

Antiangiogenic Effect of As₄O₆ on the Angiogenesis Induced by Vascular Endothelial Growth Factor (VEGF) in the Rat Cornea

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Abstract : The purpose of this study was to compare the antiangiogenic effects of As₄O₆ to those of As₂O₃ on the rat corneal micropocket model induced by VEGF. 20 ng VEGF impregnated pellets were used for angiogenic inducer on the rat cornea micropocket assay in this study. After ophthalmoscopic examination, Sprague-Dawley rats with normal cornea were implanted VEGF pellet. Total 60 eyes were used in this study. Control group only received VEGF pellet, As₂O₃ group followed oral administration of As₂O₃ at a dose of 50 mg/kg per day after VEGF pellet implantation and As₄O₆ group followed oral administration of As₄O₆ at a dose of 50 mg/kg per day after VEGF pellet implantation were classified. The eyes were examined under a surgical microscope daily on postoperative from day 3 to day 9 after pellet implantation. The number, length, clock hour of vascularization, and area of vessels in As₄O₆ group were significantly less evident than those of control group and As₂O₃ group ($P < 0.05$). In conclusion, As₄O₆ had better antiangiogenic effects on the new vessel induced by VEGF in the rat cornea.

Key words : As₄O₆, As₂O₃, VEGF, antiangiogenesis, rat.

Introduction

Arsenic trioxide(As₂O₃) is a common, naturally occurring substance. It is rarely found in its pure elemental state in nature²¹. The medical use of arsenic recently is reduced because of its toxicity and potential for carcinogenicity^{1,21}. Although arsenic can be poisonous, and chronic arsenic exposure from natural or industrial sources can cause serious toxicity, arsenic has been used medically for over 2,400 years¹. Since the antileukemic activity of arsenic agent was first reported in the late 1800 s, the reemerging of arsenic therapy has occurred. In the 1970 s, physicians in China began using arsenic trioxide as a part of treatment for acute promyelocytic leukemia (APL)^{5,18,19}. Arsenic trioxide was recently used for the treatment of tumors like multiple myeloma⁹, chronic B-cell leukemia¹² and acute promyelocytic leukemia.

Although the precise mechanism of As₂O₃ action is unknown, a variety of *in vitro* studies suggest that As₂O₃ contributes to the effectiveness *in vivo* by some mechanisms including induction of apoptosis, partial cellular differentiation, degradation of specific APL fusion transcripts, antiproliferation, and inhibition of angiogenesis^{17,21}. As₂O₃ interrupted a reciprocal stimulatory loop between leukemic cells and endothelial cells by causing apoptosis of both cell types and by inhibiting leukemic cell vascular endothelial growth factor (VEGF) production, and prevented capillary tubule and branch formation in an *in vitro* endothelial cell differentiation assay¹⁷.

As₄O₆ is one of the arsenic compound derivatives, which was used as the empirical anticancer agent in the Korean alternative medicine. However, there were few reports which showed the mechanism of anticancer effect of As₄O₆. Park et al¹⁵ suggested that As₄O₆ significantly decreased the proliferation, migration, invasion, and tube formation of endothelial cells induced by the angiogenic factors bFGF *in vitro*.

VEGF^{3,6,8,10,16}, basic fibroblast growth factor (bFGF)^{2,3,6,15}, nerve growth factor (NGF)²³, epidermal growth factor (EGF)⁸, and transforming growth factor α , β are known as the angiogenic positive factors. As₄O₆ inhibited angiogenic effect of bFGF on the rat corneal micropocket model¹⁵ and the reverse effects of As₄O₆ on the angiogenesis induced by NGF in the rat corneal micropocket model²³. In addition, Park¹⁴ reported angiogenic effect of VEGF on the rat corneal micropocket model. However, there have been no studies about antiangiogenic effects of As₄O₆ induced by VEGF.

The aim of this study was to compare the antiangiogenic effects between the new arsenic compound As₄O₆ and the existing As₂O₃ on neovascularization induced by VEGF in the rat micropocket cornea.

