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EVALUATION OF THE INHIBITORY EFFECTS OF MELATONIN ON ANGIOGENESIS IN THE CHICK EMBRYO CHORIOALLANTOIC MEMBRANE (CAM) MODEL

Authors

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Abstract

Introduction: Angiogenesis is a key event in cancer pathology. Current treatment targeting angiogenesis in cases of solid tumours is expensive and inaccessible and newer molecules will require a long time to be available in the market. Melatonin has shown potential in inhibiting angiogenesis.

Aim: The aim of the study was to evaluate the inhibitory effects of melatonin on angiogenesis in the chick embryo Chorioallantoic Membrane (CAM) model.

Methods: This was an *in-ovo* study on the chick embryo. Melatonin 1mM was compared with a positive control of Timolol 0.01% and a blank control of Dimethyl Sulfoxide (DMSO 99.99%).

Results: Melatonin 1mM (0.232 mg/ml) showed a 61% reduction in vessel density and a 20% reduction in vessel length which was significantly greater than DMSO ($p<0.001$), and Not significantly different from the effect of Timolol 0.01%, which was the positive control. Melatonin additionally showed embryotoxicity as a consequence of its anti-angiogenic effect.

Conclusion: Further clinical studies to the efficacy of melatonin in humans are warranted to make strides in the future of cancer therapy

1. INTRODUCTION

Angiogenesis is a key hallmark in the pathogenesis of various diseases including, but not limited to cancer, haemangioma, telangiectasia, diabetic retinopathy and age-related macular degeneration (AMD) [1–3]. Currently approved therapies targeting key players in angiogenesis such as Vascular Endothelial Growth factor (VEGF), Platelet derived Growth Factor (PDGF), etc are expensive and hence inaccessible to most of the population. Drugs such as Bevacizumab, Ranibizumab, Sorafenib and Sunitinib are associated with severe adverse effects such as haemorrhage, hypertension, endophthalmitis and posterior reversible encephalopathy syndrome (PRES). Newer molecules are still in the pipeline and may take several years to make it into clinical practice. [4–7]

Melatonin, a hormone secreted by the pineal gland, regulates the circadian rhythm among its varied physiological functions in the body. Melatonin is available as an over-the-counter supplement to regulate the sleep-wake cycle in patients with insomnia, shift workers, and individuals suffering from jet lag. Previous studies have shown melatonin to have a modulatory effect on angiogenesis: being pro-angiogenic in a hypoxic environment and in the context of wound healing, while being anti-angiogenic in the tumour microenvironment [8–10]

The chick embryo is a reliable and well-established model for screening anticancer, angiogenic, and anti-angiogenic drugs and toxicity studies. It requires minimum ethical considerations in addition to being cost-effective and quick.

The chick embryo's chorioallantoic membrane (CAM) is easily visualised and has a dense network of blood vessels exhibiting both the intussusceptive and sprouting types of angiogenesis.

The only limitations to this model are the small-time frame available for the experiment and non-specific immune responses that can interfere with the angiogenesis process.[11–15]

With regard to this study, the chick embryo CAM model has been well-established in the institute and previous research has been successfully conducted by Dr. Vijaykumar Gupta. [16,17]

The serendipitous discovery of propranolol as an effective means to regress haemangiomas has made the beta blocker a first-line therapy for haemangioma patients. Infantile haemangioma (IH) is infancy's most common vascular tumour and was predominantly treated by surgery, laser and corticosteroids. The role of Propranolol in the therapy of IH could be: Vasoconstriction Apoptosis via β-ADR signalling and the caspase pathway Inhibition of angiogenesis via the modulation of vascular growth factors (VEGF) [18,19]

Propranolol hydrochloride (Hemangeol®) was the first FDA-approved treatment for proliferating Infantile Haemangioma requiring systemic therapy. [20]

Several studies support the use of Timolol as an alternative to Propranolol in treating infantile haemangiomas. [21–23] With this in mind, timolol ophthalmic solution was chosen as the positive control.

This study was thus conducted with the aim of evaluating the inhibitory effects of melatonin on angiogenesis in the chick embryo chorioallantoic membrane and comparing the effects with Timolol.

2. OBJECTIVES

The primary objective was to effects of melatonin on angiogenesis in the chick embryo CAM model using the visual assessment for density and length of blood vessels.

Secondary objectives were to evaluate the effects of melatonin on angiogenesis in the chick embryo CAM model using the histopathological assessment of endothelial.

3. METHODOLOGY

3.1 Study Design and Setting

This was a prospective, comparative, In-Ovo study conducted in the Department of Pharmacology, Dr D.Y. Patil University School of Medicine, Navi Mumbai, Maharashtra.

3.2 Statement of Ethics

This study was done in accordance with the national and international laws, regulations and guidelines for the care and handling of laboratory animals and abided by the guidelines Committee for the Control and Supervision of Experiments on Animals (CCSEA formerly,

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CPCSEA). This study was approved by the Institutional Animal Ethics Committee (IAEC Ref. No. DYP/IAEC/2021/004; dt. 08/07/2021).

As per the Institutional Animal Care and Use Committee (IACAC), "If embryos will be sacrificed before 3 days before hatching (i.e., day <18), the research is not subject to review unless specifically requested by the investigator." Chick embryos younger than embryonic day 10 (EDD10) are assumed to be unable to experience pain and it is recommended to be euthanised by hypothermia, typically by placing the eggs in a −20°C freezer for a minimum of 4 hours. Standard Operating Procedures were followed to minimize any possible suffering by embryos.

CCSEA states 'Animals lowest on the phylogenetic scale (i.e., with the least degree of sentience), which may give scientifically valid results, should be used for any experimental procedure. Experiments should be designed with the minimum number of animals to give statistically valid results at a 95% confidence level.

3.3 Methods

The study flow is presented in Figure 1. Melatonin was supplied by HAB PHARMACEUTICALS & RESEARCH LIMITED (Dehradun, India). All Reagents and compounds used were of AR grade.

