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Short Communication
Molybdenum Salts Possess Potent Angiogenic Modulatory Properties: Validation on Chorioallantoic Membrane (CAM) of Chicken

[1]Rekha Khandia, [1]Pratibha Vishwakarma, [1]Abhinav Dwivedi, [1]Anshumala Kujur, [2]Kuldeep Dhama and [1]Ashok Munjal

[1]Department of Biochemistry and Genetics, Barkatullah University, 462026 Bhopal, Madhya Pradesh, India
[2]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, 243122 Bareilly, Uttar Pradesh, India

Abstract

Objective: Angiogenesis plays critical and essential role in various physiological processes of animals and humans. Present study reports the potential angiogenic modulatory effects of the two different molybdenum salts [molybdenum trioxide (MoO_3) and sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)] on chorioallantoic membrane (CAM) of embryonated chicken eggs. **Methodology:** The three groups of the embryonated chicken eggs (1 control and 2 treated groups) were taken and 200 µL of 0.5 M of each of MoO_3 and $Na_2MoO_4.2H_2O$ were directly introduced on to the CAM. The resealed eggs were incubated for 72 h in a humid incubator chamber at 37±1°C. Then the eggs were opened to observe the gross and histopathological alterations for angiogenesis modulation. **Results:** Gross examination revealed reduced number of secondary and tertiary blood vessels in MoO_3 treated group, while $Na_2MoO_4.2H_2O$ treated group showed reduction in number of blood vessels with occasional haemorrhages. Histopathological analysis indicated pro-angiogenic effect of MoO_3, with presence of numerous mesodermal blood vessels with normal CAM tissue architecture. However, Chorionic Ectoderm (CE) was absent at few places, with intact CE at most places, indicating requirement of critical dose optimization. The $Na_2MoO_4.2H_2O$ was observed as anti-angiogenic, causing inflammation in CAM tissue with hemorrhage and thus can't be used for therapeutics. **Conclusion:** The pro-angiogenic properties of MoO_3 can be explored to treat ailments related to insufficient angiogenesis like coronary artery disease, chronic wounds, stroke and myocardial infarction. However, to avoid any kind of side effects, further qualitative and quantitative analysis and critical dose determination is required before going for clinical trials.

Key words: Angiogenesis, chorioallantoic membrane, molybdenum salts, endothelial cells, therapeutics

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Corresponding Author: Ashok Munjal, Department of Biochemistry and Genetics, Barkatullah University, 462026 Bhopal, Madhya Pradesh, India

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The formation of new capillaries from pre-existing vascular network is termed as angiogenesis[1,2], which is a complex, multi step physiological process and relies on the interplay between macrophages, endothelial cells, fibroblasts and the surrounding extracellular matrix. The pivotal role of angiogenesis in various physiological processes not only during tissue repair after surgery or trauma and fetal development[3] but also in wound healing[4,5], the menstrual cycle, cancer and various ischemic and inflammatory diseases[3,6] is well known. It is the key process which occurs during both physiological and pathological conditions[7]. Pro-angiogenic [Vascular Endothelial Growth Factor (VEGF), Hypoxia Inducible Factor (HIF)] and anti-angiogenic factors (angiostatin, endostatin and thrombospondin) are the two controlling aspects regulating the angiogenic process[8]. An imbalance in this process contributes to numerous malignant, inflammatory and immune disorders[9-11].

The growth, invasion and metastasis of the tumors are reliant on angiogenesis process[12,13]. Uneven rate of angiogenesis (both excessive and insufficient) leads to diseased condition. Apart physiological angiogenesis, when pro-angiogenic factors are secreted in more quantity, it leads to excessive angiogenesis. Diseases such as cancer, diabetic blindness, age related macular degeneration, rheumatoid arthritis etc are the examples of excessive angiogenesis. Insufficient angiogenesis occurs in diseases such as coronary artery disease, stroke, myocardial infarction and chronic wounds. Insufficient or improper angiogenesis results in tissue loss and ultimately higher mortality rate[12].

