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Article

Copaiba Oilresin Exerts an Additive Effect to the Babassu Oil on Behavioral Changes in Human Endometriotic Cell Cultures

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Abstract: Background: Current drug for the treatment of endometriosis is not able of completely cure and significant side effects hinder the continuation of treatment. So, the search for new drug candidates is necessary, and the use of plants extracts is highlighted. Babassu oil and copaiba oilresin have several therapeutic properties. We investigated the in vitro effects of two nanoemulsions containing babassu oil (*Orbignya speciosa*) (SNEDDS-18) and/or copaiba oilresin (*Copaifera langsdorffii*) (SNEDDS-18/COPA) on cultured human eutopic endometrium stromal cells from endometrial biopsies of patients without (CESC) and with (EuESC) endometriosis, and human stromal cells from biopsies of endometriotic lesions (EctESC). Methods: CESC, EuESC and EctESC were established and treated with SNEDDS-18 and SNEDDS-18/COPA to evaluate its effects on cytotoxicity, cell morphology, proliferation, and signaling pathways. Results: After 48 hours incubation with SNEDDS-18 and SNEDDS-18/COPA, cell viability and proliferation were inhibited, especially in EctESC. The lowest concentration of both nanoemulsions reduced cell viability and proliferation and breakdown the cytoskeleton in EctESCs. After 24h treatment it was observed a decrease in IL-1, TNF-α, and MCP-1, and an increase in IL-10 production. Conclusions: Both nanoemulsions can affect the endometriotic stromal cell behaviors, thus revealing two potential candidates for new phytotherapeutic agents on the management of endometriosis.

Keywords: Endometriosis; *Orbignya speciosa*; *Copaifera lansgdorffii* nanoemulsion; self-nanoemulsifying drug delivery system

1. Introduction

Endometriosis is one of the most frequent benign gynecological disorders. According to the World Endometriosis Research Foundation the disease affects 176 million women of reproductive age [1] and 25-50% of all infertile women worldwide. The disease is an estrogen-dependent chronic inflammatory phenomenon characterized by the presence of endometrium-like tissue outside of the uterine cavity. Women with endometriosis generally suffer with dysmenorrhea, chronic pelvic pain, dyspareunia, and infertility, resulting in a compromised quality of life [2,3]. The ideal treatment remains unknown. Moreover,

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the effectiveness of several drug options is still a source of controversy, since none of them can eradicate the foci of the disease [4].

Medicinal plants have become popular for treating the symptoms of various gynecological diseases [5], including endometriosis [6,7]. Copaifera species are widely used in folk medicine. The oilresin obtained from the tree is used for centuries by indigenous and forest Amazonian people as an anti-inflammatory drug, to cicatrize epidermal wounds, as an urinary antiseptic, and to treat ulcer, bronchitis and cancer [8]. It was also demonstrated several therapeutic properties, such as anti-inflammatory, antitumor and cell death-inducing activities [9-13]. In relation to endometriosis, a study evaluated the effect of *C. langsdorffii* oilresin and demonstrated a significant reduction in the disease [14].

In Brazilian ethnobotany, babassu, is the popular name of *Orbignya speciosa* Mart. Barbosa Rodrigues (Arecaceae, Palmae). Native to the north, northeast, and central regions of Brazil the ethnopharmacological studies demonstrated their use for chronic wounds, ulcerations, dysmenorrhoea, menstrual pain, constipation, obesity, rheumatism, leukaemia, and inflammatory and venous diseases [15,16]. Due to its antiproliferative and apoptotic effects, some studies have suggested the babassu oil as a new and potentially efficient therapy for benign hyperproliferative and inflammatory diseases. Furthermore, it has already been demonstrated that babassu nanosystems show activity on cell cultures derived from benign prostatic hyperplasia [17]. Babassu, in nanostructured systems, also reduce the viability of macrophage-like cell line and was not toxic to human colon epithelial cancer cell line indicating its potential use for the treatment of highly inflammatory processes and for oral administration [18]. Our group demonstrated that a nanocomposite containing copaiba oilresin induced morphological and behavioral changes in human endometriotic stromal cell cultures, with decrease of cell viability and proliferation and cell death induction [19].

It has been widely proposed to apply nanotechnology to plant extracts, because nanostructured systems can potentialize the action of plant extracts, promoting the controlled release of active constituents, reducing the required dose and side effects, improving its pharmacological activity [20,21]. Self-nanoemulsifying drug delivery systems (SNEDDS) are used to improve the solubility of hydrophobic drugs, the permeability and oral bioavailability of lipophilic drugs. SNEDDS are isotropic mixtures of oil, surfactant, drug and cosurfactant [22-24].

Given the known pharmacological properties of *Orbignya speciosa* and *Copaifera langsdorffii*, as well as the effectiveness of nanostructured of vegetable oils the aim of this study was to analyze, in vitro, the effects exerted by two nanoemulsions containing only *Orbignya speciosa* (SNEDDS-18) or containing *Orbignya speciosa* oil and *Copaifera langsdorffii* oilresin (SNEDDS-18/COPA) against ectopic focus of human endometriotic lesions.

2. Results

2.1. SNEDDS-18 and SNEDDS-18/COPA does not show toxicity in vivo

Daily oral treatment of mice with 100 mg/kg of both nanoformulations did not induce neither behavioral alterations nor signs of intoxication. Post-mortem examination did not demonstrate gastric lesions, bleeding, or haematological alterations (Data not shown).

2.2. SNEDDS-18 and SNEDDS-18/COPA reduce cell viability in EctESC cultures

Incubation of CESC, EuESC or EctESC human endometrium stromal cells during 48 hours with increasing concentrations of SNEDDS-18 or SNEEDDS/COPA resulted in a dose-dependent effect (Figure 1 A, B, C). It is interesting to note that EctESC was the most sensitive to cell death induced by each one of nanoformulations (Figure 1C). We next evaluated the effects of SNEDDS-18 and SNEDDS-18/COPA (at 50 µg/ml) against the three different cells incubated during 24, 48 or 72 hours. It is evident that even after 72 hours incubation with CESC or EuESC cells with nanoformulations (at 50 µg/ml) any

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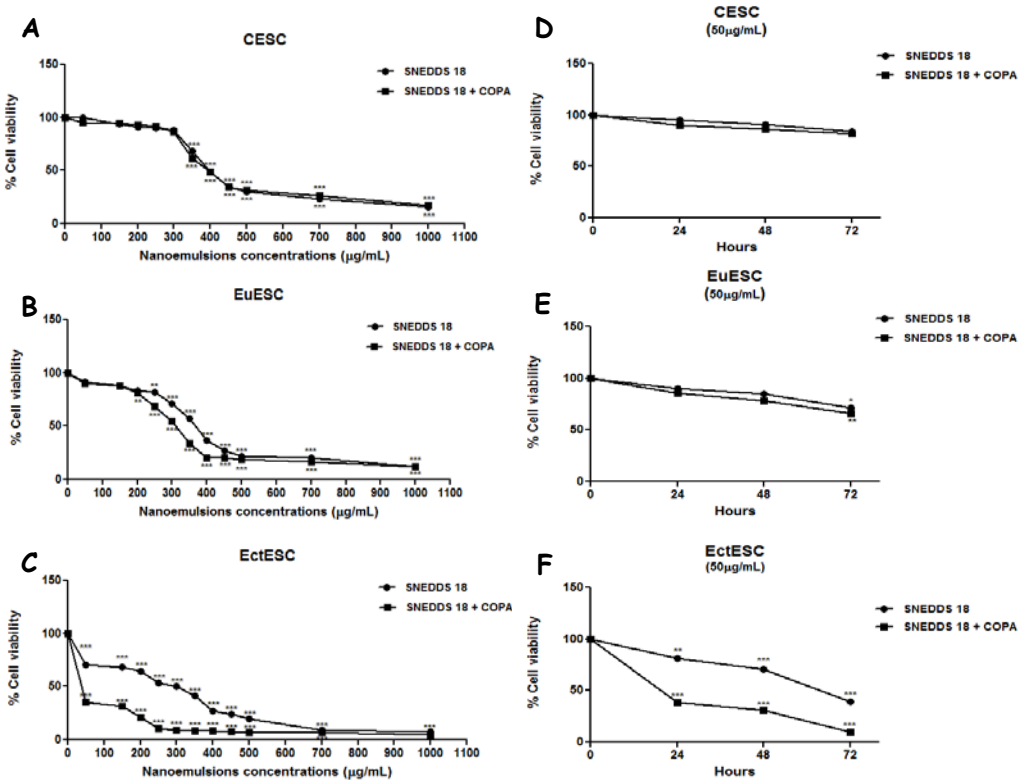
reduction in cell viability was observed. In the other hand, EctESC cells drastically reduced its viability even after 24 hours incubation (Figure 1F).