Materials and Methods

Experimental animals

Male Sprague-Dawley (SD) rats weighing 300-400 g were used in this study. Animals were housed in cages at a controlled temperature (22±2°C, 55±10% humidity) with a 12 h light/dark cycle, and fed with commercial rat pellet, and free access to water at all times throughout the study. The rats were acclimatized for 7 days prior to commencement of

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experiments. All animal experimentation was performed in accordance with guideline for the use and care of laboratory animal in Seoul National University. After ophthalmoscopic examination, rats with normal cornea only were performed VEGF pellet implantation. Total 60 eyes were used in this study.

Experimental design

The entire groups were randomly divided into three groups ; control group only received VEGF pellet, As₂O₃ group followed the trans-oral administration of As₂O₃ at a dose of 50 mg/kg per day after VEGF pellet implantation, and As₄O₆ group followed the trans-oral administration of As₄O₆ at a dose of 50 mg/kg per day after VEGF pellet implantation.

VEGF pellet preparation

Pellets were prepared according to the method previously described^{6,14,15,23}. Sterile casting solution was prepared by dissolving the poly-2-hydroxyethylmethacrylate (Hydron, Sigma Co, USA) powder in absolute ethanol (12% w/v) at 37°C with continuous stirring for 24 hrs. An equal volume of Hydron and sucralfate (12% w/v, Sigma Co, USA) were combined because the Hydron can stabilize the VEGF and sucralfate induces slow release of VEGF. Both materials do not affect the angiogenic response. Then 20 ng of VEGF were mixed with 2 µl of Hydron and sucralfate combined solution. These solutions were pipetted onto the surface of sterile teflon rods glued to the surface of petri dish to make a pellet with diameter of 2 mm. Pellets were dried at room temperature for 1 to 2 hrs in sterile environment, then stored at -20°C until used.

VEGF pellet implantation

VEGF pellet implantation into rat corneas was conducted by the method previously described^{6,14,15,23}. Rats were anesthetized with a combination of ketamine hydrochloride (20 mg/kg, IM, Yuhan Ketamine®, Yuhan Co., Korea) and xylazine hydrochloride (6 mg/kg, IM, Rompun®, Bayer Co., Korea). The eyes were topically anesthetized with 0.5% proparacaine (Alcaine®, Alcon Co., USA), and gently proptosed and secured by clamping the upper eyelid with a nontraumatic hemostat. Under a surgical microscope, 1.5 mm incision was made approximately 1 mm from the center of cornea into stroma with Beaver's blade (Beaver™, Becton Dickinson Co., USA) but not through it. A microdissector approximately 1.5 mm in width was inserted under the lip of the incision and gently blunt-dissected through the stroma toward limbus of the eye. Slight finger pressure against the globe of the eye was kept steady it during dissection. The distance between the limbus and base of the pocket was kept 1-1.5 mm in length. The VEGF pellet was rehydrated with a drop of sterile saline. The pellet was positioned down to the base of the pocket should be occupied with the implant material. After pellet implantation, antibiotic ointment (Terramycin®, Pfizer Co., Korea) was pasted around rat cornea and rat was marked on its tail.

Biomicroscopic examination

The eyes were examined under a surgical microscope (Leica M651) daily on postoperative from day 3 to day 9 after pellet implantation. Images were captured using a digital camera. The number of vessels, the length of vessels, and the angle of neovascularization were evaluated using image analyzing program (Image tools ver 3.0, the University of Texas Health Science Center in San Antonio, USA). The contiguous circumferential zone of neovascularization was measured as clock hours with a 360° reticule (where 30° of arc equals 1 clock hour). The area of neovascularization was determined by measuring with a reticule the vessel length (L) from the limbus and the number of clock hours (C) of limbus involved. A formula was used to determine the area of a circular band segment: $C/12 \times 3.1416 [r^2 - (r-L)^2]$, where r = 2.5 mm, the measured radius of the rat cornea².

Statistical analysis

For a intention verification of this study, all numerical variables (number of vessels, length of vessels, clock hour of neovascularization, and area of neovascularization) were assessed by use of repeated ANOVA test with two factors; time and treatment (control, As₂O₃, As₄O₆). And their interactions were examined. When significant difference was determined, multiple comparisons at each time point were made using Scheffe test. Values of $P < 0.05$ were considered significant.

Results

The number of vessels

New vessels began sprouting into the cornea on postoperative day 3. The number of vessels increased in all groups with time. The number of vessels in As₄O₆ group were significantly fewer than control group and As₂O₃ group from day 3 to day 6 ($P < 0.05$). However, there was no significant difference of the vessel number in As₂O₃ group and As₄O₆ group after day 7. They were significantly fewer than control group as ever (Fig 1).

