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Artículo Técnico | Tecnical Article

Isolated aorta model and its contribution to phytopharmacology

[Modelo de aorta aislada y su contribución a la Fitofarmacología]

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Abstract
Since the early 50's until now the isolated thoracic aorta has been a traditional and productive model for pharmacological studies. This experimental model has been closely related to Dr. Robert Furchgott's research. The discovery of the role of endothelium in the vasorelaxation induced by acetylcholine (ACh), represented a milestone in biological sciences and also had an important consequence on the isolated aorta preparation. In this work, we describe the isolated aorta technique and the improvements made in Dr. Penna's laboratory at Facultad de Medina, Universidad de Chile, as well as the Mexican contribution. Since endothelium plays a key role on vascular relaxation and its dysfunction is one of first indicators (biomarker) of cardiovascular diseases, the isolated aorta model is a valuable preparation. Considering the great amount of phytochemical present in many natural sources, like vegetables, fruits and medinal plants, we expect this model to continue delivering significant contributions to the knowledge in pharmacology and phytopharmacology.

Keywords: aorta, aortic rings, phytochemicals, phytopharmacology.

Resumen
Desde principios de los años 50 hasta ahora la aorta torácica aislada ha sido un modelo tradicional y productivo para estudios farmacológicos. Este modelo experimental ha estado estrechamente relacionado con la investigación realizada por el Dr. Robert Furchgott. El descubrimiento de la función del endotelio en la vasodilatación inducida por la acetilcolina (ACh), representó un hito en las ciencias biológicas y también tuvo una consecuencia importante en la preparación de aorta aislada. En este trabajo se describe la técnica de aorta aislada y las mejoras realizadas en el laboratorio del Dr. Penna en la Facultad de Medicina, Universidad de Chile, así como la contribución de investigadores mexicanos. Puesto que el endotelio juega un papel clave en la relajación vascular y su disfunción es uno de los primeros indicadores (biomarcadores) de enfermedad cardiovascular, el modelo de aorta aislada es una valiosa preparación. Teniendo en cuenta la gran cantidad de fitoquímicos presentes en muchas fuentes naturales como verduras, frutas y plantas medicinales, podemos esperar que este modelo continúe entregando importantes aportes al conocimiento en farmacología y fitofarmacología.

Palabras Claves: aorta, anillos de aorta, fitoquímicos, fitofarmacología.

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INTRODUCCIÓN

We would like to dedicate this work to the memory of Dr. Mario Penna (1924 – 1994) (Martinez, 1995) and Dr. Gianni Pinardi (1929 – 2009) for their invaluable contribution to Chilean Pharmacology.

In 1987, Dr. Pinardi introduced the isolated aorta preparation in the laboratory of Dr. Penna allowing the generation of several investigations (Vinet and Pinardi, 1988; Vinet *et al*., 1991a, Vinet *et al*., 1991b; Brieva *et al*., 1992; Illanes *et al*., 1993; Lutz *et al*., 1994; Lutz *et al*., 1995; Zamorano *et al*., 1995; Martinez and Andai, 1996; Martínez *et al*., 1997).

Since the early 50's until now isolated aorta has been a traditional and productive model for pharmacological studies. The story of isolated thoracic aorta as an experimental model has been closely related to Robert Furchgott's research. Furchgott was awarded the Nobel Prize 1998 in Physiology or Medicine, shared with Louis Ignarro and Ferid Murad for their findings concerning nitric oxide (NO) as a signalling molecule in the cardiovascular system. The discovery of the role of endothelium in the vasorelaxation induced by acetylcholine (ACh), considered "an accidental finding" represented a milestone in biological sciences and also had an important consequence on isolated aorta preparation (Furchgott and Zawadzki, 1980; Furchgott, 1998).

What does explain ACh failed to induce relaxation of isolated thoracic aorta preparations? Now, the answer is well known: any kind of preparation (strip or ring), gentle rubbing of the intimal surface (endothelium), whether deliberately or accidentally eliminated the relaxing response to ACh and other muscarinic agonists. Strips as well as rings show good relaxation when caution is taken not to rub the endothelial surface. We will describe both preparations, as well as the improvements made in the laboratory of Dr. Mario Penna at Facultad de Medina, Universidad de Chile.