Figure 1: Endometrial stromal cells viability after incubation with nanoemulsions. In A, B and C,



CESC, EuESC and EctESC were treated with different concentrations of SNEDDS-18 or SNEDDS-18/COPA for 48 h in complete medium. In D, E and F CESC, EuESC and EctESC were treated with 50 µg/ml of SNEDDS-18 or SNEDDS-18/COPA for 24, 48 or 72 hours. Cell viability was analysed by MTT method. Data are expressed as mean ± standard deviation of cell viability (in %).* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when comparing nanoformulation-treated groups with non-treated group. CESC: Endometrial stromal cells obtained from topic endometrium of patients without endometriosis; EuESC: Endometrial stromal cells obtained from topic endometrium of endometriosis patients; EctESC: Endometrial stromal cells obtained from ectopic endometrium of endometriosis patients (endometriotic lesions); SNEDDS-18: Nanoemulsion containing Babassu oil; SNEDDS-18/COPA: nanoemulsion containing Babassu oil and Copaiba oilresin.

2.3. SNEDDS-18 and SNEDDS-18/COPA reduce cell proliferation in EctESC cultures

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Considering the proliferative nature of endometriosis, we investigated whether both formulations could interfere with the proliferative status of the endometrial stromal cell cultures. Figure 2 (A, B, C) shows that different concentrations of both nanoemulsions significantly reduce in a dose-dependent manner the proliferative activity of CESC, EuESC and EctESC. It is worth highlighting that EctESCs proliferation was significantly decreased over the treatments period (Figure 2F).

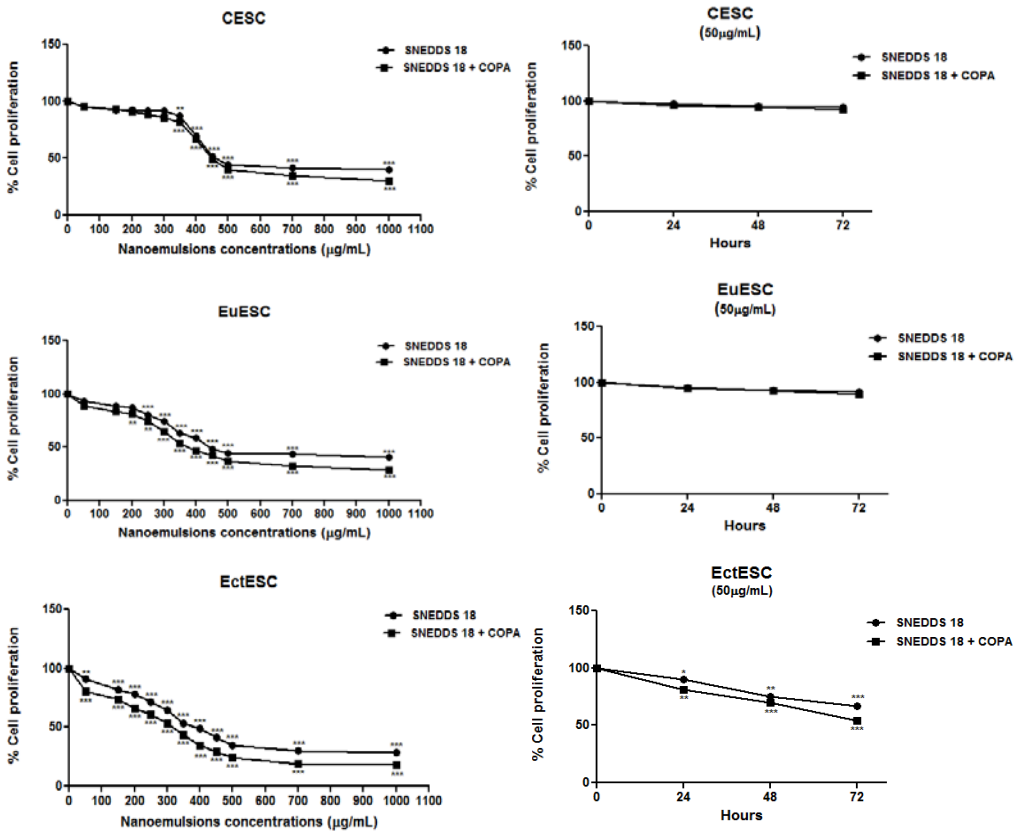

Figure 2: Endometrial stromal cells viability after incubation with nanoemulsions. In A, B and C, CESC, EuESC and EctESC were treated with different concentrations of SNEDDS-18 or SNEDDS-18/COPA for 48 h in complete medium. In D, E and F CESC, EuESC and EctESC were treated with 50 µg/ml of SNEDDS-18 or SNEDDS-18/COPA for 24, 48 or 72 hours. Cell proliferation was analysed by crystal violet method. Data are expressed as mean ± standard deviation of cell viability (in %).* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when comparing nanoformulation-treated groups with non-treated group. CESC: Endometrial stromal cells obtained from topic endometrium of patients without endometriosis; EuESC: Endometrial stromal cells obtained from topic endometrium of endometriosis patients; EctESC: Endometrial stromal cells obtained from ectopic endometrium of endometriosis patients (endometriotic lesions); SNEDDS-18: Nanoemulsion containing Babassu oil; -18/COPA: nanoemulsion containing Babassu oil and Copaiba oilresin.

To confirm the reduction in cell proliferative activity induced by SNEDDS-18 and SNEDDS-18/COPA, we performed immunostaining for cell proliferation antigen, Ki-67. In control groups (CESCs, EuESCs and EctESCs without treatment), it is possible to observe Ki-67-positive immunostaining (strong or faint, in red) (Figure 3A-I, white arrows). After 24 hours of SNEDDS-18 and SNEDDS-18/COPA treatments (50 µg/ml), it can be observed that CESCs and EuESCs cultures preserved a strong immunostaining for ki-67 (Figure 3A-C and 3D-F, respectively). In contrast, EctESCs cultures presented a reduction in Ki-67-positive staining (Figure 3G-I). When quantifying the percentage of positive Ki-67 cells, EctESC cells treated with SNEDDS-18 or SNEDDS-18/COPA