The length of vessels

Vessel length changes in each group showed a similar pattern to the number of vessels. The length of vessels in As₄O₆ group was significantly shorter than that of control group and As₂O₃ group ($P < 0.05$). The vessel length in As₂O₃ group was somewhat longer than control group, but there is little point in it (Fig 2).

The clock hours of neovascularization

Clock hour changes of neovascularization in each group showed a growth pattern that was similar to that of the length of vessels. As₄O₆ group showed significantly narrower clock hours of neovascularization than in control group and As₂O₃ group from day 3 to day 7 ($P < 0.05$; Fig 3).

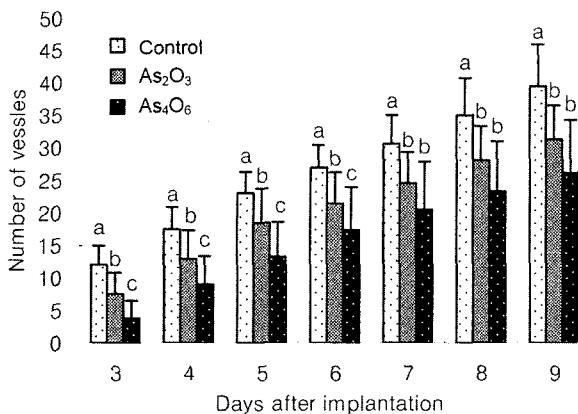


Fig 1. Change of the number of vessels after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P < 0.05$.

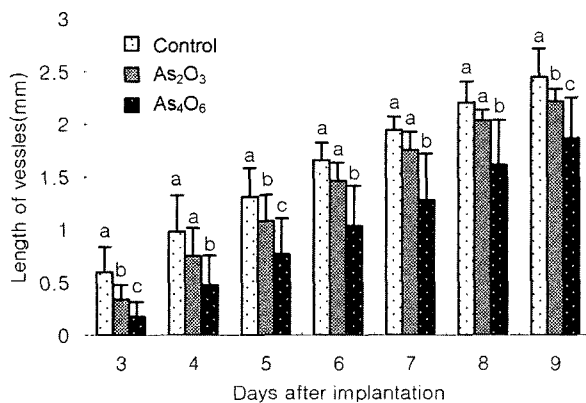


Fig 2. Change of the length of vessels after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P < 0.05$.

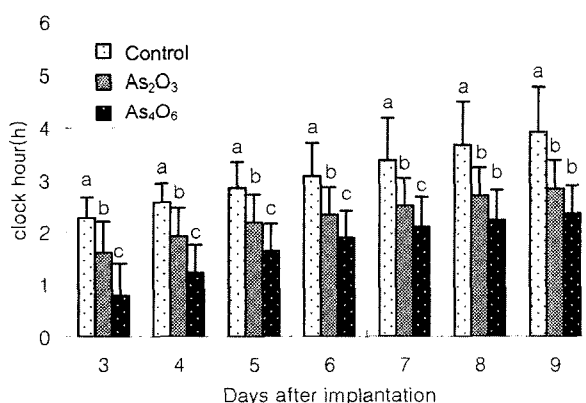


Fig 3. Change of the the clock hours of neovascularization after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P < 0.05$.

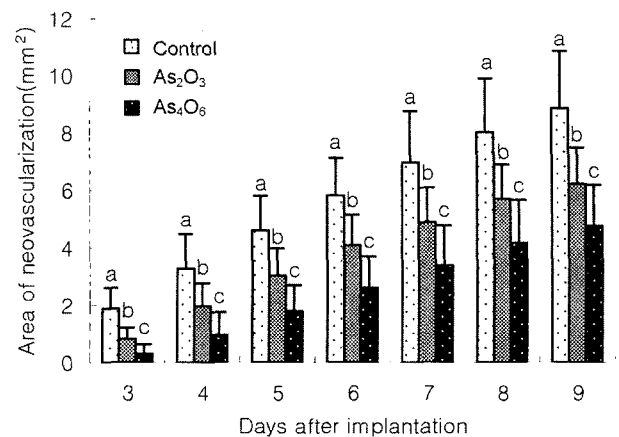


Fig 4. Change of the area of neovascularization after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P < 0.05$.