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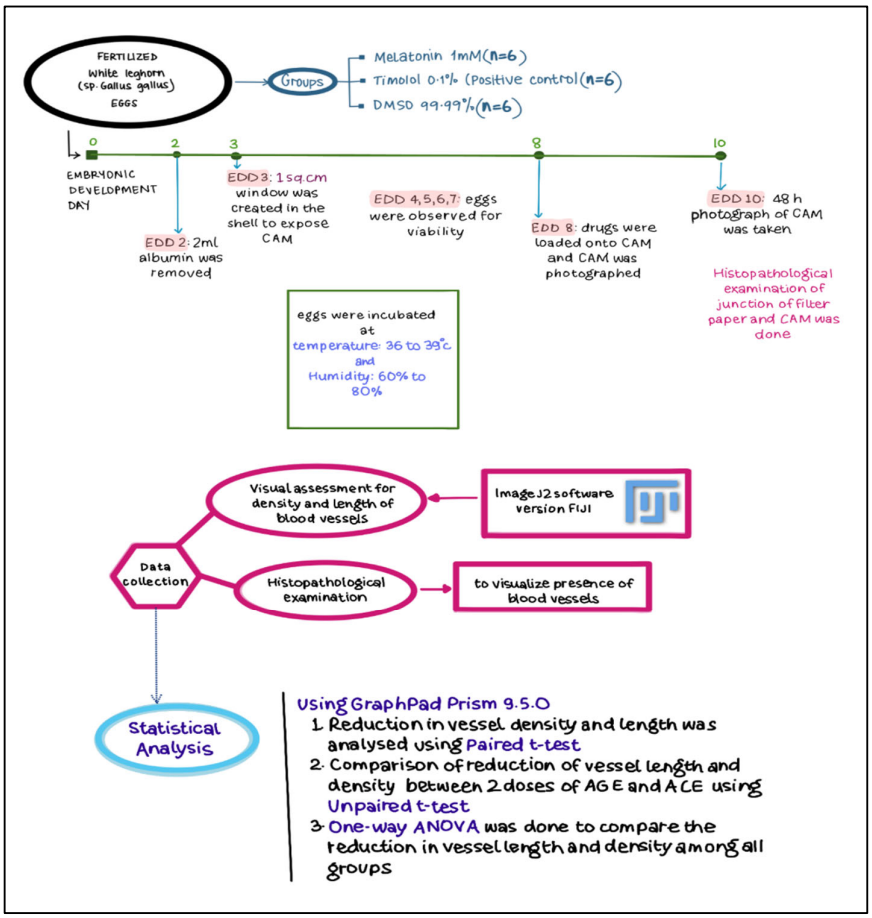

Figure 1: Flowchart showing study design

Abbreviations: ANOVA- Analysis of Variances, CAM- Chorioallantoic Membrane, EDD- Embryonic Developmental Day.

3.4 Chorioallantoic Membrane Assay

Fertilised chicken eggs (White Leghorn) were procured from More Poultry (Thane, Maharashtra) on day 0 of Embryonic Development Day (EDD). After cleaning with 70% ethanol, eggs were incubated at a temperature of 36° C - 38° C and humidity of 60-80%. Fertilization was confirmed using the egg candling instrument; Fertilised eggs have a reddish hue. For the next 2 days, the eggs were turned every 2 hours. Incubation was carried out in the laboratory.

On the 3rd day (EDD 2), a hole is made at the narrow, lower end of the egg using a sterile dental drill bit 1mm and 1-2 ml of albumin was extracted. The hole was sealed with the help of micropore and the embryo is placed back in the incubator, still kept in their original egg structure. On the 4th day (EDD 3), a small window of about 1.5 cm diameter is created at the broader upper end using the dental drill and the shell is removed using sterile forceps. The window is made to visualise the CAM and sealed with micropore and scotch tape to avoid moisture escaping. The embryos were observed daily till EDD 8. The eggs were divided into three groups: 1) test group

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– melatonin (1mM- 0.2mg/ml); 2) positive control – timolol 0.01%; and 3) Vehicle control – 99.99% DMSO. The drugs are applied onto the CAM by loading 2mm Whatman filter paper discs with 20 microliters of the sample (figure 2). All steps were carried out using aseptic conditions. After allowing the disc to completely absorb the sample, it is placed on the CAM using sterile forceps. The CAM was photographed at 0 hours and 48 hours with a Canon EOS-80D digital single-lens reflex DSLR camera and analysed using the ImageJ Fiji application.

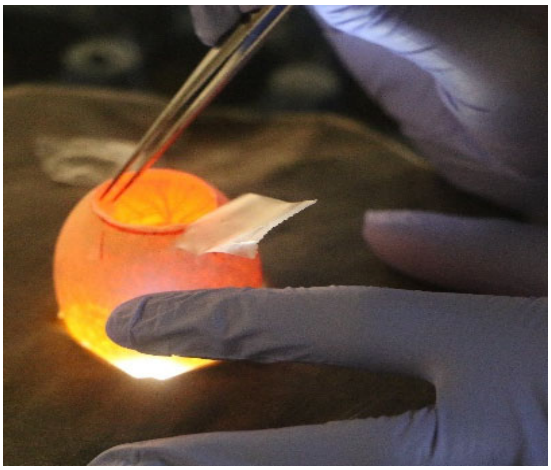

Figure 2 Placing the Whatman paper loaded with the test compound

3.5 Image Analysis

Once a high-resolution image of the CAM was obtained, it was uploaded onto the Windows-based software FIJI version 2.9.0 (ImageJ, developed by NIH).[24] A square of size 700 × 700 pixels was selected on the image and used for analyses (figures 3 and 4). The number of blood vessels was counted and vessel length was measured using ImageJ software version FIJI. Figure 3 depicts the process of analysis of an image in FIJI. ImageJ is a free software plugin that has been previously utilised similarly to measure bone density (BoneJ), in the analysis of microvasculature in retinal angiogram and in the analysis of neurite density (MyelinJ).[25–27]