Metals are required for several essential functions for life viz., zinc, copper and manganese, where selenium and molybdenum previously considered as harmful[13]. However, metals in both overload and insufficient quantity could be detrimental[14,15]. The metals are progressively becoming an important part of therapeutics to treat an array of human ailments[14]. Role of some metals in the process of angiogenesis is well known[16]. Barbucci *et al.*[17] revealed that the compounds of Cu(II) and Zn(II) ions in complexes with polysaccharides induce and inhibit endothelial cell adhesion respectively[17]. Copper (Cu) is an essential requirement for several biological functions including cell growth, redox reactions, development and angiogenesis[18,19].

Molybdenum (Mo) is known to have anti-angiogenic property but sometimes it also promotes angiogenesis process and increases oesophageal and gastric cancers in human. The Mo is known to enhance lung tumor in mice model[20]. But, the deficiency of Mo has been correlated with the increase of esophageal and gastric cancers in human and animal diets because Mo is thought to act in cancer prevention[21,22]. The Mo is also recognized to encompass anti-neoplastic properties and also prevents the formation of some experimentally induced cancer. The Mo probably exerts its anticancer effect by reducing nitrosamines and their precursors, i.e., nitrates, nitrite and secondary amines in the diet[23]. In rats, it has been observed that molybdocene dichloride and molybdenum dichloride administered in drinking water has anticancer effect against oesophageal and fore-stomach cancer[24]. Molybdenum dichloride has anti-tumor agent properties[23]. Studies revealed that repeated exposures of experimental animals to molybdenum trioxide (MoO_3) solutions can lead to increased numbers of lung tumors[25]. Wilson's disease (an autosomal recessive disorder) that leads to abnormal copper accumulation[26] can be treated with tetrathiomolybdate (TM) by chelating Cu[27].

The Mo being natural antagonist of copper (important cofactor for angiogenesis)[28] and suppose to have anti-cancer effect possibly through inhibiting Cu mediated angiogenesis. However, Mo showed both the anticancer as well as cancer proliferating role, it can be speculated that its effect on cancer via angiogenesis alteration is dependent upon the molecule, conjugated with it and determines the deeds of any compound as anti-cancerous or cancer provoking compound. Hence, the evaluation of different Mo salts is essential for defining their role as pro-angiogenic or anti-angiogenic material. The present study aimed to elucidate the angiogenesis modulatory effects of two Mo salts (molybdenum trioxide, MoO_3 and sodium molybdate dihydrate, $Na_2MoO_4.2H_2O$) on chorioallantoic membrane (CAM) model of chicken.

MATERIALS AND METHODS

Two molybdenum salts [Molybdenum trioxide (MoO_3) and sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)] were used in present study. Their effects on the process of angiogenesis were evaluated using the chicken chorioallantoic membranes (CAM) model[29,30]. The embryonated chicken eggs (n = 30; 61±3 g of 9-11 days of age) were procured from State poultry farm, Raisen Road, Bhopal (MP). The eggs were divided into three experiment groups (10 eggs in each), comprising of one control group (Group I) and two treatment groups (Group II: Treated with MoO_3, group III: Treated with $Na_2MoO_4.2H_2O$). The egg surfaces were cleaned and wiped with 70% ethanol. About 200 µL of 0.5 M of each Mo salt (MoO_3 and $Na_2MoO_4.2H_2O$) were directly introduced onto the CAM of

respective group of embryonated eggs using 26 gauge hypodermic syringe along with antibiotics (Ampicillin 50 μg mL^{-1}, streptomycin 10 μg mL^{-1}) and antimycotic solution (Amphotericin 10 μg mL^{-1}). Phosphate Buffered Saline (PBS) with antibiotic and antimycotic solution was given to the control group. The eggs were resealed with cellophane tape and incubated for 72 h in a humid incubator chamber at 37±1°C. The eggs were then opened to observe the effects of different Mo salts for angiogenesis modulation. The CAM was observed and further it was harvested and subjected to histopathological analysis using hematoxylin and eosin stain.

