

# Assessment of Ketamine's Influence on *In Vitro* Angiogenesis

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**Objective:** Angiogenesis is associated with angiogenic therapy and wound healing processes. It is important for anesthesiologists to understand the effects of perioperative and long-term use of anesthetics on angiogenesis. This study aimed to determine the effects of ketamine on *in vitro* angiogenesis: the proliferation of human umbilical vein endothelial cells (HUVEC) and normal human diploid fibroblasts (NHDF), HUVEC migration, and *in vitro* capillary tube formation in cocultured HUVEC and NHDF.

**Methods:** The effects of ketamine at concentrations of 1, 10, and 50  $\mu\text{M}$  on the proliferation of HUVEC and NHDF for 48 hours were determined by using a water-soluble tetrazolium salt reagent. Quantitation of migration for 22 hours was achieved by measuring the fluorescence of migrating HUVEC exposed to ketamine using an angiogenesis system. The effects of ketamine on capillary tube formation with or without vascular endothelial growth factor (VEGF) were investigated in cocultured HUVEC and NHDF incubated for 3 and 10 days.

**Results:** Ketamine did not show any enhancing or suppressive effects on the *in vitro* proliferation of HUVEC and NHDF, HUVEC migration, or capillary tube formation in cocultured HUVEC and NHDF for either 3 or 10 days in the presence or absence of VEGF.

**Conclusion:** Ketamine had no effects on *in vitro* angiogenesis using cultured HUVEC and NHDF. Ketamine can potentially be used as an anesthetic agent with no influence on angiogenesis.

**Key Words:** Ketamine; Angiogenesis; Human umbilical vein endothelial cells; Migration; Cell Proliferation.

Ketamine is an ionotropic, glutamatergic, N-methyl-D-aspartate receptor antagonist often used in total intravenous anesthesia<sup>1</sup> that also has anti-inflammatory effects and is a beneficial antidepressant for treatment-resistant depression.<sup>2,3</sup> The frequency of its anesthetic use may be increasing due to COVID-19-related restrictions on the use of some intravenous anesthetics. It is important for anesthesiologists to understand the effects of perioperative and long-term use of anesthetics and analgesics on angiogenesis. We recently reported about the effects of propofol and benzodiazepines on *in vitro* angiogenesis and showed that high-dose midazolam significantly impaired *in vitro* capillary tube formation by suppressing the proliferation and migration of endothelial cells via activation of extracellular signal-regulated

kinase (ERK).<sup>4</sup> Propofol and diazepam did not affect *in vitro* angiogenesis.<sup>4</sup> However, the effects of ketamine on *in vitro* angiogenesis have not yet been clarified.

In this study, we aimed to determine the effects of ketamine on the proliferation of human umbilical vein endothelial cells (HUVEC) and normal human diploid fibroblasts (NHDF), HUVEC migration, and the area and the length of tubes along with the number of joints and paths within *in vitro* capillaries.

## MATERIALS AND METHODS

HUVEC and NHDF were each incubated for 48 hours in 96 well plates (BD Biosciences) with growth medium (Kurabo Industries Ltd.) in a 5% CO<sub>2</sub> incubator at 37°C and stimulated with ketamine (s-(+)-ketamine hydrochloride, Sigma Chemical Co.) at 1, 10, and 50  $\mu\text{M}$ . HUVEC and NHDF incubated without ketamine were used as controls. The effects of ketamine on the proliferation of HUVEC and NHDF at 48 hours were determined by measuring the absorbance at 450 nm using a water-soluble tetrazolium salt reagent (Dojindo Molecular Technologies).

