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## *In Vitro* Coagulation Effects of Ophthalmic Doses of Bevacizumab

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## *In Vitro* Coagulation Effects of Ophthalmic Doses of Bevacizumab

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### Abstract

**Purpose:** *In vitro* coagulation effects of bevacizumab, a drug with potential risks for severe hemorrhagic and arterial thromboembolic events (ATEs), are unknown. The aim of this study was to evaluate the effects of bevacizumab, including the common ophthalmic dose of 1.25 mg, on the coagulation cascade.

**Methods:** Bevacizumab doses of 0.25, 0.5, 1.0, 1.25, 2.0, 2.5, and 4.0 mg were incubated at 37°C in the presence of pooled normal plasma (PNP) to determine its biological activity via activated partial thromboplastin time (aPTT) and prothrombin time (PT) at 30 min, 1 h, and 2 h. The control consisted of 40% normal saline and 60% PNP. All evaluations were conducted in triplet. Coagulation at each time point was compared with the control group by analysis of variance with Tukey's *post hoc* test. A *P* value of  $\leq 0.05$  was considered significant.

**Results:** Mean bevacizumab aPTT ranged from 38.4 to 43.9 s, 37.4 to 43.1 s, and 39.0 to 43.2 s at 30 min, 1 h, and 2 h, respectively. Mean bevacizumab PT ranged from 15.7 to 16.8 s at 30 min, 14.6 to 16.2 s at 1 h, and 14.0 to 15.8 s at 2 h. For the control, aPTT was similar over time (40.1, 40.0, and 40.5 s), while PT decreased from 16.5 at 30 min to 15.4 s at 2 h. Bevacizumab decreased PT significantly at 2 h, compared with the PNP control, for the following doses: 0.25 mg [difference between means 1.04 s, 95% confidence interval (CI) 0.12–1.96], 1.25 mg (1.16 s, 95% CI 0.16–2.15), 2.5 mg (0.94 s, 95% CI 0.02–1.86), and 4 mg (1.41 s, 95% CI 0.41–2.40). Significant differences were not observed in PT at 30 min and 1 h as compared with controls. For all doses of bevacizumab, aPTT did not vary from controls at the 3 measured times.

**Conclusions:** A common ophthalmic dose of bevacizumab 1.25 mg significantly increased *in vitro* coagulation. Further examination of the impact of ophthalmic bevacizumab on coagulation is warranted to provide insight into any putative link between ophthalmic bevacizumab and the risk for severe ATEs.

### Introduction

BEVACIZUMAB IS A humanized monoclonal antibody to anti-vascular endothelial growth factor (VEGF) and is approved by the Food and Drug Administration to treat a variety of neoplastic diseases.<sup>1</sup> When given intravenously (5 mg/kg) for chemotherapy, bevacizumab induces systemic effects, which carries a risk for both hemorrhagic and arterial thromboembolic events (ATEs).<sup>2,3</sup> Hemorrhage risk includes minor bleeding, most commonly Grade 1 epistaxis, and severe hemorrhage, including hemoptysis and gastrointestinal bleeding.<sup>4</sup> Major ATEs associated with

bevacizumab chemotherapy include cerebral infarction, transient ischemic attacks, myocardial infarction, and angina.<sup>4</sup>

Bevacizumab is also used off-label as an intraocular injection (1.25 mg/0.05 mL) to treat a variety of ophthalmic diseases, such as age-related macular degeneration, retinal vein occlusion, and diabetic retinopathy.<sup>1</sup> It is unknown, however, whether ophthalmic doses of bevacizumab increase the risk of severe hemorrhagic events or ATEs, as well-controlled clinical trial research has yet to be conducted.<sup>4,5</sup> Recent research suggests that adverse events associated with ophthalmic bevacizumab include ATEs and

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other serious adverse reactions.<sup>6,7</sup> The present study investigated the impact of ophthalmic doses of bevacizumab on the coagulation cascade to provide further insight into its potential systemic effects.

## Methods

Commercially available pharmacy stock of bevacizumab (Genentech, South San Francisco, CA; lot #761158) was reconstituted with sterile water for injection according to the manufacturer's instructions. Bevacizumab doses of 0.25, 0.5, 1.0, 1.25, 2.0, 2.5, and 4.0 mg were incubated at 37°C in the presence of pooled normal plasma (PNP) to determine its biological activity via activated partial thromboplastin time (aPTT) and prothrombin time (PT) at 30 min, 1 h, and 2 h. The control consisted of 40% normal saline and 60% PNP. To obtain a dilution with 60% PNP, ophthalmic doses were achieved in pharmacological testing dose equivalents per milliliter. All evaluations were conducted in triplet.

Mean coagulation was calculated for each dose, as was the difference between mean bevacizumab coagulation and mean control coagulation. Within-dose changes between time points were assessed using the paired *t*-test. Bevacizumab coagulation at each time point was compared with the control group by analysis of variance with Tukey's *post hoc* test, and verified with Bonferroni methods. A *P* value of  $\leq 0.05$  was considered significant. All statistical analyses were performed using SAS (SAS Institute, Inc., Cary, NC; Version 9.2).

## Results

Mean bevacizumab aPTT ranged from 38.4 to 43.9 s, 37.4 to 43.1 s, and 39.0 to 43.2 s at 30 min, 1 h, and 2 h, respectively, as shown in Table 1. Mean bevacizumab PT ranged from 15.7 to 16.8 s at 30 min, 14.6 to 16.2 s at 1 h, and 14.0 to 15.8 s at 2 h. For the control, aPTT was similar over time (40.1, 40.0, and 40.5 s), while PT decreased from 16.5 to 15.4 s ( $P=0.004$ ). Within bevacizumab doses, aPTT measurements were similar over time, except for 4.0 mg, which decreased significantly ( $P=0.006$ ). Alternatively, PT decreased significantly over time for all bevacizumab doses.

