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Evaluating the Effects of Enrofloxacin on Angiogenesis Using the Chick Embryo Chorioallantoic Membrane Model

Hadi Tavakkoli[*1], Javad Tajik[1], Mahdi Zeinali[2]

Abstract

Objective- Because little information was available in the literature about the angiogenic property of enrofloxacin, this investigation was undertaken to evaluate this aspect using an *in vivo* model.
Design- Experimental study.
Animals- Twenty fertile chicken eggs (Ross 308) with the average egg-weight of 55 ± 0.4 g were randomly divided into experimental and control groups.
Procedures- Agar pellets were prepared with enrofloxacin concentrations at a dosage of 10 mg/Kg egg-weight. A window was carved on the eggshell and drug-impregnated pellet applied on day 8 of incubation on the surface of the chick chorioallantoic membrane. The response of vascular plexus to the drug application was evaluated.
Results- Our results showed that enrofloxacin decrease angiogenesis as shown by a reduction in the morphometric parameters of vascular plexus including total vessel's length, the number of vascular branch, vascular complexity and capillary density ($p<0.05$).
Conclusion and Clinical Relevance- Based on findings, it is suggested that enrofloxacin offer a new class of anti-angiogenic agents that might augment the drugs available for the clinical treatment of angiogenesis related diseases.
Keywords- Angiogenesis, Chorioallantoic membrane, Enrofloxacin.

Introduction

The blood vessel network arises through two distinct processes, vasculogenesis and angiogenesis. Vasculogenesis refers to the de novo generation of blood vessels from vascular progenitor cells during embryogenesis, whereas angiogenesis refers to the formation of new capillaries from pre-existing blood vessels. Angiogenesis is a complex event that requires an exquisite interplay between different cell types. The angiogenic process is critical for the maintenance of oxygen homeostasis and is required in many physiological and pathological conditions, including embryonic development, wound healing, tissue regeneration and tumor growth.[1,2] Chick embryo chorioallantoic membrane (CAM), rabbit cornea, and rodent mesenteric are the most commonly used *in vivo* models of angiogenesis. The CAM model is often used due to its low-cost and simplicity, which facilitates experiment reproducibility.[3]

Enrofloxacin drug has been used across the globe for many years. It is effective against most gram-positive and gram-negative bacterial agents such as Salmonella, Aeromonas, Proteus, Brucella, Vibrio, Staphylococcus, Mycoplasma, Chlamydia, Campylobacter, Shigella and Mycobacterium. It is a bactericidal agent. The bactericidal activity of enrofloxacin is concentration-dependent and susceptible bacterial cell death occurring within 20–30 minutes of exposure.[4,5] Its mechanism of action is not thoroughly understood, but it is believed to act by inhibiting bacterial DNA gyrase (a type-II topoisomerase), thereby preventing DNA supercoiling and DNA synthesis.

Although increasing consumption of enrofloxacin compounds is predicted in veterinary medicine, there is little information about the angiogenic property of this drug. Thus, the current study was performed in CAM as a model to investigate the angiogenic and/or anti-angiogenic properties of enrofloxacin.

[1]Department of Clinical Science, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran
[2]Student of Veterinary Medicine, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran
Address all correspondence to Hadi Tavakkoli (PHD)
E-mail: Tavakkoli@uk.ac.ir
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Materials and Methods

Egg

Fertile chicken eggs (Ross 308) with the average egg-weight of 55 ± 0.4 g were purchased from a local breeder farm with standard condition of breeding.

Drugs

Enrofloxacin 5% injectable solution was obtained from the Razak Pharmaceutical Company, Iran. Each milliliter of drug contains 50 mg enrofloxacin.