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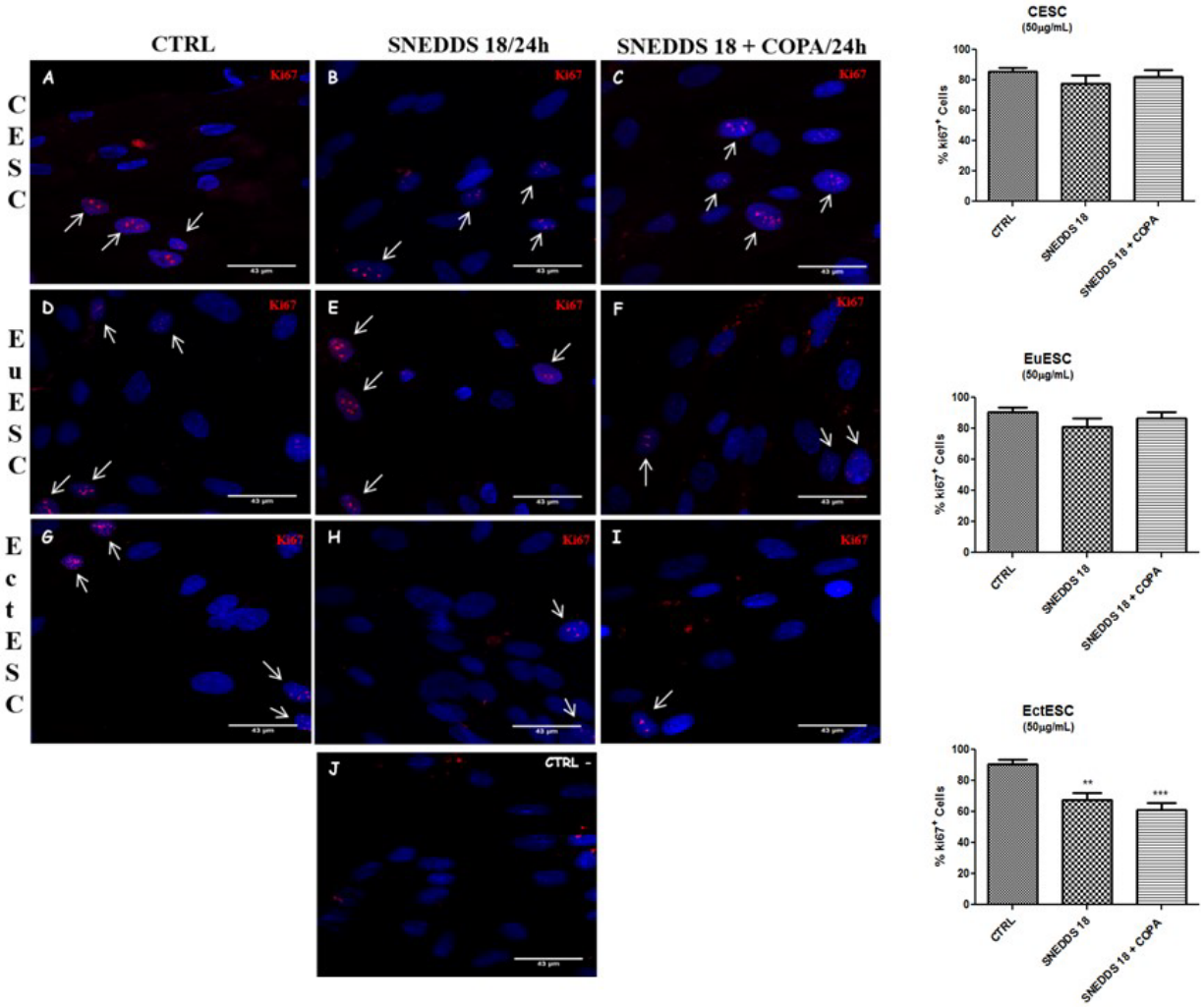
presented a reduction in 30 and 38% in cell proliferation, respectively (Figure 3, right graphs).

Figure 3: Immunostaining of Ki-67 positive cells (left photos). CESC (A, B, C); EuESC (D, E, F) and



EctESC (G, H, I) were incubated with Ki-67 and treated or not with SNEDDS-18 or SNEDDS-18/COPA (50 µg/ml) for 24 hours. In J is demonstrated the Negative reaction. In red are Ki-67 labelling and in blue are DAPI (nuclear marker) labelling. Representative images of three independent experiments performed in triplicate. Scale bar = 43 µm. Percentage of Ki-67 positive cells (right graphs). Results are expressed as mean ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when comparing SNEDDS-18 or SNEDDS-18/COPA with vehicle group. CESC: Endometrial stromal cells obtained from topic endometrium of patients without endometriosis; EuESC: Endometrial stromal cells obtained from topic endometrium of endometriosis patients; EctESC: Endometrial stromal cells obtained from ectopic endometrium of endometriosis patients (endometriotic lesions); SNEDDS-18: Nanoemulsion containing Babassu oil; SNEDDS-18/COPA: nanoemulsion containing Babassu oil and Copaiba oilresin.

2.4. SNEDDS-18 and SNEDDS-18/COPA alters the EctESC motility

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Next, we asked whether SNEDDS-18 and SNEDDS-18/COPA could influence on cell motility. CESC, EuESC and EctESC motility were evaluated through videomicroscopy after 72 hours exposed to 50 μg/ml of SNEDDS-18 or SNEDDS-18/COPA. It was observed that the cells exhibited an elongated and, spread morphology with an intense proliferation, metabolic activity, and accelerated cell movement. No significant differences in motility were found between the three untreated cell cultures (Data not shown). On the other hand, when CESCs, EuESCs and EctESCs were treated with SNEDDS/COPA only the last ones presented morphological alterations and reduced motility. Notably, over the 72 hours, we observed several events indicative of cell damage and death in EctESCs cultures treated with both SNEDDS-18 and SNEDDS-18/COPA, such as plasma membrane and cytoplasm retraction, loss of cell-cell contact, loss of cell-substrate adhesion and presence of intracytoplasmic vacuoles, leading to the deconfiguration of the EctESCs monolayer. In fact, within the first 6 hours of treatment with SNEDDS-18/COPA, we can observe an intense cellular breakdown culminating in the disruption of the EctESCs monolayer (Figure 4).

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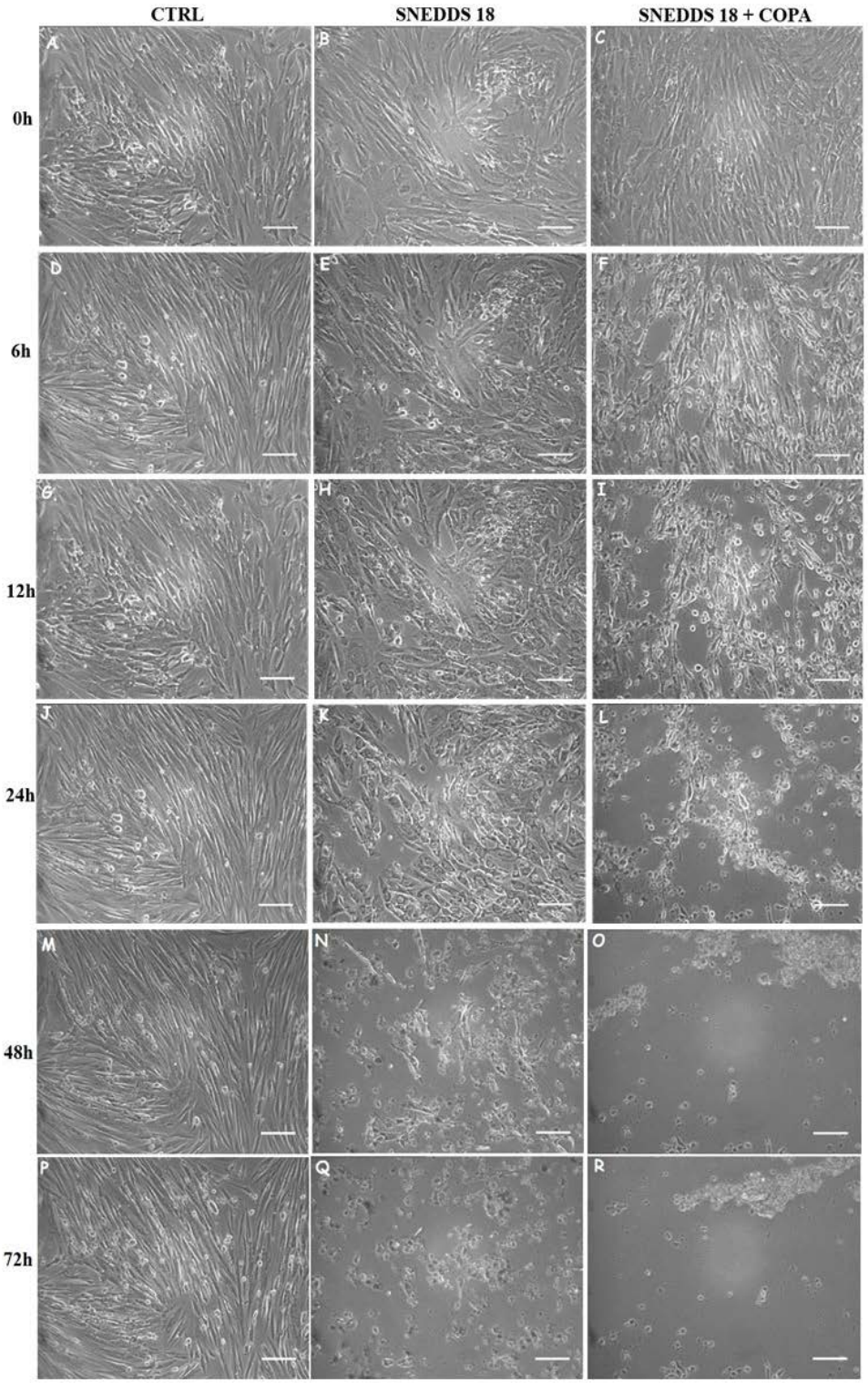