Area of neovascularization

As₄O₆ group was found to have significantly lesser vessel area than in control group and As₂O₃ group from day 3 to day 9 ($P < 0.05$; Fig 4).

Discussion

The purpose of this study was to compare the antiangiogenic effects of As₄O₆ to those of As₂O₃ on the rat corneal micropocket model induced by VEGF. With application of cornea micropocket assay, the confusion could be avoided between new vessels and previously exiting vessels^{10,14,15,23}.

20 ng VEGF impregnated pellets were used for angiogenic inducer on the rat cornea micropocket assay in this study. Park¹⁴ used the same dose as the optimal effective dose of VEGF, and reported that the dose did not induce side effects such as corneal edema or inflammation after operation. We also found VEGF had no significant side effects in rats at the above same dose.

This study showed that As₄O₆ inhibited angiogenesis induced by VEGF in the rat cornea. As₄O₆ had a better antiangiogenic activity than As₂O₃ on VEGF. Park *et al*¹⁵ and You *et al*²³ reported that As₄O₆ had a better antiangiogenic effects on the neovascularization induced by bFGF and NGF in the rat cornea. They suggested that there was different antiangiogenic mechanism between As₂O₃ and As₄O₆. In contrast to the severe gastrointestinal toxicities of oral administration of As₂O₃¹⁸, they also observed that oral administration of As₄O₆ at the dose of 50 mg/kg per day for 7 days to SD rats and for 22 days to C57BL/6 mice caused no remarkable side effects. In this study, we also found As₄O₆ had no noticeable side effects in SD rats at the above same dose for 9 days. The oral rat LD₅₀ of As₂O₃ for investigation as mutagen, tumorigen and reproductive effector was known as 14.6 mg/kg²⁰.

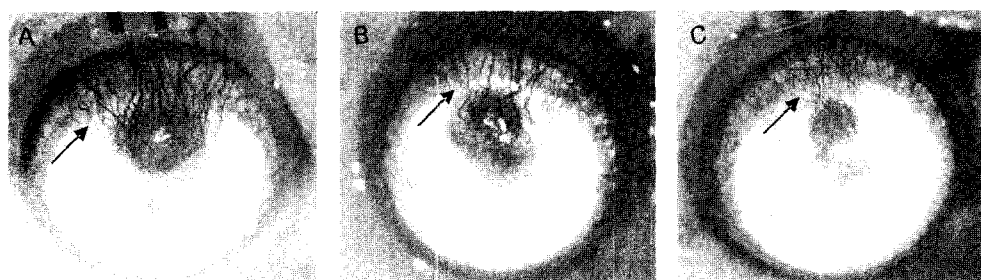


Fig 5. Appearance of angiogenesis on day 9 after VEGF pellet implantation on the rat corneal stroma and administration of arsenic compounds per oral. A. control group. B. As₂O₃ group. C. As₄O₆ group. °E: New vessels.

VEGF has reported to have an endothelial cell-specific mitogen and an angiogenesis inducer released by a variety of tumor cells and expressed in human tumors *in situ*⁸. Angiogenic effect of VEGF in this study is in agreement with the Kenyon's research⁷ who observed angiogenesis at the limbus of rat cornea 5 to 6 days after post-implantation using VEGF.

Lee *et al*¹⁰ reported that VEGF had the dose-dependent relationship between VEGF-dose and matrix metalloproteinase (MMP) -2, 9 (gelatinase) in rat cornea. Park *et al*¹⁵ demonstrated that As₄O₆ appeared to be a novel antiangiogenesis and antimetastasis chemical agent that can be orally administered in animal models. They also observed that induction of cell cycle arrest at G₂/M phase and inhibition of MMP-2 secretion might be possible antiangiogenic mechanisms of As₄O₆¹⁵. In this study, As₄O₆ had better antiangiogenic effect than As₂O₃ in length, and area of vessels. As As₄O₆ inhibited the angiogenesis induced by VEGF, it was hypothesized that As₄O₆ might suppress autocrine secretion.

In recent years, arsenic trioxide (As₂O₃) has been used successfully in the treatment of patients with acute promyelocytic leukemia (APL). Some recent studies showed anti-vascular¹¹ and antiangiogenic activity of As₂O₃¹⁷. As₂O₃ acts on cells through a variety of mechanisms as influencing numerous signal transduction pathways and resulting in a vast range of cellular effects that include apoptosis induction, growth inhibition, promotion or inhibition of differentiation, and angiogenesis inhibition²².