RESULTS

The treated resealed embryonated chicken eggs with different Mo salts and control were opened and examined following 72 h of incubation after treatment. Modulatory effects of different Mo salts on the process of angiogenesis were observed by visualizing the gross and histopathological alterations in chorioallantoic membranes (CAM). At gross level, well arborized vasculature with major and minor blood vessels was revealed in the control (Group I; Fig. 1a). In CAMs of MoO_3 treated (Group II), few branch point has been observed with less number of secondary and tertiary blood vessels (Fig. 1b); however, among $Na_2MoO_4.2H_2O$ treated (Group III), reduction in number of branch point along with occasional hemorrhage was observed (Fig. 1c).

Histopathology of the control (Group I) revealed normal thickness of CAM, with concordant dispersed mesodermal blood vessels (BV) and vein (V) with loose connective tissue (Fig. 2a). The CAM of Group II treated with MoO_3 revealed the absence of chorionic epithelium at some places (Fig. 2b) whilst at most of the places; both the CE and AE were present. Number of blood vessels was also increased remarkably. The CAM of Group III treated with $Na_2MoO_4.2H_2O$ has reduced number of blood vessels with scanty haemorrhages at numerous places. Highly accumulated fibroblast cells were also observed (Fig. 2c).

DISCUSSION

Angiogenesis is the formation of new blood vessels from existing one. Excess and subsided angiogenesis, both conditions are responsible for pathological conditions[31]. Its inhibition could be a strategy to combat disorders like tumor, diabetic retinopathy, arthritis, age related macular degeneration etc.[19,32], where enhanced angiogenesis might be helpful in improving conditions of ischemic chronic wound[33], coronary artery diseases[34] and bone integration and fracture repair[35]. Angiogenesis research has now caught the pace and it will probably change the face of medicine in the forthcoming decades, with many more people worldwide predicted to be benefited from angiogenesis modulatory treatments. Many chemicals like metals, non-metals, metalloids and their salts play great role in physiological functioning and maintenance of body. Metals have role in sustaining and promoting or declining the angiogenesis too. The Cu is known to induce angiogenesis[36], whereas Ag[32], gold[37], Zn[38] inhibit angiogenesis. The Mo has also been

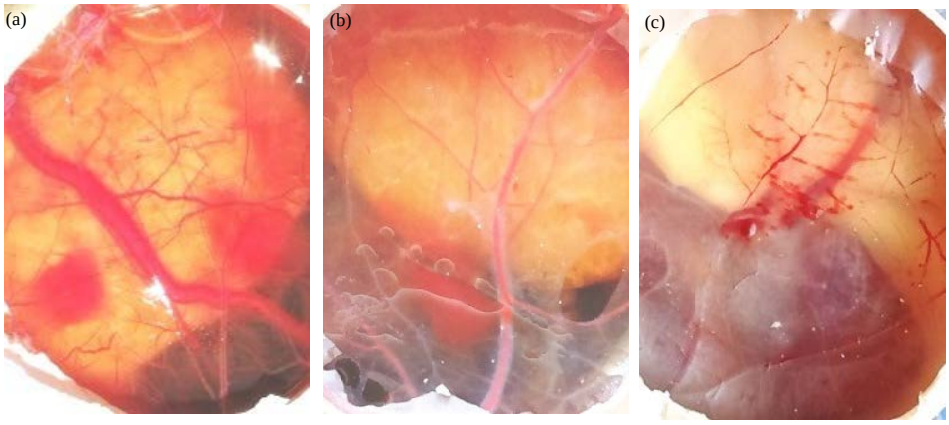

Fig. 1(a-c): (a) CAM of control eggs (Group I) with well arborized vascular system, (b) CAM of MoO_3 treated eggs (Group II) showing primary blood vessel with less visible secondary and tertiary blood vessels and (c) CAM treated with $Na_2MoO_4.2H_2O$ revealed thin blood vessels with less branch points and occasional bleeding

