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Development of in vitro and in vivo tools to evaluate the antiangiogenic potential of melatonin to neutralize the angiogenic effects of VEGF and breast cancer cells: CAM assay and 3D endothelial cell spheroids

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ABSTRACT

Melatonin is a molecule with different antitumor actions in breast cancer and has been described as an inhibitor of vascular endothelial growth factor (VEGF). Despite the recognition of the key role exerted by VEGF in tumor angiogenesis, limitations arise when developing models to test new antiangiogenic molecules. Thus, the aim of this study was to develop rapid, economic, high capacity and easy handling angiogenesis assays to test the antiangiogenic effects of melatonin and demonstrate its most effective dose to neutralize and interfere with the angiogenic sprouting effect induced by VEGF and MCF-7. To perform this, 3D endothelial cell (HUVEC) spheroids and a chicken embryo chorioallantoic membrane (CAM) assay were used. The results showed that VEGF and MCF-7 were able to stimulate the sprouting of the new vessels in 3D endothelial spheroids and the CAM assay, and that melatonin had an inhibitory effect on angiogenesis. Specifically, as the 1 mM pharmacological dose was the only effective dose able to inhibit the formation of ramifications around the alginate in the CAM assay model, this inhibition was shown to occur in a dose-dependent manner. Taken together, these techniques represent novel tools for the development of antiangiogenic molecules such as melatonin, with possible implications for the therapy of breast cancer.

1. Introduction

Although the incidence and mortality rates for breast cancer have declined in recent years, it is still the most common tumor in women worldwide. Angiogenesis, the formation of new capillaries from preexisting blood vessels, has been well recognized as a fundamental requirement to facilitate tumor progression [1]. Reciprocal molecular interactions between tumor cells and their microenvironment (endothelial cells included as part of the stroma) are considered crucial for tumor formation, progression and metastasis [2,3]. Tumor angiogenesis starts with malignant tumor cells releasing molecules that transmit signals to surrounding normal tissue [4]. Hypoxia occurs in tumors and induces an angiogenic switch in favor of the release of proangiogenic molecules [5], promoting the stimulation of growth factors such as VEGF [6], thereby encouraging the formation and growth of new blood vessels [4]. Increased VEGF mRNA expression has been found in breast cancer tissue compared to normal breast tissue [7] and high tissue VEGF levels appear to correlate with a poor prognosis in breast cancer patients

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[8,9]. VEGF secreted by breast cancer cells interacts with VEGF receptors in endothelial cells and then stimulates downstream signaling cascades involving the PKC-MEK-ERK1/2, PI3K-AKT-mTOR and JAK2-STAT3 pathways, triggering endothelial cell migration, proliferation and survival [10,11]. Thus, the inhibition of pathological angiogenesis can limit the growth and spread of tumors by starving the tumor cells of nutrients, and this has become an attractive alternative therapeutic approach in the development of anticancer therapies [4,12].

Melatonin is a hormone with different oncostatic actions, being especially effective in breast cancer [13]. Melatonin exhibits antioxidant properties through the scavenging of free radicals [14], protecting cells from carcinogen-mediated DNA modifications caused by oxidative damage, and therefore also from the initiation of malignant transformation [15,16]. Furthermore, melatonin is also found to exert antiproliferative effects on MCF-7 breast cancer cells by inducing a cell cycle G1-S transition delay, with the subsequent accumulation of the cells in G0/G1 phase [17], arresting the cell cycle in the G1 phase, leading to decreased migration and invasion of breast cancer cells [16]. Furthermore, and importantly with regard to this article, melatonin exerts oncostatic activity in breast cancer through antiangiogenic actions [18,19]. Recently, melatonin has been reported to play a role in the tumor microenvironment by regulating the paracrine interactions between malignant epithelial cells and proximal endothelial cells through a downregulatory action on VEGF expression in human breast cancer cells, which decreases the levels of VEGF around endothelial cells, thus reducing angiogenesis [20].

Given the importance of neovascularization in tumor growth and metastasis, the search and development of cost-effective in vitro and in vivo models to mimic and evaluate the angiogenesis process and test angiogenic molecules in preclinical studies is crucial.