Figure 4: Alterations in EctESC cultures after treatment with SNEDDS-18 and SNEDDS-18/COPA. Representative images of untreated and treated EctESC after incubation with nanoemulsions of SNEDDS-18 and SNEDDS-18/COPA (50 μg/ml) obtained by phase contrast microscopy at 0, 6, 12, 24, 48 and 72 h. Scale bar = 50 μm. CESC: Endometrial stromal cells obtained from topic endometrium of patients without endometriosis; EuESC: Endometrial stromal cells obtained from topic endometrium of endometriosis patients; EctESC: Endometrial stromal cells obtained from ectopic endometrium of endometriosis patients (endometriotic lesions). SNEDDS-18: Nanoemulsion containing Babassu oil. SNEDDS-18/COPA: Nanoemulsion containing Babassu oil and Copaiba oilresin.

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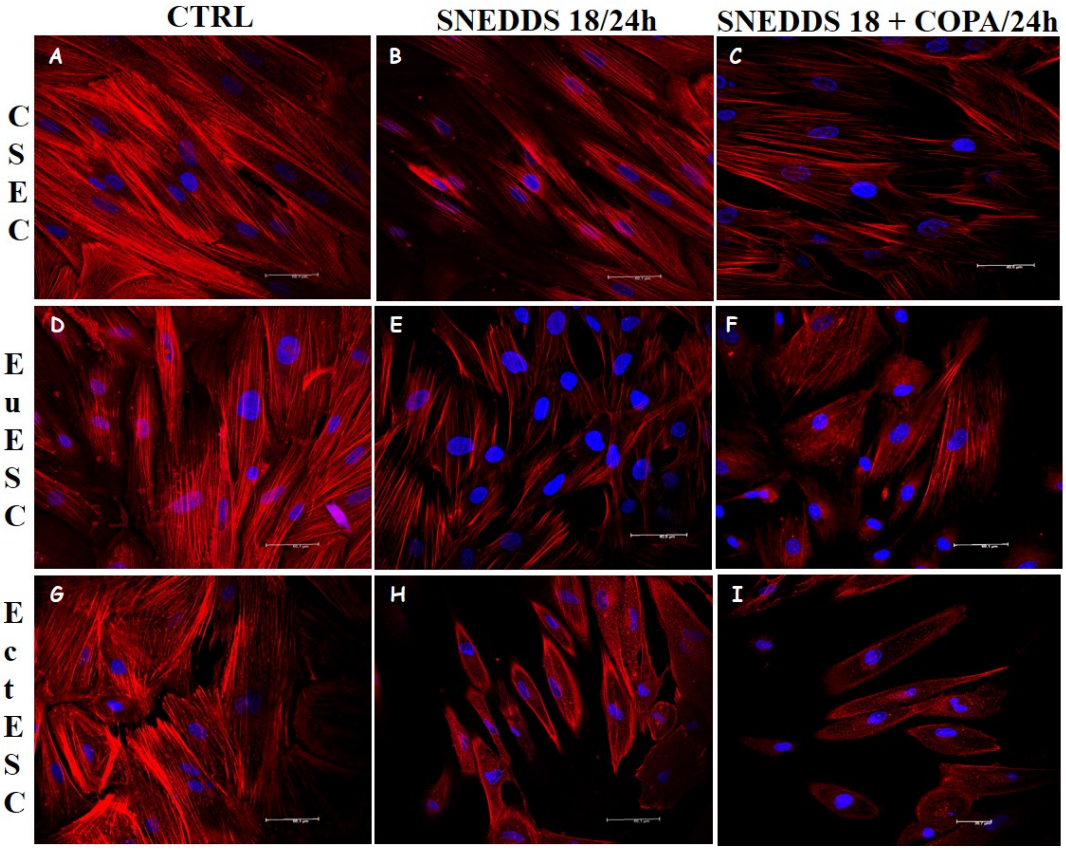
2.5. Nanoemulsions disturbs the cytoskeleton of the EctESCs cultures

In order to better understand the influence of the nanoemulsions on the structure of the stromal cells we analyzed the organization of the three cytoskeleton components (microfilaments, microtubules and intermediate filaments) after 24 hours of treatment with the nanoemulsions. In control conditions actin filaments are polymerized and organized in parallel to each other, giving the cells their scattered morphology, providing the necessary support to the plasma membrane, allowing the cell-cell and cell-substrate adhesion. (Figure 5).

Figure 5: Alterations in actin filaments of ESCs cultures after treatment with nanoemulsions. CESC,



EuESC and EctESC were labeled with Cy3 fluorochrome-coupled fungal phalloidin toxin (rhodamine, red) or DAPI (nuclear marker, blue) 24 hours after treatment with nanoemulsions. Representative images of three independent experiments performed in triplicate. Scale bar = 50 μm. CESC: Endometrial stromal cells obtained from topic endometrium of patients without endometriosis; EuESC: Endometrial stromal cells obtained from topic endometrium of endometriosis patients; EctESC: Endometrial stromal cells obtained from ectopic endometrium of endometriosis patients (endometriotic lesions).

Vimentin is a protein of the intermediate filament family, and together with actin microfilaments and microtubules it is part of the cytoskeleton. This protein is present in cells of mesodermal origin, functioning as a marker of stromal cells, and for that reason its presence and distribution were also analyzed in cell cultures treated with both

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nanoformulations. In the untreated CESC, EuESC and EctESC cultures, we can observe vimentin filaments distributed through the cytoplasm, organized in stable protein networks, forming structures similar to strings or wire ropes, conferring mechanical strength and stabilization to these cells. Likewise, we observed that treated CESCs and EuESCs cultures preserved their vimentin cytoskeleton while in treated EctESCs cultures all this organization was lost. It is important to note that, when EctESCs were treated with SNEDDS-18/COPA, this effect appears to be exacerbated and entire intermediate vimentin cytoskeleton is undone), indicating a probable additive effect of copaiba oilresin in babassu oil (Figure 6).