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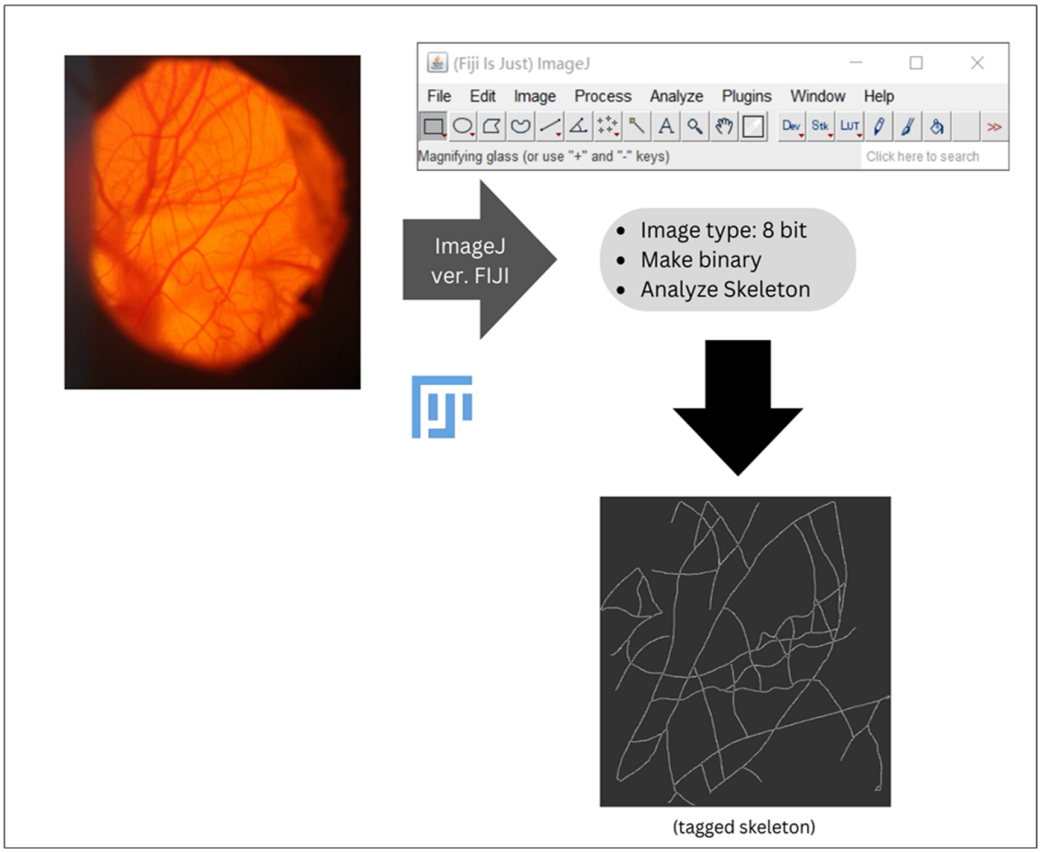

Figure 3 Flowchart showing the process of image analysis using ImageJ (FIJI)

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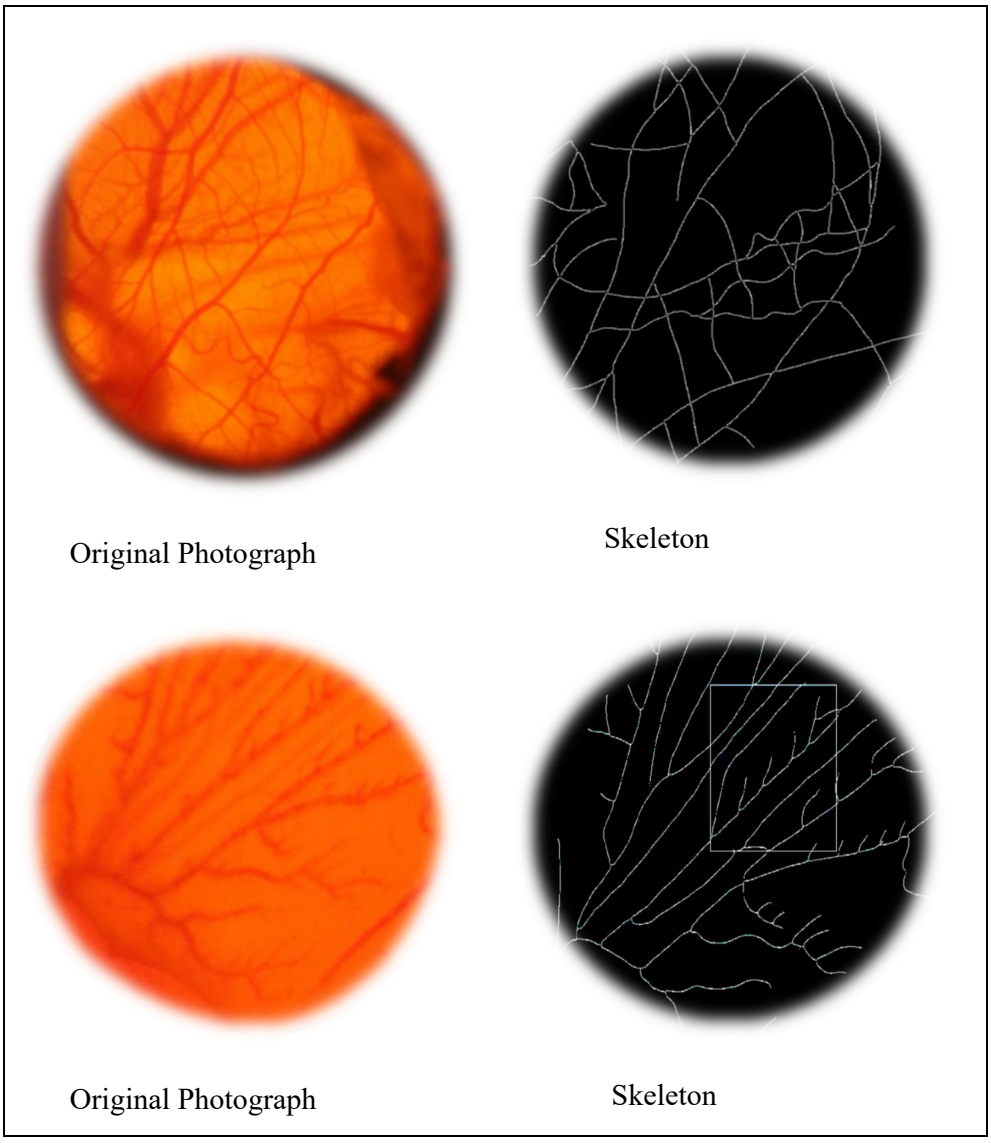

Figure 4 Skeletonised version of Chorioallantoic Membrane images

3.6 Histopathological examination

Histopathology was carried out in the pathology department of the institute. CAM tissue was immersed in formalin (for fixation) followed by alcohol (dehydration), xylene (clearing), and paraffin wax (infiltration). After the specimen is infiltrated with wax, it is formed into a "block". This step is carried out using an embedding centre where a mould is filled with molten wax and the specimen placed into it. A cassette is placed on top of the mould, topped up with more wax, and the whole thing is placed on a cold surface to solidify.