Experimental protocol

Eggs were incubated in horizontal position at 37.7ºC and 80% relative humidity. On day 3 of incubation period, the egg surface and all instruments used to handle the eggs were disinfected with 70% ethanol. Then 4 ml of albumin was taken from the pointed end of the eggs, using 5-ml syringe with a 21-gage needle, to allowing detachment of the embryo from the eggshell. A 1×1.5 cm window was carved to open a window on the other side of the eggs on the day 4 of incubation. Normal embryonic development was verified and embryos with malformations or dead embryos were excluded from the study. The window was sealed with a sterile drape and thereafter, the eggs were returned to the incubator to have CAM reached approximately 2 cm in diameter. On Day 8, the sterile drape was removed and agar pellet impregnated with enrofloxacin was placed on the CAM of each egg. The drape was replaced and the eggs were allowed to incubate for 72 hours. The level of angiogenesis around the pellet was evaluated after the incubation period. Ten eggs were used for drug-treatment group, and pellets containing only agar comprised the negative control group. Pellets that caused an inflammatory response resulting in embryo toxicity were excluded from the study. Although the result of the first trial was recorded, the experiment was repeated for a total of two trials with similar findings. The experiment was performed according to the suggested European ethical guidelines for the care of animals in experimental investigations.

Pellet preparation

Agarose (Merck, Darmstadt, Germany) was added to distilled water to make a 2.5% weight of agarose to the total volume of solution mixture. The solution was sterilized in the autoclave and then cooled in a sterile container to 37 °C. After cooling, the drug was added into the mixture. Enrofloxacin at a dosage of 10 mg/Kg egg-weight was used. Then a 10 µl drop of the agar-enrofloxacin mixture at the desired concentration was pipetted into previously sterilized cylindrical stainless steel rods of 5 mm diameter to form circular pellets. The pellets were allowed to further solidify at a sterile condition.

Angiogenesis evaluation

The vascular plexus in the CAM in response to the drug-impregnated pellet was evaluated. The evaluation specifically accounts for changes in total vessel's length, the number of vascular branch, vascular complexity and capillary density around the pellet and were previously described. [6-9] Briefly, en face images of the CAM were captured using a stereoscopic microscope (Olympus, Japan). Images at high magnification (instrumental magnification·2.5×1×10) and in good focus were used. Four areas (each approximately 19 mm^2 containing 500·×500 pixels) were identified around the drug-impregnated pellet. Adobe Photoshop® (Version CS5 extended), ImageJ® (Version 1.48) and MATLAB® (Mathworks Matlab R2015a) software were used for the quantification and morphometric evaluation of vascular plexus in obtained images. The mean of evaluated parameters in all areas of captured image was calculated in each group. Vascular complexity was determined by fractal dimension (*D*) with the box-counting method. The value of the slope yields *D* value. The *D* value is used as an indicator of vascular complexity that combines branching and tortuosity of vessels but does not discriminate among these two parameters.[6, 9] For capillary density analysis, areas containing only capillary plexus were selected for quantification. The percentage of the area containing red pixels (i.e. blood) was quantified. [8, 10]

Statistical analysis

Statistical analysis was performed using SPSS version 20. The analysis of variance was used to determine the significant differences in vascular morphometric parameters between experimental groups. A P-value of <0.05 was considered as statistically significant.

Results

The images of the surface of the CAM are presented in figure 1. An apparent decrease in the vascular composition in enrofloxacin-treated group (Fig.1a) in comparison to control group (Fig.1b) was evident. The results of quantification and morphometric evaluation of vascular plexus are listed in table 1. A significant decrease was observed in the total vessel's length, the number of vascular branch and capillary density of enrofloxacin-treated group compared to control group ($p<0.05$). Figure 2 shows the influence of enrofloxacin on vascular complexity. A reduction in this parameter is also observed in enrofloxacin-treated group (Fig.2a) compared to control group (Fig.2b).

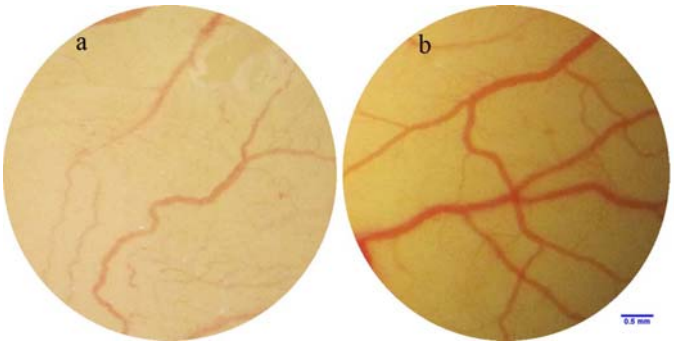

Figure 1. En face images of the surface of the chick embryo chorioallantoic membrane. An apparent decrease was observed in the vascular composition of enrofloxacin-treated group (a) compared to control group (b).

