Microscopy and Microanalysis (2019), **25**, 961–970
doi:10.1017/S1431927619000588

Microscopy AND Microanalysis

Original Article

Live Imaging and Analysis of Vasoactive Properties of Drugs Using an *in-ovo* Chicken Embryo Model: Replacing and Reducing Animal Testing

Akila Swaminathan[1], Uma Maheswari Balaguru[1], Reji Manjunathan[1], Srinivasan Bhuvaneswari[1,2], Dharanibalan Kasiviswanathan[1,2], Bandi Sirishakalyani[3], Prasunpriya Nayak[3,4] and Suvro Chatterjee[1,2]*

[1]Vascular Biology Lab, AU-KBC Research Centre, Anna University, MIT Campus, Chennai, India; [2]Department of Biotechnology, Anna University, Chennai, India; [3]Department of Physiology, NRI Medical College & General Hospital, Andhra Pradesh, India and [4]Department of Physiology, All India Institute of Medical Sciences, Jodhpur, India

Abstract

Vasodilation occurs as a result of the relaxation of the smooth muscle cells present in the walls of blood vessels. Various suitable models are available for the analysis of the vasoactive properties of drugs with therapeutic applications. But all these models have limitations, such as ethical issues and high cost. The purpose of this study is to develop an alternative model for studying the vasoactive properties of drugs using an *in-ovo* chicken embryo model. In the preliminary experiment, we used a well-known vasoconstrictor (adrenaline) and a vasodilator (spermine NoNoate) in the chick embryo area vasculosa and evaluated their concentration-response curve. Adrenaline (10 μM) and spermine NoNoate (10 μM) were administered in different arteries and veins and different positions of the right vitelline artery of the chick embryo. Results showed the middle of the vessel bed of the right vitelline artery having the best vasoactive effect compared to others. Finally, anti-hypertensive drugs, calcium channel blockers, and NOS agonists were administered in the chick embryo area vasculosa to validate the model. Results demonstrate that the chick embryo area vasculosa can be an alternative, robust, and unique *in-ovo* model for screening of anti-hypertensive drugs in real time.

Key words: alternative model, antihypertensive drugs, chick embryo area vasculosa, live-imaging, vasorelaxation

(Received 5 March 2018; revised 25 March 2019; accepted 14 April 2019)

Introduction

Vascular tone is defined as the control over the relaxation and constriction of blood vessels. Arteries and venous vessels exhibit some degree of smooth muscle-dependent constriction in basal conditions that determine the diameter of the blood vessels (Klabunde, 2011). Homeostasis of blood flow is very critical to survival (Nemeno-Guanzon et al., 2012). An increase in arterial blood pressure manifests in a condition called hypertension, which leads to several clinical manifestations such as angina and congestive heart failure (Klabunde, 2011). Intrinsically, vasorelaxation is regulated by several mediators such as extracellular matrix components, metabolic factors, secreted growth factors, and nitric oxide (NO) (Rensen et al., 2007). Deregulation of vasomotor tone leads to a wide range of vasculopathies including hypertension. Therefore, proper maintenance and treatment of hypertension is a key step to a healthy life. Various animal-based vasorelaxation models have been reported using rat, mouse, and dog (Su et al., 2000; Wenzel et al., 2006). Further, an *in-vitro* contraction assay, using cell lines, is also available for the screening of the vasodilation properties of drugs used in the treatment of hypertension (Lijnen et al., 2001). However, these assays are technically challenging since they require the use of animals and sophisticated infrastructural support.

The current study aims at the development of an alternative model for screening vasoactive drugs using chick embryos on the 5th day of incubation. In the chick embryo, the networks of blood vessels develop in the inner part of the area opaca which then becomes the area vasculosa on the 2nd and 3rd days of incubation (Sheng, 2010). The chick embryo area vasculosa is composed of the central vascular region of the vitelline membrane and extends around the yolk. The main function of the vessel is to transport blood and to absorb nutrients from the yolk (Stewart et al., 1989). The rationale for using the chick embryo area vasculosa as an alternative model includes its extensive vascularization, easy accessibility, its rapid development, comparatively large size during the early phase of development (Ribatti et al., 2000; Vergara & Canto-Soler 2012), and fewer ethical concerns. The chick embryo vitelline arteries and vitelline veins were treated with vasodilator and vasoconstrictor with different doses and variations in the duration of the incubation. The model was validated using three types of experiments: (1)

***Author for correspondence:** Suvro Chatterjee, E-mail: soovro@yahoo.ca

Cite this article: Swaminathan A, Balaguru UM, Manjunathan R, Bhuvaneswari S, Kasiviswanathan D, Sirishakalyani B, Nayak P, Chatterjee S (2019) Live Imaging and Analysis of Vasoactive Properties of Drugs Using an *in-ovo* Chicken Embryo Model: Replacing and Reducing Animal Testing. *Microsc Microanal* **25**, 961–970. doi:10.1017/S1431927619000588

Measurement of the vasorelaxation and the vasoconstriction activity of spermine NoNoate and adrenaline in the chick embryo area vasculosa. (2) Identification of the specific blood vessel that responds most to vasoactive treatment in the chick embryo area vasculosa. (3). Search for specific areas of the right vitelline vein that responds most to vasoactive treatment in the chick embryo area vasculosa. Further, we used anti-hypertensive drugs, calcium channel blockers, and recently reported NOS agonist for validating the *in-ovo* model.

Materials and Methods

Materials

Reagents

Spermine NoNoate was purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). Amlodipine and Atenolol were purchased from Sigma Chemicals (St. Louis, Missouri, USA). Allyl sulfide was purchased from ChemSpider (Raleigh, North Carolina, USA). L-Theanine was purchased from Sigma Chemicals (India). Adrenaline, Diltiazem and Acetazolamide drugs were obtained from local medical suppliers.

In-ovo *Model*

Fertilized White Leghorn chick (*Gallus domesticus* L.) eggs were obtained from the Poultry Research Station, Potheri, Chennai, TN, India and incubated at 37–38°C (Southern egg incubators), and at a relative humidity of 60%.

Methods

Preparation of Windowed Egg Model

The eggs were wiped with 70% ethanol. A small hole was made in the air cell of the egg using an egg driller on the 5th day of incubation. A small part of the eggshell was cut open and then the outer shell membrane was removed using sterile forceps (blunt end) to expose the entire developing chick embryo area vasculosa for video recording. (Siamwala et al., 2013).

Preparation of Test Solutions and Mode of Administration

Different concentrations of drugs were prepared using 1X phosphate buffer saline (PBS). A sterile Whatman No.1 filter paper disc was soaked in drug solution under test and was placed on the right vitelline artery using sterile forceps. The disc absorbed approximately 20 μL of the drug solution and the rest of the drug diffused from the disc and finally reached the surrounding blood vessels. Control embryos were treated with 1X PBS. Each drug concentration was tested in triplicate.

Vasoactivity Assay

The eggshell was broken gently under the sterile condition. A sterile Whatman No.1 filter paper disc was soaked in spermine NoNoate (0.01–100 μM) and adrenaline (0.01–100 μM) drug solution and was placed on the right vitelline artery using sterile forceps. Next, videos were recorded with a Sony Handycam attached to a stereomicroscope at a 1X magnification for a period of 20 min. Snapshot images were taken from the videos and the width of the targeted blood vessels was measured from these images using Image J software and Adobe Photoshop (Siamwala et al., 2013).