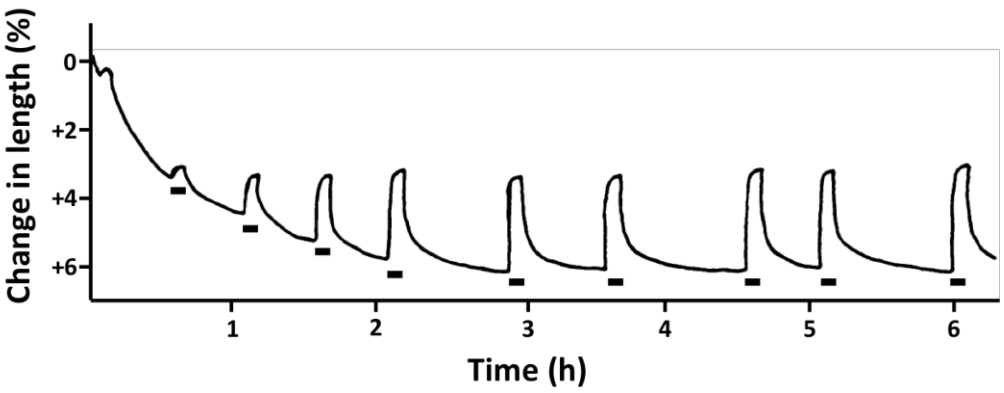
Furchgott and Bhadrakom (1953) introduced the helical strip of the rabbit aorta as a useful preparation for quantitative pharmacological studies on vasoactive drugs and drug-receptor interactions. They showed that strips never exhibit rhythmic contractions and when is mounted in muscle chambers they undergo a gradual elongation and increase in sensitivity to stimulating drugs over a period of about 2 to 3 hr. Drugs, which were found to give only contraction of aortic strips fall in the following order of potency: norepinephrine and epinephrine > histamine > ACh. A surprising finding was that ACh, well recognized as a very potent vasodilator in whole animal and perfused organ studies, elicited no relaxation but only contraction of the aortic strip, whether or not the strip was tested at rest or precontracted with a vasoconstrictor like norepinephrine.

Preparation of rabbit aortic strips

Here we describe the original method used for Furchgott and Bhadrakom (1953) in rabbit. For each experiment a rabbit weighing 2.5 to 3.5 k is rapidly decapitated. The descending thoracic aorta is removed and placed in a Petri dish containing Krebs bicarbonate solution at room temperature. Excess fat and connective tissue are trimmed off. The whole length of aorta is then cut along a close spiral. During the cutting, which is done with a small, sharp-pointed scissors, the uncut portion of the aorta, held gently between the thumb and fingers of the operator's free hand, is gradually rotated and moved forward toward the scissors in such a manner as to permit a continuous spiral incision. The resulting strip is usually about 0.4 mm thick, 2 mm wide and 20 cm long, and contained smooth muscle fibers (the circular fibers of the intact aorta) oriented at about 15 degrees relative to its long axis. From this strip shorter strips of 2 to 4 cm length are cut for use in experiments. During the whole procedure of preparing the strips, the tissue is kept moistened with Krebs solution. Then strips are mounted in organ chambers of 20-30 mL working volume with the aid of stainless steel S-hooks, under 4 g of basal tension. The Krebs bicarbonate solution is bubbled with 95% O_2 and 5% CO_2 and maintain at 37° C. The isotonic recording is carried out with an adequate force transducer attached to a polygraph.

When aortic strips are first attached under 4 g tension they immediately stretch about 40 per cent (Figure 1). They then undergo a further gradual increase in length, which usually amounts to about 5 per cent, but varies from 2 to 10 per cent. This gradual increase approximately follows an exponential curve with a half-time of 10 to 25 minutes, so that elongation is essentially complete in 2 to 3 hours.

Figure 1



Change in length and sensitivity of rabbit aortic strips with time. Abscissa is time after strip was attached to lever exerting 4 g tension. Ordinate is change in length in per cent of initial length under tension. Typical experiment showing gradual increase in sensitivity to epinephrine with time, with attainment of maximal sensitivity in 2 to 3 h. At each horizontal bar, the strip was exposed to 3 x 10^{-9} epinephrine (modified from Furchgott and Bhadrakom, 1953).

