typical feature in BPH specimens. As reported by others authors, isolated BPH stromal cells can induce epithelial cell proliferation *in vivo*, which mimics the natural history of these diseases (Barclay et al., 2005). When treated with *NanoOSE* 300 $\mu\text{g/mL}$, the morphology of these cells was altered, as the actin microfilaments were disorganized, disassembled, or disrupted; and the cell viability was inhibited by 75% in a 24 h exposure period of treatment. These results demonstrated that actin filament disorganization might play a key role in the *NanoOSE*-induced decrease in viability of BPH stromal cells. These observations were similar to other studies that shown a proliferation inhibition (Boulbès et al., 2006) and an apoptosis induction (Quiles et al., 2010) of cultured human prostatic myofibroblasts and fibroblasts treated with *Pygeum africanum* extract (from the bark of the African plum tree). **In addition, an increasing LDH release into the extracellular medium by cells treated with *NanoOSE* was also observed**, showing that cell membrane integrity has become damaged. The presence of the exclusively cytosolic enzyme, LDH, in the cell culture medium is indicative of cell membrane damage (Allen et al., 1994). In corroboration, it was also demonstrated that *NanoOSE* showed no toxicity in animals and their

effects were not dependent on the nanoparticle carrier material. These results showed that *NanoOSE* treatment was remarkably effective to reduce cell viability induced a specific cytotoxicity on human BPH stromal cells, yet extremely safe with regard to its toxicity.

BPH organ cultures maintained their glandular organization as well as epithelial cell differentiation as observed by the PSA expression. PCNA immune assay revealed also that 24 h *NanoOSE* treatment was able to promote a significant reduction of cell proliferation: PCNA immunoreactivity was almost 50% lesser than that observed in fresh tissue and controls.

Following 24 h treatment with *NanoOSE*, a significant atrophy and diffuse apoptosis of prostate glands and a statistically significant increase in the apoptotic index in BPH tissue cultures was evident by TUNEL assay. These results are similar to those obtained by other authors that showed a selectivity of the action of *Serenoa repens* (Permixon®) for prostate cells, promoting morphological changes and an increase in the apoptotic index along with an inhibition in the activity of the nuclear membrane bound 5 α -reductase isoenzymes (Bayne et al., 2000). **In prostatic tissue from men with symptomatic BPH treated for 3 months with Permixon®, an increase of molecular markers involved in the apoptotic process, Bax-to-Bcl-2 expression ratio and caspase-3 activity was also reported (Vela-Navarrete et al., 2005).** A previous study using P69 prostate epithelial cell line indicated that *S. repens* treatment may relieve symptoms of BPH by inhibiting specific components of the IGF-1 signalling pathway, and inducing JNK activation, thus mediating antiproliferative and proapoptotic effects on prostate epithelia (Wadsworth et al., 2004). IGF-1 action is extremely crucial for prostate growth and development. Therefore, we can assume by analogy that *NanoOSE* treatment could also affect this signalling pathway. Finasteride treatment of BPH tissues also induced a caspase-dependent apoptotic process restricted to epithelial cells by activating effectors caspases-3 and -6 (Bozec et al., 2005). **There is increasing evidence for *S. repens* and finasteride** that indicate an improvement in urinary tract symptoms and urinary flow with fewer adverse treatment events (Bent et al., 2006; Marks et al., 2000). Similarly to *S. repens* and finasteride, *NanoOSE* could

also have these same effects on BPH treatment. Further research is needed using standardized preparations of *NanoOSE* to determine its long-term effectiveness and ability to prevent BPH complications.

Many studies have demonstrated that androgens directly stimulate VEGF secretion in prostate cells, promoting angiogenesis and contributing to the progression of BPH condition (Joseph et al., 1997). Decreased expression of VEGF by finasteride inhibits angiogenesis and significantly decreases micro vessel density in prostatic sub urethral tissue (Pareek et al., 2003). These observations provide new insights for *NanoOSE* effects into the angiogenesis process, using BPH tissue cultures.