Analysis of the Vasoactive Effect of Drugs at various Arteries and Veins of the Chick Embryo Area vasculosa

The eggshell was broken gently under the sterile condition and effective concentration of spermine NoNoate (10 μM) and adrenaline (10 μM) was applied as described earlier. The drug was applied to different blood vessels, namely (A) Anterior vitelline vein, (B) Right vitelline artery, (C) Right lateral vitelline vein, (D) Posterior vitelline vein, (E) Left vitelline artery, (F) Left lateral vitelline vein (Fig. 2a), and videos were recorded at an original1X magnification from 0 to 20 min as described above. The brightness and contrast [blood vessel in red color and chick embryo area vasculosa in yellow color (Fig. 1a)] were adjusted in the snapshot images using Image J software. The modified images were opened in Image J. The width measurement tool for Image J was used for the calculation of the width of the blood vessels. Finally, the percentage of the width of the blood vessels was calculated and a vasoactivity graph was plotted. For the identification of the most responsive location of the blood vessels, the drugs were applied to the different location of the vessels; vessel near to the embryo (VNE) vessel middle of the bed (VMB) and vessel at the edge of the bed (VEB) (Fig. 3a).

Histology of the Chick Embryo Area vasculosa

Fertilized eggs incubated at 37°C in a humidified atmosphere and at a relative humidity of 60%, were used for the histology study of the smooth muscle cells in the chick embryo area vasculosa. On the 5th day of incubation, area vasculosa of the fertilized egg was exposed to the drugs. The chick embryo area vasculosa was detached along with the developing embryo without any damage using butter paper to avoid tearing and folding of the membrane. The membranes were cut into three pieces in different areas VNE, VMB, and VEB, and were fixed in neutral formalin. The membranes were put into dehydrating fluid (60–70% of ethanol) and embedded in paraffin. The specimens were sliced into 5-μm sections and stained with Haematoxylin and Eosin according to the standard protocol. Images were taken at 20X original magnification using an Olympus fluorescence inverted microscope. (Manjunathan & Ragunathan, 2015).

Masson's Trichrome Staining

On the 5th day of incubation, the area vasculosa of the fertilized egg was exposed by removing the shell in aseptic conditions. The membrane was fixed in neutral formalin and was put into dehydrating fluid (60–70% of ethanol) followed by embedding in paraffin. The specimen was sliced into 5-μm sections and stained with Masson's trichrome staining according to the standard protocol. The images were taken at 400X original magnification using an Olympus fluorescence inverted microscope.

Measurement of the Heartbeat of the Chick Embryo as a Heart Function Parameter After Treatment

The eggshell was broken gently under sterile conditions as described above. Next, Whatman No.1 paper disc was soaked in the drug solution (spermine NoNoate and adrenaline) and was placed on the right vitelline artery using sterile forceps. The chick embryo heart was focused and video-graphed for 5 min using a Sony Handycam attached to a stereomicroscope at 1X original magnification. The heartbeat was counted manually and calculated as beats/min.

https://doi.org/10.1017/S1431927619000588 Published online by Cambridge University Press

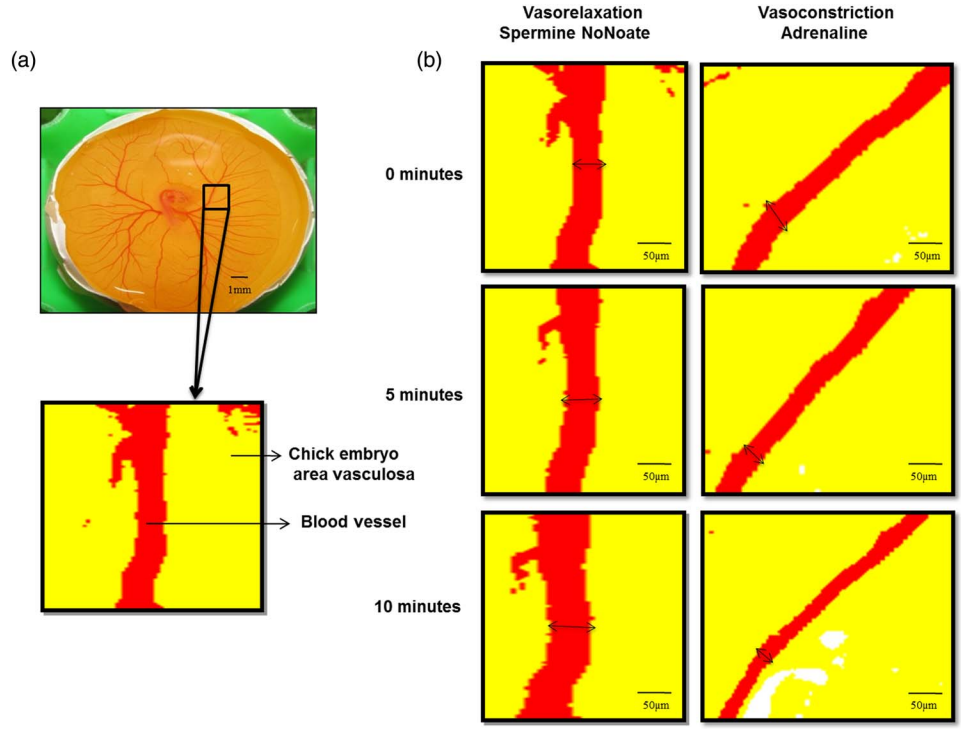

Fig. 1a. Chick embryo area vasculosa shows concentration and time-dependent vasodilatory and constrictory effect upon treatment. **a:** Representative images of the early chick embryo area vasculosa highlighted with a single large vessel. **b:** Chick embryo area vasculosa shows increased vasodilation under spermine NoNoate treatment and increased vasoconstriction under adrenaline treatment. **c:** Determination of effective time of the drug-treatments. **d:** Fold change in the percentage of the diameter of the blood vessel after spermine NoNoate treatment. The vessel was treated with Spermine NoNoate of 0.01, 0.1, 1, 5, 10, and 100 μM concentrations for 10 min. The result showed the effect *of* Spermine NoNoate of 10 μM concentration with the maximum vasodilatory after 10 min of incubation. **e:** Fold change in the percentage of the diameter of the blood vessel after Adrenaline treatment. The vessel was treated with Adrenaline of 0.01, 0.1, 1, 5, 10, and 100 μM concentrations for 10 min. The result showed *the effect of* Adrenaline of 10 μM concentration with the maximum vasoconstrictory after 10 min of incubation. Experiments were performed in triplicate and *the* data have been presented as mean + SEM, (**$p \leq 0.001$, *$p = 0.003$; ¥ $p = 0.012$ and #$p = 0.004$ compared to control). Images are representative of five sets of experiments with 1X magnification. Scale bars: 1 mm in 1(**a**) and 50 μm in 1(**b**).

Assessment of Drug Activity on the Chick Embryo Area vasculosa Under various Levels of Treatment

The eggshell was broken gently under sterile condition. Videos of the vascular activities were taken for 0–10 min before the drug treatment. Next, sterile Whatman No.1 paper disc was soaked in the drug solution (Spermine NoNoate) and was placed on the right vitelline artery using sterile forceps. Videos were recorded for 10 min. A 500 μL of PBS was added on the right vitelline artery and PBS was taken using a pipette (washing step repeated twice). Videos were recorded for 10 min. After washing, spermine NoNoate was applied on the same right vitelline artery and videos were recorded for 10 min, followed by rinsing with PBS. Videos were taken again for 10 min. The same procedure was followed for the adrenaline treatment in the chick embryo area vasculosa. In all the cases, videos were recorded using a Sony Handycam camera attached to a stereomicroscope at a 1X original magnification continuously for the specified duration. Images were obtained from the videos using snapshots. The vessel width was quantified from images using Image J and Adobe Photoshop.

