

## Anti-angiogenic response of ketamine in the *in vitro* and *in vivo* settings

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### ABSTRACT

**Objectives:** This study aims to investigate the effect of ketamine on angiogenesis.

**Materials and methods:** Between June 2021 and August 2021, chorioallantoic membrane (CAM) assay cell viability assay protocols and cell viability assay protocols were performed to investigate angiogenesis. While *in vivo* angiogenic property of ketamine was studied in the chick CAM model, its effect on *in vitro* cell proliferation was evaluated in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

**Results:** A significant anti-angiogenic effect of ketamine was observed in chick CAM in the *in vivo* setting, compared to the control group ( $p < 0.001$ ). It showed a strongly dose-dependent inhibitory and cytotoxic effect on endothelial cells.

**Conclusion:** Our study results indicate that ketamine has an anti-angiogenic effect *in vitro* and *in vivo*. However, further research is needed to investigate its use in human vascular system as a new anti-angiogenic therapy.

**Keywords:** Angiogenesis; chorioallantoic membrane assay, ketamine.

Vasculogenesis is a process of blood vessel formation from progenitor cells and refers to earliest stages of vascular development. The process involves differentiation and expansion of vascular endothelial cell precursors to form a network of primitive tubules. The structure, consisting of interconnected vessels, is referred as primary capillary plexus. This primary capillary plexus is remodeled by branching and differential growth of vessels to form a more mature structure. Process of differentiation from primary plexus to more mature vascular patterns is referred as angiogenesis.<sup>[1]</sup> It also takes place in mature tissues by endothelial progenitor cells. It is de novo capillary growth stimulated by hypoxia, ischemia or injury.<sup>[2]</sup> It also plays an important role in pathogenesis diseases such as malignancies and chronic inflammatory diseases.<sup>[3]</sup>

The process of angiogenesis requires a balance between both different cell types undergoing

differentiation, and angiogenic/angiostatic factors.<sup>[4]</sup> Vascular endothelial growth factor A (VEGF-A) is the first specific example and a dominant growth factor for controlling the balance in angiogenesis.<sup>[5]</sup> It is a member of the VEGF family.<sup>[1]</sup> It also involves factors such as VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PLGF)<sup>[5]</sup> Behaviors of VEGF-A include endothelial mitogenesis stimulation, control of vascular permeability, regulation of cell migration by degradation of extracellular matrix and regulation of newly formed vascular structures.<sup>[5]</sup> A study of embryos lacking one VEGF allele has demonstrated that angiogenesis and vascular network formations are impaired.<sup>[6]</sup> Impairment of the balance in angiogenesis causes several developmental anomalies and diseases.

Ketamine is a widely preferred intravenous anesthetic agent, since its approval in 1970. It is an N-methyl-d-aspartate (NMDA) receptor antagonist

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that has dissociative anesthetic and analgesic effects.<sup>[7]</sup> Its effect of analgesia even in sub-narcotic doses makes it preferable in patients with opioid tolerance and opioid resistant pain.<sup>[8]</sup> It also induces vasorelaxation by reducing myofilament  $Ca^{2+}$  sensitivity and intracellular  $Ca^{2+}$  concentration.<sup>[9]</sup> Recent studies have shown that ketamine has an influence on cell cycle. It can cause neurotoxicity by neuronal apoptosis and neural stem progenitor cell deaths.<sup>[10]</sup> Moreover, its regulatory effect on proliferation in several cancers are shown in several studies.<sup>[11-13]</sup> Although its regulatory effects on vascular resistance and proliferation in cancer cells are described in many studies, its role on angiogenesis is still unclear.

Although several studies have shown the inhibitory effects of ketamine on VEGF in abusers and vascular smooth muscle cell proliferation,<sup>[14,15]</sup> there is no study investigating the effect of ketamine on angiogenesis. In the present study, we aimed to investigate the effect of ketamine on angiogenesis.

## MATERIALS AND METHODS

This study was performed in Vascular Biology Laboratory of Gulhane Education and Research Hospital Department of Medical Biochemistry between June 2021 and August 2021. Chorioallantoic membrane (CAM) assay and cell viability assay protocols were performed to investigate angiogenesis.

### Ketamine solution

Ketamine solutions were prepared in two different concentrations containing 1.25 and 2.5 mg of ketamine to be used in the experiments.

### Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method was used to assess the cell viability. Human umbilical vein endothelial cells (HUVECs) which had  $4 \times 10^4$  cells/well concentration were incubated in 96-well plates.