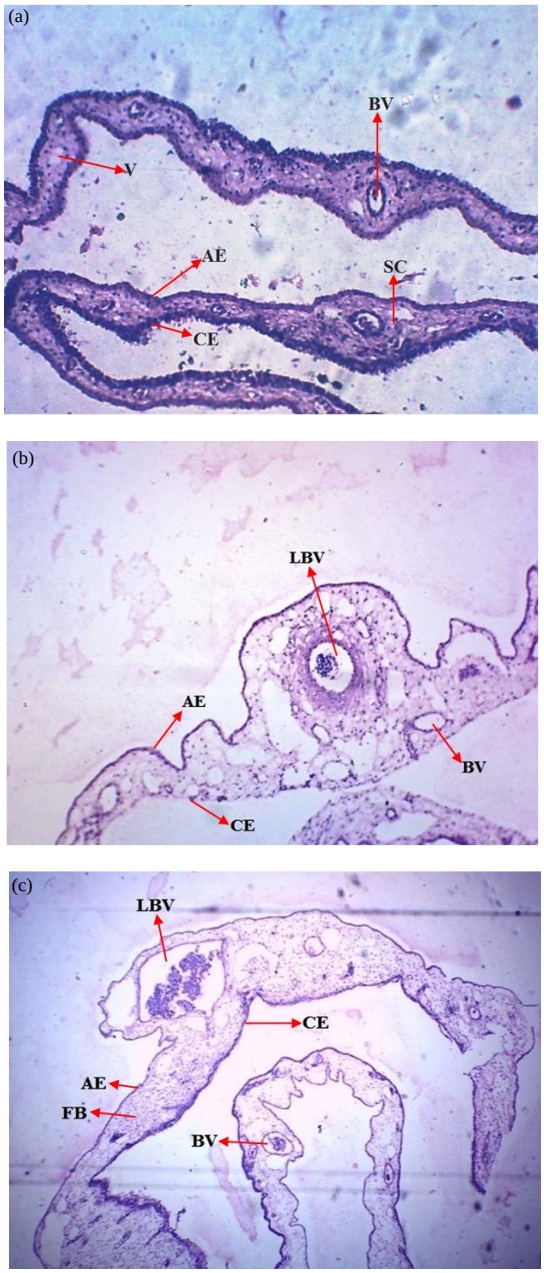

Fig. 2(a-c): Histopathology of CAM (100X), (a) Control CAM showing AE and CE of even thickness, (b) MoO_3 treated group II CAM showing reduced thickness in CE, hypodermic LBV, numerous BV indicative of the pro-angiogenic activity and (c) $Na_2MoO_4.2H_2O$ treated group III CAM showed less number of blood vessels and LBV with densely accumulated FB cells, indicative of anti-angiogenic activity, AE: Allantoic epithelium, CE: Chorionic epithelium, LBV: Large blood vessels, BV: Blood vessels, V: Vein, FB: Fibroblast, SC: Small capillary

reported to decrease the angiogenesis process by chelating Cu, a well known co-factor of several proteins significantly associated with angiogenesis by promoting proliferation and migration of endothelial cells[39,40]. In fact, ammonium tetrathiomolybdate, initially used for the treatment of an autosomal disorder Wilson's disease, which is associated with the unusual accumulation of Cu and later it was realized that it may act as anti-angiogenic therapy. In human clinical trials, ammonium tetrathiomolybdate stabilized the disease, while reducing Cu content through chelation during the period of treatment[41,42] with only emergence of mild anaemia. Treatment with ammonium tetrathiomolybdate brought stabilizing disease in 5 out of 6 patients[41]. Colon, lung, pancreas cell lines were found to inhibit proliferation with maximum efficiency against leukemic cells via activation of caspases and apoptosis induction[43]. Four dioxomolybdenum (VI) complexes had anti-proliferative roles against human colorectal carcinoma (HCT 116) cell line with unique symptoms of apoptosis like cell membrane blabbing, nuclear condensation and vesicle formation[44]. It has been observed that sodium molybdate administered in drinking water has a protective action against the induction of cancer in rats[24].