Some of these models are based on 2D endothelial cell culture on plastic surfaces to generate a monolayer that mimics many functional properties of the endothelium in vivo. However, this model has limitations that include the progressive loss of CD34 expression from the differentiated endothelial cell phenotype under these conditions. Also, as endothelial cells can grow, they do not normally support the quiescent state, a resting phenotype that these cells maintain in vivo [21]. Herein, we developed 3D endothelial cell spheroids to solve this limitation, allowing the endothelial cells to acquire a resting phenotype within cell number-defined aggregates. This model was based on spheroids generated from HUVECs mixed with methylcellulose [22]. Despite much study of this technique, the capacity of this in vitro angiogenesis assay to prove the antiangiogenic properties of melatonin to avoid capillary sprout formation has not yet been studied.

On the other hand, regarding the in vivo model, we used a CAM assay, which is normally used for the purpose of seeking antiangiogenic molecules as it possesses various advantages, including its rapid, cost-effective, high-capacity easy handling, since it readily accepts and supports xenogenic transplants [23,24]. This method evaluates angiogenesis by implanting alginate beads containing the treatment to be tested on the chorioallantoic membrane of the egg and subsequently quantifying the number of blood vessels formed around it [25]. The CAM model has been used successfully to assess the invasion of cells through the basement membrane, mimicking early invasive stages of carcinomas and melanoma [26–28]. The CAM is comprised of upper chorionic epithelium, intervening mesenchyme, and lower allantoic epithelium. The chorionic epithelium is structurally similar to human epithelium in that the collagen-IV-rich basement membrane simulates the basement membrane that separates the epithelium from the underlying connective tissue [28]. Since the first use of the CAM assay technique, many adaptations of the method have been developed to allow for the assessment of angiogenesis [29] but, so far, melatonin has not been tested and breast cancer cells have not been used as an angiogenic stimulus in this model.

Accordingly, since melatonin regulates the tumor microenvironment by decreasing the secretion of VEGF by tumor cells [20], the aim of the present study was to investigate the antiangiogenic capacity of melatonin in inhibiting the proangiogenic effects of VEGF, as well as human breast cancer cells (MCF-7). In this context, we characterize a novel approach in which the efficacy of melatonin as an antiangiogenic molecule is tested using implantation onto the in vitro 3D HUVEC cell spheroids and the in vivo CAM assay model, through the sprouting formation around the implant.

2. Materials and methods

2.1. Cells and culture conditions

HUVECs were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). They were maintained as monolayer cultures in 58.1 cm^2 plastic culture plates in Vascular Cell Basal Medium (VCBM) (ATCC, Rockville, MD, USA) supplemented with Endothelial Cell Growth Kit-BBE (ATCC, Rockville, MD, USA), which contains 2% fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria), 0.2% bovine brain extract, 5 ng/ml rhEGF, 10 mM L-glutamine, 0.75 units/ml heparin sulfate, 1 μg/ml hydrocortisone hemisuccinate, 50 μg/ml ascorbic acid, penicillin (20 units/ml) and streptomycin (20 μg/ml) (Sigma-Aldrich, Madrid, Spain) at 37ºC in a humid atmosphere with 5% CO_2 (Table 1). Cells were passaged at ~ 80% confluence and, to avoid genetic mutation and low viability, no more than six passages of HUVECs were used for the experiments.

MCF-7 human breast cancer cells were also purchased from the American Tissue Culture Collection (ATTC; Rockville, MD, USA). They were maintained as monolayer cultures in 58.1 cm^2 plastic culture plates in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Madrid, Spain) supplemented with 10% FBS (PAA Laboratories, Pasching, Austria), penicillin (20 units/ml) and streptomycin (20 μg/ml) (Sigma-Aldrich, Madrid, Spain) at 37ºC in a humid atmosphere containing 5% CO_2 (Table 1). The medium was changed twice per week and cells were passaged once a week, at a subcultivation ratio of 1:3.