Preparation of rat aortic rings

We describe a modified method for the determination of aorta contractility (Vinet and Pinardi, 1988; Vinet *et al.*, 1991a; Illanes *et al.*, 1993). Rats from 250 to 290 g are killed by decapitation and the thoracic aorta is carefully excised and placed in a Petri dish containing Krebs-Henseleit modified buffer (in mM: NaCl 122; KCl 4.7; $NaHCO_3$ 15.5; KH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 2.0; glucose 11.5; EDTA 0.026; pH 7.4) at room temperature. Aorta is dissected, clean of connective tissue and divided into 5 mm rings segments.

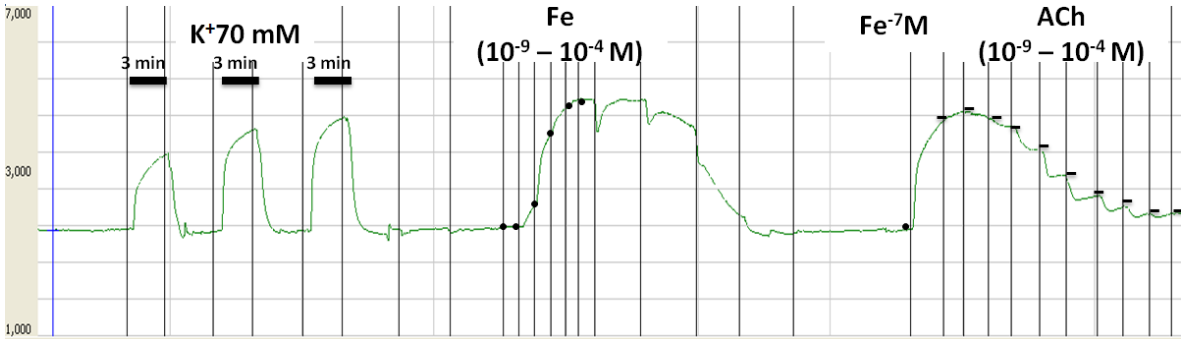
The rings are suspended between two L-shaped stainless steel hooks and placed in a 20-30 mL organ chambers containing modified Krebs-Henseleit buffer, maintained at 37 °C and oxygenated continuously with a 95% O_2 - 5% CO_2 gas. Isometric tensions are measured using a force displacement transducer connected to a polygraph. The rings are allowed to equilibrate in the tissue bath for 60 min under an optimal resting tension of 1.5 g. Rings are progressively stretched at least three times with a depolarizing 70 mM KCl solution (in mM: NaCl 52; KCl 70.0; $NaHCO_3$ 15.5; KH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 2.0; glucose 11.5; EDTA 0.026; pH 7.4) until the contractile response is maximal (optimal and reference tension).

Aortic rings are repeatedly washed and allowed to re-equilibrate for an additional 30 min. The preparation is ready to evaluate relaxation or contraction activities. The analysis of the effect of drug or extract on aortic reactivity included maximal relaxation (Rmax) and the concentration causing 50% of the maximal response (EC_{50}), expressed as pD_2 (- log EC_{50}). Integrity of endothelium may be assessed by testing the relaxation produced by the addition of acetylcholine (1 μM) in phenylephrine (0.1-1 μM) precontracted rings. When relaxation is evaluated, rings are usually precontracted with an $alpha_1$ adrenergic agonist (i.e. phenylephrine 0.1-1 μM) and once a stable contraction is achieved, cumulative concentration-response curve is obtained by a stepwise increase in the drug or extract concentration (usually in the range from 10^{-9} to 10^{-4} M). Response is measured as a percentage of relaxation from the precontracted level, considering the baseline as 100% relaxation.

When contraction is evaluated, drug or extract is added directly on aortic rings under basal tension by cumulative addition to obtain a concentration-response curve. The analysis of the effect of drug or extract also included maximal contraction (Cmax) and EC_{50}, as previously described.

The experiments were performed in male rat because females in response to differents drugs depends on the rat estrous cycle (Zamorano *et al.*, 1994).

Figure 2



Typical recording showing rat aortic tension (mg) with time. After equilibration at 1.5 g of tension, aotic ring was exposed three times to 70 mM KCl and then a cumulative concentration-curve was constructed using phenylephrine. Finally, on the precontracted ring a cumulative concentration-curve was constructed using acetylcholine (Flores et al., 2011).