Very little has been studied about the use of different parts of this palm tree for the treatment of different types of cancer. Babassu is widely used in Brazil for a variety of conditions including leukemia, other cancers, urinary symptoms etc. The fruit is used in so many different ways and each part seems to play a different role for the treatment of one specific condition from one specific community. To the best of our knowledge, the study published by Renno et al. in 2008 was the first to evaluate one part of the fruit (mesocarp) for the treatment of leukemia. With the present study, now analyzing the kernels and their use for the treatment of different symptoms of the lower urinary tract, we could establish the effects of a particulate extract prepared with them over benign prostate hyperplastic cells and tissues.

5. Conclusions

Following ethnopharmacological information about the use of babassu kernels for the treatment of lower urinary symptoms, this study is the first that shows in vitro evidence that *NanoOSE* exerts antiproliferative and apoptotic effects on BPH primary cell and tissue cultures. We propose that cultured human prostatic cells and tissues provide a reliable model for

preclinical screening of therapeutic agents, and to study the mechanisms underlying the inhibition of proliferation and the induction of apoptosis. We can also indicate that *NanoOSE* may be an option for the treatment of BPH, although clinical studies (already underway), need to be completed so that they evaluate the efficacy and dose to be given to each patient.

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Conflicts of interest:

Nothing to disclose.

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Legends of Figures

Figure 1. BPH stromal cell characterization

(A) Phase contrast microscopy of BPH primary cell culture, 27 days after tissue dissociation, showing elongated fibroblastoid-like cells, and some round epithelial-like cells (dashed arrow). (B) Phase contrast microscopy of confluent established BPH stromal cell culture, fourth passage, with no epithelial-like cells. (C-H) Immunocytochemistry of BPH stromal cells, fifth passage, showing an immunolabeling of vimentin (C) and α -smooth muscle actin (α -SMA) (D) cytoskeleton microfilaments; and an immunodistribution of fibronectin (E), laminin (F), type IV collagen (G) and chondroitin sulfate (H) extracellular matrix components. A, B X250; C-H X400.

Figure 2. BPH stromal cell MTT viability assay.

BPH stromal cells treated with varying concentrations of *NanoOSE* for 24 h in DMEM, showing that 300 μ g/mL *NanoOSE* was able to induce about 75% reduction in cell viability (A); BPH stromal cells treated with 300 μ g/mL *NanoOSE* for 72 h in DMEM and compared with non treated cells in DMEM and DMEM + 10% FBS, showing that reduction in cell viability was maintained over 72 h (B), and even with the addition of 10% FBS, 300 μ g/mL *NanoOSE* was able to maintain its inhibition activity (C); BPH stromal cells treated with the crude, oily *O. speciosa* extract at 300 μ g/mL in comparison with 300 μ g/mL *NanoOSE* for 72 h. demonstrating superior inhibition of viability produced by *NanoOse* over the crude extract (D). Data are expressed as the percentage of cells in the control treatment at 24 h. Bars $\pm$ SE, n = 3. Means with *different letters* are significantly different, $P < 0.05$.

Figure 3. Phalloidin actin staining.

BPH stromal cells were stained with phalloidin after been maintained with complete medium for 4h (A), 8 h (C) and 12 h (E) or treated with 300 $\mu\text{g/mL}$ *NanoOSE* in complete medium for 4 h (B), 8h (D) and 12 h (F), showing an intense disorganization of the actin cytoskeleton in a time-progressive manner. A-F X200

Figure 4. LDH cytotoxicity assay.

LDH release dose-response curve of BPH stromal cell culture treated with varying concentrations of *NanoOSE* in 24 and 48 h showing a progressive LDH release, reaching 70% as compared to cell lysis by 2% Triton (A), and treated with 300 $\mu\text{g/mL}$ *NanoOSE* for 4, 8 and 12 h (B), demonstrating an early effect on cell death. Dotted line represents normal spontaneous LDH release, while continuous line represents complete LDH release after Triton exposure.

Figure 5. BPH tissue culture characterization.

A 72 h BPH tissue culture stained with hematoxylin and eosin demonstrating a typical BPH histological appearance, with enlarged prostatic glands and a folded epithelial line with eosinophilic luminal secretions and an intense stromal cellularity (A-B). Immunohistochemistry revealed PSA expression in the epithelium as well as in the glands lumen (C); p63 positive basal epithelial cells (D); and PCNA epithelium (E) and stroma (F) immunolabeling. A-F X200

Figure 6. PCNA immunohistochemical staining

PCNA immunolabelling of BPH tissue culture maintained 24 h in complete culture medium (A), 72 h in DMEM (B), 72 h in complete culture medium (C) and treated with 300 $\mu\text{g/mL}$ *NanoOSE* for 72 h in complete culture medium (D). Observe that the treatment produces a

dramatic decrease in PCNA immunostaining, both in glandular epithelial and stromal cells.