Evaluation of Vasodilation Activity of Drugs on the Right Vitelline Artery of the Chick Embryo Area vasculosa

Vasodilation activity of the various drugs such as antihypertensive drugs [Atenolol (0.1 μM) and acetazolamide (100 μM)], calcium channel blockers [amlodipine (10 μM) and diltiazem (10 μM)], and NOS agonist [L-theanine (0.1 μM) and allyl Sulfide (20 μM)] were evaluated using the 5th day incubated chick embryo area vasculosa. The experimental procedure was followed as mentioned earlier.

Comparative Study of Vasoactivity of Drugs Using Chick Embryo Area vasculosa

In a comparative study, a known vasoconstrictor (adrenaline) was applied to the middle of the right vitelline artery of the 5-day chick embryo area vasculosa. Videos were recorded for 10 min. A 500 μL of PBS was added on the middle of the right vitelline artery and PBS was taken using a pipette (washing step was repeated twice). The same blood vessel was treated with atenolol, a known vasodilator, and videos recorded as described earlier.

Statistical Analysis

Data were analyzed using the one-way ANOVA test, Student t-test, and Turkey post hoc tests using sigma stat software. All data are presented as mean ± standard error mean (SEM). Data with a p-value less than or equal to 0.05 was considered as statistically significant.

https://doi.org/10.1017/S1431927619000588 Published online by Cambridge University Press

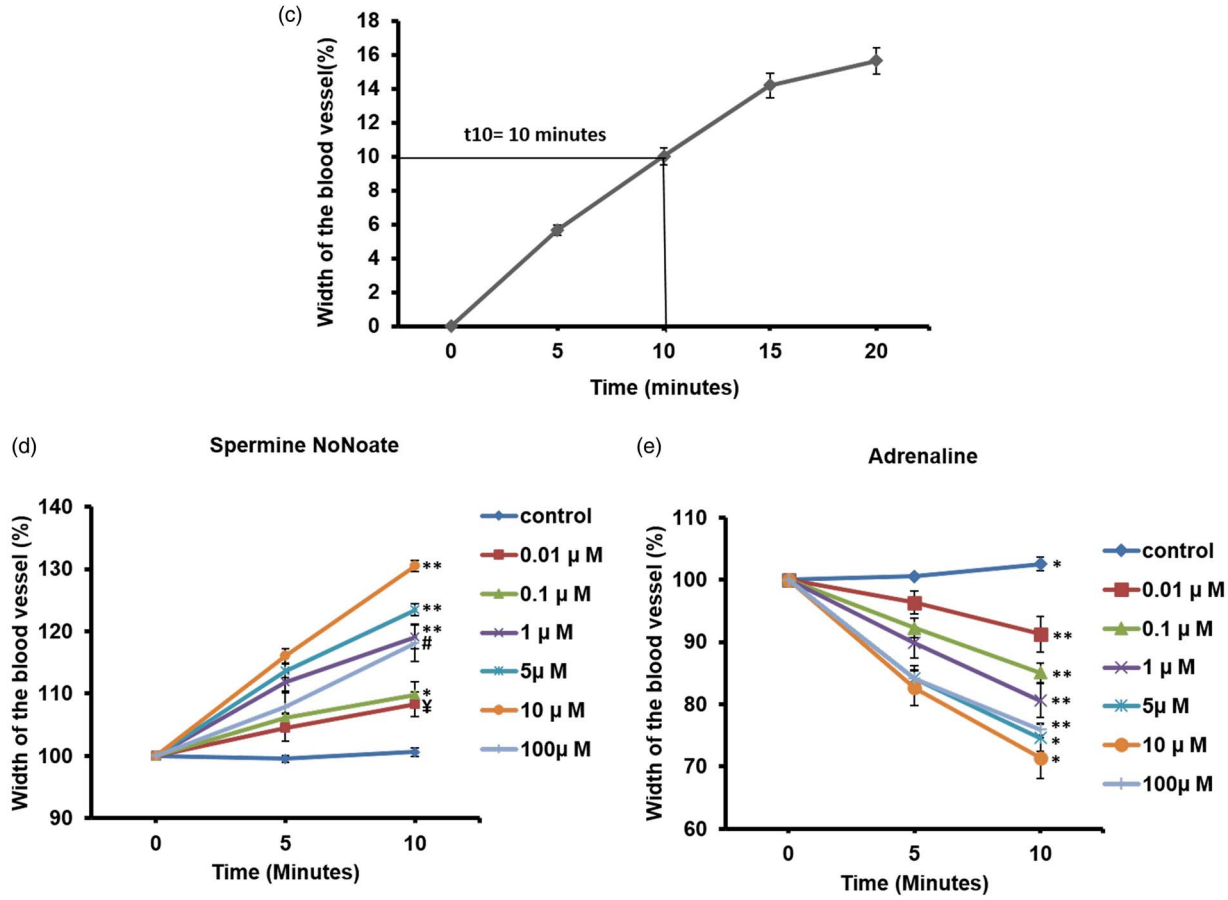

Fig. 1. Continued.

Results

Chick Embryo Area vasculosa as a Suitable Model to Analyze Vasoactive Properties of Drugs

Each drug was prepared at various concentrations; spermine NoNoate (0.01–100 μM) and adrenaline (0.01–100μM), and the chick embryo area vasculosa was treated with the drugs. This was done for the purpose of determining the vasorelaxation and the vasoconstriction activity of spermine NoNoate and adrenaline respectively. The vasoactive effects of the drugs were measured using Image J software from the images taken at 0, 5, and 10 min of incubation (Figs. 1a, 1b). Chick embryo blood vessel was treated with 10 μM of spermine NoNoate and the vascular effect was measured from the images taken at 0, 5, 10, 15, and 20 min after the treatments initiated. We identified the effective treatment time as 10 min for the drug (Fig. 1c). Results showed spermine NoNoate induced concentration depending on the vasodilation in the chick blood vessel compared to the control. These data demonstrated 10 μM of spermine NoNoate showing a remarkable vasodilation effect at 10 min of incubation (Fig. 1d), while treatment with adrenaline (10 μM) induced concentration depended on the vasoconstriction in the chick blood vessel (Fig. 1e). Altogether, the data showed both spermine NoNoate and adrenaline induced concentrations and time-dependent vasodilatory and vasoconstrictive effects, respectively, on the chick blood vessel. The data clearly established a link between the rates of the heartbeat and the drug treatments of the embryo (Table 1).

Analysis of Vasoactive Properties of Drugs with Respect to various Arteries and Veins of Chick Embryo

The aim of this experiment was to identify the most responsive blood vessel in the area vasculosa after treatment with spermine NoNoate and adrenaline. (Fig. 2a) The results showed that spermine NoNoate and adrenaline exerting maximum vasoactive effects on the right vitelline artery (Figs. 2b, 2c). Altogether, the data showed spermine NoNoate induced vasorelaxation 8.9% of the width of the right vitelline artery when compared to other arteries and veins (Figs. 2b, 2c).