Sections of 4-µm-thickness were taken and stained with haematoxylin and Eosin (H&E) for histopathological analysis. The slides were analysed by histopathology experts under 100X magnification.[28]

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3.7 Study Outcomes:

Primary outcomes were the vessel density and vessel length measured by ImageJ software using the images of chorioallantoic membrane taken pre and 48 hours post-treatment with Melatonin 1mM. Secondary outcomes were the histopathological changes seen at the junction of the chorioallantoic membrane and the Whatman filter paper loaded with test compound.

3.8 Statistical Analysis

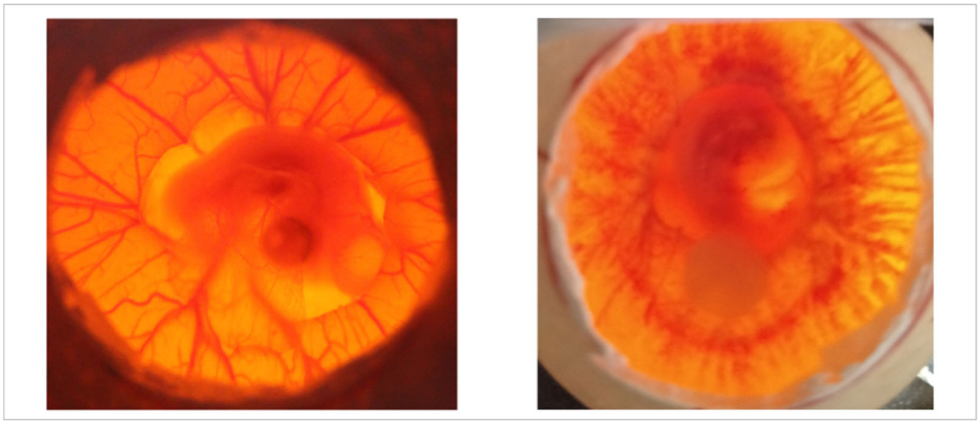
Statistical analysis was performed using the trial version GraphPad Prism 9.5.0: Statistical software (GraphPad Software, CA). Descriptive data are presented as mean with standard deviation (SD). Two-tailed Paired t-test was used to compare vascular density before and after treatment. A comparison between all compounds was done using one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test for pairwise comparisons. [29]

4. RESULTS

4.1 Effect of Melatonin 1mM (0.232 mg/ml) on vessel density and vessel length

Melatonin significantly reduced vascular density (61%) and vessel length (20%) post-treatment, comparable to the control Timolol 0.1%. Melatonin also showed haemorrhage in the CAM followed by embryotoxicity (figure 5).

Table 1 and Figure 6 present the descriptives for vascular density and vessel length in different groups.



Pre-treatment 48 hours post-treatment

Figure 5 Chorioallantoic Membrane photographed before treatment and 48 hours after treatment

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Table 1 A)	*Pre-treatment*	*Post-treatment with Melatonin 1mM*	*Post-Pre*
No. of eggs (N)	6	6	6
I) Vessel Density: No. of vessels/700 x 700 sq pixels			
Mean	11.00	4.33	-6.67
Std. Deviation	3.57	2.06	2.34
Median	9.50	4.50	-6.500
Minimum	8.00	2.00	-10.00
Maximum	16.00	7.00	-3.00
II) Vessel length in pixels			
Mean	245.70	197.90	-47.79
Std. Deviation	29.31	36.630	9.915
Median	246.10	192.50	-47.59
Minimum	203.60	145.10	-58.53
Maximum	286.10	244.60	-32.96

Table 1 B)	*Melatonin1mM*	*Timolol 0.01%*	*DMSO 99.99%*
No. of eggs (N)	6	6	6
I) Vessel Density			
Mean	-0.61	-0.62	-0.01
Std. Deviation	0.15	0.15	0.01
Median	-0.78	-0.81	-0.01
Minimum	-0.76	-0.74	-0.01
Maximum	-0.38	-0.43	0.0
II)Vessel Length			
Mean	-0.20	-0.21	-0.01
Std. Deviation	0.06	0.06	0.01
Median	-0.21	-0.23	0.0
Minimum	-0.29	-0.26	-0.02
Maximum	-0.12	-0.12	0.0

Table 1A) Pre and post-treatment effects of Melatonin on I) Vessel density and II) Vessel length and Table 1B) Comparison between Melatonin and control groups with respect to reduction in I) Vessel density and II) Vessel length