Histomorphometry analysis of PCNA immunolabelling (E). A-D X200

Figure 7. TUNEL assay.

TUNEL staining of BPH tissue culture maintained for 72 h in DMEM (A), in complete medium (B), treated with 300 µg/mL *NanoOSE* for 72 h in DMEM (C) and treated with 300 µg/mL *NanoOSE* in complete medium (D). Note that the treatment caused an intense and diffuse immunolabelling indicating increasing apoptosis induction. Arrows are indicating some apoptotic nucleus. Histomorphometry analysis of TUNEL assay (E). A-D X200.

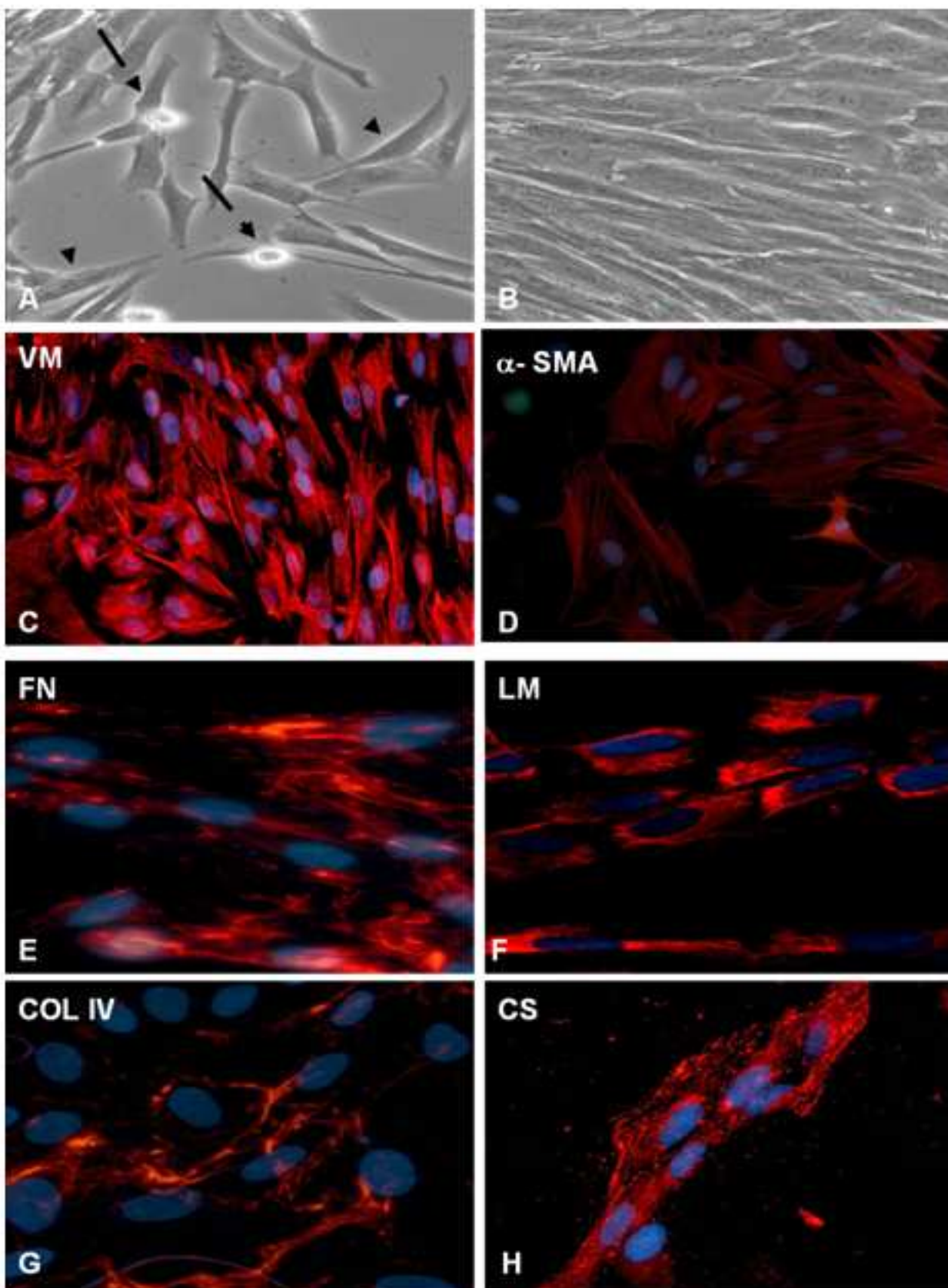
Souza et al., 2010

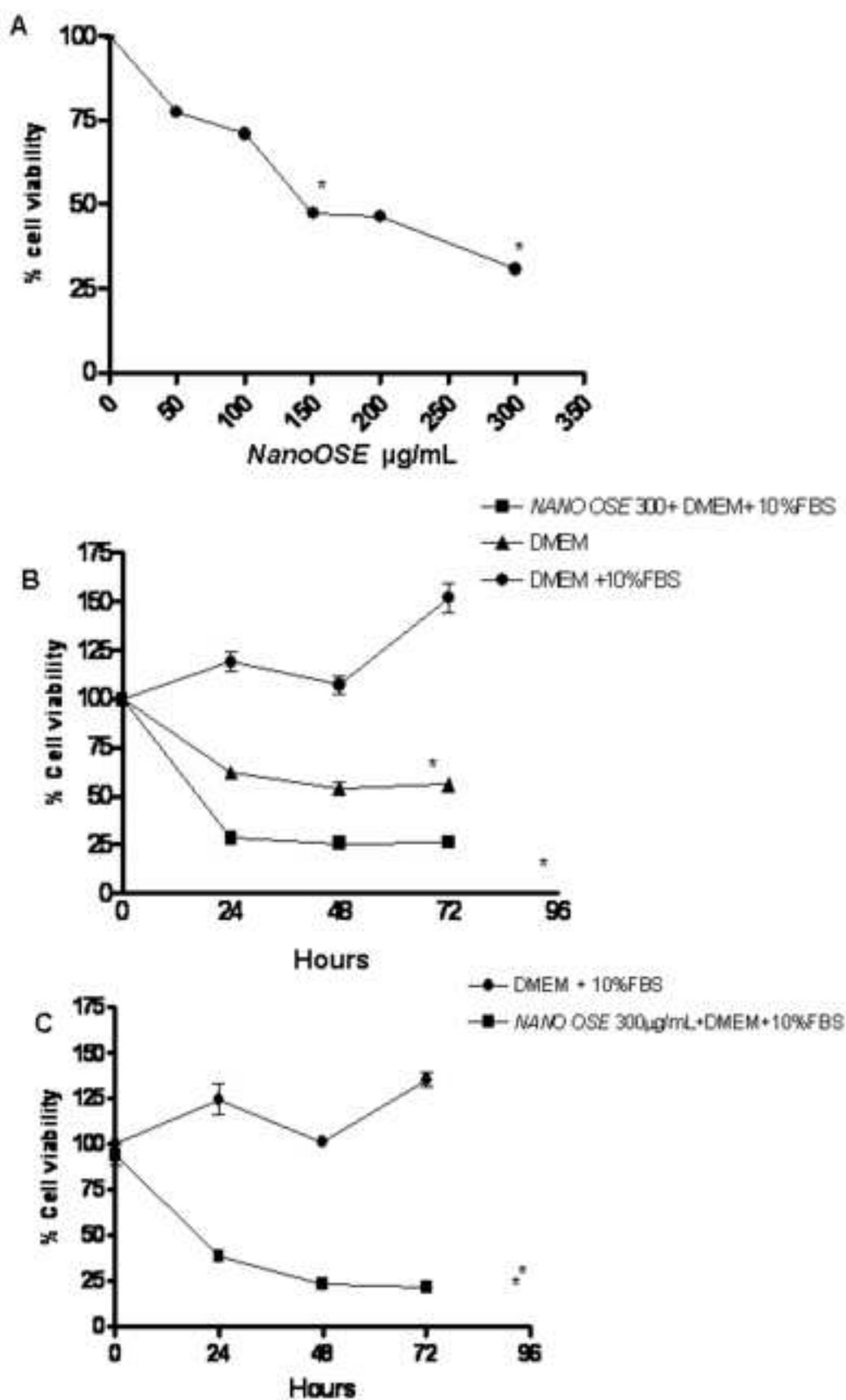
Table 1: Hematological evaluation of *NanoOse* toxicity assay in mice
Total count of leukocytes ($\times 10^6/\text{mL}$)