Chick Embryo Area Vasculosa Demonstrated the Area-Specific Vasoactive Effect

The chick embryo area vasculosa was divided into three major zones on the basis of the position of the blood vessel from the developing embryo; VNE, VMD, and VED (Fig. 3a). The results demonstrated spermine NoNoate and adrenaline exerts maximum effects on blood vessels in the middle area of the vascular bed defined as VMD (Figs. 3b, 3c). The data showed spermine NoNoate relaxed 19% of the width of the vessel, while adrenaline constricted 26% of the width of the vessel (Figs. 3b, 3c).

https://doi.org/10.1017/S1431927619000588 Published online by Cambridge University Press

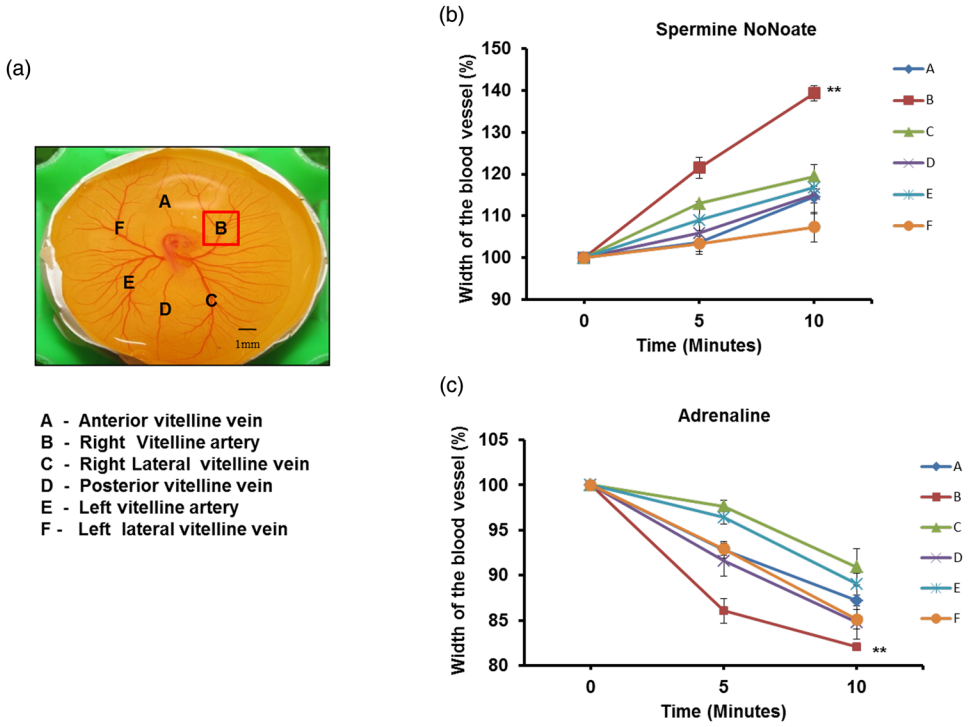

Fig. 2. Chick embryo area vasculosa shows vessel specific vasodilatory and constriction effect upon treatment. **a:** Representative images of the early chick embryo area vasculosa highlighted with various arteries and veins. Denotation stands for A—Anterior vitelline vein, B—Right Vitelline artery, C—Right Lateral vitelline vein, D—Posterior vitelline vein, E—Left vitelline artery and F—Left lateral vitelline vein. **b:** Fold change in the percentage of the diameter of various vessels after 10 μM concentration spermine NoNoate treatment. The result showed the right vitelline artery among the vessels analyzed exerting the maximum vasodilatory effect at 10 min of incubation. **c:** Fold change in the percentage of the diameter of various vessels after 10 μM concentration of Adrenaline treatment. The result showed the right vitelline artery among the vessels analyzed exerting the maximum vasoconstrictory effect after 10 min of incubation. Experiments were performed in triplicate and the data have been presented as mean + SEM, (**$p \leq 0.001$compared to the Right lateral vitelline vein; Anterior vitelline vein; Posterior vitelline vein; left vitelline artery and left lateral vitelline vein treated with Spermine NoNoate and treated with Adrenaline respectively; $n = 5$). Scale bars: 1 mm in 2(**a**).

Analysis of Vasoactivity Regulated by Smooth Muscle Cells in the Chick Embryo Area Vasculosa

Vascular smooth muscle cells play a key role in maintaining the volume of the blood vessel and local blood pressure (Zhao et al., 2010). Extraembryonic tissue is produced by the animal embryo to provide protection and nutrition. A developing embryo contains four membranes namely yolk sac, amnion, chorion, and allantois (Sheng, 2010). Hence, we performed a histological evaluation of the extraembryonic tissues, which was categorized into three major zones based on the blood vessel position from the embryo namely (1) Membrane near to the embryo, (2) Membrane at the middle of the bed, and (3) Membrane at the edge of the bed as marked in the chick embryo vascular bed given in (Fig. 4a). Figure 4b presents a clear distinction between small blood vessels and large blood vessels (Fig. 4b).

Histological evaluation of the membrane section very close to the embryo showed the presence of both large and small blood vessels which were clearly identified by the presence of trapped nucleated RBC. Blood islands or blood sinus occupied a large area of the membrane. Epithelial cells showed a loose pattern of cellular arrangement at both the ectodermal and endodermal layers. Epithelial cells around the blood vessels were highly compressed and irregularly arranged. It was very difficult to distinguish between the arteries and the veins due to lack of smooth muscles at the arteries in this specific histological section (Fig. 4c).

Histological evaluation of the membrane section middle of the vascular bed (Fig. 4d) showed a number of large and small blood vessels compared to the other two sections. Irrespective of the size of the vessels, most of the vessels showed the presence of a layer of smooth muscle cells in the wall. Veins were clearly distinguished from the arteries due to the presence of a large number of smooth muscle cells in the arteries (Fig. 4d). Epithelial cells were highly differentiated and arranged in a compact fashion (Figs. 4c, 4e).

Histological evaluation of the membrane section at the edge of the bed of the developing embryo shows a large number of blood vessels compared to the membrane section near the embryo (Fig. 4e). It was difficult to distinguish between the arteries and the veins situated near the embryo in the sections of the membrane. There were fewer or no blood islands or blood sinuses observed in this section. Epithelial cells were tightly packed both at ecto and endodermal layers when compared to the membrane section very near to the embryo.

The membrane section at the middle of the bed section was stained with Masson's trichrome staining to enable identification of smooth muscle cells. Masson's trichrome image confirmed the presence of smooth muscle cells (purple color) around the blood vessel wall (Fig. 4f).

Analysis of the Impact of Vasoactive Drugs Repeated Conformation Using Chick Embryo Area Vasculosa

The developing chick embryo area vasculosa had the ability to respond to the chemicals spontaneously and could maintain its

https://doi.org/10.1017/S1431927619000588 Published online by Cambridge University Press