2.2. HUVEC sprouting assay

HUVECs were used at early (I–IV) passages and grown on a plastic surface coated with 1 ml gelatin (Sigma-Aldrich, Madrid, Spain) in M199 medium supplemented with 10% FCS (GIBCO Life Technologies (Grand Island, NY)). Spheroids (800 cells/spheroid) were generated by mixing HUVECs (1000,000 cells/ml) with 20 ml 2% methylcellulose (Sigma-Aldrich, Madrid, Spain) in 80 ml M199 medium containing 10% FBS, making the final volume 100 ml (ratio 1:4). Cells (200 μl/well) were then seeded onto U-bottom 96-well plates (Greiner Bio-One, Kremsmünster, Austria) and incubated at 37ºC, 5% CO_2 overnight. A falcon tube was prepared for fibrinogen solution (3.1 mg/ml) (Sigma-Aldrich, Madrid, Spain) and aprotinin was added (5 μl/ml) (Sigma-Aldrich, Madrid, Spain). Subsequently, the solution was filtered with a 0.45 μm diameter filter. Formed spheroids were transferred from the 96-well plates to falcon tubes and the medium was aspirated, leaving the spheroids at the bottom. The fibrinogen solution was added to the falcon tubes and thrombin (Sigma-Aldrich, Madrid, Spain) was added for 1 min. The mix was then seeded onto a 48-well plate and placed in the incubator at 37ºC for 2 min to polymerize.

The following treatments were then added to the wells, with 32 spheroids per group: M199 medium with 10% FCS for control, 1 mM

Table 1
Cell lines used in the experiments. HUVEC: Human umbilical vein endothelial cells; MCF-7: Michigan Cancer Foundation; VCBM: Vascular Cell Basal Medium; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal Bovine Serum; ER+ : Positive estrogen receptor.

Cell line	Origin	Culture medium
HUVEC	Human umbilical vein endothelial cells	VCBM + 2% FBS
MCF-7	Human mammary adenocarcinoma cells (ER+)	DMEM + 10% FBS

melatonin diluted in ethanol, 1 nM melatonin diluted in ethanol, 30 ng/ml VEGF165-A (starting from a stock of 100 ng/μl in BSA) (PSI, Villigen, Switzerland) or MCF-7 supernatant in the absence or presence of different concentrations of melatonin (1 nM or 1 mM) (Sigma-Aldrich, Madrid, Spain). After 24 h incubation at a constant temperature (37ºC) and pCO2 (5%) in the incubator, the number of sprouts grown in each spheroid was counted by an observer, who was blinded, using an inverted microscope (Axiovert 200 M equipped with objective LD A-Plan 20X/0.30PH1 (Zeiss)) [22] and photomicrographs were taken of the formed spheroid sprouting.

2.3. Chick embryo chorioallantoic membrane (CAM assay)

The CAM assay was used as an in vivo model to study the angiogenic response and blood vessel count, as previously described with minor modifications [4]. A total of 72 fertilized White Leghorn chicken eggs from Granja Santa Isabel (Córdoba, Spain) were incubated for 3 days at 37ºC in a humidified incubator. 4 ml of albumin were aspirated from the acute end of the egg with a hypodermic needle to allow detachment of the developing CAM shell. At day 4 the shells were covered with a transparent adhesive tape and a small window on the eggshell above the air sac was opened with scissors on the broad side directly over the avascular portion of the embryonic membrane. The opening on the eggs was sealed with paraffin to prevent dehydration. The eggs were then re-incubated for another 7 days. During the incubation, the viability of the eggs was assessed daily. At day 11 of incubation, the eggs were divided into 9 groups (8 eggs per each group): control, melatonin 1 nM, melatonin 1 mM, VEGF, VEGF and melatonin 1 nM, VEGF and melatonin 1 mM, MCF-7, MCF-7 and melatonin 1 nM, and MCF-7 and melatonin 1 mM. Alginate beads (4.0 μl) containing PBS (vehicle), VEGF (100 ng per embryo) or MCF-7 (10.000 cells/bead) in the presence or absence of melatonin (1 mM or 1 nM) were polymerized with $CaCl_2$ and were placed onto the CAM of a subset of eggs. VEGF 1.57 pM was used as a positive control of angiogenic compound whereas PBS was used as a negative control. Chemical agents were dissolved in 1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Madrid, Spain). After 72 h, at day 14, when the vascularization potential of the CAM reached its maximum, newly formed blood vessels around the alginate were counted at 5 × magnification using a STEMI SR stereomicroscope equipped with a 100 mm lens with adapter ring 47070 (Zeiss), by 2 observers in a double-blind fashion, and fixed with 7% buffered formalin and photographed (Fig. 1).

