Original Article

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Effects of Gold Nanoparticles on Angiogenesis in A Chick Chorioallantoic Membrane Model

Tavuk Chorio Allantoik Membran Modelinde Altın Nanopartikülünün Anjiogeneze Etkisi

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ABSTRACT

Objective: Today, nanotechnology is widely used in many fields because of its ability to alter the structure of molecules at the atomic level. Metallic nanoparticles attracted great attention from researchers because of their unique properties. Based on the notable features of gold nanoparticles (AuNPs), they have long been evaluated as a potential diagnostic tool for several cancers and for drug delivery applications. Angiogenesis is the development of new vessels that support embryonic up growth and critically modulate many biological processes through adulthood. The inhibition of angiogenesis causes regression of development and metastasis of malign tumours. In this study we aim to examine the effects of AuNPs on angiogenesis in an *in vivo* chick chorioallantoic membrane (CAM) model.

Methods: We applied 20 mL concentrations of AuNPs solution to 24 eggs of the CAM on the fifth day. After then, we evaluated results macroscopically on the 6th and 7th days.

Results: In our study, we observed that AuNPs induced angiogenesis.

Conclusion: We suggest that AuNPs may not be ideal nanoparticles to make biosensors due to increasing angiogenesis during the course of cancers.

Keywords: Angiogenesis, chorioallantoic membrane, gold nanoparticles, cancer

ÖZ

Amaç: Günümüzde nanoteknoloji, atom yapısındaki moleküllerin yapısını değiştirme kabiliyeti nedeniyle birçok alanda yaygın olarak kullanılmaktadır. Metalik nanopartiküller, benzersiz özellikleri nedeniyle araştırmacılardan büyük ilgi görmüştür. Altın nanoparçacıkların (AuNP) belirgin özelliklerine dayanarak, uzun zamandır birçok kanserin teşhisi ve ilaç verme uygulamaları için potansiyel bir araç olarak değerlendirilmiştir. Yeni damarların gelişimi olan anjiyogenez, embriyonik büyümeyi destekler ve yetişkinlik dönemindeki birçok biyolojik süreci kritik bir şekilde düzenler. Anjiyogenezin inhibisyonu, gelişme regresyonuna ve malign tümörlerin metastazına neden olur. Bu çalışmada AuNP'lerin *in vivo* civciv koryoallantoik membran (CAM) modelindeki anjiyogenez üzerindeki etkilerini incelemeyi amaçladık.

Yöntemler: Beşinci gün yirmi dört yumurtanın CAM'ye 20 mL konsantrasyonda AuNP çözeltisi uyguladık. Ardından 6. ve 7. günlerde makroskopik olarak sonuçları değerlendirdik.

Bulgular: Çalışmamızda AuNP'lerin anjiyogenez oluşturduğunu gözlemledik.

Sonuç: AuNP'lerin kanser seyri sırasında artan anjiyogenez nedeniyle biyosensör yapımında ideal nanoparçacıklar olamayabileceğini düşünüyoruz.

Anahtar Sözcükler: Anjiogenez, korioallantoik membran, altın nanopartikülleri, kanser

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Introduction

Nanotechnology is the engineering of very small structures having a size of 0.1 to 100 nm that are called "nanoparticles" (NPs). Nanotechnology means any technology that uses things on a nanometre scale and has real-world applications. Today, nanotechnology has been extensively used in many areas thanks to its ability to alter the structure of atomic molecules. Metallic NPs attracted great attention from researchers by their unique properties. Some of these features include thermal features [1], catalytic features (2-4), electrical conductivity (5), biological applications (6) and optical features (7). Their advantageous characteristic features are affected by high surface areas and relatively very small dimensions (8). In the polymer medium, the synthesis of NPs is encouraging due to their solubility. The processing is simplified, has less toxicity and allows controlling the growth of emerging NPs.

Gold NPs (AuNPs) are the most suitable and efficient inorganic structures for various applications. They can conform to living tissue due to the virtue of controllable stability and low virulence, small dimensions and opportunity to interact with several substances (9). Based on the notable features of AuNPs, they have long been evaluated as a potential diagnostic tool for several cancers and drug delivery applications (10). The physical size of AuNPs is important for its uptake into the cell (Figure 1) (11). Although AuNPs are biologically inactive and have less toxicity, they are relatively difficult to clean from the circulation and tissues, which can cause some diseases (12). Due to the large surface area of AuNPs, in addition to their stability, they can accommodate multiple binders that provide better binding sites for drugs or cancer-targeting groups. The typical physicochemical properties of AuNPs are used to bond with several biomolecules with or without a polymer functionalised for diagnostic and therapeutic applications (13).