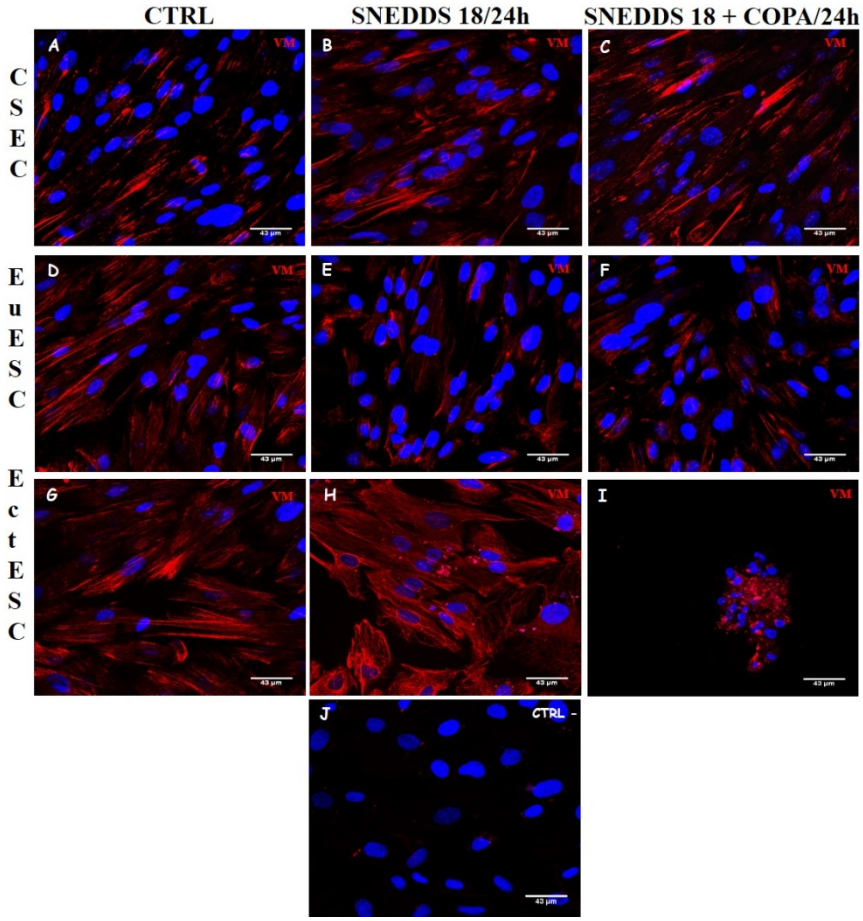

Figure 6: Morphological analysis of vimentin intermediate filaments the ESCs cultures treated with SNEDDS-18 and SNEDDS-18/COPA. CESC, EuESC and EctESC were labeled with anti-vimentin antibody (red) and DAPI (nuclear marking - blue) after 24 hours of treatment with of nanoemulsions (50 μg/ml). Representative images of three independent experiments performed in triplicate. Scale bar = 43 μm. CESC: Endometrial stromal cells obtained from topic endometrium of patients without endometriosis; EuESC: Endometrial stromal cells obtained from topic endometrium of endometriosis patients; EctESC: Endometrial stromal cells obtained from ectopic endometrium of endometriosis patients (endometriotic lesions).

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Figure 7 shows that untreated CESC, EuESC and EctESC cultures presented an organized α-tubulin cytoskeleton, creating a polarized, elongated and scattered characteristic morphology of these stromal cell cultures. After 24 hours of treatment with both nanoformulations, the CESC and EuESC cultures preserved the structure of the microtubule cytoskeleton. In contrast, a clear disruption of protofilaments can be observed in EctESCs cultures, culminating in cytoplasmic retraction and, consequently, changes in their morphology.

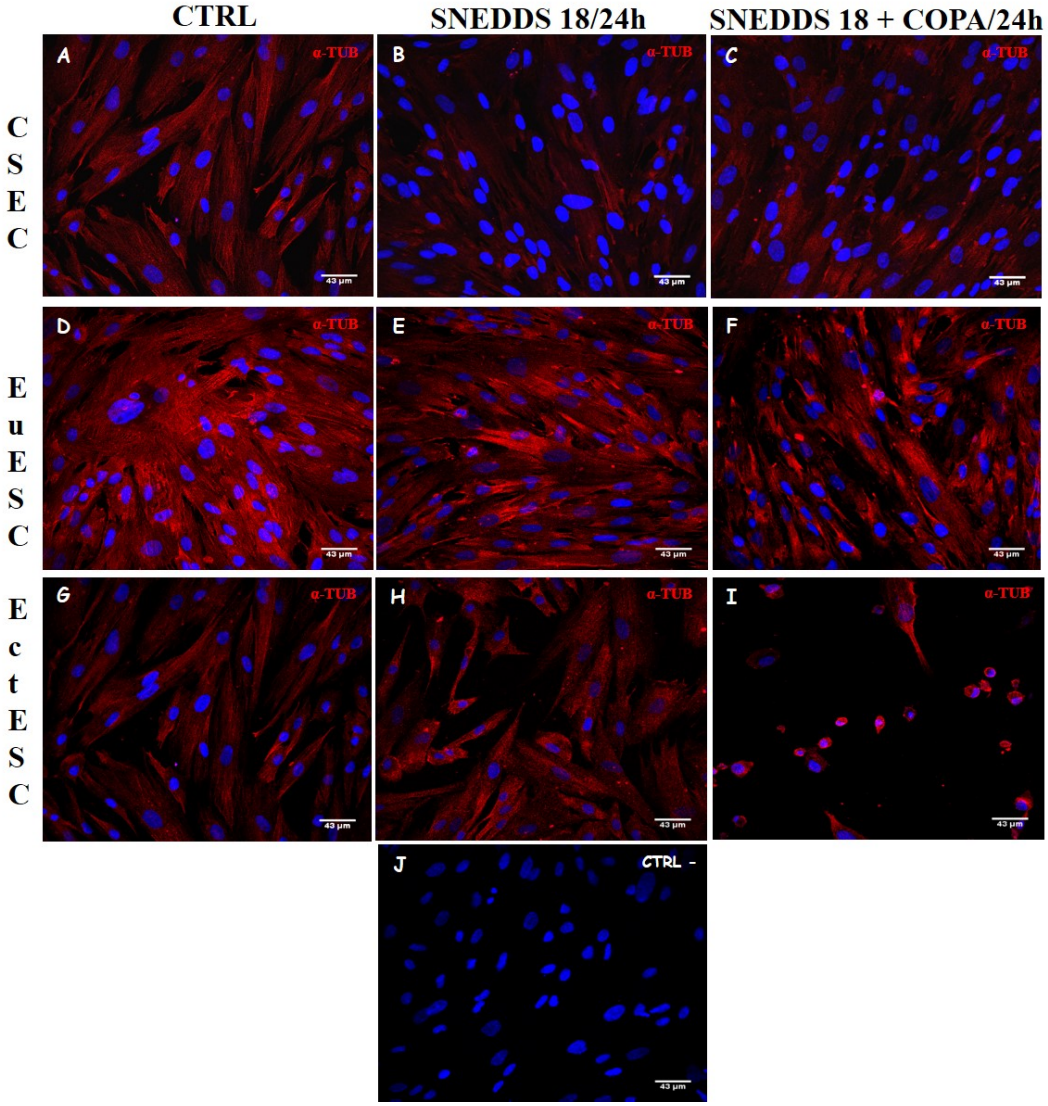

Figure 7: Analysis of tubulin structure of the ESCs cultures after treatment with nanoemulsions. CESC, EuESC and EctESC were labeled with anti-α-tubulin antibody (red) or DAPI (nuclear marker - blue) after 24 hours of treatment with nanoemulsions (50 µg/ml). Scale bar 43 = µm. CESC: Endometrial stromal cells obtained from topic endometrium of patients without endometriosis; EuESC: Endometrial stromal cells obtained from topic endometrium of endometriosis patients; EctESC: Endometrial stromal cells obtained from ectopic endometrium of endometriosis patients (endometriotic lesions).

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2.6. Nanoemulsions induce changes in cell adhesion process in EctESCs cultures

Due to the structural changes observed, we decided to evaluate the extent to which both nanoemulsions could interfere with the cell adhesion process. For this, we evaluated the distribution of β-catenin, an integral structural component of cadherin-based adherens junctions. In the untreated CESC, EuESC and EctESC cultures, the location and distribution of β-catenin are preserved, the protein remains located in the cell cytoplasm (as fine granules) and, mainly in the plasma membrane at the points of cell-cell adhesion. After 24 hours of treatment with nanoemulsions, we noticed that only the cultures of EctESCs presented changes in the distribution and location of β-catenin, which concentration on the cell cytoplasm. A remarkable nuclear localization of β-catenin was also observed EctESCs treated with SNEDDS-18/COPA). Thus, both SNEDDS-18 and SNEDDS-18/COPA modified the distribution of β-catenin exclusively in EctESCs cultures (Figure 8, white arrows).