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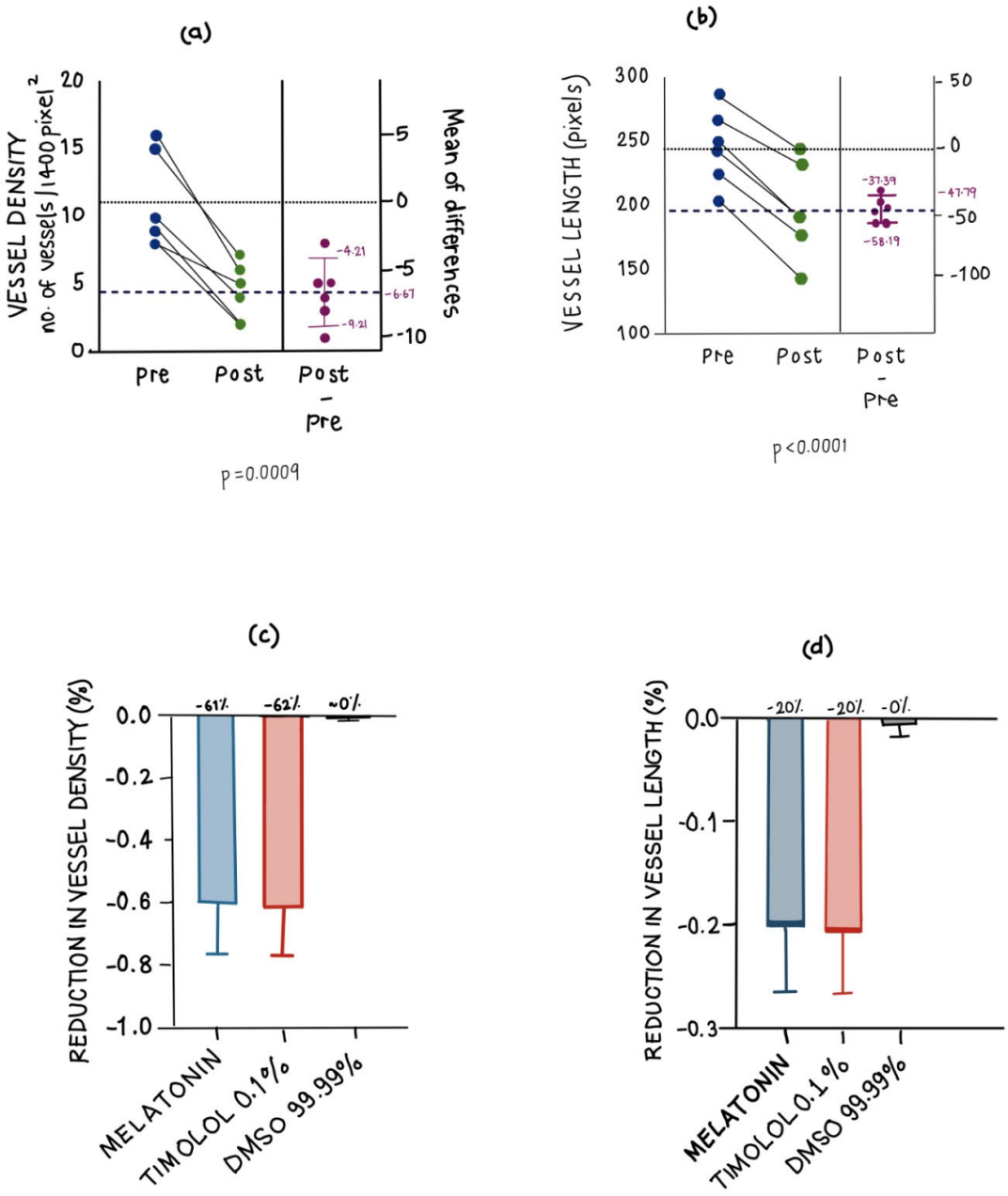

Figure 6 Graphical depiction of changes in a) vessel density and b) vessel length Melatonin 1mM and comparison between Melatonin 1mM, Timolol 0.1% and DMSO 99 % on reduction in c) vessel density and d) vessel Length

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4.2 Histopathological examination

In addition to inhibition of blood vessels, melatonin also causes necrosis and embryotoxicity (Figure 7)

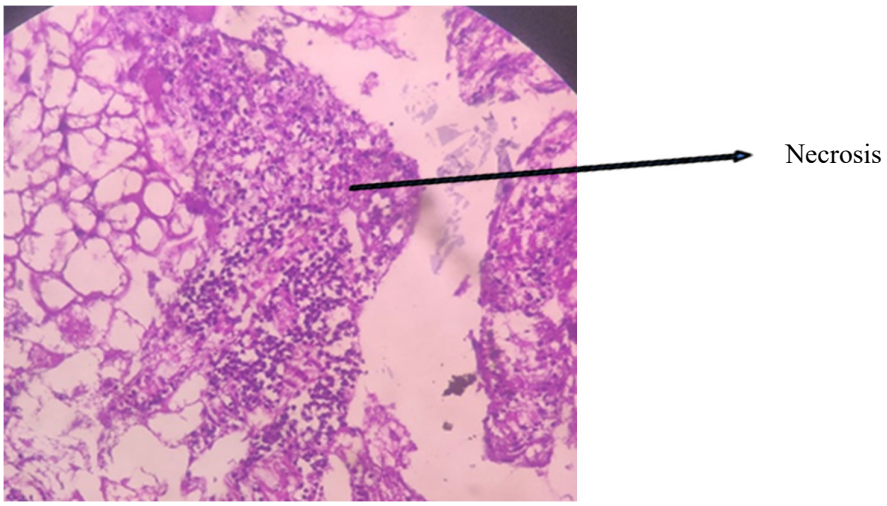

Figure 7 Necrosis seen on HPE of CAM with Melatonin

5. DISCUSSION

Melatonin is most peculiar in its role in angiogenesis. Melatonin (N-Acetyl-5-Hydroxytryptamine), is a pleiotropic hormone capable of altering various biologic processes and its levels in the body are tightly regulated by the suprachiasmatic nuclei and exposure to light (Figure 8). Melatonin levels also vary based on age, gender and season. Physiological functions of Melatonin are thought to be carried out via three receptors (MT1, MT 2 and MT3) of the G-Protein Coupled Receptor family and also via orphan nuclear receptor RZR/RORγ. [10,30,31]

