The Investigation of the Antiangiogenic Potential of Amiodarone HCl in the Chick Embryo Chorioallantoic Membrane Model

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Abstract

Angiogenesis, which plays a significant role in a variety of physiological processes, such as embryonic growth and wound healing, is strictly delimited and finely tuned by a balance of proangiogenic and antiangiogenic factors. Cardiac rhythm disorders are diseases that are often accompanied by vascular pathologies. As such, the purpose of this study was to investigate the antiangiogenic effects of Amiodarone HCl in the chorioallantoic membrane model. In this study, the antiangiogenic effect of Amiodarone HCl was compared with a positive control group that was given pure paraffin and the vascular endothelial growth factor inhibitor Bevacizumab, as well as a negative control group in which clearly antiangiogenic activity was shown in this model previously. Concentrations of $10^{-4}$, $10^{-5}$, and $10^{-6}$M of each drug were administered. For the purpose of determining the antiangiogenic effects of the drugs, blood vessels of the chorioallantoic membranes were evaluated using a stereoscopic microscope. The antiangiogenic effect scores of Amiodarone HCl at the dose of $10^{-4}$ molar (M) were higher than those of $10^{-5}$M and $10^{-6}$M, but that result was statistically insignificant. The antiangiogenic effect scores of Bevacizumab at the concentrations of $10^{-4}$M and $10^{-5}$M were significantly higher than that of $10^{-6}$M. This effect of Amiodarone may be important for determining routine antiarrhythmic doses.

Keywords: Chorioallantoic membrane; angiogenesis; Amiodarone HCl; Bevacizumab

Materials and Methods

Preparation of the pellets
In this trial, the possible antiangiogenic effect of Amiodarone HCl (Cordarone®, 150 mg/3ml Sanofi-Dogu Ilac AS, Istanbul, Turkey) was studied. The Amiodarone used was in its commercially available form as a soluble infusion. Agarose (Merck, Damstadt, Germany) was added to distilled water to obtain a 2.5% (w/v) solution, which was autoclaved at 121°C and under one atmos-
pheric pressure to provide dissolution and sterilization. Subsequently, it was cooled to 37°C in a sterile container, at which point the study drug was added. The positive control group was constructed with the antiangiogenic agent Bevacizumab, which is known as a specific vascular endothelial growth factor inhibitor [7].

Appropriate volumes of solutions were used to achieve three different concentrations of the drug (10^{-5}, 10^{-4}, and 10^{-3}M). Approximately sixty pellets were used for each study set. Therefore, approximately 1 ml of combined agar and drug solution (10 μl×100 =1 ml) was prepared initially for the drug. The drug solutions with 100 IU, 10 IU and 1 IU/10 μl concentrations were prepared by diluting these initial mixtures ten-fold with the agarose solution again.

Using a micropipette, 10-μl drops of this mixed solution were placed on previously sterilized, vertical, cylindrical, stainless steel rods 5 mm in diameter to obtain circular pellets with the same diameter. The pellets were then left to solidify at room temperature in a sterile setting.

**Chicken chorioallantoic membrane (CAM) assay**

Ross 308 strain fertilized hen eggs were obtained from Yemsel Poultry Company (Kayseri, Turkey). The study protocol was approved by the Local Animal Ethics Committee.

The fertilized hen eggs were incubated in a horizontal position, with environmental conditions of 37.5°C temperature and 80% relative humidity. On the fifth day of the incubation period, 5 ml of albumen were taken through the eggshell with a syringe (Figure 1A), and a shell piece 2-3 cm in diameter was removed from the opposite side of the eggs. Normal development of the CAM was verified (Figure 1B), and malformed or dead embryos were excluded. The windows on the eggshells were sealed with gelatin, and the eggs were incubated for 72 more hours, until the CAM reached 2 cm in diameter. Subsequently (on day 8), the seal was removed and the pellets were placed on the chorioallantoic membrane of each egg (Figure 1C). The seal was replaced and the eggs were incubated for 24 hours. The angiogenesis level was evaluated after that period.

There were five group created; pellet group without drug were utilized as negative control group (Group I), Bevacizumab was used for positive control group (group II), three different dosage of drug (10^{-5}, 10^{-4}, and 10^{-3}M) groups were classified as group III-IV-V respectively. For each tested drug solution, 20 eggs were used. All the tests were duplicated. Eggs in which the pellets caused inflammation and embryo toxicity were excluded. Totally 200 eggs were used for five created groups (20 for each group, 20 for confirmation each test). Negative control group consisted with pellet without drug, did not demonstrate any antiangiogenic behaviour.