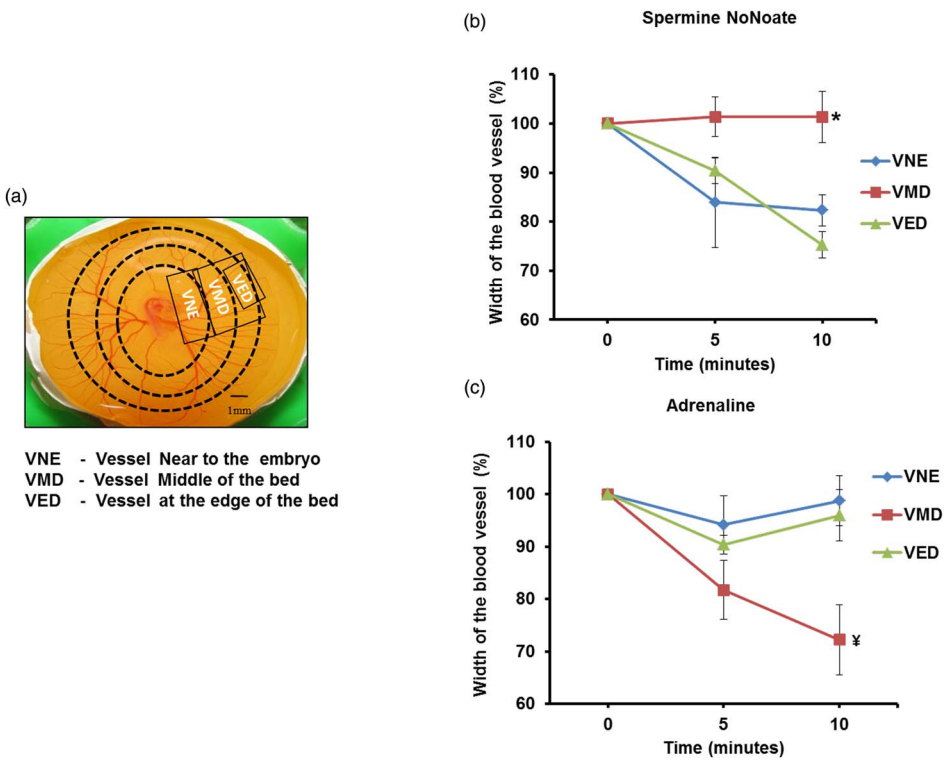

Fig. 3. Chick embryo area vasculosa shows area specific vasodilatory and constrictory effect upon treatment. **a:** Representative images of the early chick embryo area vasculosa marked as three different zones. Denotation stands for VNE—Vessels near to the embryo, VMD—Vessels at the middle of the bed and VED—Vessels at the edge of the bed. **b:** Fold change in the percentage of the diameter of a different zone after 10 μM concentration of spermine NoNoate treatment. The result showed that the vessels, among these in the analyzed zone present at the middle zone of the membrane exerting maximum vasodilatory effect at 10 min of incubation. **c:** Fold change in the percentage of the diameter at different *zones* after 10 μM concentration of Adrenaline treatment. The result also showed the vessel which was present at the middle zone of the membrane among these in the analyzed zone exerting *the* maximum vasoconstrictory effect at 10 min of incubation. Experiments were performed in triplicate and *the* data have been presented as mean + SEM, (*$p = 0.010$ for spermine NoNoateand ¥$p = 0.046$ for adrenaline compared to the vessel near to the embryo; $n = 3$). Scale bars: 1 mm in 3(**a**).

Table 1. Observation of Heat Beats of the Chick Embryos in Different Drug Treatment.

Incubation Period of Eggs	No. of Embryos Observed	Treatment Groups	Beats Per Min
4–5th day	6	Control	205
		Spermine NoNoate	206.333
		Adrenaline	213.533

normal vascular tone after the treatment. The spermine NoNoate treated blood vessels showed an increase in vasodilation up to 14%, while, adrenaline treated vessels showed a 6% increase in vasoconstriction ($p = 0.019$ versus second washes) when compared to the pretreatment conditions. Then, the blood vessel was rinsed (1st wash) with PBS. After the first wash, the result showed a decrease of almost 15% of the width of the blood vessels compared to the post-spermine NoNoate treatment and an increase of almost 15% of the width of the blood vessels compared to the post-adrenaline treatment. After washing, the same blood vessel was treated a second time with spermine NoNoate and adrenaline. The spermine NoNoate treated blood vessels showed an increase in vasodilation up to 8%, while, the adrenaline treated vessels showed a 7% increase in vasoconstriction compared to the first wash effects. Further, the blood vessel was rinsed (2nd wash) with PBS. After the second wash, there was an almost a 4% decrease in the width of the blood vessels compared to the second time-spermine NoNoate treatment and an 8% increase in the width of the blood vessels happened in response to the second time-adrenaline treatment. (Figs. 5a, 5b).

Evaluation of the Vasoactive Potential of Various Drugs in Chick Vascular Blood Vessel

The present study aims at developing an alternative model for vasoactive using a chick extravascular blood vessel. It envisages the validation of a model for drug screening and antihypertensive research work. The vascular bed was treated with various chemicals of different types such as atenolol and acetazolamide (antihypertensive drugs), amlodipine, and diltiazem (calcium channel blockers) and also with allylsulfide and L-Theanine (NOS agonist). The data indicated a dilation of the blood vessels by 22 and 18% for atenolol ($p = 0.041$ and $p \leq 0.001$ versus control) and acetazolamide ($p \leq 0.001$ versus control), respectively. The calcium channel blockers (amlodipine and diltiazem) were able to increase vessel dilation up to 4 and 10%, respectively. Allyl sulfide and L-theanine showed 12 and 21% ($p = 0.018$ versus control) increase in the dilation of the blood vessel, respectively, in

https://doi.org/10.1017/S1431927619000588 Published online by Cambridge University Press

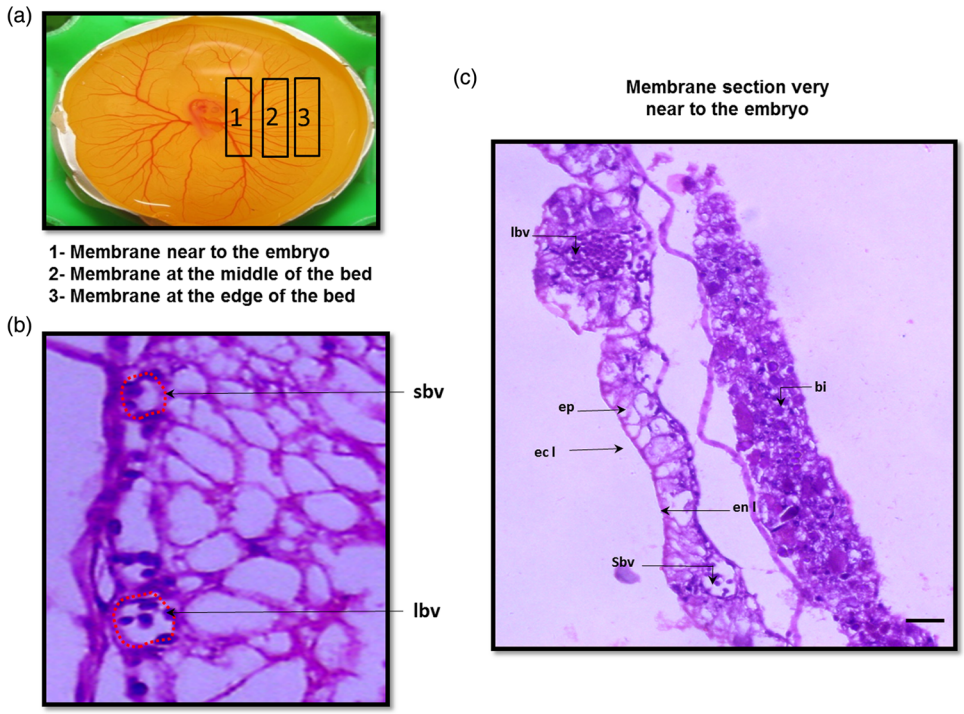

Fig. 4. Vascular tones of the vessels at the middle zone of the chick embryo area vasculosa regulated by smooth muscle cells. **a:** Representative images of the early chick embryo area vasculosa marked as three different parts of extraembryonic tissues. Denotation stands for (1) Membrane near to the embryo, (2) Membrane at the middle of the bed, and (3) Membrane at the edge of the bed. Paraffin-embedded specimens were sectioned into *5- μ*m size and then slides were stained with Haematoxylin and Eosin. **b:** This figure presents a clear distinction between small blood vessels and large blood vessels. **c:** The membrane section close to the embryo showed the presence of both large and small blood vessels with irregularly arranged epithelial cells around the blood vessels. **d:** The membrane section *with* the middle of the vascular bed shows a greater number of large and small blood vessels. Irrespective of the size of the vessels, most of the membranes *showed* the presence of a thin layer of smooth muscle cells around the arteries (arrowhead) with highly differentiated and compactly arranged epithelial cells around the vessels. **e:** *The* membrane section at the edge of the bed of the developing embryo showed blood vessels of a large number than in the membrane section in the middle of the vascular bed. Some of the large blood vessels showed a very thin layer of smooth muscle cells around the wall. Epithelial cells were tightly packed both in ectodermal and endodermal layers when compared to the membrane section close to the embryo. Experiments were performed in triplicates. Scale bar: 50 *μ*m and magnification is 20 X. Arv-Area Vasculosa, Lbv- large blood vessels, Sbv- small blood vessel, Ec l- ectodermal layer, En l- endodermal layer, V- vein, Ep- epithelial cells, Bi- blood islands or blood sinus. The arrowhead indicates the presence of a smooth muscle layer around the vessels. *n* = 3 Scale bars: 1 mm in 4(**a**). **f:** The membrane sections were stained with Masson's trichrome. A thin layer of smooth muscle cells (blackish purple color) was seen around the blood vessel wall (VW). One single red blood cell (R) is trapped in the vascular lumen (L). Scale bar: 540 *μ*m in 4(**f**).