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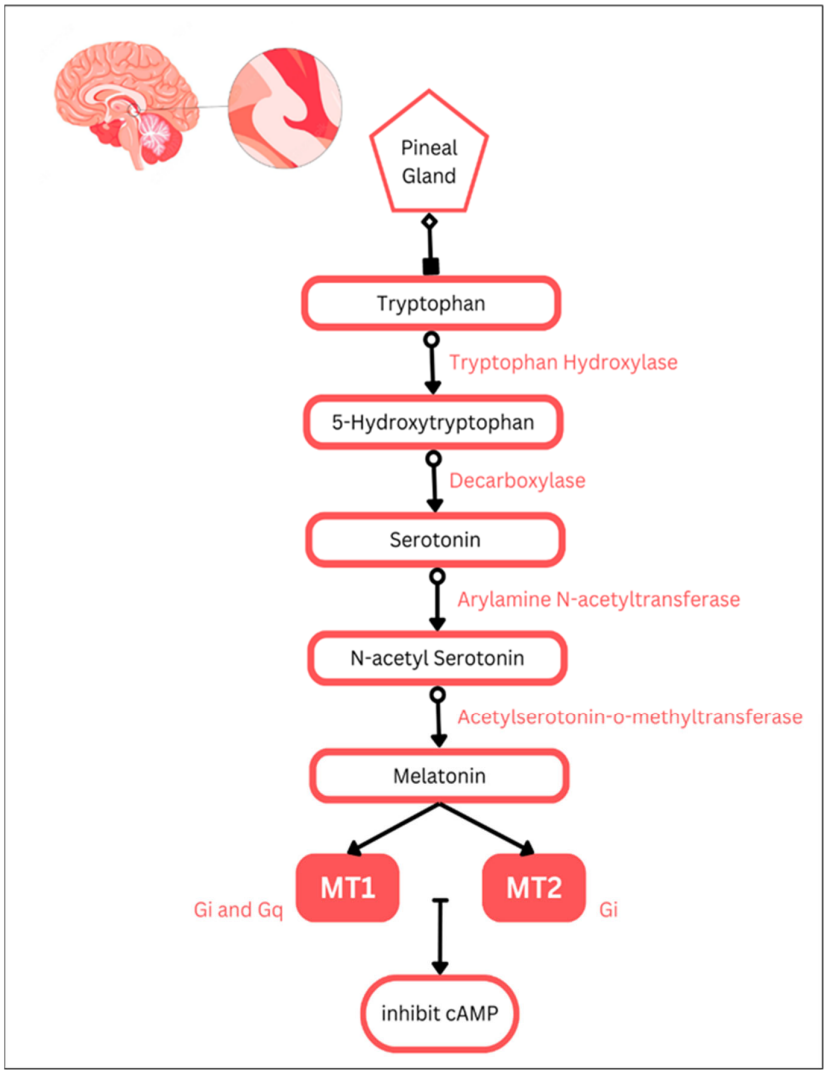

Figure 7 Physiology of Melatonin

Abbreviations: cAMP: cyclic Adenosine monophosphate, MT: Melatonin receptors.

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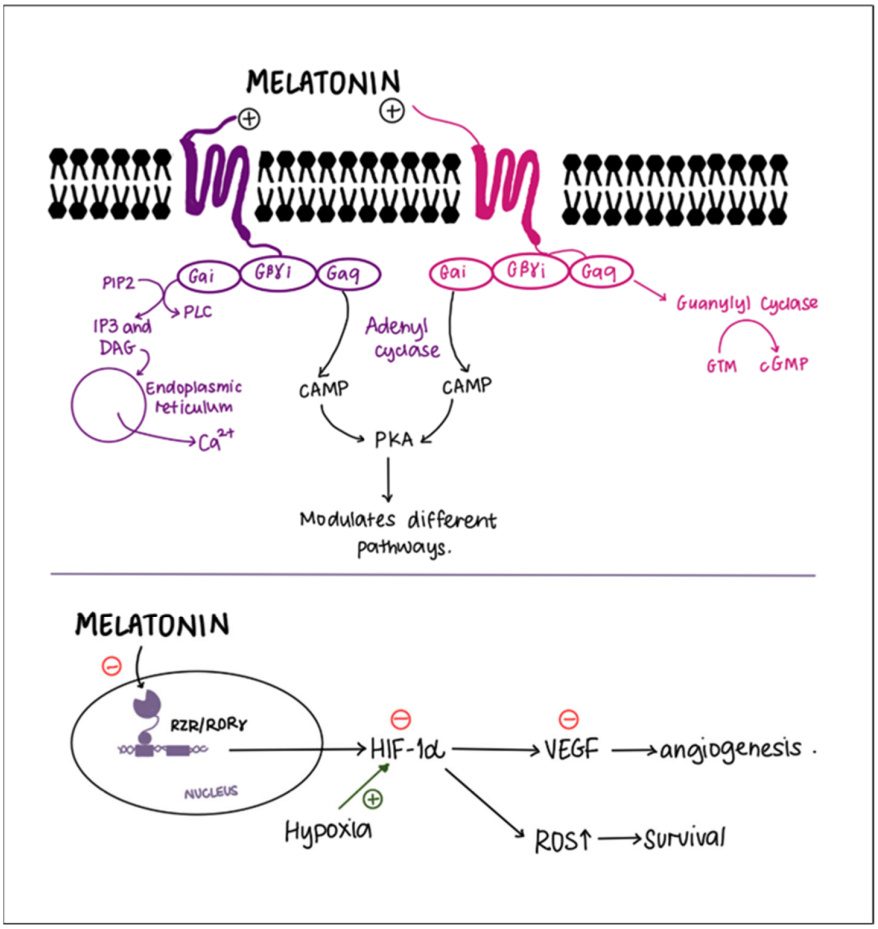

Figure 8 Schematic representation of the melatonin function. Recreated from Rahbarghazi et al (2021)[10]

Abbreviations: cAMP- cyclic Adenosine monophosphate, GMP- Guanosine monophosphate, cGMP- cyclic GMP, HIF-1α-Hypoxia-inducible Factor 1 alpha, IP3/ DAG- Inositol triphosphate/ Diacyl Glycerol, PIP2- Phosphoinositol triphosphate, PLC Phospholipase C, PkA-Phosphokinase A, ROS-Reactive Oxygen Species, VEGF-Vascular Endothelial Growth Factor.