All experiments were performed with the approval of the Bioethics Committee at the Virgen de la Victoria University Hospital (Málaga, Spain) and carried out in full accordance with the guidelines of the European Community (2010/63/EU) and Spanish regulations (RD 53/2013).

2.4. Statistical analysis of the data

All statistical analyses were performed using GraphPad Prism 6 program (Graphpad Software, Inc. California, USA) and statistical significance was evaluated using Student's t-test or one-way ANOVA followed by Bonferroni multiple comparison posttest. Results were considered statistically significant for p-values < 0.05. All experiments were repeated 3 times under identical conditions and results are reported as mean ± standard error of the mean (SEM).

3. Results

3.1. Melatonin prevents VEGF- and MCF-7-triggered sprouting cell spheroids

In order to evaluate the antiangiogenic effects of melatonin on the formation of sprouts around the spheroids, we performed the 3D endothelial cell spheroid assay. We used different doses of melatonin to determine the most effective. As seen in Fig. 2(a), melatonin alone had no apparent effect on sprout number. Nevertheless, the addition of

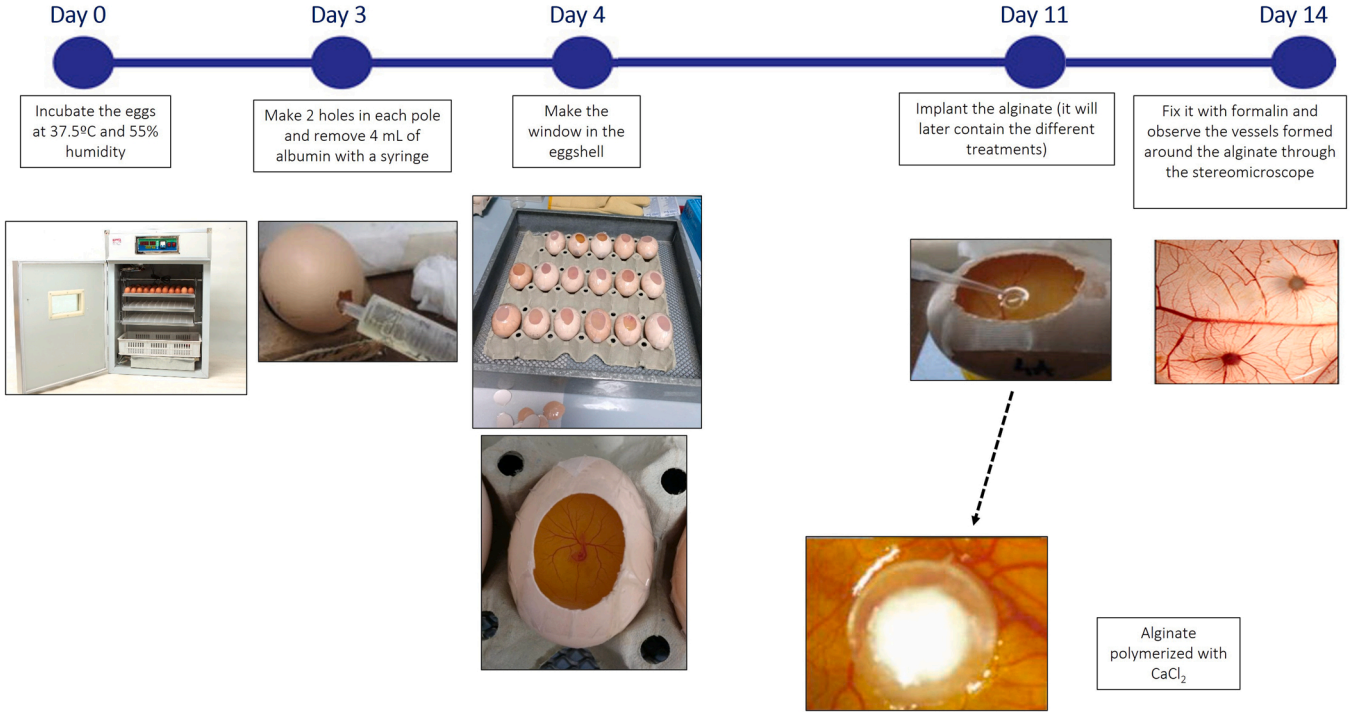