Angiogenesis, that is the development of new vessels supports embryonic up growth and critically modulates many biological processes through adulthood (14). However, angiogenic leakage plays a role in many pathologic processes including diabetic retinopathy, haemangiomas, psoriasis, arthritis, solid tumour growth and fibrosis (15-17). For instance, the hypoxic microenvironment that is created by discomposed tumour perfusion may support the selection of more aggressive and invasive tumour cells (18). In this process, endothelial cells lose their apical-basal polarity and adhesion properties to form highly aggressive, migratory, extended mesenchymal cells and induce different pathological processes (19). This allows AuNPs to pass through the enlarged wall of the vessel more easily. If AuNPs have a potential-inducing effect on angiogenesis, they may more easily pass through the enlarged vessel walls and infect the tissue.

The mesodermal layers of the allantois and chorion conjugate to form the chorioallantoic membrane (CAM) throughout avian development. This structure quickly expands, generating a rich vascular network that supplies an interface for gas and waste exchange. The CAM allows studying tissue grafts, tumour growth and metastasis, drugs delivery and toxicologic analysis and angiogenic and anti-angiogenic molecules (20). The CAM model is appropriate for studying cancer invasion because the basement membrane of the chorionic epithelium mimics the human epithelial tissue (21). It is cheap and easy to observe, as well. This is the reason why it makes this model useful. In this study, we purposed to determine the effects of AuNPs on angiogenesis in the CAM model.

Method

Preparation of AuNPs Solutions

The synthesis of 20 nm AuNPs uses the sodium citrate approach. In brief, 20 mL of 0.01 M $AuCl_2$ solution is prepared with (20 mL of pure H_2O and 4.08 mg of $AuCl_2$) and left on a magnetic stirrer until it reaches boiling (around 15 mins at 150 rpm at ambient temperature). After this process, 8 mL of 0.1 M sodium citrate solution was prepared and added to the reaction mixture. The colour of the mixture turned from purple to red. Thus, the obtained AuNPs size was approximately 20 nm (22-23).

Preparation of Atak-S Type Fertilised Chicken Eggs

Atak-S fertilised chicken eggs were obtained from Poultry Institution (Tokat, Turkey) and incubated at 37 °C, and 85%-90% relative humidity was maintained throughout the experiment. Experiments were performed to examine the mode of action of AuNPs on vascular development on the 5th, 6th and 7th day on CAM. Forty-five eggs were used and kept on hold in an incubator for five days (at 37 °C, 85%-90% humidity). Five days later, the eggs were perforated after the eggshells were cleaned with an antiseptic solution.The eggs were kept under suitable heat and humidity until the day that the application was performed. A solution can be applied to eggs. They are kept under appropriate conditions until the evaluation of the results. Results were evaluated at the 24th and 48th hour after application, as stated in the literature, and visual data were recorded using digital cameras (24).

Application of Solutions on Fertilised Chick CAM

On the 5th day, between 11 a.m. and 4 p.m., a hole was opened on each egg to see to the CAM, and 20 mL of AuNPs solution was applied to the surface of each CAM. After that, all the holes on the eggs were covered with transparent tape. On the 6th and 7th days, CAMs were screened and photographed twice using a Canon 80d, 18-135 mm. Finally, the change in vessel formation was determined.

Knighton et al. (25) s scoring methodology was applied in consideration of vascularisation in our study. According to this scoring protocol, CAM blood vessels were observed after 24 hours by two different blinded observers. Observation of the vessels at the end of 24th hour was enough for accurate scoring. In addition, a 48 hour observation was made for to detect embryos for viability based on the literature. In Knighton et al. (25) s scoring methodology, CAM blood vessels near the treatment points were recorded after 24 hours by two different observers. This vascular response was graded as 0.1+ and 2+. As a score, 0 means no change in vessel formation. 1+, and 2+ reflect an

increased density and length of vessels converging toward the treatment point (26). This protocol describes the state of angiogenesis in CAM experiments.

Results

Although we used 45 eggs for this study, on the 5th day, when we perforated the eggshells, we saw that nine eggs had died before application of AuNPs solutions. Twelve of the remaining 36 eggs were used as controls. We applied 20 mL concentrations of AuNPs solution to the CAM of the remaining 24 eggs on the 5th day (27). Finally, we assessed the results of vessel formation and growth on the 6th and 7th days.

On 6th day, the CAM with AuNPs-treated solutions caused a remarkable development in CAM vascular region of 16 eggs. Furthermore, we observed a yellow tint on the CAM of nine eggs from the treatment group. In the control group, physiological angiogenesis was observed in the form of some allantoic vessels. In contrast, the macroscopic increase in the CAM area of the eggs treated with AuNP solution was observed (Figure 2).