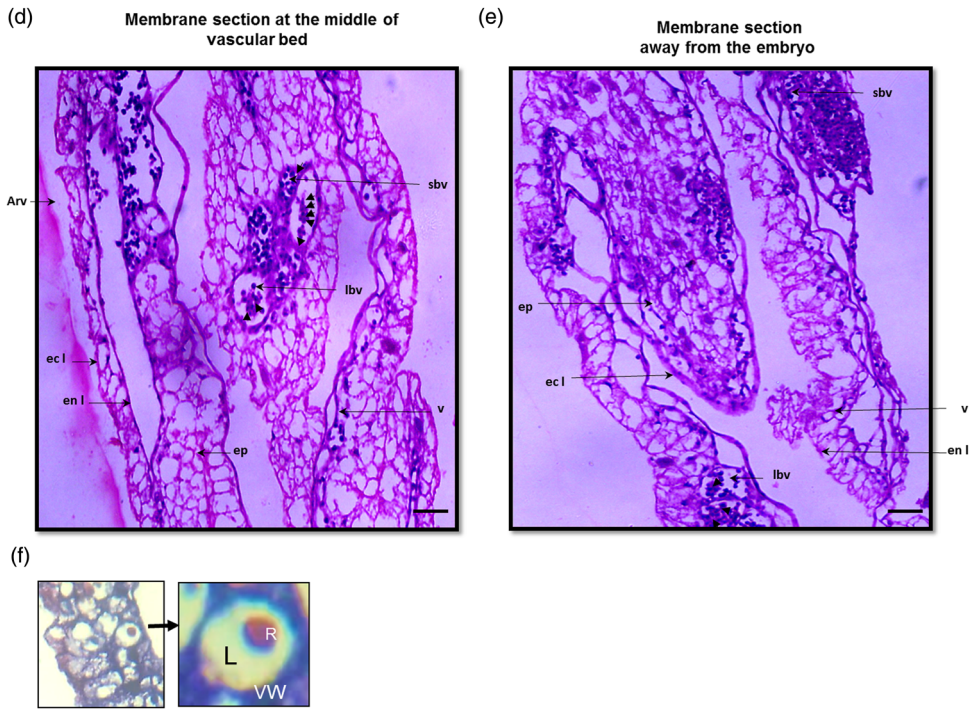

Fig. 4b. Continued.

https://doi.org/10.1017/S1431927619000588 Published online by Cambridge University Press

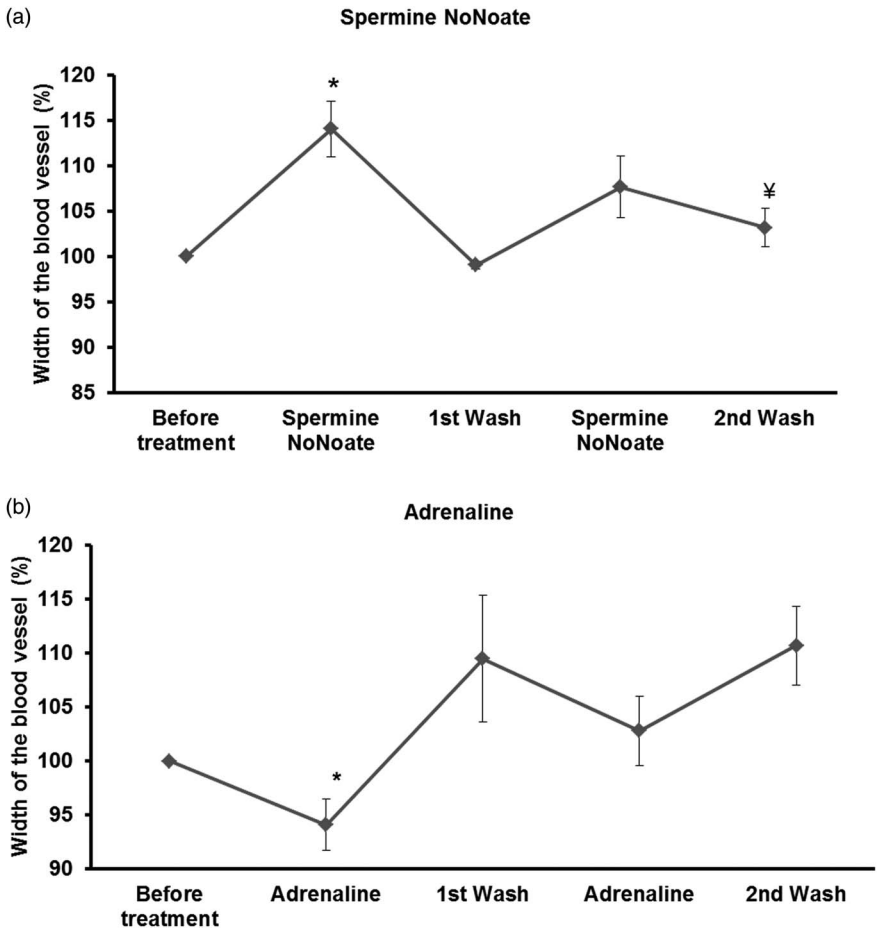

Fig. 5. Blood vessels of the chick embryo area vasculosa show spontaneous as well as reversible vasoactivity upon treatment. **a:** The vasodilatory effect of the blood vessel under spermine NoNoate treatment has been categorized into before and after the treatment. *The* vessel treated with spermineNoNoate showed a 14% increase in vasodilation compared to pre-treatment, 15% reduction after the first wash compared to first treatment, 8% increase compared to first wash, and 4% decrease compared to first treatment. **b:** The vasoconstrictory effect of the blood vessel under Adrenaline treatment *has been* categorized into before and after the treatment. *The* vessel treated with Adrenaline showed a 6% increase in vasoconstriction compared to pre-treatment, a 15% reduction after the first wash compared to first treatment, a 7% increase compared to the first wash, and a 4% decrease compared to the second level of treatment. Experiments were performed in triplicate and the data has been presented as mean + SEM, (*$p = 0.009$ compared to first wash, ¥$p = 0.045$ compared to first treatment for Spermine NoNoate and *$p = 0.019$ compared to the second wash for Adrenaline; $n = 3$).

the chick embryo area vasculosa (Fig. 6a). We further treated the same blood vessel in the chick embryo area vasculosa with adrenaline, followed by treatment with atenolol, a known potent vasodilator. This experiment was performed for demonstrating the multiple uses of the single chick embryo area vasculosa for the screening of the vasoactive effect of various chemicals. An increase in the vasoconstrictory property of the vessel up to 12% ($p = 0.037$ versus 10 min of control) was observed under adrenaline within 10 min of incubation and the same vessel showed a hike in the vasodilation up to 11% under atenolol treatment for another 10 min of incubation (Fig. 6b).