Based on the data available, there seems to be an inverse relationship between endogenous melatonin and cardiovascular diseases, especially myocardial infarction (MI). Thus, it may play a supporting role in ameliorating or preventing cardiovascular injury in the event of an attack. A schematic diagram of the pro-angiogenic mechanism is presented below. Several studies have also demonstrated the pro-angiogenic potential of melatonin. However, the role of melatonin is said to be dependent on the dose, context, and distribution of MT receptors.[31] At pharmacological doses, melatonin typically inhibits angiogenesis by inhibiting VEGF. In MCF-7 cells, melatonin reduced VEGF-C, VEGFR2, VEGFR3, MMP9, and angiotensin. [32]

Several studies have also demonstrated the anti-angiogenic properties; however, the mechanism still eludes researchers. One of the possible mechanisms is that melatonin inhibits the stability of Hypoxia-Induced Factor 1α (HIF-1α) by downregulating nuclear receptors RZR/ RORγ and thereby reduces VEGF (Figures 9 and 10). A novel molecule, KC7F2 which is an HIF-1α

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inhibitor is also being studied as an antiangiogenic agent and in combination with melatonin may synergistically reduce VEGF more effectively.[33]

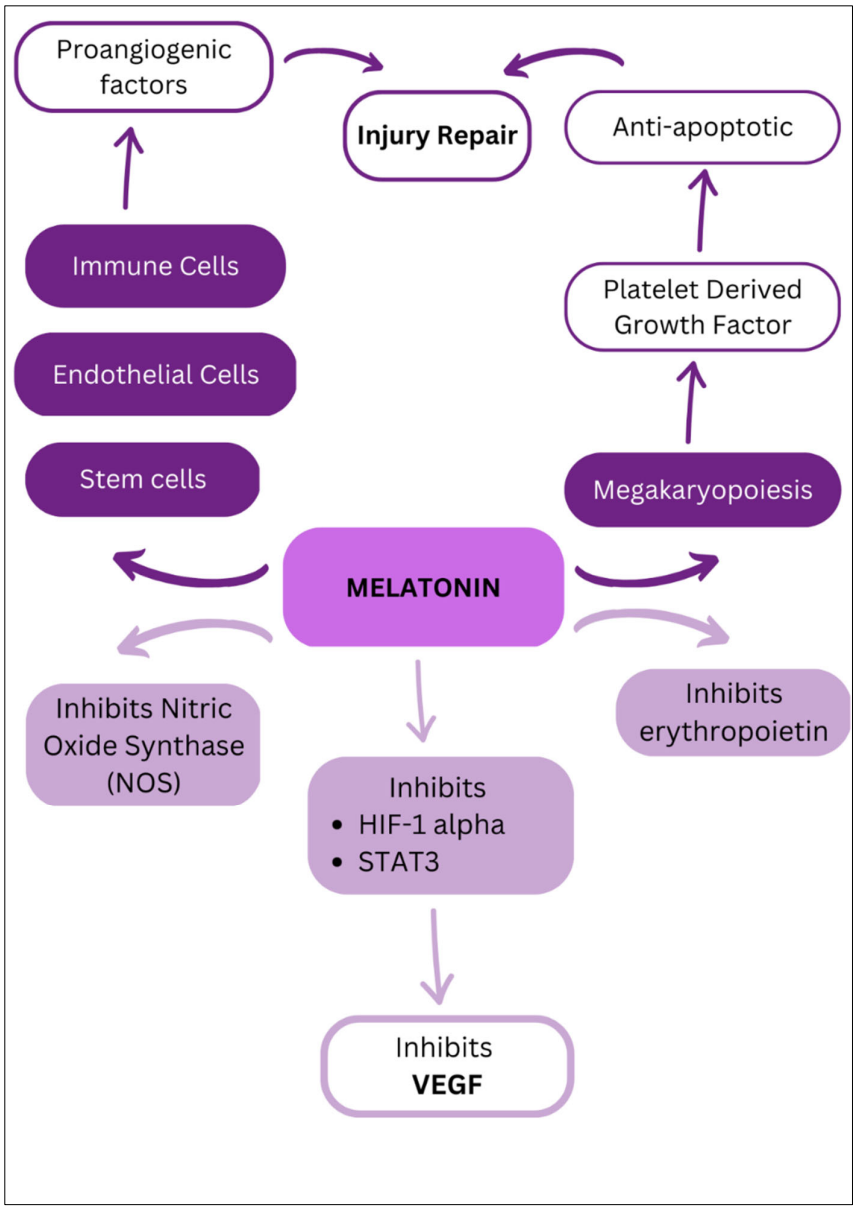

Figure 9 Mechanisms of action of Melatonin

Abbreviations: HIF-1α-Hypoxia-inducible Factor 1 alpha, STAT-Signal Transduction and Activator of Transcription, VEGF-Vascular Endothelial Growth Factor

5.1 Melatonin has an inhibitory effect on angiogenesis in the chick CAM model

Studies of melatonin in chick embryo have been scarce. In the case of cardiovascular dysfunction during the hypoxic development of chick embryos, melatonin was found to enhance antioxidant capacity and restore vascular endothelial growth factor expression and nitrous oxide (NO)

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bioavailability. On the contrary, melatonin was found to inhibit micro-vessel formation and suppress gastric cancer growth in the chick embryo tumour xenograft model at concentrations of 0.0014, 0.0041, 0.012, 0.037, 0.11, 0.33, 1 and 3 mM (significantly at 1mM and 3mM). In this study, we studied the effect of melatonin 1mM in the context of normal development of the chick embryo. The physiological and rhythmic secretion of melatonin in the chick embryo by cAMP begins only after EDD 16.

In this study, melatonin showed an inhibitory effect on blood vessel length and density in the CAM model, which was significant and comparable to Timolol, the control. Melatonin also caused embryonic death and histopathological examination of the membrane showed areas of necrosis consistent with the macroscopic examination. These findings may be supported by a study done by Beedie et al (2016) to investigate the possible mechanisms of teratogenesis of antiangiogenic drugs in the chick embryo. Their results suggested that anti-angiogenic drugs are likely to be teratogenic to the embryo, causing limb defects and death, similar to thalidomide. [34]