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**Table 1.** Proliferation of HUVEC and NHDF at 48 hours and Effects of Ketamine<sup>a</sup>

Group	HUVEC		NHDF	
	Absorbance	<i>P</i> value for comparison with control	Absorbance	<i>P</i> value for comparison with control
Control	0.74 ± 0.03	n/a	0.70 ± 0.02	n/a
1 μM Ketamine	0.79 ± 0.03	.412	0.73 ± 0.03	.704
10 μM Ketamine	0.80 ± 0.04	.658	0.73 ± 0.06	.580
50 μM Ketamine	0.66 ± 0.04	.186	0.70 ± 0.03	.477

<sup>a</sup> Data are expressed as mean ± SD.

Abbreviations: HUVEC, human umbilical vein endothelial cells; NHDF, normal human diploid fibroblasts.

Quantitation of endothelial cell migration was achieved by measuring the fluorescence of migrating HUVEC incubated for 22 hours using an angiogenesis system (BD Biosciences) after labeling with calcein AM (4 μg/mL). The effects of ketamine (50 μM) on endothelial cell migration were investigated using HUVEC with media containing the anesthetic with or without 2% fetal bovine serum (FBS; BD Biosciences). HUVEC incubated without ketamine were used as controls. The fluorescence of the invaded cells was read with a Tecan Infinite F200PRO microplate reader (Tecan Group Ltd.). Experiments were subsequently conducted on cells within 8 passages.

The effects of ketamine on capillary tube formation were investigated in cocultured HUVEC and NHDF (Kurabo Industries Ltd.). Cells were incubated for 3 days with medium with or without 10 ng/mL vascular endothelial growth factor (VEGF)-A as the positive control and stimulated with ketamine (50 μM) or the VEGF inhibitor

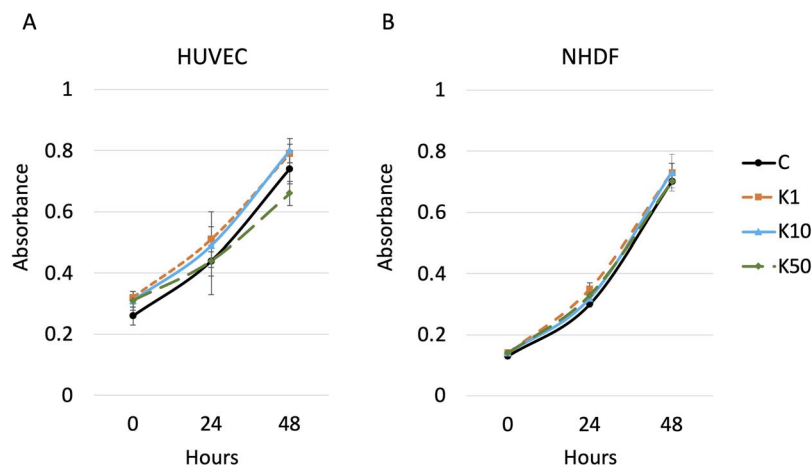
**Table 2.** FBS Stimulated Migration in HUVEC With or Without Ketamine<sup>a</sup>

Group	FBS	Fluorescence intensity	<i>P</i> value compared with control	
			without FBS	with FBS
Control	–	3076.3 ± 444.1	n/a	<.001
	+	34853.3 ± 3076.7	<.001	n/a
Ketamine	–	2663.5 ± 296.2	1.000	.001
	+	38641.0 ± 9023.0	<.001	.858

<sup>a</sup> Control: Fluorescence intensity of calcein AM in HUVEC incubated without FBS (FBS-) or with 2% FBS (FBS+) for 22 hours. Ketamine: Fluorescence intensity of calcein AM in HUVEC stimulated by 50 μM ketamine with FBS- or FBS+ for 22 hours. Data of Fluorescence intensity are expressed as mean ± SD.