Compared with the PNP control, bevacizumab decreased PT significantly at 2 h for the following doses: 0.25 mg [dif-

ference between means 1.04 s, 95% confidence interval (CI) 0.12–1.96], 1.25 mg (1.16 s, 95% CI 0.16–2.15), 2.5 mg (0.94 s, 95% CI 0.02–1.86), and 4 mg (1.41 s, 95% CI 0.41–2.40). No significant differences were observed in PT at 30 min and 1 h as compared with controls. For all doses of bevacizumab, aPTT did not vary from controls at the 3 measured time points.

## Discussion

The human coagulation cascade is a series of amplification and activation processes that occur in sequence to generate fibrin monomers, which aggregate spontaneously to form insoluble clots that trap platelets, red blood cells, and other particles to form a thrombus.<sup>8</sup> We chose to measure the effects of bevacizumab on the clotting tendency of blood using the contact activation pathway and extrinsic coagulation pathway, due to the ease and accessibility of the aPTT and PT tests. The present study found that ophthalmic doses of bevacizumab shortened PT relative to control at 2 h. Decreased PT indicates increased *in vitro* coagulation, and therefore this may be a possible mechanism for ATEs at ophthalmic doses of bevacizumab.

VEGF is a signal protein produced by cells that stimulate the growth of new blood vessels. VEGF induces vascular hyperpermeability, which allows plasma proteins, such as coagulation factors, to leak into the extracellular matrix. Bevacizumab's effects on vasoconstrictive and vasodilatory factors may explain our findings that demonstrate a lower mean PT compared with controls. Bevacizumab influences factors such as endothelin-1 and nitric oxide, which are influenced by VEGF.<sup>9</sup> When bevacizumab affects vasoconstrictive and vasodilatory factors, it may increase coagulation.<sup>9</sup>

A potential limitation of the present study is the stability of the PNP, which can be affected as time progresses in the processor, especially when incubated. This may explain the observed findings of decreased 2h PT at 0.25, 1.25, 2.0, 2.5, and 4.0 mg, but not at 0.5 or 1.0 mg doses. However, mixing each sample immediately before analysis and running several controls helped to minimize error. Our study compared PT and aPTT between control and experimental samples within each time point of interest (30 min, 1 h, and 2 h), to control for degradation over time. All the platings and

TABLE 1. TRIAL TIMES OF ACTIVATED PARTIAL THROMBOPLASTIN TIME AND PROTHROMBIN TIME AT VARYING DOSES OF BEVACIZUMAB

	aPTT in seconds, mean $\pm$ standard deviation			PT in seconds, mean $\pm$ standard deviation		
	0.5 h	1 h	2 h	0.5 h	1 h	2 h
Control, 40% normal saline	40.1 $\pm$ 1.0	40.0 $\pm$ 1.0	40.5 $\pm$ 0.9	16.5 $\pm$ 0.9	15.5 $\pm$ 0.0	15.4 $\pm$ 0.9
Bevacizumab						
0.25 mg	38.4 $\pm$ 1.2	37.4 $\pm$ 0.3	40.0 $\pm$ 3.5	15.7 $\pm$ 0.7	14.9 $\pm$ 0.1	14.4 $\pm$ 0.3 <sup>a</sup>
0.5 mg	39.6 $\pm$ 3.2	37.6 $\pm$ 0.6	39.5 $\pm$ 2.8	15.7 $\pm$ 0.4	15.3 $\pm$ 0.2	14.8 $\pm$ 0.1
1.0 mg	43.9 $\pm$ 2.3	41.9 $\pm$ 4.1	43.2 $\pm$ 2.3	16.5 $\pm$ 0.1	16.2 $\pm$ 0.1	15.8 $\pm$ 0.4
1.25 mg	40.1 $\pm$ 2.7	43.1 $\pm$ 2.6	42.1 $\pm$ 2.5	15.8 $\pm$ 0.5	15.1 $\pm$ 0.6	14.3 $\pm$ 0.5 <sup>a</sup>
2.0 mg	39.0 $\pm$ 0.8	39.6 $\pm$ 2.1	40.4 $\pm$ 3.7	—	—	—
2.5 mg	40.1 $\pm$ 1.5	40.6 $\pm$ 1.7	40.5 $\pm$ 1.6	16.1 $\pm$ 0.6	15.9 $\pm$ 0.3	14.5 $\pm$ 0.6 <sup>a</sup>
4 mg	40.6 $\pm$ 1.3	39.1 $\pm$ 0.4	39.0 $\pm$ 0.5	16.8 $\pm$ 1.2	14.6 $\pm$ 0.3	14.0 $\pm$ 0.1 <sup>a</sup>

<sup>a</sup>Statistically significant difference compared with control ( $P \leq 0.05$ ).

—, no data; aPTT, activated partial thromboplastin time; PT, prothrombin time.

readings were performed within 1 h, which is well within the experimentally derived window of 2 h. This procedure ensures that results accurately reflect the drug's effects on the PNP and not the breakdown of PNP from temperature and time.

In summary, the present study suggests that bevacizumab significantly affects the human coagulation cascade by increasing coagulation at a common ophthalmic dose of 1.25 mg, and at both lower and higher doses. These findings may provide a mechanism for thrombosis and underscore the importance of further research on the effect of ophthalmic bevacizumab on the coagulation cascade.

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### Disclaimer

The views expressed in this article are those of the authors and do not necessarily reflect the position or policy of the Department of Veterans Affairs or the U.S. government.

### Author Disclosure Statement

No competing financial interests exist.

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