Roboz *et al*¹⁷ reported that As₂O₃ inhibited capillary tubule and branch formation in an *in vitro* endothelial cell-differentiation assay. They believed that As₂O₃ interrupted a reciprocal stimulatory loop between leukemic cells and endothelial cells by causing apoptosis of both cell types and by inhibiting leukemic cell VEGF production. It is known that impaired production or expression of VEGF will disrupt angiogenesis¹³.

As₄O₆ significantly decreased the proliferation, migration, invasion, and tube formation of endothelial cells induced by the angiogenic factors bFGF. As₄O₆ also inhibited angiogenesis in the rat corneal micropocket model induced by bFGF and lung metastasis in the mouse model *in vivo* and migration and invasion of bovine capillary endothelial(BCE) cells, and tubular structure formation of human umbilical vein endothelial cells(HUVECs) on Matrigel¹⁵. As₄O₆ also had antiangiogenic

effects on the new vessels induced by VEGF in the rat cornea. However there are almost never antiangiogenic effects in As₂O₃ group²³.

The different angiogenic factors may control the formation of new vessels via either similar or distinct signaling pathways in angiogenesis. There were a total of 94 human genes with differential expression patterns in response to mitogen treatment. And the thirty-two gene expression patterns in 94 genes were similarly regulated by either VEGF or bFGF, whereas those of the remaining sixty-two genes were regulated by only one of them⁴.

It may be suggested that there were different mechanisms between As₄O₆ and As₂O₃ in antiangiogenic activity and As₄O₆ had more antiangiogenic effects than As₂O₃ in similar signaling pathways.

Conclusion

To evaluate the antiangiogenic effects of As₄O₆ and As₂O₃ on the angiogenesis induced by VEGF, cornea micropocket assay in the rat was used. The number, length, the clock hour of vascularization, and the area of the vessels in As₄O₆ group were significantly less evident than those of control group and As₂O₃ group during experimental period ($P < 0.05$). In conclusion As₄O₆ had better antiangiogenic effects on the new vessel induced by VEGF in the rat cornea.

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References

1. Antman K. Introduction : The history of arsenic trioxide in cancer therapy. *Oncologist* 2001; 6: 1-2.
2. D'Amato R, Loughnan M, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci* 1994; 91: 4082-4085.
3. Folkman J, D'Amore P. Blood vessel formation : what is its molecular basis? *Cell* 1996; 87: 1153-1155.
4. Jih YJ, Lien WH, Tsai WC, Yang GW, Li C, Wu LW. Distinct regulation of genes by bFGF and VEGF-A in