Molybdenum dichloride has anti-tumor agent properties[24]. A deficiency in molybdenum has been cited as a possible factor in the causation of oesophageal cancer. It is known to have anti-neoplastic properties and together with manganese prevents the formation of some experimentally induced cancer. Molybdenum probably exerts its anti-cancer effect by reducing nitrosamines and their precursors, i.e., nitrates, nitrite and secondary amines in the diet[24]. Also, it is associated with increased incidence of lung cancer upon inhalation. When administered intramuscularly, MoO_3, increase the incidence of lung tumor but through undefined mechanism[20]. Therefore, it is worth speculation that pro or anti-angiogenic effects are dependent on its conjugated counterpart. Hence, in the present study, two Mo salts were evaluated for their role in angiogenesis. Molybdenum compound $[(\eta_3\text{-}C_3H_5)Mo(CO)_2(phen)Cl]$ in a concentration of 10 μmol L^{-1}, when applied on 14 cancer cell lines of various origins like leukemia, breast, ovary, cervix, stomach[43]. In higher concentrations, the copper is toxic for sheep and it is highly susceptible for higher concentrations of Cu depending upon the breed, age of animal, other minerals uptake and presence of several feed additives in the diet and if in the diet Mo is present at 3 ppm concentration, up to 20-25 ppm Cu may be tolerated well by sheep[45]. In feedlot cattle accidental inclusion of sodium molybdate at a concentration of 1.9% of the total ration, caused severe toxicity with massive hepatic acute renal tubular necrosis[46].

In the present study, two salts of molybdenum (MoO_3 and $Na_2MoO_4.2H_2O$) were investigated for the assessment of the modulation of angiogenesis in CAM of embryonated chicken eggs. In the present study, though at gross level, number of secondary and tertiary blood vessels was reduced but at histopathological level, it showed numerous mesodermal blood vessels with loose connective tissue and normal tissue morphology, indicative of pro-angiogenic activity of MoO_3. Whilst at maximum place normal CAM architecture appeared, at some places, the CE was absent. This is an indicative of that the requirement of critical optimization of the MoO_3 dose. CAM treated with $Na_2MoO_4.2H_2O$ revealed remarkable decrease in blood vessel formation with regular AE and CE but scanty hemorrhage was observed, which limits its utility as anti-angiogenic agent. Therefore, from the present observations, it can be inferred that MoO_3 may be used as pro-angiogenic therapeutic drugs after fine tuning for critical dose decision.

CONCLUSION

Metal salts have very distinctive effects on the process of angiogenesis as co-factors. Molybdenum is having important role in functioning of several enzymes including ethyl benzene dehydrogenase, glyceraldehyde-3-phosphate ferredoxin oxidoreductase and respiratory arsenate reductase. It also determines the activities of several endothelial cell proliferation, differentiation and migration associated enzymes in humans and animals. In the present study, two Mo salts (MoO_3 and $Na_2MoO_4.2H_2O$) were evaluated and former was found to be pro-angiogenic, whereas the later was anti-angiogenic. The MoO_3 exerted pro-angiogenic activity along with no detrimental effects on normal CAM tissue morphology of chicken and hence can be used as therapeutics against coronary artery disease, stroke, myocardial infarction and chronic wounds. The $Na_2MoO_4.2H_2O$ showed anti-angiogenic activity along with accumulation of numerous inflammatory cells in CAM with scanty haemorrhages and thus can't be used in therapeutics.

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