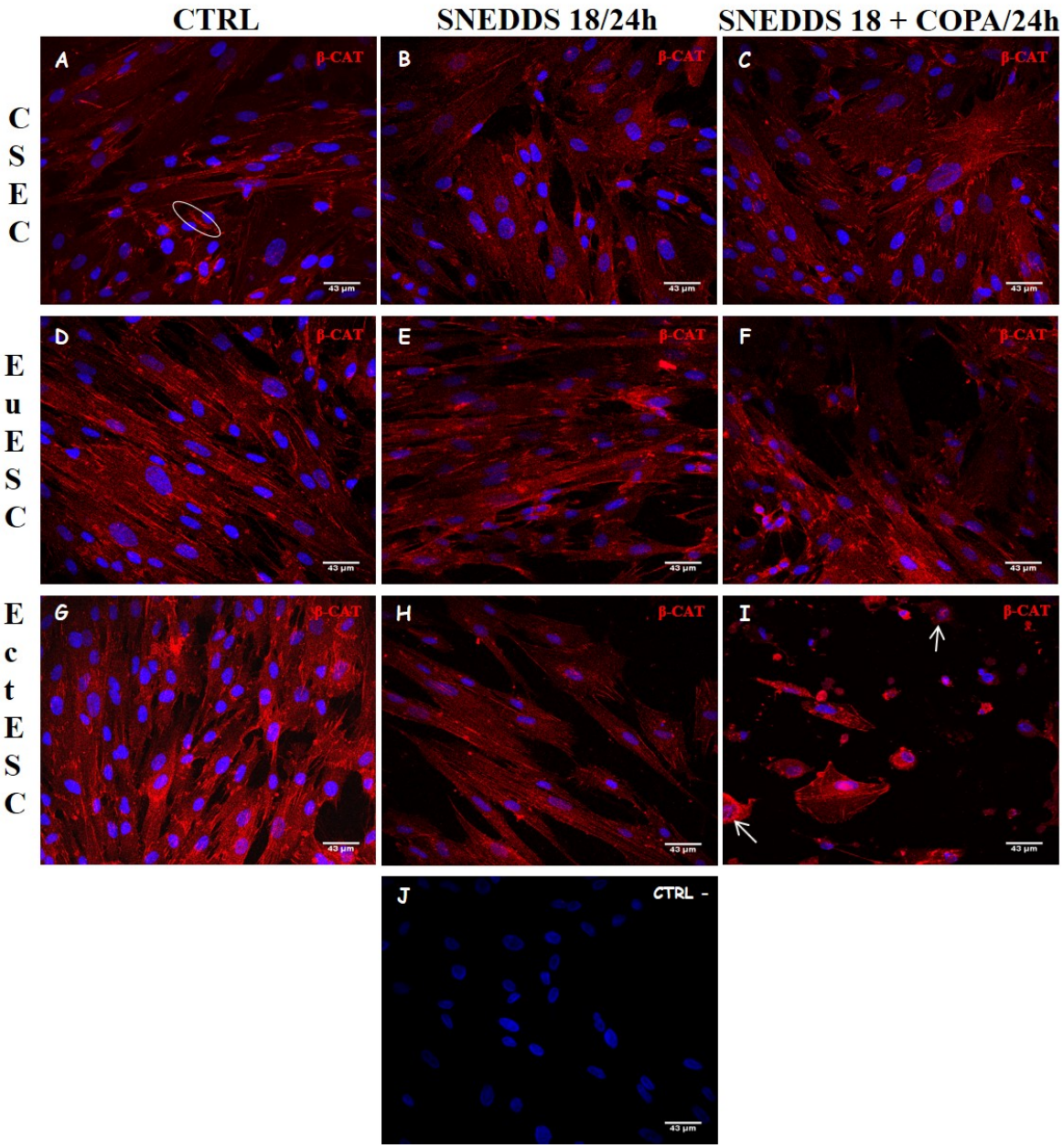

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Figure 8: Distribution of β-catenin on ESCs cultures treated nanoemulsions. by immunocytochemistry using anti-β-catenin antibody. CESC, EuESC and EctESC were labeled with anti-β-catenin antibody and DAPI (nuclear marking - blue) after 24 hours of treatment with nanoformulations (50 μg/ml). Representative images of three independent experiments performed in triplicate. Scale bar = 43 μm. CESC: Endometrial stromal cells obtained from topic endometrium of patients without endometriosis; EuESC: Endometrial stromal cells obtained from topic endometrium of endometriosis patients; EctESC: Endometrial stromal cells obtained from ectopic endometrium of endometriosis patients (endometriotic lesions).

2.7. SNEDDS-18 and SNEDDS-18/COPA affect the secretion of cytokines by EctESCs cultures

Next, we evaluated the effects of nanoemulsions in regulating cytokine production by cell cultures. CESC, EuESC and EctESC were treated with SNEDDS-18 and SNEDDS-18/COPA and production of IL-1β, TNF-α, IL-10 and MCP-1 were analysed. EctESCs cells do secrete more cytokines than cell cultures obtained from topical endometrium's (CESC and EuESC). After 24 hours of treatment with one of the nanoemulsions, there is a significant decrease in the secretion of IL-1β and TNF-α in the EuESCs and EctESCs cultures. As expected, SNEDDS-18/COPA reduced IL-1β secretion more intensely in EctESCs cultures. (Figure 9). Any significant differences were observed between CESC, EuESC and EctESC untreated cultures. In contrast, both treatments decrease MCP-1 secretion in EctESCs cultures. In the other hand, nanoemulsions increased IL-10 production by EuESCs and EctESCs cultures. Moreover, we can notice that SNEDDS-18 and, especially SNEDDS-18/COPA, significantly increased the secretion of IL-10 by EctESCs cultures when comparing with EuESCs (Figure 9).

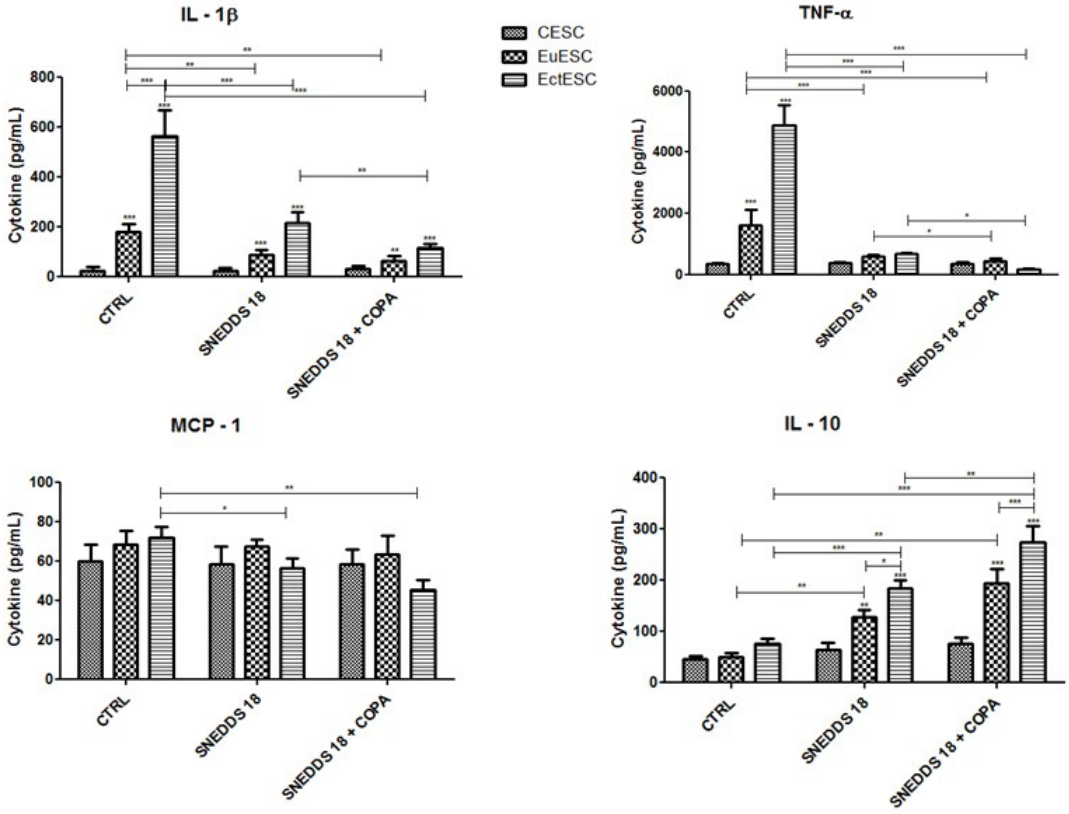

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Figure 9: Production of cytokines by CESC, EuESC and EctESC cells after treatment with nanoemulsions. After 24 hours incubation with nanoemulsions (50 μg/ml) supernatant were collected to measurement of indicated cytokines. Results are expressed as mean ± SD (n=9). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when comparing CESC-, EuESC- and EctESC-nanoemulsions treated groups with vehicle-treated group. CESC: Endometrial stromal cells obtained from topic endometrium of patients without endometriosis; EuESC: Endometrial stromal cells obtained from topic endometrium of endometriosis patients; EctESC: Endometrial stromal cells obtained from ectopic endometrium of endometriosis patients (endometriotic lesions).