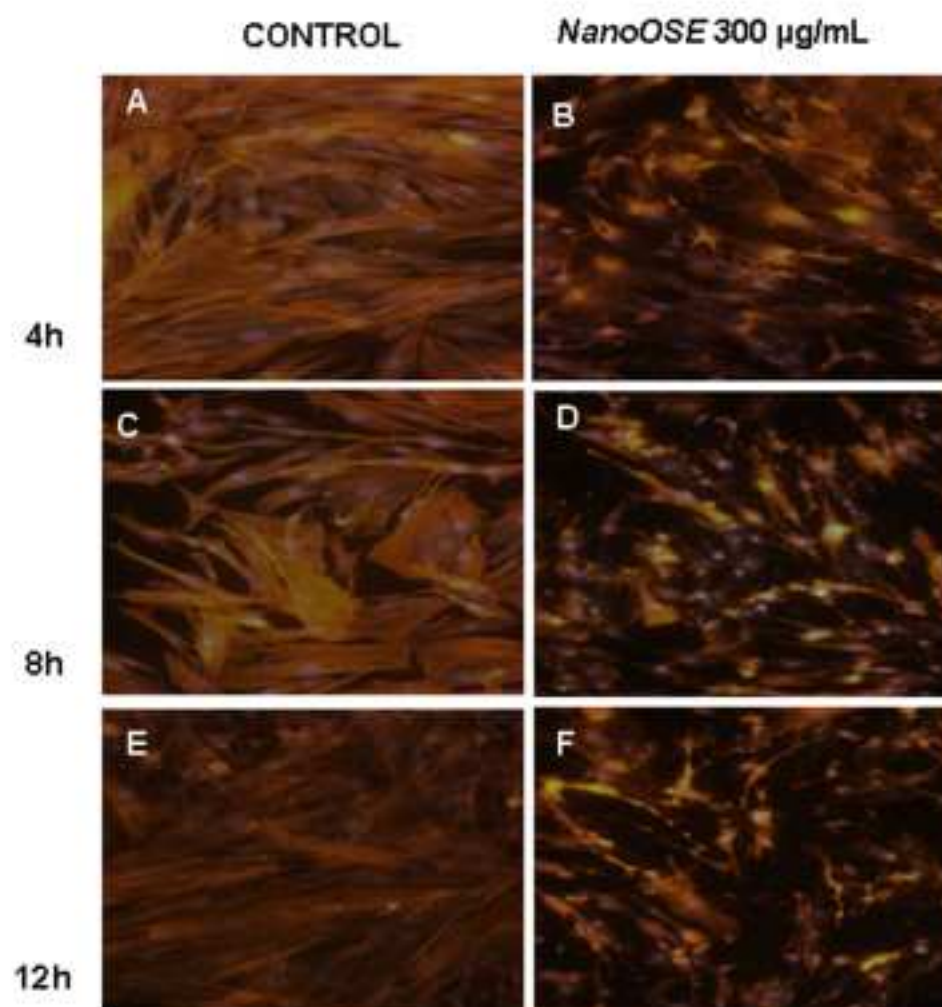
Group	30 min	1h	4h	6h	24h	10 days
Vehicle	7.8 $\pm$ 1.	7.1 $\pm$ 1.	6.7 $\pm$ 0.	6.9 $\pm$ 1.	6.6 $\pm$ 0.	6.3 $\pm$ 3.
<i>NanoOse</i>	8.1 $\pm$ 0.	7.4 $\pm$ 0.	7.6 $\pm$ 0.	7.3 $\pm$ 1.	7.0 $\pm$ 1.	7.3 $\pm$ 2.
	4	1	5	0	7	8
	9	9	8	2	0	1