Author(s)	*Title*	*Model*	*Outcomes*
González A et al (2017)[35]	HUVECs-human neuroblastoma cells SH-SY5Y co-culture in a paracrine and juxtracrine manner	In vitro	Paracrine VEGF↓, Tubulogenesis↓, Migration of HUVECs↓
González-González A et al (2019) [36]	Combined effect of ionizing radiation and melatonin on HUVECs and MCF-7	In vivo and in vitro	VEGF↓, ANG-1↓, and -2↓, HUVECs survival↓, Migration↓, Tubulogenesis↓, Estrogen biosynthesis↓, VE-cadherin↓, Chorioallantoic membrane angiogenesis assay↓
Cerezo AB et al (2019) [37]	Effect of Melatonin on VEGF-exposed HUVECs	In vitro	VEGFR-2 phosphorylation↓, PLCγ1 phosphorylation↓, pAkt/Akt ratio↓, p-eNOS/eNOS ratio↓
Kumari R et al (2017) [38]	Effect of melatonin on Dalton's lymphoma angiogenesis potential	In vitro and in vivo	Dalton's lymphoma-induced endothelial cells proliferation↓, Migration↓, Chick chorioallantoic membrane angiogenesis↓, Mouse mesentery peri-vascularization↓, TIMP3↑, VEGF↓, VEGFR↓, FGF↓
Sohn EJ et al (2015) [39]	Effect of melatonin on hypoxic PC-3 prostate cancer cells	In vitro	miRNA3195↑ and miRNA374b↑, HIF-1α↓, HIF-2α↓, VEGF↓

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Author(s)	*Title*	*Model*	*Outcomes*
Goradel NH et al (2017)[9]	Effect of melatonin on tumour cells under normal condition	In vitro	phosphor-STAT3↓ and CBP/p300↓, VEGFR2↓, HIF-1α↓
Carbajo-Pescador S et al (2013) [40]	Effect of melatonin on hypoxic HepG2 hepatic cancer cells	In vitro	p-STAT3/STAT3↓, HIF-1α↓, VEGF↓, CBP/p300↓, Tubulogenesis↓
Wang R-X et al (2016) [30]	Effect of melatonin on SGC-7901 gastric cancer cells	In vivo and in vitro	VEGF secretion↓, RZR/RORγ↓, SUMO-specific protease↓, HIF-1α↓
González-González A et al (2020) [41]	The combined effect of Melatonin and Docetaxel and Vinorelbine on angiogenesis	In vitro and in vivo	VEGF-B↓, VEGF-C↓, VEGFR-1↓, VEGFR-3↓, ANG1↓, ANG-2↓, VE-cadherin↓, Chick Chorioallantoic membrane angiogenesis↓
Marques JH et al (2018) [42]	Effect of melatonin on angiogenesis potential of MDA-MB-468 breast cancer	In vivo and in vitro	miR-152-3p↑, IGFR↓, VEGF↓, HIF-1α↓

Table 2 In-vivo and in-vitro studies showing the effects of Melatonin on angiogenesis

Abbreviations: HUVECs Human umbilical vein endothelial cells, VEGF Vascular endothelial growth factor, ANG-1 and 2 Angiopoietin-1 and -2, Vascular endothelial-cadherin: VE cadherin, PLCγ1 Phospholipase Cγ1, Endothelial nitric oxide synthase: eNOS, TIMP-3 Tissue inhibitor of metalloproteinase-3, VEGR: Vascular endothelial growth factor receptor, FGF Fibroblast growth factors, HIF-1 and 2α Hypoxia-inducible factor1α and 2α, STAT3 Signal transducer and activator of transcription 3, CBP CREB binding protein, ROR RAR-related orphan receptor gamma, SUMO Small ubiquitin-like modifier

Pravastatin, a tumour-suppressor statin when given alone, increased VEGF in rat mammary carcinogenesis model. However, this effect was reversed by melatonin, suggesting that Statins co-administered with melatonin should be further evaluated for tumour-preventive properties. Melatonin when combined with a ketogenic diet, was able to overcome cisplatin and vincristine drug resistance in breast carcinoma syngraft with a cure rate of 70%. Several such studies corroborate these results. [36,41,43] Such findings are most encouraging for the repurposing of Melatonin and its role as an alternative to the currently used anti-angiogenic agents in solid tumours. [9,38,44–46]

Theiler et al stated that melatonin supplementation improved sleep patterns in adults treated with beta-blockers. One of the postulated mechanisms for disturbed sleep with beta-blockers is the suppression of melatonin secretion during the night. Since its serendipitous discovery as an anti-angiogenic agent in 2008, Propranolol has shown significant improvement in to complete

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resolution of hepatic haemangioma as well as head and neck haemangiomas in addition to infantile haemangiomas. We can hypothesise that melatonin can serve to improve the efficacy of beta-blockers in treating haemangiomas while also improving sleep. [47]

While we have considered melatonin to be useful only in alleviating sleep disturbances, it is time for future studies to focus on other potential pharmacological benefits in therapeutics.

5.2 Limitations of the study

The ex-Ovo method has an advantage over the in-Ovo method because it allows the quantification of the response over a wider area of the CAM, a large number of samples can be tested simultaneously and the time required for a response to occur is shorter. The small sample size is also a drawback of the study however, as per the ethical guidelines for animal experimentation, the minimum number of eggs to give a statistically significant result was used. The initial choice for positive control in this study was Bevacizumab, a known anti-angiogenic drug used in cancers. However high cost and inaccessibility prevented its use. Oral Propranolol (Brand name Hemangiol), approved by the FDA for Infantile Haemangioma (IH) was another choice but was difficult to obtain. Thus, the available alternative, Timolol 0.01% eye drops was chosen. Though IH is not an approved indication for the use of Timolol, the beta-blocker is used off-label to treat superficial haemangiomas.

While the objective of this study was to see if the test compounds had the potential to inhibit angiogenesis, it did not aim to identify the exact mechanisms by which the compounds act. To identify possible mechanisms more advanced techniques such as Human umbilical vein endothelial cell (HUVEC) culture, Endothelial cell tube formation assays, Angiogenic factors (FGF2 and VEGF) release assays, and CAM tumour implant model assay etc., are required which needed technical expertise and were costly. The chick CAM model is simply used for the purpose of screening and further studies are necessitated for detailed results.

6. CONCLUSION

Melatonin showed significant anti-angiogenic activity on the chick embryo chorioallantoic membrane. Real-world data is available for all the alternatives considered in this study and their safety profiles are well documented. This will reduce the economic burden on the pharmaceutical industries if they invest in repurposing these drugs and the cost reduction will trickle down to the consumer. The broader goal is to make healthcare more accessible and feasible for patients suffering from cancer in a smaller time frame.

The prospect of off-label use in cancer or regulatory approval of these alternatives following randomised controlled trials is truly encouraging.

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