The cells were left overnight to develop a well formation. Some of these cells were, then, separately treated with 50  $\mu$ L of solutions involving 2.5 mg and 1.25 mg ketamine for 24 h, while other parts were left as the control group. After 24-h desired interval was completed, 20  $\mu$ L of MTT reagent solution (Biological Industries, Kibbutz Beit-Haemek, Israel) was added to each well. All plates were incubated at 37°C for 2 to 4 h in a carbon dioxide incubator. Following the incubation process, 100  $\mu$ L of solubilization buffer added into each well and incubated at room

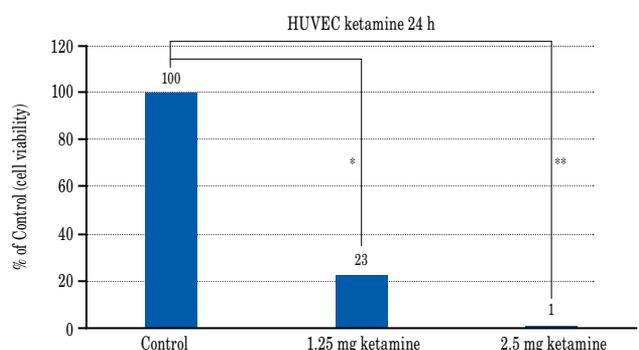
temperature for 2 to 4 h. The results were expressed in percentage variation compared to controls. The cell viability was repeated in triplicate with three independent experiments.

### Chorioallantoic Membrane assay

The CAM assay was used to evaluate angiogenesis *in vivo*. The same steps as indicated in our previously published article were followed for CAM assay.<sup>[16]</sup> Fertilized eggs obtained from Atak-S chickens produced in National Poultry Institution (Ankara, Turkey). Eggs kept at constant temperature (37°C) and humidity (85 to 90% of relative humidity) throughout experiment. On Day 6, between 12.00 to 14.00, each fertilized chicken eggs were fenestrated for access. While phosphate-buffered saline (PBS) applied to control group, 50  $\mu$ L of ketamine solution prepared at a concentration of 2.5 mg was placed on the surface of each CAMs. Finally, all eggs were sealed until Day 8. The effect of ketamine on vascular areas of CAMs was photographed with a computerized stereomicroscope (model S6D; Leica Microsystems, Heerbrugg, Switzerland) and scores evaluated with the software Leica Application Suite V4 Automated Image Analysis (Leica Microsystems, Wetzlar, Germany).

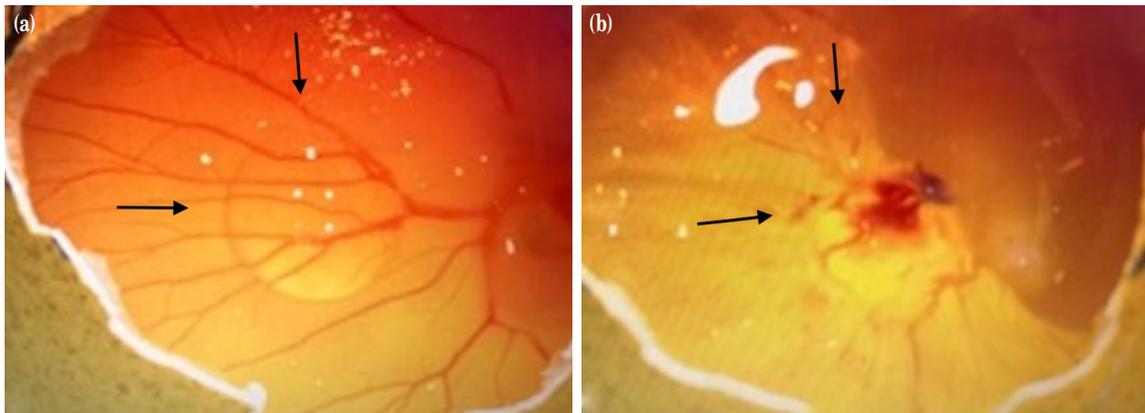
### Statistical analysis

Statistical analysis was performed using the MedCalc for Windows version 12.5 (MedCalc Software, Ostend, Belgium). Descriptive data were expressed in number and frequency. The chi-square test was used for the non-parametric tests. The Spearman correlation test was used to measure the statistical relationship between the continuous variables. A *p* value of <0.05 was considered statistically significant.



**Figure 1.** Cell proliferation of HUVECs under ketamine (1.25 and 2.5 mg) incubation (cell viability was indicated as percentage of control).