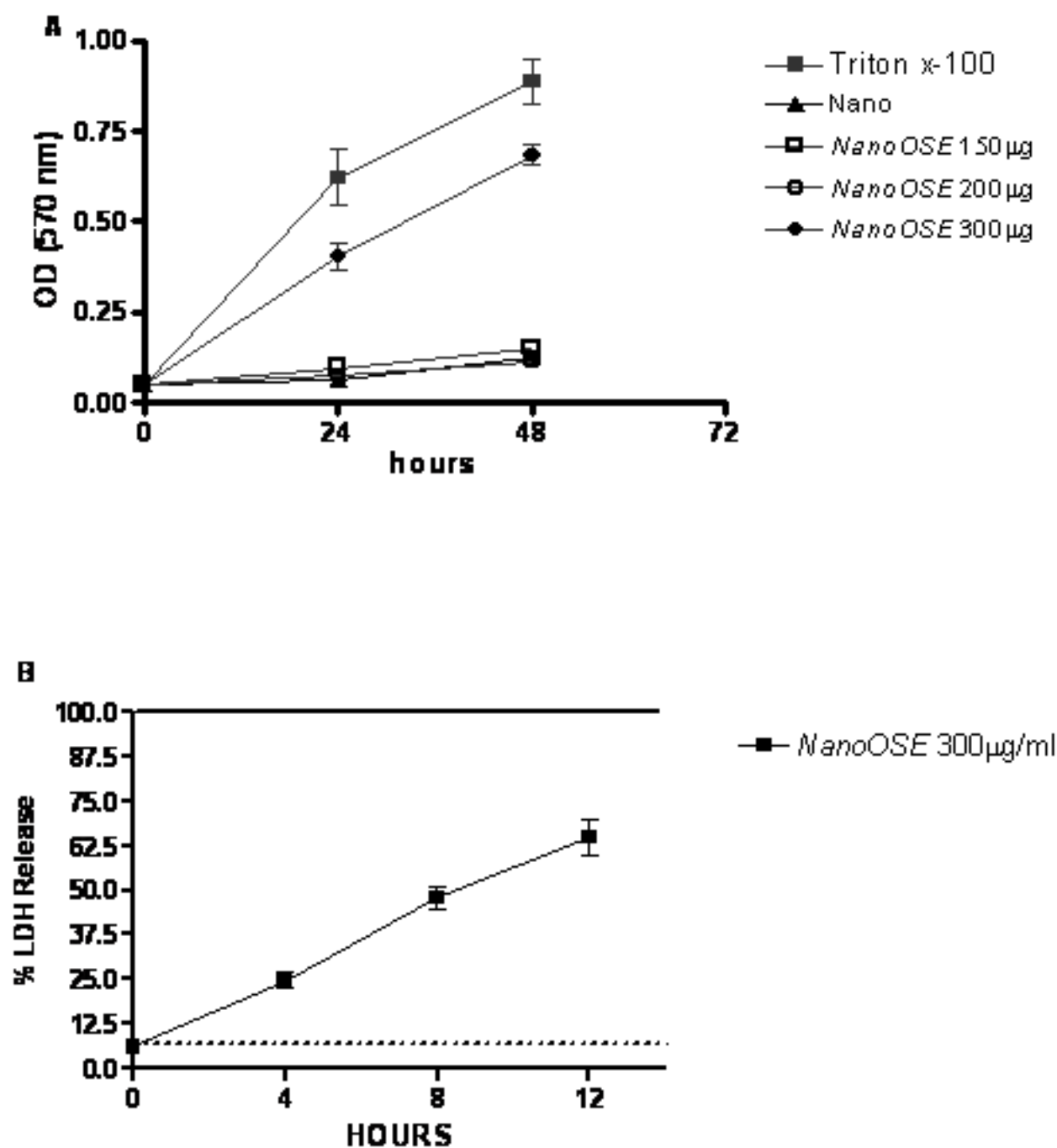
There were no significant changes in the assessment of white blood cells of treated animals compared to controls.

Results are presented as mean $\pm$ SD. Statistical differences (* $p < 0.05$) between mean values were determined by one-way ANOVA followed by Bonferroni post-test.









Souza et al, 2010

Figure 6