- endothelial cells. *Angiogenesis* 2001; 4: 313-321.
5. Jing Y, Dai J, Chalmers-Redman R, Tatton W, Waxman S. Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. *Blood* 1999; 94: 2102-2111.
 6. Kenyon B, Voest E, Chen C, Flynn E, Fokman J, D'Amato R. A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci* 1996; 37: 1625-1632.
 7. Kenyon B, Browne F, D'Amato R. Effect of thalidomide and related metabolites in a mouse corneal model of neovascularization. *Exp Eye Res* 1997; 64: 971-978.
 8. Kim K, Li B, Winer J, Armanini M, Gillett N, Phillips H, Ferrara N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* 1993; 362: 841-844.
 9. König A, Wrazel L, Warrell Jr R, Rivi R, Pandolfi P, Jakubowski A, Gabrilove J. Comparative activity of melarsoprol and arsenic trioxide in chronic B-cell leukemia Lines. *Blood* 1997; 90: 562-570.
 10. Lee JB, Jung SE, Lee HC, Kwon HM, Hong BK, Park HY, Kim EK, Oh JH. Effect of vascular endothelial growth factor(VEGF) on the corneal neovascularization and expression of MMP-2,9,TIMP-1,2 and flk-1. *J Korean Ophthalmol Soc* 2001; 42: 1053-1062.
 11. Lew Y, Brown S, Griffin R, Song C, Kim J. Arsenic trioxide causes selective necrosis in solid murine tumors by vascular shutdown. *Cancer Res* 1999; 59: 6033-6037.
 12. Munshi N. Arsenic trioxide : An emerging therapy of multiple myeloma. *Oncologist* 2001; 6: 17-21.
 13. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor(VEGF) and its receptors. *FASEB J* 1999; 13: 9-18.
 14. Park CH. Angiogenic effect of vascular endothelial growth factor on the rat cornea. Kangwon national university. 2003.
 15. Park MJ, Park IC, Bae IJ, Seo KM, Lee SH, Hong SI, Eun CK, Zhang W, Rhee CH. Tetraarsenic oxide, a novel orally administrable angiogenesis inhibitor. *International Journal of Oncology* 2003; 22: 1271-1276.
 16. Risau W. Mechanisms of angiogenesis. *Nature* 1997; 386: 671-674.
 17. Roboz G, Dias S, Lam G, Lane W, Soignet S, Warrell Jr R, Rafii S. Arsenic trioxide induces dose- and time-dependent apoptosis of endothelium and may exert an antileukemic effect via inhibition of angiogenesis. *Blood* 2000; 96: 1525-1530.
 18. Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY, Zhu J, Tang W, Sun GL, Yang KQ, Chen Y, Zhou L, Fang ZW, Wang YT, Ma J, Zhang P, Zhang TD, Chen SJ, Chen Z, Wang ZY. Use of arsenic trioxide(As₂O₃) in the treatment of acute promyelocytic leukemia(APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood* 1997; 89: 3354-3360.
 19. Soignet S, Maslak P, Wang ZG, Jhanwar S, Calleja E, Dardashti L, Corso D, DeBlasio A, Garbrilove J, Scheinberg D, Pandolfi P, Warrell R. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. *NEJM* 1998; 339: 1341-1348.
 20. Univerisy of Tennessee, Department of EHS. 1997. Arsenic trioxide. <http://web.utk.edu/~ehss/arsenic/arsenicb.htm>.
 21. Waxman S, Anderson K. History of the development of arsenic derivatives in cancer therapy. *Oncologist* 2001; 6: 3-10.
 22. Wilson H. Miller, Jr., Hyman M. Schipper, Janet S. Lee, Jack Singer, and Samuel Waxman. Mechanisms of action of arsenic trioxide. *Cancer Research* 2002; 62: 3893-3903.
 23. Yoo MH, Kim JT, Rhee CH, Park MJ, Bae IJ, Yi NY, Jeong MB, Jeong SM, Nam TC, Seo KM. Reverse effects of tetraarsenic oxide on the angiogenesis induced by nerve growth factor in the rat cornea. *J Vet Med Sci* 2004; 66: 1091-1095.

랫드 각막에서 Vascular Endothelial Growth Factor(VEGF)로 유발시킨 신생혈관에 대한 As₄O₆의 혈관신생 억제효과

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요약 : 본 연구는 VEGF로 유발된 랫드 각막 미세낭 모델에서 As₄O₆와 As₂O₃의 혈관신생 억제효과를 비교하기 위해 실시하였다. 20 ng VEGF가 함유된 펠렛을 이식하여 혈관신생을 유도하였다. 안과 검사후 정상적인 각막을 가진 Sprague-Dawley 랫드의 안구 60안을 이용하여 각 20안씩 무작위로 선정하여 대조군(비투약군), As₂O₃군(50mg/kg As₂O₃, 경구투여, 1일 1회), 그리고 As₄O₆군(50 mg/kg As₄O₆, 경구투여, 1일 1회)으로 나누어 실시하였다. 이식이 완료된 후, 신생혈관의 개수, 길이 및 신생혈관의 각도를 미세수술현미경하에서 이식 3일째부터 9일째까지 매일 검사하였다. 신생혈관의 넓이는 신생혈관의 길이와 각도를 수학적인 공식에 적용하여 계산하였다. 실험 기간동안 As₄O₆군의 신생혈관 개수, 길이, 각도 및 넓이가 대조군과 As₂O₃군에 비하여 유의성있게 억압되었다(P<0.05). 이러한 결과 As₄O₆는 랫드 각막에서 VEGF에 의해 유도된 신생혈관에 As₂O₃보다 더 뛰어난 억제효과를 가지고 있음을 보여주었다.

주요어 : As₄O₆, As₂O₃, VEGF, 혈관신생억제, 랫드