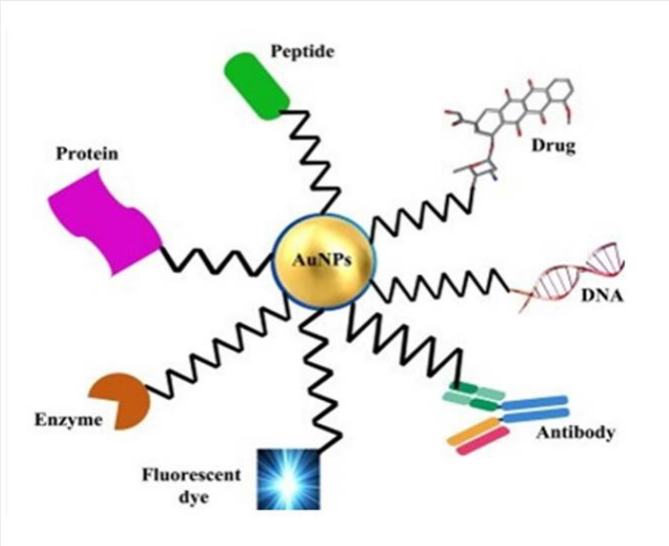

Figure 1. Properties of AuNPs as drug carriers. AuNPs are covalently bound to drugs, DNA, an antibody, an enzyme, a peptide, a fluorescent dye or a protein

On 7th day, likewise, we observed a significant improvement in the CAM vascular area of nine eggs; we observed a yellow tint on the CAM of the other seven eggs. The vessels of the effected CAM were thicker and also showed more branching. There was a notable increase in angiogenesis in comparison with the control group. The results of the macroscopical evaluation of CAMs are shown in Table 1.

Discussion

The CAM is appropriate, especially for studying the effects of angiogenic or anti-angiogenic molecules. Although we used 45 eggs for this study, on the 5th day, when we have perforated the eggshells, we saw that nine eggs died before the application of AuNPs solutions. Some possible explanations for the deaths should be suggested. A few of them had not been fertilised, and/or the membranes of a few of them were perforated when we tried to open the eggshell. Also, the balance of heat did not provide some of them adequate warmth because they stayed in the corner of the incubator. Nevertheless, the remaining eggs were adequate for obtaining preliminary information related to the effects of AuNPs on angiogenesis on CAM.

Angiogenesis is a period of new blood vessel formation. Current studies show that the induction of angiogenesis is a strategy, especially for the treatment of ischaemic diseases, instead of repressing angiogenesis for the treatment of cancers (28). The growth of solid tumours is accompanied by the stimulation of angiogenesis. So, the inhibition of angiogenesis causes the regression of development and metastasis of malignant tumours (29).

The CAM assay of chick embryos is widely used as an *in vivo* model for angiogenesis. The CAM assay is preferable because it is highly sensitive and cheaper than other *in vivo* or *in vitro* models (26). AuNPs have long been evaluated as a potential tool for the diagnosis of several cancers and drug delivery applications. Up to the present, no study on the angiogenic or anti-angiogenic effect of AuNPs on the CAM model has been reported whereas there are several studies on some other *in vivo* and *in vitro* models (30-31).

Al-Trad et al. (30) and Shen et al. (31) used a rat retinal model to determine the effects of AuNP on angiogenesis. They reported

Table 1. Macroscopic evaluation of the effects of gold nanoparticles application on chorioallantoic membrane

	Group		Efficiacy			
			Ineffective	+1	+2	Total
Day 6	Control	n	10	2	0	12
		%	83.3	16.7	0.0	100.0
	20 mM	n	0	13	11	24
		%	0.0	54.2	45.8	100.0
Day 7	Control	n	9	3	0	12
		%	75.0	25.0	0.0	100.0
	20 mM	n	0	4	7	11
		%	0.0	36.3	43.7	100.0