3. Discussion

Endometriosis is a benign, chronic, and inflammatory gynecological condition commonly seen in women of reproductive age [25]. Although much progress has been made in the treatment, there is a lack of direct drugs with fewer side effects. Given this scenario, the discovery of new therapeutic alternatives seems to be very promising.

The use of plants and herbal medicines have proved stands out as important sources of new molecules and bioactive compounds with pharmacological activity. Both copaiba oil-resin and babassu oil has extensive popular use in traditional medicine. Copaiba exuded oleoresin is used as an anti-inflammatory, to healing wounds, as an urinary antiseptic, and to treat ulcer, bronchitis and cancer [8]. Pharmacological studies confirmed the anti-inflammatory [8,10,13,18, 26], antitumoral [12,27,28] and analgesic [12].

In this work, we analyzed the effects of two nanoformulations containing babassu oil (*Orbignya speciosa*) and copaiba oilresin (*Copaifera langsdorffii*) on CESC, EuESC and EctESC cells cultures obtained from normal or ectopic focus of human endometriotic lesions.

The role of nanometric systems as therapeutic carriers has been extensively investigated. In fact, the application of nanomedicine is increasing with the promise of improving the efficiency of the substance retention in lesions, optimization of pharmacokinetic properties [29]. In addition, nanostructured systems favored the in vitro immunomodulatory response of the peritoneal macrophages of the women with endometriosis [30], besides compromising the behavior of the cell cultures from human endometriotic lesions, reducing cell viability, cell proliferation and altering the cell morphology [19]. Withal, the inhibiting the in vivo endometriosis progression via regulation of the murine peritoneal macrophages has also been shown [31]. Many studies address phytotherapy as a therapeutic alternative for the endometriosis treatment. In this sense, some studies corroborate our findings, evidencing anti-endometriotic effects of various extracts and plant actives [32-40]. Likewise, our results are in accordance with a reduction in the cell viability found by Cao et al.[41], as well as a decrease in the expression of several molecules involved in the pathophysiology of endometriosis (VCAM-1, ICAM- 1, TNF-α, IL-1, IL-6, IL-8, and MCP-1), in the cell cultures obtained from endometriotic lesions, after treatment with curcumin [36].

There are a few reported cases in the literature that analyze the treatment of endometriosis with the oil or extract of copaiba [14,19] and there are practically no studies evaluating the effects of babassu oil or extract on this gynecological disorder. Our research group had already demonstrated that a nanocomposite containing oilresin from copaiba (*C. langsdorffii*) modified cell cultures obtained from endometriotic lesions of ovary capsule; in which the viability of these cell was reduced to 46% [19]. We observed that the nanoemulsion composed by the oil extracted from both species developed a more intense effect than the nanoemulsion consisting only of babassu oil, suggesting a possible additive effect of the two combined. These results are corroborated by other studies in which the effect of vegetable preparations consisting of several types of medicinal plants with

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antiendometriotic properties [42-44], revealing a greater effect of herbal formulations when the assets are combined in the same preparation [43,45]. Our analysis allowed us to verify that the nanoformulations reduced the viability and proliferation of EctESCs, without compromising the behavior of CESC and EuESC. It is important to note that the cell cultures from topical endometrium (CESC and EuESC) had no change in monolayers configuration after treatments. Corroborating these results other studies have shown reduction in contractility [46], invasive capacity [33,47,48] and cell migration [36,38] of human endometriotic cell cultures, after therapeutic treatment. So, our findings indicate that different behaviors are exhibit by the stromal cell cultures obtained from the topical and ectopic endometrium. Additionally, changes in cell morphology of EctESCs treated with SNEDDS-18/COPA were clearly observed, in which the three strands of the cell cytoskeleton (actin, α-tubulin and vimentin) were disorganized and unstructured. It is possible that the disorganization of these cytoskeleton filaments is associated with the reduction of the viability of EctESCs cultures induced by both nanoemulsions. Corroborating our analyzes, studies shown a significant reduction in cell viability and changes in the morphology of the endometriotic cell cultures treated with different extracts, oil and plant formulations [19,33,36,37,46].

Interestingly, treatment with nanoemulsions reduced the proliferative activity of EctESC. These data are consistent with other studies on phytotherapy and endometriosis [35,37,49-52]. In this context, Wieser et al. [52] and Zhou and Qu [44] reported proliferation inhibition and apoptosis induction of the stromal cell cultures derived from endometriotic lesions by a Chinese preparation of nine medicinal plants with antiendometriotic properties. Further, confirming our analyzes, Lian et al. [42] evaluated the effectiveness of the Quyu Jiedu Recipe (QJR), a Chinese herbal preparation in the treatment of endometriosis, and revealed that this formula was able to significantly reduce the VEGF expression and the cell proliferation antigen Ki-67.

The multifunctional protein β-catenin is one of the most important binding partners in the cell-cell adhesion process, besides being the central signal-transducing molecule of the WNT pathway. When located in the plasma membrane, β-catenin participates in cell-cell adhesion. Our data indicated that both nanoemulsions modified the distribution and location of β-catenin exclusively in EctESCs cultures, inducing the translocation of β-catenin from the plasma membrane to the cytoplasm and resulting in intense loss of cell-cell adhesion, morphologic changes, decreasing cell viability and cell proliferation. Corroborating our results, several studies revealed a decrease in the expression of adhesion proteins, induced by therapeutic strategies, compromised the development and progression of endometriosis *in vivo* and *in vitro* [36,53-56].

Endometriosis is a chronic inflammatory disease with the production of several cytokines [57-59]. It has also been observed an increase in the amount of these cytokines in the peritoneal fluid of patients with endometriosis [57,59,60-64]. Our data indicate that EctESCs released a greater amount of IL-1β and TNF-α than EuESC and CESC and both nanoemulsions significantly reduced IL-1β and TNF-α secretion in EuESCs and EctESCs. This is in line with an additive effect of copaiba oilresin to babassu oil in the endometriosis pathogenesis. We also showed that the combination of both oil (babassu and copaiba) decreased MCP-1 secretion and increased IL-10 production by CESC, EuESCs and EctESCs. High concentrations of MCP-1 have been detected in the peritoneal fluid of women with endometriosis and in vitro and in vivo models [65-67]. IL-10 is an immunomodulatory cytokine that can inhibit the synthesis of cytokines such as Interferon-γ (IFN-γ), IL-2, IL-3 and TNF-α [69]. Interestingly, studies have revealed elevated levels of IL-10 in the peritoneal fluid of women with advanced endometriosis [69-71]. Thus, local deregulation of cytokines allows endometrial fragments to adhere and implant in the peritoneal cavity.

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Taken together our data is extremely relevant for the therapeutic endometriosis treatment since the existence of a compound that can reduce inflammation component in both the uterine (topical endometrium) and extrauterine (ectopic endometrium) environment would assist in the control of the endometriosis growth and progression.

4. Materials and Methods

4.1. Endometrial tissue and endometriotic lesions samples

This study was approved (protocol no. 23002513.7.0000.5257, 2014) by the Research Ethics Committee from Hospital Universitário Clementino Fraga Filho of the Federal University of Rio de Janeiro/Brazil. Samples of normal endometrial tissue were obtained from three patients, without endometriosis, submitted to a total hysterectomy for myoma treatment. Samples of topic endometrium and endometriotic lesions were collected from three patients with endometriosis undergoing videolaparoscopy. All tissue samples were isolated according to Olivares et al. [72].