Fig. 1. (a) Schematic representation of the workflow of the CAM assay experiment. First, the eggs are placed in an incubator at 37.5ºC and 55% humidity. On the third day, two holes are made, and albumin is extracted so that the chorioallantoic membrane does not stick to the shell. On day 4, windows are made and the eggs are incubated again. On day 11, the alginates are implanted with the different treatments to be tested, and three days later the blood vessels formed around the alginate are counted to evaluate angiogenesis.

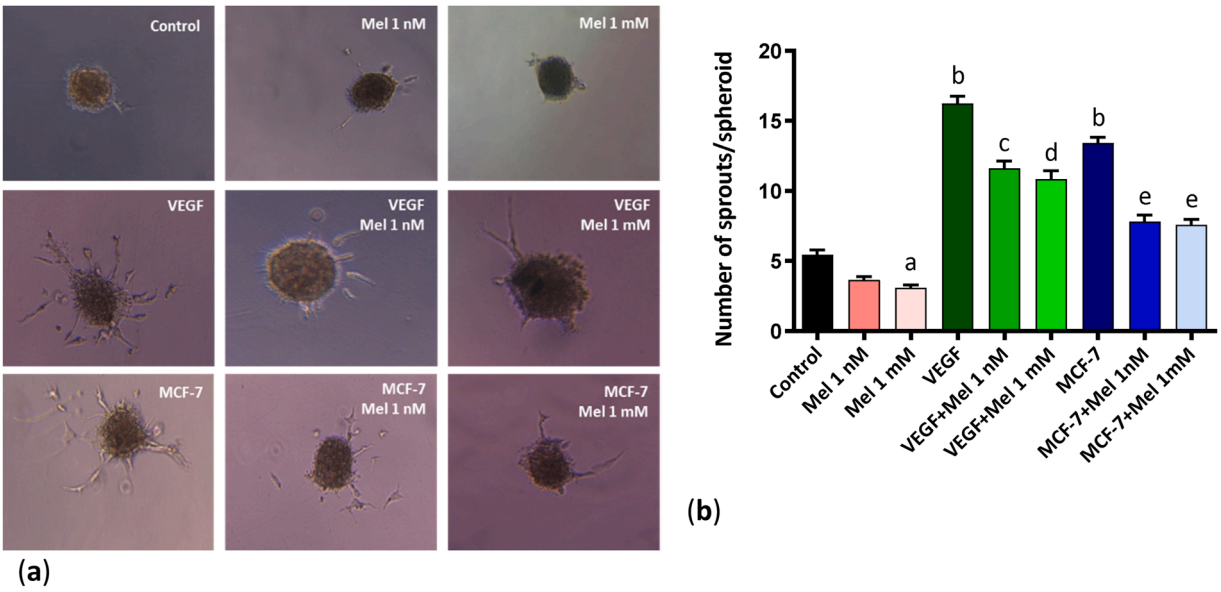

Fig. 2. In vitro effect of melatonin-induced changes in VEGF- or MCF-7-stimulated angiogenesis in the 3D spheroid model. Spheroids were generated by mixing HUVECs with 20 ml 2% methylcellulose in M199 medium with 10% FCS. Different treatments were added and after 24 h of incubation, the number of formed sprouts was counted using an inverted microscope. The protocol is detailed in the Material and Methods section. (a) Images of the spheroids with the different added treatments; (b) VEGF and MCF-7 supernatant induced an increase in sprouting formation around the spheroid. Melatonin (1 mM or 1 nM) neutralized the stimulating effect on sprout formation. a, $p < 0.01$ vs Control; b, $p < 0.001$ vs Control; c, $p < 0.01$ vs VEGF; d, $p < 0.001$ vs VEGF; e, $p < 0.001$ vs MCF-7. Mel: melatonin.