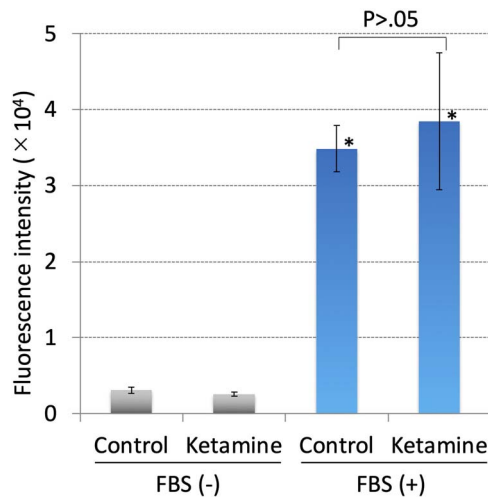
Abbreviations: FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells.

suramin (50 μM) as a negative control. Cells were also incubated for 10 days with medium with or without 10 ng/mL VEGF-A as the positive control and stimulated with ketamine (10, 50 μM) or suramin (50 μM) as a negative control. After 3 or 10 days, the cells were incubated with mouse anti-human CD31 antibody for 1 hour at 37°C. The cells were washed with phosphate buffered saline, then incubated with goat anti-mouse IgG alkaline phosphatase conjugate as a secondary antibody for 1 hour and then for approximately 10 minutes more with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium solution until the cells expressed a deep purple fluorescence. The area and the length of tubes along with the number of joints and paths within *in vitro* capillaries were assessed using the software for *in vitro* capillary tube formation (Kurabo Industries Ltd.). These assessments were performed in a blinded manner.

**Figure 1.** Effects of Ketamine on Cell Proliferation

HUVEC (A) and NHDF (B) were stimulated with ketamine at 1, 10, and 50 μM (K1, K10, and K50, respectively; C, control) and incubated for 48 hours. Ketamine did not significantly influence HUVEC and NHDF proliferation at 48 hours. Abbreviations: HUVEC, human umbilical vein endothelial cells; NHDF, normal human diploid fibroblasts.

**Figure 2.** Effects of Ketamine on Endothelial Cell Migration



Control: Fluorescence intensity of calcein AM in HUVEC incubated with medium containing 2% FBS (FBS+) or no FBS (FBS-) for 22 hours. Ketamine: Fluorescence intensity of calcein AM in HUVEC stimulated by 50 μM ketamine with FBS- or FBS+ for 22 hours. Ketamine did not significantly influence migration in non-stimulated or FBS-stimulated HUVEC. \* indicates  $P < .001$  compared with the migration of HUVEC in Control group without FBS. Abbreviations: FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells.

The results were expressed as mean ± SD. Data were compared by means of a one-way analysis of variance followed by the Scheffé test. A  $P < .05$  was considered statistically significant.

**Table 3.** The Effects of Ketamine on Capillary Tube Formation After Incubation for 3 Days<sup>a</sup>

Factors of <i>in vitro</i> angiogenesis	Group	Mean ± SD (pixels)	P value compared with	
			No stimulation	VEGF
Area	No stimulation	63649.1 ± 7528.4	n/a	<.001
	+ 50 μM Ketamine	90385.4 ± 6839.4	.936	<.001
	VEGF	321442.3 ± 85475.5	<.001	n/a
	+ Suramin	277380.6 ± 53285.2	<.001	.705
	+ 50 μM Ketamine	368613.4 ± 105774.6	<.001	.071
Length	No stimulation	4539.7 ± 1137.4	n/a	<.001
	+ 50 μM Ketamine	6326.2 ± 1979.9	.917	<.001
	VEGF	21909.5 ± 5804.3	<.001	n/a
	+ Suramin	18009.1 ± 2956.7	<.001	.358
	+ 50 μM Ketamine	25223.5 ± 6094.5	<.001	.526
Joint	No stimulation	13.9 ± 7.2	n/a	<.001
	+ 50 μM Ketamine	33.9 ± 11.8	.845	<.001
	VEGF	132.7 ± 54.1	<.001	n/a
	+ Suramin	84.4 ± 34.9	.005	.108
	+ 50 μM Ketamine	144.8 ± 53.8	<.001	.972
Path	No stimulation	83.6 ± 18.8	n/a	<.001
	+ 50 μM Ketamine	121.5 ± 33.9	.840	<.001
	VEGF	340.1 ± 102.0	<.001	n/a
	+ Suramin	277.4 ± 55.3	<.001	.434
	+ 50 μM Ketamine	370.1 ± 102.1	<.001	.925