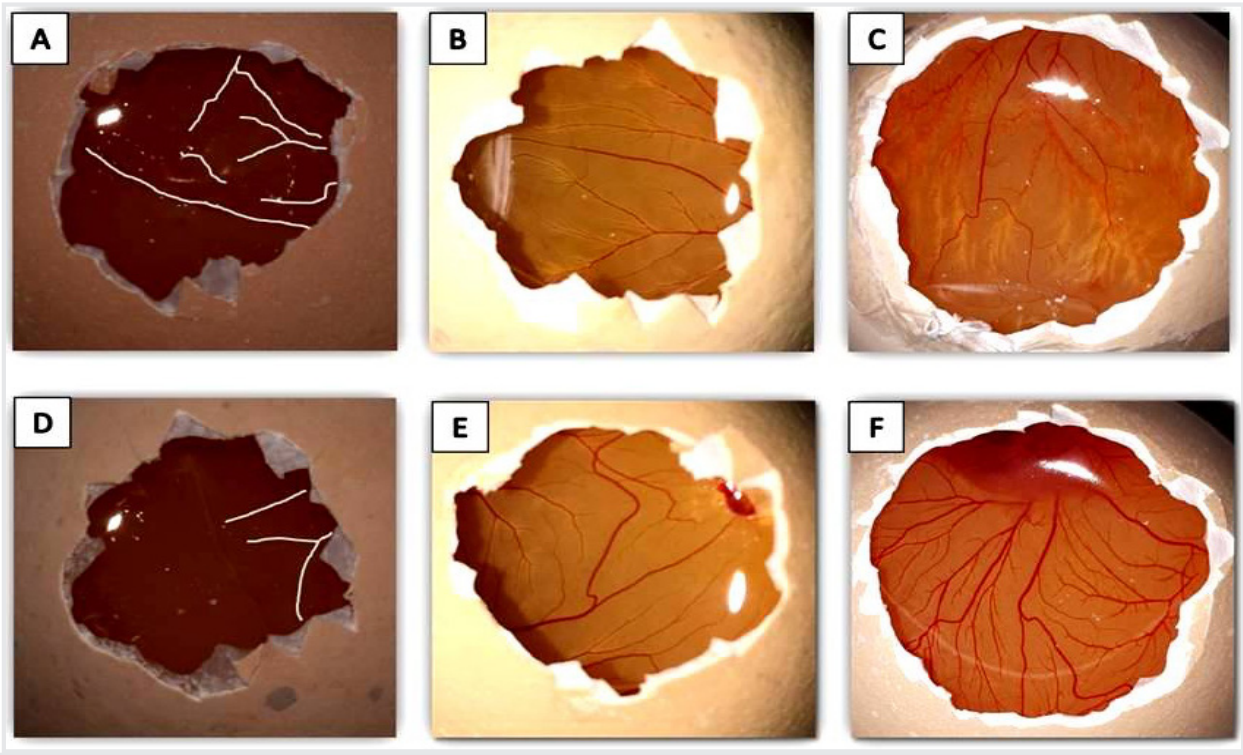

Figure 2. Effects of AuNPs on angiogenesis in the CAM model
A, B, C: Controls. A: On the 5th day (A), the eggshell was opened and then covered with parafilm. Macroscopic appearances on the 6th day (B) and 7th day (C) are shown.
C, D, E: AuNPs. On 5th day before AuNPs was not applied (D), on the 6th (E) and 7th day (F). Macroscopic appearances are shown.

Group			Efficiacy			
			Ineffective	+1	+2	Total
Day 6	Control	n	10	2	0	12
		%	83.3	16.7	0.0	100.0
	20mM	n	0	13	11	24
		%	0.0	54.2	45.8	100.0
Day 7	Control	n	9	3	0	12
		%	75.0	25.0	0.0	100.0
	20mM	n	0	4	7	11
		%	0.0	36.3	43.7	100.0

the inhibitory effect of AuNPs on angiogenesis of the rat retina. Conversely Marza et al. (32) by using a wound-healing model reported the stimulation effect of AuNPs on angiogenesis. Similarly, we detected that AuNPs induced angiogenesis on the CAM assay as *in vivo* model. We suggest that these contradictory results of the studies are not enough to certify the real effect of AuNPs on angiogenesis. However, based on the results we have obtained, we suggest that AuNPs may not be ideal NPs to make biosensors because of the risk of inducing angiogenesis in cancers. Conversely, since AuNPs increase angiogenesis, they should be beneficial in the treatment of various diseases characterised by ischaemia (33). Based on the notable features of AuNPs, they have long been evaluated as a potential tool for the diagnosis of several cancers (10). As a side effect, they may induce angiogenesis and secondarily may induce growth or metastasis of tumours.

Conclusion

As a result of this preliminary study, we suggest that further detailed studies are needed to assess the effect of AuNPs on angiogenesis and tumour growth and metastasis.

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Authorship Contribution

Surgical and Medical Practices: S.C., M.El., E.Y., E.A., M.Er., F.Y., S.M., R.Ç., M.Y.İ., M.E., Design: S.C., M.E., Concept: S.C., E.Y., M.E., Data Collection or Processing: S.C., M.Y.İ., O.F., Analysis or Interpretation: S.C., M.El., E.Y., E.A., M.Er., F.Y., S.M., R.Ç., M.E., Literature Search: S.C., M.El., E.A., M.Er., F.Y., S.M., R.Ç., M.Y.İ., Writing: S.C., M.El., E.Y., E.A., M.Er., F.Y., S.M., R.Ç., M.Y.İ., M.E.

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