HUVECs: Human umbilical vein endothelial cells; \* *p*<0.001; \*\* *p*<0.001.



**Figure 2.** Effect of ketamine solution on CAM (a) before and (b) after 48 h. A significant fading and slimming are visible in figure (b). CAM: Chorioallantoic membrane.

## RESULTS

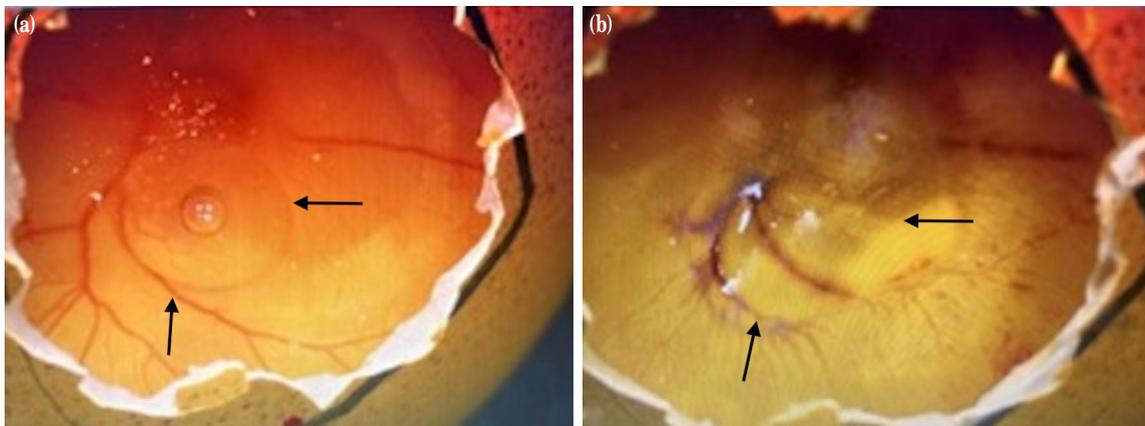
### Cell viability assay of ketamine

The HUVECs were incubated with increasing doses of ketamine for 24 h, and cell viability was observed with the MTT assay. Ketamine-related cytotoxic effect was observed on HUVECs and significantly higher than the control wells ( $p < 0.001$ ). When the efficacy of increasing doses was evaluated with Spearman correlation test, it was observed that a very strong negative correlation ( $r = -1.000$ ,  $p < 0.001$ ) and indicating the dose-dependent cytotoxic effect of ketamine on angiogenesis in the *in vivo* setting.

### Ketamine inhibits angiogenesis on CAM

To determine the effects of ketamine, we applied 2.5 mg of ketamine solution to CAMs on Day 6 and

evaluated its effect on vessel growth angiogenesis on Day 8. There was a significant decrease in CAM vascularity in CAM vascular area on Day 8. The formation of vascular network in CAMs was scored. In the control group, physiological angiogenesis was observed (Score 0). However, there was a macroscopically reduction of vascular network, seen as slimming and fading in CAM areas of eggs treated with ketamine in Figure 2 (Score 0: none; Score 1: vascular formation impaired in some of the vessels; Score 2: vascular formation totally disrupted and include necrosis). Necrotic areas can be also clearly seen in some CAMs in Figure 3. A significant decrease on CAM vascularity was seen for 2.5 mg of ketamine solution ( $p < 0.001$ ). Macroscopic results are also shown in Table 1.



**Figure 3.** Effect of ketamine solution on CAM (a) before and (b) after 48 h. Necrotic areas are visible in figure. CAM: Chorioallantoic membrane.

Table 1. Macroscopic evaluation of the effect of ketamine treatment on CAM

Groups	Efficacy								p
	Ineffective (Score 0)		Score 1		Score 2		Total		
	n	%	n	%	n	%	n	%	
Control	10	100	0	0	0	0	10	100	<0.001
Ketamine 2.5 mg	0	0	3	30	7	70	10	100	

CAM: Chick chorioallantoic membrane.

## DISCUSSION

The current study has shown that ketamine has both cytotoxic and anti-angiogenic effects, increasing in a dose-dependent manner. Angiogenesis is a requirement for tissue development and repair. It also has a role in neoplastic and chronic inflammatory diseases. Angiogenesis is controlled by a complex mechanism and a balance between angiogenic/angiostatic factors.<sup>[4]</sup> Impairment among these factors and existence of tumor-derived factors are responsible for tumor growth and development of a neovascular supply.