Discussion

The present work endorses the possibility of chick embryo area vasculosa as an alternative model for screening chemicals mainly for its vasoactive properties. The main advantage of this model is the visual identification of the vascular effect of the drugs on the blood vessel as a result of the treatment of a short duration which could be recorded easily. The recorded images could be subsequently analyzed with the help of Image J for quantitative analysis. The other major advantage of this model is the system allows direct contact of the blood vessels with the drugs enabling a more promising effect at the right vitelline artery. The chick embryo area vasculosa could be targeted for screening the vasoactive nature of the drugs with minimal usage compared to other systems such as murine. The circulatory homeostasis of the chick embryo area vasculosa are also regulated by smooth muscle cells and β adrenoreceptor mediated mechanisms (Chruscinski et al., 2001). The present study confirms the presence of vascular smooth muscles (VSM) in chick embryo area vasculosa (Fig. 4). Hence, the study deems the role of VSM in the vaso-activities of the area vasculosa as described in other animal models (Su et al., 2000; Wenzel et al., 2006). The chick embryo area vasculosa has good efficacy (Figs. 1c–1e) and therefore, can be used for evaluation of various antihypertensive drugs.

Regulation of vascular resistance is critical for maintaining circulatory homeostasis, which is associated with the vasomotor functions of smooth muscles as described by earlier reports (Su et al., 2000; Chruscinski et al., 2001; Lijnen et al., 2001). The vascular membrane is a chorioallantoic membrane. It is found in embryonated eggs of birds and reptiles (Makanya

https://doi.org/10.1017/S1431927619000588 Published online by Cambridge University Press

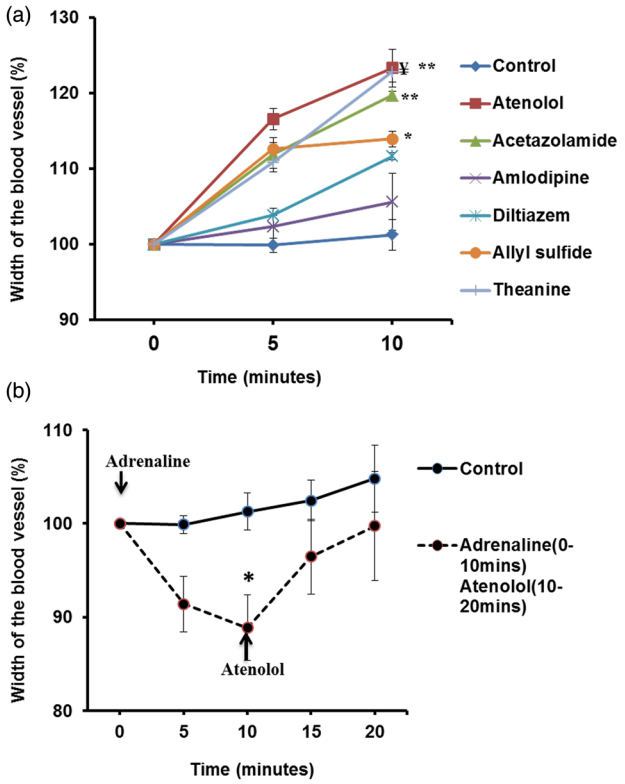

Fig. 6. Chick embryo area vasculosa enables the screening of vasodilation properties of multiple drugs. **a:** The chick embryo area vasculosa were treated with various chemicals namely, Atenolol and acetazolamide (antihypertensive drugs), amlodipine and diltiazem (calcium channel blockers) and with allylsulfide and L-Theanine (NOS agonist) for its vasodilation properties. The graph shows that dilation of the blood vessels increased by 22% for atenolol, 18% for acetazolamide, 4% for amlodipine 10% for diltiazem, 12% for allylsulfide and 21% for L-Theanine at 10 min of incubation. Experiments were performed in triplicate and the data has been presented as mean + SEM, (¥$p = 0.041$ and **$p \leq 0.001$ for Atenolol, **$p \leq 0.001$ for acetazolamide and *$p = 0.018$ for allylsulfide compared to controls). **b:** Screening of vasoactive effect of multiple drugs on the single blood vessel. A single blood vessel *was* treated with a known vasoconstrictor (Adrenaline) and with a known potent vasodilator (Atenolol). The graph indicates the single vessel showing an increase in the vasoconstrictory property of the vessel up to 12% under Adrenaline treatment and an increase in the vasodilation up to 11% for Atenolol treatment. Experiments were performed in triplicate and the data has been presented as mean + SEM, (*$p = 0.037$ compared to control for Adrenaline at 10 min of incubation; $n = 3$).

et al., 2016). These reports include chicken chorioallantoic artery ring assay (Ogut & Brozovich, 2003), chicken chorioallantoic membrane imaging method using late CAM (Tay et al., 2012), and *in vitro* contraction assay using various cell lines (Ribatti et al., 2000). All these vascular tone assays have several technical restrictions that include isolation and maintenance for a long period, and invasive procedures. The present model enables a fast screening of antihypertensive drugs with the use of the least invasive imaging technique. Other advantages of the chick vasoactive model over on other available models are detailed in Table 2 (Table 2). Many reports on the use of arteries obtained from Hamburger Hamilton Stage (HH stage) 17 of chick embryo for vasodilation analysis have been documented (Lindgren et al., 2010). These reports highlight the higher elastic character of the vessels that allow them to stretch unconditionally. Our data showed both spermine NoNoate and adrenaline acting on the model in a dose-dependent manner (Figs. 1c, 1d). These observations are in accordance with other available data that demonstrated that the treatments with spermine NoNoate (10 μM) and adrenaline (10 μM) acted on rat aorta rings (Gittenberger-de Groot et al., 1999; Tay et al., 2012). These observations validate the chick embryo area vasculosa as an alternative model for vasodilation studies. The relationship between the effects of the drugs used on various vessels and their locations in relation to embryo position was also analyzed. We identified the middle zone of the right vitelline artery as the best location to apply drugs. This finding will help researchers in performing experiments with minimal use of drugs within a short incubation period. Further, the model was validated from a different perspective of the washing effects (Figs. 5a, 5b). A medium washing could reduce both the contraction and the relaxation effects by 50%. This observation further endorses this model for the pharmacological testing of drugs. Graham and Lamb (1968) have reported the adrenaline-inducing contracture and twitches of the ventricles of the frog. Then the addition of 200 mM potassium Chloride (KCL) to ringer solution showed adrenaline causing some increase in the decay of contracture tension in the presence of excess KCL. This indicates the continuing effect of adrenaline on reducing contracture tension. Next, the addition of the ringer solution alone was seen inducing the resting potential in the ventricles of the frog. A similar kind of result (repeated vasoactive stimuli effects after washing out in Fig. 5) was observed in our alternative model.

Vasodilation of blood vessels is the outcome of the relaxation of smooth muscle cells which present within the inner wall of large arteries and smaller arterioles (Swaminathan et al., 2012). A vascular smooth muscle cell induces the vasodilation process mediated through β adrenoceptor coupled with Gs-protein and whose stimulation induces an increase in cAMP formation which, in turn, leads to smooth muscle relaxation (Nemeno-Guanzon et al., 2012). An earlier report revealed that the blood vessel wall of CAM incubated for 5 days as consisting of a single layer of endothelial cells and a thin-walled lamina with smooth muscle cells around it (Webb et al., 2003). This study has also endorsed the presence of a thin layer of smooth muscle cells at the lumen of the blood vessels, especially those which lie in the middle zone of the membrane. This reveals the exertion of the pumping pressure on the vessels of the cellular system in the part of the membrane close to the embryo through its pattern of arrangement and the pumping pressure provided by the smooth muscles action present in the inner wall of the arteries when it moves from the embryo. This leads to the conclusion that the murine model of vasomotor function and the vasodilator potential of the chick embryo area vasculosa against the drug treatments are the direct manifestation of the smooth muscle induced relaxation.