either VEGF or MCF-7 supernatant strongly induced the formation of sprouts around the spheroid. In both cases, in combination with melatonin, the stimulating effect induced by VEGF or MCF-7 was neutralized and reduced (Fig. 2(b)). Specifically, the pharmacological dose (1 mM) was even more effective than the physiological dose (1 nM).

3.2. *Melatonin inhibits angiogenesis induced by VEGF and MCF-7 tumor cells in vivo*

To demonstrate the antiangiogenic effects of melatonin in vivo, we performed a CAM assay to evaluate the formation of new vessels. In this set of experiments, melatonin (1 nM and 1 mM), VEGF, and MCF-7 were put into the alginate beads and implanted on developing CAM at day 11. After 3 days, the angiogenic response was measured by counting the number of newly formed microvessels converging toward the alginate

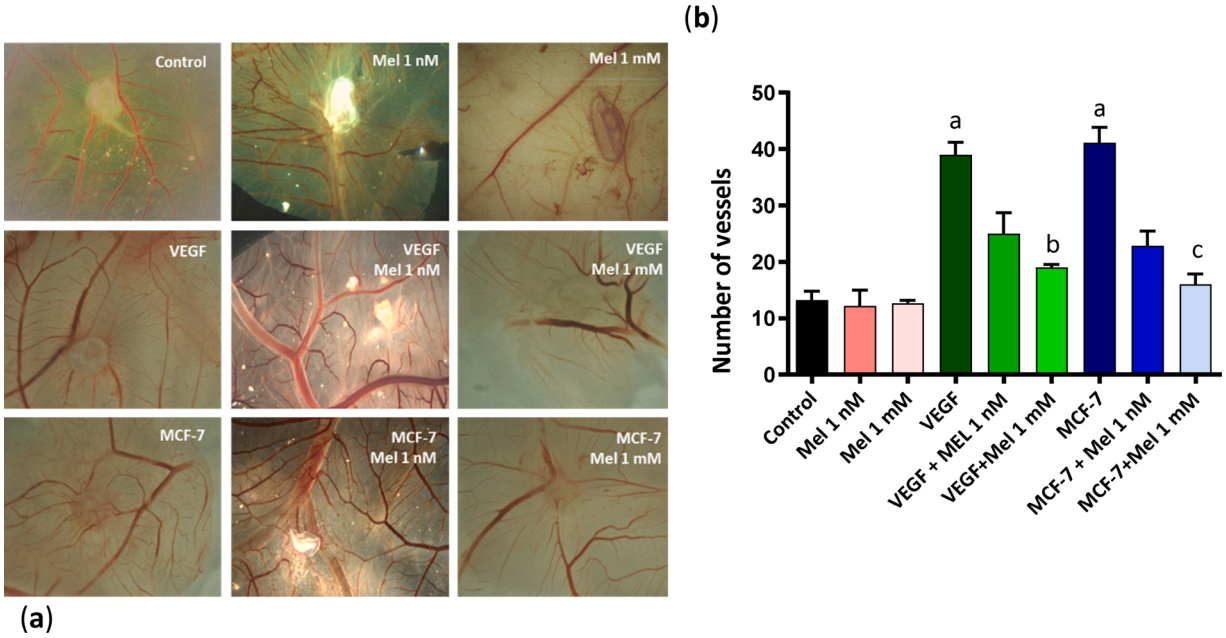

Fig. 3. In vivo effect of melatonin-induced changes in VEGF- or MCF-7-stimulated angiogenesis in the chorioallantoic membrane assay. VEGF or MCF-7 were put into the alginate bead on top of the chicken CAM at day 11 postfertilization in the absence or presence of melatonin (1 mM or 1 nM). CAMs were photographed under a stereomicroscope at day 14, and blood vessels around the alginates were counted. Data are the mean ± SD of 8 eggs per group. (a) Representative CAM images; (b) VEGF and MCF-7 cells induced an increase in sprouting formation around the alginate. Melatonin (1 mM) neutralized this stimulatory effect on the formation of new microvessels. a, $p < 0.001$ vs Control; b, $p < 0.01$ vs VEGF; c, $p < 0.01$ vs MCF-7. Mel: melatonin.