<sup>a</sup> We assessed the area and length of tubules and the numbers of joints and paths to determine the effects of ketamine on *in vitro* capillary tube formation. VEGF: 10 ng/mL vascular endothelial growth factor-A, Suramin: 50 μM suramin (a VEGF inhibitor). Data are expressed as mean ± SD.

Abbreviation: VEGF, vascular endothelial growth factor.

**RESULTS**

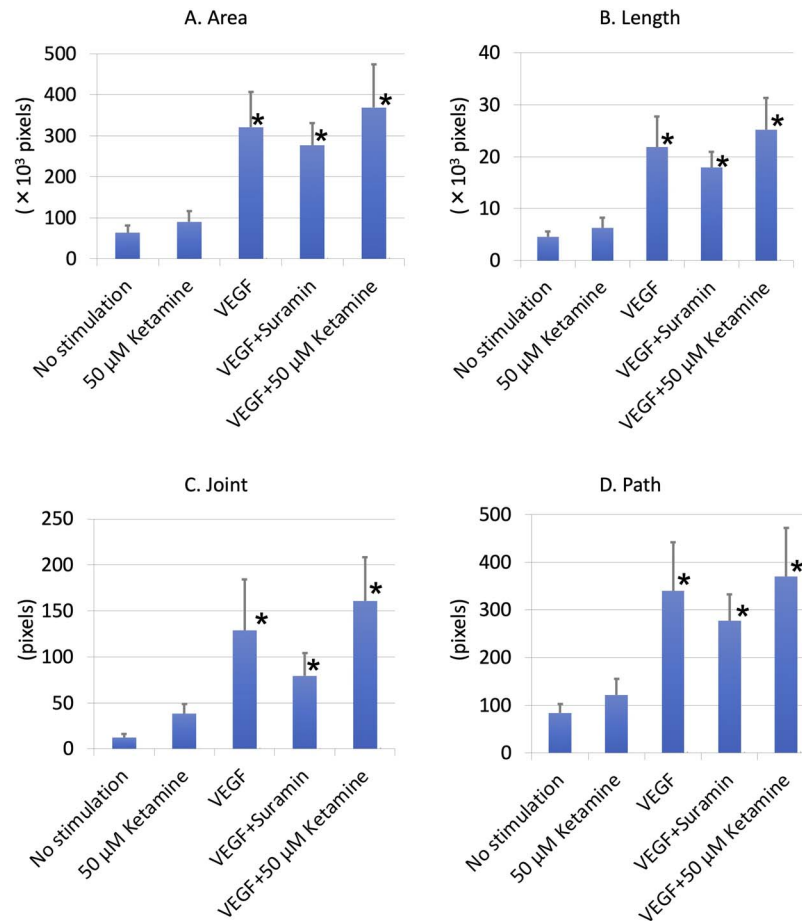
Ketamine (1, 10, and 50 μM) did not significantly influence HUVEC/NHDF proliferation relative to controls at 48 hours ( $P > .05$ ); Table 1, Figure 1).

Migration of HUVEC was significantly stimulated with FBS in the control (incubated in the medium without ketamine) and ketamine group (incubated with 50 μM ketamine) compared to each without FBS ( $P < .001$ ; Table 2). However, ketamine (50 μM) did not affect either nonstimulated or FBS-stimulated endothelial cell migration relative to the control ( $P > .05$ ; Figure 2).