The work of Tay et al. (2012) relates to the diameter of the blood vessel using chick embryo area vasculosa with various vasoactive drugs and screened for irritancy on the basis of a semi-automatic image processing and the analysis of the techniques. Earlier studies used different imaging and measuring techniques, such as MATLAB used for the measurement of the blood vessel diameter (Ausprunk et al., 1974; Moens et al., 2005). Our group has used Image J software for the analysis of the vasomotor functions in the chick embryo area vasculosa (Siamwala et al., 2013). Following a similar protocol, we could easily assess the changes in the diameter of the right vitelline artery for further validation in the present study. We also found the quick changes in the diameter of the right vitelline artery against the drug. The main advantage of the chick embryo area vasculosa is that the same right vitelline artery can be used for the analysis of two different drugs within a minimal acclimatization period after a wash.

https://doi.org/10.1017/S1431927619000588 Published online by Cambridge University Press

Table 2. Comparison Between Different Species Used in Vasoactive Assay.

Species	Experimental Time	Isolation of Aorta	Complication in Experimental Procedure	Infrastructure	Price	Ethical
Dog	Long	Difficult	High	Required high	High	Strict ethical norms
Rodent	Long	Difficult	High	Required high	High	Strict ethical norms
Amphibian	Long	Difficult	High	Required high	High	Strict ethical norms
Avian	Short	Simply treating directly on vascular blood vessel	Less	Minimal	Low	Minimal ethical norms

Hence, the present study offers an alternative way of screening the drugs in the chick embryo area vasculosa.

Conclusion

The *in-ovo* chick embryo area vasculosa system could be a potent and useful model for the screening of drugs for their vasoactive properties. This model also enables screening of vasoactive properties of pharmacological and antihypertensive drugs of a wide range with the help of the Image J software.

Author ORCIDs. Suvro Chatterjee, 0000-0003-4413-9760.

Acknowledgment. This work was partially supported by grants from the University Grant Commission-Faculty Recharge Programme (UGC-FRP), Government of India to SC, and the Council of Scientific and Industrial Research (CSIR) -Research Associate Fellowship, Government of India to Akila Swaminathan (grant no. 09/468/0525-2018).

There is no conflict of interest.

References

Ausprunk DH, Knighton DR & Folkman J (1974). Differentiation of vascular endothelium in the chick chorioallantois: A structural and autoradiographic study. *Dev Biol* **38**, 237–248.

Chruscinski A, Brede ME, Meinel L, Lohse MJ, Kobilka BK & Hein L (2001). Differential distribution of beta-adrenergic receptor subtypes in blood vessels of knockout mice lacking beta(1)- or beta(2)-adrenergic receptors. *Mol Pharmacol* **60**, 955–962.

Gittenberger-de Groot AC, DeRuiter MC, Bergwerff M & Poelmann RE (1999). Smooth muscle cell origin and its relation to heterogeneity in development and disease. *Arterioscler Thromb Vasc Biol* **19**, 1589–1594.

Graham JA & Lamb JF (1968). The effect of adrenaline on the tension developed in contractures and twitches of the ventricle of the frog. *J Physiol* **197** (2), 479–509.

Klabunde RE (2011). *Cardiovascular Physiology Concepts*, 2nd ed. Philadelphia, USA: Lippincott Williams, Wilkins.

Lijnen P, Petrov V & Fagard R (2001). In vitro assay of collagen gel contraction by cardiac fibroblasts in serum-free conditions. *Methods Find Exp Clin Pharmacol* **23**(7), 377–382.

Lindgren I, Zoer B, Altimiras J & Villamor E (2010). Reactivity of chicken chorioallantoic arteries, avian homologue of human fetoplacental arteries. *J Physiol Pharmacol* **61**, 619–628.

Manjunathan R & Ragunathan M (2015). Chicken chorioallantoic membrane as reliable model to evaluate osteosarcoma – An experimental approach using SaOS2 cell line. *Biol Proced Online* 17–10.

Makanya AN, Dimova I, Koller T, Styp-Rekowska B & Djonov V (2016) Dynamics of the developing chick chorioallantoic membrane assessed by stereology, allometry, immunohistochemistry and molecular analysis. *PloS One* **11**(4), e0152821.

Moens AL, Goovaerts I, Claeys MJ & Vrints CJ (2005). Flow-mediated vasodilation: A diagnostic instrument, or an experimental tool? *Chest* **127**, 2254–2263.

Nemeno-Guanzon JG, Lee S, Berg JR, Jo YH, Yeo JE, Nam BM, Koh YG & Lee JI (2012). Trends in tissue engineering for blood vessels. *J Biomed Biotechnol* **2012**, 956345.

Ogut O & Brozovich FV (2003). Regulation of force in vascular smooth muscle. *J Mol Cell Cardiol* **35**, 347–355.

Rensen SS, Doevendans PA & van Eys GJ (2007). Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. *Neth Heart J* **15**, 100–108.

Ribatti DA, Vacca L, Roncali L & Dammacco F (2000). The chick embryo chorioallantoic membrane as an in vivo assay to study antiangiogenesis. *Curr Pharm Biotechnol* **1**, 73–82.

Su JB, Hoüel R, Héloire F, Barbe F, Beverelli F, Sambin L, Castaigne A, Berdeaux A, Crozatier B & Hittinger L (2000). Stimulation of bradykinin B_1 receptors induces vasodilation in conductance and resistance coronary vessels in conscious dogs. *Circulation* **101**, 1848–1853.

Sheng G (2010). Primitive and definitive erythropoiesis in the yolk sac: A bird's eye view. *Int J Dev Biol* **54**(6–7), 1033–1043.

Siamwala JH, Dias PM, Majumder S, Joshi MK, Sinkar VP, Banerjee G & Chatterjee S (2013). L-theanine promotes nitric oxide production in endothelial cells through eNOS phosphorylation. *J Nutr Biochem* **24**, 595–605.

Stewart R, Nelson J & Wilson DJ (1989). Epidermal growth factor promotes chick embryonic angiogenesis. *Cell Biol Int Rep* **13**, 957–965.

Swaminathan A, Sridhara SR, Sinha S, Nagarajan S, Balaguru UM, Siamwala JH, Rajendran S, Saran U & Chatterjee S (2012). Nitrites derived from *Foneiculum Vulgare* (Fennel) seeds promotes vascular functions. *J Food Sci* **77**, 273–279.

Tay SL, Heng PW & Chan LW (2012). The chick chorioallantoic membrane imaging method as a platform to evaluate vasoactivity and assess irritancy of compounds. *J Pharm Pharmacol* **64**, 1128–1137.

Vergara MN & Canto-Soler MV (2012). Rediscovering the chick embryo as a model to study retinal development. *Neural Dev* **7**(1), 22.

Webb RC (2003). Smooth muscle contraction and relaxation. *Adv Physiol Educ* **27**(1–4), 201–206.

Wenzel DA, Schmidt K, Reimann J, Hescheler G, Pfitzer W & Bloch BK (2006). Endostatin, the proteolytic fragment of collagen XVIII, induces vasorelaxation. *Circ Res* **9**, 1203–1121.

Zhao TJ, Sakata I, Li RL, Liang G, Richardson JA, Brown MS, Goldstein JL & Zigman JM (2010). Ghrelin secretion stimulated by {beta}1-adrenergic receptors in cultured ghrelinoma cells and in fasted mice. *Proc Natl Acad Sci USA* **107**, 15868–15873.

https://doi.org/10.1017/S1431927619000588 Published online by Cambridge University Press