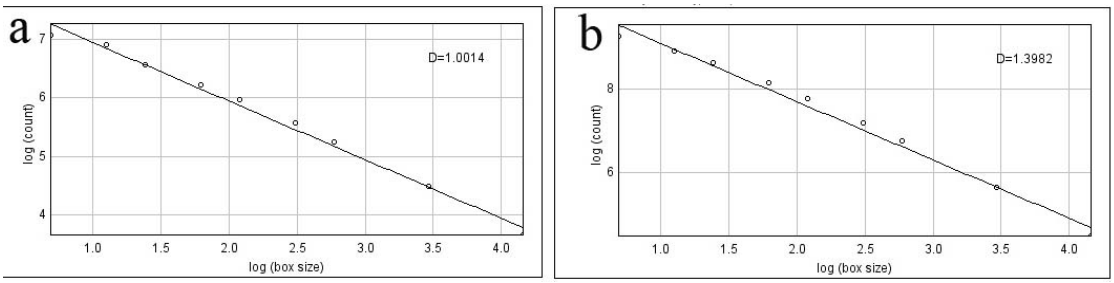

Figure 2. Effect of enrofloxacin on the vascular complexity parameter. A reduction was observed in the vascular complexity of the enrofloxacin-treated group (a) compared to control group (b). The vascular complexity is determined by fractal dimension (*D*) with the box-counting method. The value of the slope yields *D* value. The *D* value was used as an indicator of vascular complexity that combines branching and tortuosity of vessels but does not discriminate among these two parameters.

Table 1. Quantification and morphometric evaluation of vascular plexus in enrofloxacin-treated and control groups

Group	Quantification and morphometric parameters*		
	Total vessel's length (mm)	Number of vascular branch	Capillary density (%)
Enrofloxacin group	58.08 [a]	38 [a]	6.33 [a]
Control group	83.07 [b]	86 [b]	10.20 [b]

* Values are mean. Values in a column followed by different superscripts differ significantly ($p<0.05$).

Discussion

Fluoroquinolone have an increased role as therapeutic agents against animal and avian pathogens. They have bactericidal effect and a wide antibacterial spectrum. Most gram-positive and gram-negative organisms are susceptible. [11] Enrofloxacin belongs to the fluoroquinolone pharmacological group. It has been used successfully for several decades in many countries such as Canada, Spain, France, Austria, Polish, Denmark, Germany, Turkey, Africa, United States and China. In recent years, its use has increased rapidly in the Iranian animal and poultry industry, but there is little information available about the angiogenic property of enrofloxacin.

Our results showed that enrofloxacin decreased angiogenesis in the CAM model as shown by a reduction in the morphometric parameters of vascular plexus. The use of the CAM to evaluate the effect of enrofloxacin on angiogenesis provides a number of

unique advantages over other systems, and including the ability to observe and quantify changes on an ongoing basis and evaluation of the effects on rapidly growing or in comparatively mature blood vessels.[3]

It has been demonstrated that control and modulation of angiogenic activity are important for the development, repair, and growth of normal and abnormal tissues. Determining the angiogenic and/or anti-angiogenic properties of drugs on the CAM of chicken embryo is a useful method for studying the biological properties of drugs. Angiogenic antagonists reduce or diminish the tumor tissue neovasculature and prevent the establishment and growth of tumor cells. They can be developed for anti-angiogenic therapy and play an important role in the clinical treatment of a number of diseases including cancer, arthritis, psoriasis, and diabetic retinopathy. Anti-angiogenic property of few antibiotics has been reported in some researches.[8,12-20]

The cause of anti-angiogenic property of enrofloxacin is not clearly defined. This behavior may be due to the direct influence of the antibiotic on endogenous angiogenic agents. For example, angiogenin is a potent inducer of neovascularization and has been demonstrated to induce the formation of new blood vessels in the chicken CAM.[21] Hu (1998) suggested that neomycin, and its analogs are a class of antibiotics that inhibit nuclear translocation of human angiogenin in human endothelial cells and decrease angiogenesis. [13]