Stimulation with VEGF significantly increased all the factors of the *in vitro* capillary tube formation assay ( $P < .001$ ). However, ketamine (50 μM) did not influence any factors in the absence or presence of VEGF in the *in vitro* capillary tube formation assay at 3 days ( $P > .05$ ; Table 3, Figure 3). VEGF also significantly stimulated *in vitro* angiogenesis at 10 days ( $P < .001$ ), and suramin significantly inhibited *in vitro* angiogenesis ( $P < .001$ ). However, none of the concentrations of ketamine increased or impaired any factors in the absence or presence of VEGF in the *in vitro* capillary tube formation assay for 10 days ( $P > .05$ ; Table 4, Figure 4).

**DISCUSSION**

Angiogenesis contributes to numerous diseases that are characterized by insufficient or excessive angiogenesis,

**Figure 3.** Effects of Ketamine on Capillary Tube Formation After Incubation for 3 Days

We assessed the area (A) and length (B) of tubules and the numbers of joints (C) and paths (D) to determine the effects of ketamine on *in vitro* capillary tube formation. VEGF significantly stimulated *in vitro* capillary tube formation in all aspects. Ketamine (50  $\mu\text{M}$ ) did not significantly influence any of the components of *in vitro* capillary tube formation stimulated with or without VEGF. Data are expressed as mean  $\pm$  SD; \* indicates  $P < .001$  compared with no stimulation. Abbreviation: VEGF, vascular endothelial growth factor.

including ischemic, inflammatory, and infectious disorders, and malignant tumors. Therapeutic antiangiogenic therapy has been applied in patients with diseases such as diabetic retinopathy or malignant tumors. Treatments with therapeutic angiogenesis using basic fibroblast growth factor, hepatocyte growth factor, other growth factors, or autologous cultured bone marrow-derived mesenchymal stem cells also have been undertaken and are expected to be effective for patients with severe coronary artery disease,<sup>5,6</sup> critical limb ischemia, or peripheral arterial disease.<sup>7–9</sup> These therapies could offer a safe, effective strategy to improve microcirculation and assist regeneration after infarction. It is critical for anesthesiologists to understand the angiogenic effects of anesthetics used perioperatively as well as sedatives chronically used in patients undergoing therapeutic angiogenesis treatments.

Angiogenesis involves the following processes: the release of regulatory factors such as VEGF, cell proliferation, cell migration, and tube formation. VEGF, a main regulator of angiogenesis, is produced by macrophages, smooth muscle

cells, fibroblasts, tumor cells, and others. Among the several isoforms of VEGF, the most important is VEGF-A. VEGF-A stimulates endothelial cells by binding VEGF receptor-1 and receptor-2,<sup>10,11</sup> which mediate angiogenesis. Suramin, a polysulfonyl-naphthyl-urea compound, inhibits the activation of VEGF receptor-2.<sup>12</sup>

It was previously reported that midazolam and diazepam—but not ketamine—stimulated the release of VEGF from rat vascular smooth muscle cells.<sup>13</sup> In our results, ketamine did not influence *in vitro* angiogenesis with or without the presence of 10 ng/mL VEGF-A. Furthermore, ketamine did not influence the cell proliferation of human endothelial cells and fibroblasts as well as the migration of endothelial cells in our study. Others reported that propofol or midazolam but not ketamine or dexmedetomidine suppressed platelet-derived growth factor-BB-induced migration in A10 cells, which is used as a model of rat vascular smooth muscle cells.<sup>14</sup> In contrast, other reports demonstrated ketamine inhibited human aortic smooth muscle cell proliferation at a clinical concentration