4.2. Acute toxicity test

The toxicity assays were performed on male and female Swiss Webster mice (18-25g). The animals were kept in a temperature-controlled room (22 ± 2ºC) in light/dark cycles for 12 hours, with free access to food and water. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation. The protocol for animal use was approved (code DFBCICB-015) by the animal experimentation ethics committee of the Health Sciences Center of UFRJ, Rio de Janeiro. Parameters of acute toxicity were determined according to Lorke [73], with some modifications. The daily oral dose (100 mg/kg) of the two nanoemulsions (SNEDDS-18 and SNEDDS-18/COPA) was administered separately to groups of 15 male and female mice for 15 consecutive days. Daily, after 0.5, 1, 4, 6 and 24 hours the Lorke´s parameters were observed. After the fifteenth day, the animals were sacrificed by cervical dislocation, stomachs removed, and an incision along the greater curvature was made. Next, the number of ulcers (single or multiple erosions, ulcers or perforations) and hyperemic areas were counted, and blood samples were collected by the tails and analyzed on a CellPouch automatic counter (pocH-100iV Diff, Sysmex).

4.3. Endometrial and endometriotic stromal cell cultures treatment with SNEDDS-18 and SNEDDS-18/COPA

Once developed in Laboratório de Tecnologia Industrial Farmacêutica Pharmacy Scholl Federal University of Rio de Janeiro, SNEDDS-18 and SNEDDS-18/COPA were diluted directly in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (from now on named complete medium), to provide working solutions (50 or and 1000 μg/mL). The SEDDS formulations were prepared according to a protocol described by Singh et al. [23], which included a fractional factorial design approach (Type 24) that generated 25 different formulations. Babassu oil (*O. speciosa*; Tobasa, Brazil), Copaiba oilresin (*C. langsdorffi*; Beraca, Brazil), oleic acid (Sigma Aldrich, USA), oleylamine (Sigma Aldrich, USA), Labrafac® (Gattefossé, France), Labrasol® (Gattefossé, France) and isopropyl myristate (Vetec, Brazil) were used as components of oil phase, which was combined with the co-surfactants of transcutol HP (Sigma Aldrich, USA),

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ethanol and propyleneglycol. The surfactants consisted of Tween® 20 (Vetec, Brazil), Tween® 80 (Vetec, Brazil), Span® 80 (Vetec, Brazil), lecithin (Sigma Aldrich, USA) and Kolliphor RH 40 (Sigma Aldrich, USA). The surfactants were mixed under magnetic stirring before the oil/co-surfactant phase was added. The SNEDDS controls were exposed to either complete medium or the nanoemulsion vehicle. Incubation varies between 24 and 72 hours.

4.4. Cell viability assay

Cellular survival was obtained using the 3-[4,5-dimethylthiazol-2y]-2,5-diphenyltertrazolium bromide (MTT) assay. Cells ($5x10^3$) obtained from CESCs, EuESCs and EctESCs were seeded into 96-well cell culture plate and exposed for 48 h to different concentrations of SNEDDS-18 and SNEDDS-18/COPA. After treatment, a MTT solution (0.5 mg/ml) was added and after 4 hours of incubation at 37oC, supernatant was discarded and dimethylsulfoxide was added. Formazan crystals formed was quantified at 570 nm in a spectrophotometer (BIO-RAD iMARKE).

4.5. Evaluation of cell morphology and proliferation

Morphological changes and cellular proliferation were assessed using laser scanning confocal microscopy. CESCs, EuESCs and EctESCs ($1x10^4$) were seeded on 12-mm round coverslips and treated with SNEDDS-18 and SNEDDS-18/COPA (50 μg/ml) for 24 hours. Subsequently, the cells were washed with sterile PBS and fixed with cold ethanol for 20 minutes at room temperature. To assessing intermediate filament vimentin and microtubules, the cells were incubated with the monoclonal primary antibodies anti-vimentin (1:100 dilution, Sigma-Aldrich) or anti-α-tubulin (1:100 dilution, Sigma-Aldrich) for 2 hours at room temperature followed by additional 2 hours incubation with Alexa 546 secondary antibody (1:300 dilution, Invitrogen). Actin microfilaments were evaluated by staining with phalloidin conjugated to rhodamine (1:100 dilution, Sigma) for 60 minutes at room temperature. To detect Ki-67, the cells were incubated with the ki67 antibody (1:100 dilution, Dako) for 18 hours at room temperature followed by Alexa 546 secondary antibody (1:300 dilution, Invitrogen). Cell nuclei were labeled with DAPI (5 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Coverslips were mounted onto histological slides with N-propylgalate (Sigma-Aldrich) and images obtained by fluorescence microscopy (Nikon, Tokyo, Japan) attached to a digital camera (Coolpix 990; Nikon). For each case, immunostaining-negative controls were performed by omitting the primary antibody. The images shown are representative of at least three separate experiments.

The possible effects of both nanoemulsions on cell proliferation was also accessed by the crystal violet incorporation assay. The three ESCs cultures (5x103) were seeded and treated for 24, 48 and 72 hours. Then the cells were washed, fixed in ethanol for 10 minutes, stained with crystal violet 0.05% (Vetec, Brazil) for 10 minutes, and solubilized with methanol. The supernatant was measured by spectrophotometer (BIO-RAD iMARKE) at 570nm. All assays were performed in triplicate.

4.6. Analysis of the cellular motility

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To evaluate the in vitro motility of the CESC, EuESC and EctESC over to 72 hours of treatment with 50 μg/ml of SNEDDS-18 and SNEDDS-18/COPA, we used videomicroscopy assay. To do that, each cell culture was plated, and samples were moved to a culture chamber, with controlled conditions of temperature and CO2 (37°C and 5%, respectively), adapted to an inverted microscope Nikon Eclipse TE 300 (Nikon). During 72 h, phase contrast images of treated and untreated cell cultures were captured every minute using a Hamamatsu C2400 CCD camera (Hamamatsu, Japan) and, in the end, the movies were assembly.

4.7. Analysis of the cellular adhesion

To determine whether the nanoemulsions could interfere with cellular adhesion, we performed a cell adhesion test by immunocytochemistry, as described for morphological analysis. Briefly, the CESCs, EuESCs and EctESCs cultures in equal number ($1x10^4$ cells/well) were treated for 24 hours, fixed, permeabilized and incubated with anti-β-catenin primary antibody (1:100, Sigma-Aldrich), followed by Alexa 546 secondary antibody (1:300, Invitrogen). Then, the cells were incubated with DAPI, the coverslips mounted onto histological slides and photographed in a fluorescence microscope (Nikon, Tokyo, Japan). The images shown are representative of at least three separate experiments.

4.8. Cytokine measurement

Conditioned medium was obtained 24 hours after incubation of CESC, EuESC and EctESC with 50 μg/ml of nanoemulsions (SNEDDS-18 and SNEDDS-18/COPA). Specific ELISA kits were used (BD OptEIA™ Set Mouse ELISA) for IL-1β, TNF-α, MCP-1 and IL-10, and their levels were determined according to the manufacturer's recommendations (BD Biosciences). Absorbance was measured at 450 nm using a microplate reader (BIO-RAD) and cytokine concentrations were calculated using a standard curve.

4.9. Data analysis

The statistical analyses were performed using the GraphPad Prism 5.0 (GraphPad Software Inc., USA). The results of cell viability, cell proliferation and cell death assays are expressed as the mean ± standard deviation from values of independent experiments. The differences between groups were analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test, as a post test. The $p<0.05$ (*) value was considered statistically significant.

5. Conclusions

To the best of our knowledge this work is the first to show in vitro evidence that a nanoemulsion containing *O. speciosa* oil or a combination with *C. langsdorffii* oilresin reduce cell viability and proliferation and modify cell morphology in primary cultures of human ectopic endometrium stromal cells suggesting these nanoemulsions as a new option for the endometriosis treatment.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee from Hospital Universitário Clementino Fraga Filho of the Federal University of Rio de Janeiro/Brazil (protocol no. 23002513.7.0000.5257, 2014) for studies involving human samples. The animal study protocol was approved by Animal Experimentation Ethics Committee of the Health Sciences Center of UFRJ, Rio de Janeiro (code DFBCICB-015).

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