Another hypothesis to account for the anti-angiogenic activity of enrofloxacin is interaction of antibiotic with endothelial cells. Intervention in the interaction between agents and its target cells is involved in the inhibition of angiogenesis. In this way, several compounds of antimicrobial drugs have been reported to inhibit endothelial cell function and angiogenesis. These include doxycycline[8], anthracycline[22], 15-deoxyspergulin[23], D-penicillamine[24], eponemycin[25], fumagillin[12], herbimycin[25], and streptomycin.[8]

An additional explanation that leads to the inhibition of angiogenesis, is the specific structural features of antibiotics. For example, it is showed that among the aminoglycoside antibiotics, neomycin is the only one that shows inhibitory activity to angiogenin-induced cell proliferation, while, it is noteworthy that the structurally very similar analog, paromomycin, does not exhibit any inhibitory activity. It is therefore suggested that the amino group on the carbon 6 of the glucose ring plays an important role in this inhibition.[13] It is therefore possible that some specific structural aspects of enrofloxacin, which remain to be investigated, will also contribute to its anti-angiogenesis activity. Certain inherent properties of enrofloxacin have also been postulated to be associated with its effects. Now, new efforts are required to find new mechanisms that might be of great relevance in anti-angiogenic activity of enrofloxacin.

In conclusion, it is suggested that enrofloxacin offer a new class of anti-angiogenic agents that might augment the drugs available for the clinical treatment of angiogenesis related diseases. The anti-angiogenic property of enrofloxacin may be due to different factors including the influence on the angiogenic agents, interaction with endothelial cells, specific structural features and mechanisms, which contribute to the overall direct effect of the drug.

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چکیده

ارزیابی اثر داروی انروفلوکساسین بر رگ زایی با استفاده از مدل پرده کوریوآلانتوئیک جوجه

هادی توکلی [1*]، جواد تاجیک [1]، مهدی زینلی [2]

[1]بخش علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه شهید باهنر کرمان، کرمان، ایران
[2]دانشجوی دامپزشکی، دانشکده دامپزشکی، دانشگاه شهید باهنر کرمان، کرمان، ایران

هدف- از آنجا که اطلاعات کمی در مورد خصوصیات رگ زایی داروی انروفلوکساسین در دسترس می‌باشد، این تحقیق به منظور بررسی این جنبه با استفاده از یک مدل زنده انجام شد.

طرح- مطالعه تجربی

حیوانات- تعداد ۲۰ عدد تخم مرغ نطفه دار (راس ۳۰۸) با میانگین وزن ۰/۴ ± ۵۵ گرم به طور تصادفی به دو گروه آزمایش و کنترل تقسیم شدند.

روش کار- پلت آگار حاوی داروی انروفلوکساسین با دوز ۱۰ میلی‌گرم برکیلوگرم وزن تخم مرغ آماده شد. یک دریچه روی سطح پوسته تخم مرغ ایجاد گردید و پلت حاوی دارو در روز ۸ دوره انکوباسیون بر روی سطح پرده کوریوآلانتوئیک جوجه به کار برده شد. پاسخ شبکه عروقی به درمان دارویی بررسی گردید.

نتایج- نتایج نشان داد که داروی انروفلوکساسین باعث کاهش رگ‌زایی و کاهش پارامترهای مورفومتریک شبکه عروقی از جمله طول کل عروق، تعداد شاخه های عروقی، پیچیدگی و تراکم شبکه مویرگی گردید ($p<0.05$).

نتیجه‌گیری و کاربرد بالینی- بر اساس یافته‌ها، پیشنهاد می‌شود که داروی انروفلوکساسین یک گروه جدید از عوامل ضد رگ‌زایی را ارائه می‌دهد که می‌تواند به لیست داروهای موجود برای درمان بالینی بیماری های ناشی از عوامل رگ‌زایی اضافه گردد.

کلمات کلیدی- رگ‌زایی، پرده کوریوآلانتوئیک، انروفلوکساسین.