**Table 4.** The Effects of Ketamine on Capillary Tube Formation After Incubation for 10 Days<sup>a</sup>

Factors of <i>in vitro</i> angiogenesis	Group	Mean ± SD (pixels)	P value compared with	
			No stimulation	VEGF
Area	No stimulation	106949 ± 58154	n/a	<.001
	+ 10 μM Ketamine	108199 ± 34547	.000	<.001
	+ 50 μM Ketamine	99160 ± 20171	.000	<.001
	VEGF	411766 ± 106732	<.001	n/a
	+ Suramin	196278 ± 41957	.003	<.001
	+ 10 μM Ketamine	409623 ± 84002	<.001	1.000
Length	+ 50 μM Ketamine	437382 ± 87599	<.001	.986
	No stimulation	8053 ± 4259	n/a	<.001
	+ 10 μM Ketamine	7703 ± 2264	1.000	<.001
	+ 50 μM Ketamine	7774 ± 1726	1.000	<.001
	VEGF	29893 ± 8798	<.001	n/a
	+ Suramin	12755 ± 2564	.161	<.001
Joint	+ 10 μM Ketamine	29431 ± 5944	<.001	1.000
	+ 50 μM Ketamine	34511 ± 7530	<.001	.532
	No stimulation	26.9 ± 18.8	n/a	<.001
	+ 10 μM Ketamine	26.3 ± 10.0	1.000	<.001
	+ 50 μM Ketamine	24.6 ± 9.6	1.000	<.001
	VEGF	182.4 ± 86.9	<.001	n/a
Path	+ Suramin	61.8 ± 21.8	.396	<.001
	+ 10 μM Ketamine	187.2 ± 61.8	<.001	1.000
	+ 50 μM Ketamine	235.1 ± 75.7	<.001	.242
	No stimulation	98.2 ± 48.4	n/a	<.001
	+ 10 μM Ketamine	97.6 ± 28.2	1.000	<.001
	+ 50 μM Ketamine	98.9 ± 33.9	.998	<.001
Path	VEGF	407.1 ± 153.2	<.001	n/a
	+ Suramin	194.1 ± 47.5	.310	<.001
	+ 10 μM Ketamine	421.3 ± 103.6	<.001	1.000
	+ 50 μM Ketamine	508.9 ± 131.0	<.001	.175

<sup>a</sup> We assessed the area and length of tubules and the numbers of joints and paths to determine the effects of ketamine on *in vitro* capillary tube formation. VEGF: 10 ng/mL vascular endothelial growth factor-A, Suramin: 50 μM suramin (a VEGF inhibitor). Data are expressed as mean ± SD.

Abbreviation: VEGF, vascular endothelial growth factor.

via the protein kinase C pathway<sup>15</sup> and that ketamine (10 and 100 μM) stimulated DNA synthesis via activation mitogen-activated protein kinase ERK2 and nuclear accumulation of c-Fos in human aortic smooth muscle cells.<sup>16</sup> The effects of ketamine on cell proliferation and cell migration are different in various cells. Our study also showed that ketamine did not influence *in vitro* angiogenesis using human endothelial cells and fibroblasts. Although ketamine may not affect angiogenesis therapy, it may affect the human smooth muscle cell proliferation associated with vascular occlusive disorders.