Ketamine is a non-competitive NMDA receptor antagonist that has been widely used in dissociative anesthesia and opioid resistant pain since 1970s.<sup>[7]</sup> As it has less effect on heart rate and respiratory system compared to other anesthetics, it is commonly preferred in intensive care units, operating rooms, and on battlefield where mechanical ventilation is not available. It is also used among cancer patients, due to its analgesic effect in sub-narcotic doses.<sup>[8]</sup> However, ketamine may induce a dose-dependent toxicity over neural system and neural stem progenitor cells.<sup>[8]</sup> Immune system components are also affected by ketamine. It interferes immune system by suppressing natural killer cell activity, inducing lymphocyte apoptosis, and reducing the production of pro-inflammatory cytokines.<sup>[17]</sup> Some reports also indicated that ketamine has regulatory and inhibitory effects on several cancers such as hepatocellular carcinoma, pancreatic cancer, and lung adenocarcinoma.<sup>[11-13]</sup> A study by Li et al.<sup>[18]</sup> indicated that ketamine induced ovarian cell cycle arrest and inhibited colony formation in ovarian cancer. In such studies, there is no recommendation for the use of ketamine as a treatment option in cancer treatment. Despite the wide variety of studies on the effects of ketamine, there are no studies showing its anti-angiogenic properties. In the future, by revealing different properties of ketamine, it may become an agent used effectively in cancer treatment.

Discovery of hallucinogenic effects of ketamine after 1970s has made it a recreational drug for abusers. It can provoke symptoms such as schizophrenia and cognitive impairments in both humans and animal models.<sup>[19,20]</sup> In a study, serum level of VEGF was investigated among ketamine abusers.<sup>[14]</sup> The VEGF is an important signaling protein in central nervous system functioning for long-term potentiation and neural protection. It is also a potent growth factor secreted from endothelial cells for regulation of vasculogenesis and angiogenesis. According to the study, VEGF serum levels were significantly lower in ketamine users compared to the control group. The effects of ketamine on vascular smooth muscle cells was investigated in another study.<sup>[15]</sup> According to the results, ketamine inhibited platelet-derived growth factor induced vascular smooth muscle cell proliferation in a concentration-dependent manner.

In 1995, Folkman<sup>[3]</sup> hypothesized an effective strategy of inhibiting angiogenesis to treat cancer, indicating the strong relation between tumor growth and angiogenesis. According to this strategy, preventing angiogenesis may be effective way to stop tumor growth. Ferrara and Kerbel<sup>[21]</sup> also reported a similar suggestion that inhibition of angiogenesis was a promising strategy for cancer treatment.

In the present study, *in vitro* and *in vivo* assays are used to evaluate angiogenesis. Cell proliferation assay showed that ketamine had a cytotoxic and anti-angiogenic effect of HUVECs. This result provides evidence of anti-angiogenic effect of ketamine in the *in vitro* setting. In CAM assay, similar to *in vitro* results, ketamine inhibited and reduced the vascular network formation, thus causing an anti-angiogenic effect *in vivo*. A significant fading and slimming can be seen in Figure 2, indicating the inhibition of vessel formation. Necrotic areas are also observed in some CAMs (Figure 3). Both necrosis and inhibition of vessel formation indicates the anti-angiogenic effect of ketamine. Although our study did not involve the investigation of growth

factors, it may be hypothesized that inhibitory effect of ketamine on angiogenesis is caused by impairment in angiogenic/angiostatic factors, consistent with previous studies. Further studies should be conducted to investigate the inhibitory mechanism of ketamine on angiogenesis and its relationship with the angiogenic/angiostatic factors.

In conclusion, anti-angiogenesis is a goal for treatment of neoplastic disease and vascular malformations. Based on our study results, it can be evaluated that anti-angiogenic effect of ketamine, which is a widely used anesthetic, may be benefitted to cure cancer and other proliferative diseases. However, further studies are needed for its use as a new anti-angiogenic therapy.

**Ethics Committee Approval:** Ethics committee decision is not required for poultry studies. The study was conducted in accordance with the principles of the Declaration of Helsinki.

**Patient Consent for Publication:** A written informed consent was obtained from each patient.

**Data Sharing Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Author Contributions:** Conception and design, administrative, technical, and logistical support content, collection, analysis and/or interpretation of data, statistical expertise: A.K.Y.; Provision of study materials or patients, administrative, technical, and logistical support content, collection, analysis and/or interpretation of data: E.Ö.; Conception and design, drafting of the article, critical revision of the article for important intellectual: M.E.İ.

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