**Angiogenesis scoring**

The inhibitory effects of the drugs on angiogenesis in chorioallantoic membrane were evaluated under a stereoscopic microscope and assessed according to the scoring system used previously in several studies [8,9].. In this scoring system, the change in the density of the capillaries around the pellet and the extent of the effect are evaluated (Figure 1D). In the initial scoring of each subject, a score of 0 indicated the absence of any demonstrable antiangiogenic effect (normal embryo and no difference in surrounding capillaries); 0.5 indicated a very weak antiangiogenic effect (no capillary-free area, but an area with reduced density of capillaries not larger than the pellet area); 1 indicated a weak moderate antiangiogenic effect (a small capillary-free area or a small area with significantly decreased density of capillaries, less than double the size of the pellet); and 2 indicated a strong antiangiogenic effect (a capillary-free area around the pellet equal to or more than double the size of the pellet). The equation used for the determination of the average score was as follows:

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\text{Average score} = \frac{\text{Number of eggs (Score 2) × 2 + Egg Number (Score 1) × 1}}{\text{Total number of eggs (Score 0, 1, 2)}}.
\]

According to this scoring system, a score of 0.5 meant that there was no antiangiogenic effect, a score of 0.5 to 1 indicated a weak antiangiogenic effect, and a score of N1 implied a strong antiangiogenic effect.

**Statistical analysis**

The angiogenesis scores were compared with a Kruskal-Wallis ANOVA test and a Mann-Whitney U test. A p value of less than 0.05 was considered statistically significant.

**Results**

Drug-free pellets of 10 μl agarose solution were used as negative controls; there were no significant antiangiogenic effects observed in the negative controls (average antiangiogenic score=0.1). Bevacizumab was used as the positive control and had an average antiangiogenic score of 0.95 (very good antiangiogenic effect) in 10^{-3}M concentrations. Each tested drug was evaluated separately, and the results with different solutions were compared.

Our findings indicated that antiangiogenic behaviours were relatively increased with dose escalation. There
were mildly antiangiogenic effects observed in $10^{-4}$ M (M: Molar) concentration of Amiodarone, with antiangiogenic scores of 1.0 in one egg and 0.5 in two eggs. The 0.5 antiangiogenic scores in the two eggs were observed at $10^{-5}$ M concentration of Amiodarone. The antiangiogenic score of 0.5 in the one egg was at a $10^{-6}$ M concentration of Amiodarone (Fig 2). No statistically significant differences were observed between these dose-related antiangiogenic tendencies ($p>0.05$).

$10^{-6}$ M concentrations of Bevacizumab displayed significantly higher antiangiogenic effects than all the dosages of Amiodarone HCl ($p<0.05$) (Fig 2).

**Figure 1.** A: The removal of albumin from egg; B: The appearance of the chorioallantoic membrane (CAM) through a window on the egg shell (X8); C: The placement of the pellet on the CAM (X8); D: Inhibition of the capillaries on the CAM (score: 0.5) by the drug (X8).

**Figure 2.** The anti-angiogenic scores of Bevacizumab and Amiodarone HCL (a: comparison of Amiodarone HCl and Bevacizumab. B: comparison of $10^{-6}$ M and $10^{-5}$ M concentration of Amiodarone HCL)

Discussion

Angiogenesis is a vital process that plays a role in embryonic development, menstrual cycle, and wound healing; it is controlled by proangiogenic and antiangiogenic factors [2,10]. Recently, the CAM model has long been preferred for the studies on tumour angiogenesis and metastasis, as well as the studies of the standard drug molecules with angiogenic or antiangiogenic activity [11]. The myocardium is a highly oxygen-dependent tissue, and the continuity of blood flow is critical for functional protection in acute ischaemic conditions. Thus, therapeutic angiogenesis for myocardial tissue has gained importance with recent advances. However, recent studies indicate that maintaining existing physiological angiogenesis response is important in stimulating neo-angiogenesis with therapeutic methods [12,13]. Both regulatory factors and basic mechanisms that enrolled at myocardial angiogenesis should be investigated for the defining possible therapeutic approaches [13].

Amiodarone HCl effectively controls a wide spectrum of atrial and ventricular arrhythmias. However, because of the wide spectrum of the mechanism of action of Amiodarone and the limiting possible side effects, such as thyroid dysfunction, pulmonary fibrosis, and dermatologic changes, its long-term use may be limited in some patients, and care should be taken when administering the drug [14]. These side effects have prompted researchers to observe other potential effects of Amiodarone HCl. Traupe et al. showed that Amiodarone rapidly accumulates in atherosclerotic vascular tissue, abolishes vascular autorhythmicity, and improves endothelium-dependent function in atherosclerotic arteries. The researchers suggested that these effects following Amiodarone administration might contribute to its antiarrhythmic effects [15]. Chen et al. found that Amiodarone treatment caused failure of cardiac valve formation in zebrafish embryos [6]. These complex effects have led to the notion that Amiodarone HCL can affect neo-angiogenesis in regenerating tissues such as the myocardium. As such, we investigated the possible antiangiogenic effects of Amiodarone HCL on a CAM model in this study; non-significant antiangiogenic effects were observed at a $10^{-4}$ M dose of Amiodarone HCL, with antiangiogenic scores of 1.0 in one egg and 0.5 in two eggs ($p>0.05$). The antiangiogenic activity of Bevacizumab, known as a specific vascular endothelial growth factor inhibitor, was naturally higher than that of Amiodarone HCL ($p<0.05$).

Our results revealed that Amiodarone HCl shows dose-related antiangiogenic effects. However, this effect is mild and is encountered at high doses. According to these data, the antiangiogenic effect of Amiodarone HCl seems to be safe for administration in different types of arrhythmias; however, the other possible side effects should not be ignored. Regarding antiangiogenic behaviour, Clinicians should be careful for only the continuous loading dose.
References


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