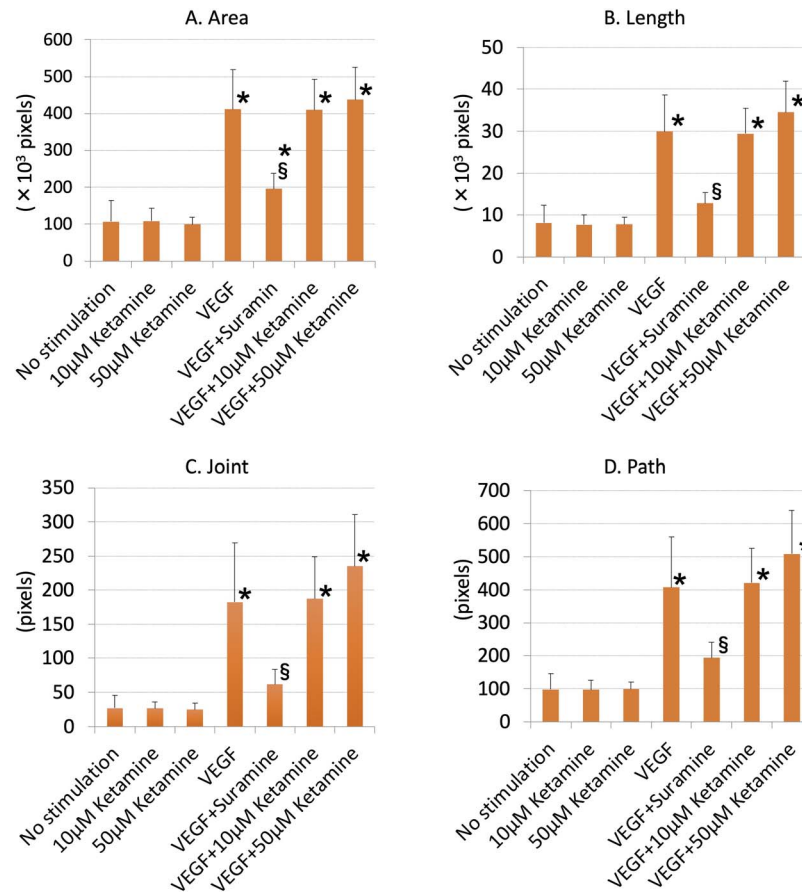
The ketamine concentrations (1, 10, and 50 μM) used in this study were 200 to 10,000 times higher than concentrations typically used clinically.<sup>17</sup> Ketamine shows a chiral structure of 2 optical isomers: s-(+)-ketamine has been reported to be 4 times more effective for analgesia and sedation by inhibition of the N-methyl-D-aspartate receptor than r-(-)-ketamine and 2 times more effective than the racemic mixture, whereas r-(-)-ketamine is more effective against depression.<sup>18–20</sup> Ketamine also inhibited hyperpolarization-activated cyclic nucleotide-gated channel 1, with greater channel inhibition being obtained with the s-(+)-ketamine isomer in neuronal cells.<sup>21</sup>

A limitation of this study is that we used s-(+)-ketamine even though we clinically use the racemic compound of ketamine containing both isomers. It may be necessary to consider the different affinities and effects of the 2 optical isomers of ketamine on VEGF receptors.

We hoped that anesthetics with positive effects on angiogenesis could be used during the perioperative period in treatments that promote angiogenesis, and vice versa. Our results suggested that ketamine could be safely used in the perioperative setting with pro- and anti-angiogenic therapies. Studies on anesthetics with and without effects on angiogenesis may lead anesthesiologists to influence patients' prognosis.

## CONCLUSION

We investigated the effects of ketamine on *in vitro* angiogenesis: cell proliferation of HUVEC and NHDF, endothelial cell migration of HUVEC, and capillary tube formation in cocultured HUVEC and NHDF. Ketamine appeared to have no influence on the proliferation of endothelial cells

**Figure 4.** Effects of Ketamine on Capillary Tube Formation After Incubation for 10 Days

We assessed the area (A) and length (B) of the tubules and the number of joints (C) and paths (D) to determine the effects of ketamine on *in vitro* capillary tube formation. VEGF significantly stimulated *in vitro* capillary tube formation in all aspects, while suramin significantly inhibited all aspects with VEGF. Ketamine (10, 50  $\mu$ M) did not significantly influence any of the components of *in vitro* capillary tube formation with or without VEGF. Data are expressed as mean  $\pm$  SD; \* indicates  $P < .001$  compared with no stimulation; § indicates  $P < .001$  compared with VEGF (positive control). Abbreviation: VEGF, vascular endothelial growth factor.

and fibroblasts, endothelial cell migration, or capillary tube formation within this *in vitro* study. The lack of *in vitro* angiogenic effects suggested that ketamine can be used as an anesthetic with no influence on pro- or anti-angiogenic therapy.

### Conflict of Interest

The authors declare no conflicts of interest.

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