bead. In controls, blood vessels formed a dense and spatially oriented branching network composed of vascular structures of progressively smaller diameter as they branched (Fig. 3(a)). We found that VEGF alone induced numerous blood vessels (Fig. 3(a)). Furthermore, MCF-7 induced an increase in the number of blood vessels around the alginate. In this case, melatonin alone did not have any effect (neither 1 nM nor 1 mM), but melatonin 1 mM exerted a significant inhibitory effect on blood vessel formation triggered by VEGF or MCF-7 cells ($p < 0.001$) (Fig. 3(b)), observed as an inhibition of the ingrowth of new vessels in the area covered by the alginate. The peripheral vessels (relative to the position of the alginate) grew centrifugally, avoiding the treated area, where a decrease in the vascular density could be observed (Fig. 3(b)).

4. Discussion

Angiogenesis constitutes a fundamental step in pathologic conditions in which uncontrolled release of angiogenic growth factors and alterations of the production of natural angiogenic inhibitors take place [30]. Tumor angiogenesis is a dynamic process that plays a crucial role in tumor initiation and progression. Tumor cells synthesize and secrete the angiogenic factors which, by stimulating vascular permeability and endothelial cell proliferation, play a fundamental role in the progression and development of malignant breast tumors [31]. VEGF represents the main angiogenic target for the development of antiangiogenic therapies in cancer patients [6,31–33].

Unfortunately, despite the recognition of the key role exerted by VEGF in the pathogenesis of pathological neovascularization, limitations exist for the development of antiangiogenic therapies. There is therefore an urgent need to develop rapid and cost-effective models to screen novel antiangiogenic molecules with the aim of transferring preclinical results to the clinical setting [22].

In this study we developed two models to evaluate the antiangiogenic efficacy of melatonin at pharmacological and physiological concentrations using VEGF or MCF-7 cells as the stimulus source of sprouting. Preclinical studies have demonstrated the benefits of the combination of antitumor treatments used in cancer, such as chemotherapy or immunotherapy and the antiangiogenic inhibitors, producing significantly greater inhibitory actions on tumor growth and angiogenesis than either agent alone [34–36]. In the last few years, melatonin has been shown to exert antiangiogenic properties by downregulating VEGF expression and protein levels [20,37]. The fact that melatonin acts in the downregulation of VEGF could be an effective therapeutic key to block angiogenesis and tumor growth. The antitumor properties of melatonin are especially relevant in hormone-dependent tumors [38,39].

Our results showed that both MCF-7 cells and VEGF could stimulate the sprouting of HUVEC spheroids and the formation of blood vessels around the alginate in the CAM assay. Nevertheless, the addition of both doses of melatonin exerted an inhibitory activity on the germination of HUVEC induced by MCF-7 and VEGF in the spheroid model, but only 1 mM was effective in the CAM assay.

Previous findings demonstrated that the pharmacological concentration of melatonin decreased HUVEC growth and only this melatonin concentration had an inhibitory effect on cell proliferation [40]. Furthermore, Álvarez-García et al. demonstrated that the most effective melatonin concentration was 1 mM to produce the most inhibitory action in endothelial cells [20]. It is known that melatonin can be concentrated at least 1000 times more in tumor and adipose tissues of mammary tissue [41]. The fact that melatonin reaches high concentrations in this tissue could explain why high levels of this hormone are necessary to obtain some antitumor effects. However, some authors maintain that physiological concentrations of melatonin induce a strong inhibitory effect on human breast cancer cells [42–44]. In line with this, in our experiments the pharmacological concentration of melatonin was effective in both models. However, the physiological dose of melatonin was only effective in the spheroid assay.

This study is relevant since we demonstrate the antiangiogenic effects of melatonin in both in vitro and in vivo assays that constitute cost-effective and rapid tools to evaluate the antiangiogenic capacity of this hormone. It is important to highlight that melatonin was tested to neutralize not only the angiogenic effects of VEGF, but also the angiogenic effects of MCF-7. For this, we chose the in vitro 3D sprouting assay [22]. This technique constitutes a novel tool that helps to corroborate the antiangiogenic impact in the formation of sprouts around the spheroids generated from endothelial cells [22]. Some studies have demonstrated the efficacy of this technique to evaluate angiogenesis. Specifically, the experiments were carried out in pancreatic cancer [45], liver cancer [46,47], lung cancer [48] and prostate cancer [49], among others. We found that melatonin abolished the angiogenic effects induced by VEGF or MCF-7 supernatant on branch formation.

Furthermore, regarding the choice of the in vivo experiment, we decided to carry out the CAM assay, which is an economical and easy-handling tool to evaluate the antiangiogenic potential of anticancer molecules [24]. The usefulness of this tool has been reported for the evaluation of angiogenesis in other studies such as pancreatic cancer [50], gynecological or urological cancer [51–53], head and neck cancer [29] or breast cancer [54]. In this study, we corroborate the efficacy of melatonin as an antiangiogenic molecule in vivo as it neutralizes the stimulating effect induced by MCF-7 or VEGF in the CAM assay.

5. Conclusions

In view of our results, the 3D endothelial cell spheroids and CAM assay constitute rapid and cost-effective experimental procedures suitable for the evaluation of the antiangiogenic efficacy of melatonin. These techniques might contribute to the 3 R concept and be an experimental alternative to a classical animal test. In addition, we have demonstrated that melatonin exerts an inhibitory activity on the germination of HUVEC induced by MCF-7 and VEGF in 3D sprouting and CAM assays and that this inhibition is dose-dependent in the latter, as only the melatonin concentration of 1 mM was able to inhibit the formation of sprouts around the alginates. Taken together, our study shows that the spheroid-based angiogenesis assay and CAM assay provide sensitive and versatile tools to study the impact of antiangiogenic molecules and we have demonstrated that melatonin constitutes a promising antiangiogenic agent that could be used in combination with other conventional therapies to improve its properties in breast cancer. Further investigations will be necessary to demonstrate these promising properties of melatonin in clinical applications.

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CRediT authorship contribution statement

Alicia González-González, Emilio Alba, María Isabel Queipo-Ortuño: Conceptualization, Visualization, Supervision, Project administration, Writing – original draft preparation. **Alicia González-González, Aurora Laborda-Illanes, Lidia Sánchez-Alcoholado, Isaac**

Plaza-Andrades, Lucía Aranega-Martín, Jesús Peralta-Linero: Methodology. **Alicia González-González, Aurora Laborda-Illanes, Lidia Sánchez-Alcoholado, Daniel Castellano-Castillo, Soukaina Boutriq, Isaac Plaza-Andrades, Lucía Aranega-Martín, Jesús Peralta-Linero**: Software. **Alicia González-González, Aurora Laborda-Illanes, Lidia Sánchez-Alcoholado, Daniel Castellano-Castillo, Soukaina Boutriq**: Validation. **Alicia González-González, Aurora Laborda-Illanes, Lidia Sánchez-Alcoholado, Daniel Castellano-Castillo, Soukaina Boutriq**: Formal analysis. **Alicia González-González, Aurora Laborda-Illanes, Lidia Sánchez-Alcoholado, Daniel Castellano-Castillo, Soukaina Boutriq, Isaac Plaza-Andrades, Jesús Peralta-Linero**: Investigation. **María Isabel Queipo-Ortuño, Emilio Alba**: Resources, Funding acquisition. **María Isabel Queipo-Ortuño, Emilio Alba**: Data curation. All authors: Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

The animal study protocol was approved by the Ethics Committee of University of Málaga (date of approval: 27/06/2019) and the experiments were carried out in full accordance with the guidelines of the European Community (2010/63/EU) and the Spanish regulations (RD 53/2013).

Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable.

Conflicts of interest statement

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

Data will be made available on request.

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