If there is interest in assessing the influence of the endothelium in relation to a particular agonist-induced response, endothelium removal is a good alternative. It is possible by gently rubbing the intimal surface of the vessel with a stainless steel rod and rolling the vessel on Whatman filter paper moistened with cold MKHB (Vinet *et al.*, 1991a).

In order to to better understand the mechanism involved in endothelium-dependent responses, is very frequent to evaluate if NO and/or prostaglandins are involved. For this purpose aortic preparation may be incubated with NG-nitro-L-arginine methyl ester (L-NAME), a selective inhibitor of nitric oxide synthase (eNOS) and/or indomethacin, a nonselective inhibitor of cyclooxygenase (COX) (Mishra *et al.*, 2000; Paredes-Carbajal *et al.*, 2001).

The Mexican experience in aortic rings

The experiments are performed on aorta rings from male rats of the Wistar strain, weighing 250-300 g (males are used in order to avoid hormonal changes during the estral cycle, but, obviously, female rats should be used in studies analyzing the effects of plants acting on the reproductive system). All animals are kept in individual cages at room temperature, exposed to 12-h light-dark cycle and with free access to food and water. However, in studies analyzing the effects of acute or chronic oral ingestion of a plant or its extract either the usual rat chow or the water is accordingly substituted. After 12 h fasting, animals are killed by cervical dislocation and following an immediate thoracotomy the thoracic aorta is removed and placed in a bath with oxygenated Tyrode´s solution. Under a dissection microscope the aorta is then cleaned of connective and adipose tissue and cut into 2 mm long rings. Special care is taken to avoid damage to the endothelium. Afterwards in every other ring, the endothelium is removed by gently rubbing the rings.

For each experiment a pair of rings from the central portion of the same aorta (one with intact endothelium, the other without a functional endothelium) is used. Each of these rings is suspended horizontally in the same miniature organ chamber (volume 0.5 ml) between a pole fixed to the bottom of the bath and a hook attached to an isometric force transducer. Both, the poles and the hooks are made from thin (0.5 mm) stainless steel insect needles. With this setup, it is possible to maintain the two rings under the same experimental conditions and record simultaneously responses mediated by a direct effect on the vascular smooth muscle and those where the endothelium is involved. The vessels are continuously superfused (2 ml/min) with prewarmed (37^0C) aerated (95% O_2 and 5% CO_2) modified Tyrode`s solution (composition in mM: NaCl, 137; KCl, 2.7; $MgCl_2$, 0.69; $NaHCO_3$, 11.9; NaH_2PO_4, 0.4; $CaCl_2$, 1.8 and glucose, 10; pH was adjusted to 7.4).

The rings are initially stretched until resting tension reaches 2 g and allowed to equilibrate for one hour; during this period the resting tension is continuously monitored and, if needed, readjusted to 2 g by further stretching.

Before starting an actual experiment, responsiveness of each pair of rings to the $alfa_1$ adrenoceptor agonist phenylephrine and to the stable cholinergic agonist carbachol (carbamoylcholine) is tested. This is achieved by switching the superfusing Tyrode`s solution for 10 min to one containing phenylephrine (10^{-5} M) and, thereafter, to one containing, in addition to phenylephrine, carbachol (10^{-5} M). Development of a vigorous (2.0 -3.0 g) and sustained contracture in response to phenylephrine evidences the functional integrity of the smooth muscle layer. Carbachol-induced relaxation of the phenylephrine precontracted vessels is taken as evidence for the preservation of an intact endothelium whereas lack of relaxation confirms the absence of a functional endothelium.

Superfusion and bath temperature control

Rings are continuously superfused with either Tyrode`s or test solutions at a rate of 2 ml/min driven by a peristaltic pump (although gravity may equally be used). Before entering at the bottom of the miniature organ bath, solutions flow through a spiral immersed in water at a thermostatically controlled temperature. When a solution is changed, the arrival of the new solution at the bottom of the bath may be monitored by the small air bubble preceding its inflow. Solutions are drained by overflow assisted by a cellulose wick to prevent small volume changes (see Figure).

Tension recording.

Tension developed by each ring is picked-up by an isometric force transducer (Grass FT03), and following appropriate amplification and filtration, continuously recorded analogically on a Grass model 79 polygraph system. Additionally, with the aid of a PowerLab/200 (ADInstruments) A/D converter, the amplified analog tension signal is digitized and stored on a hard disk, for further analysis

Experimental protocol

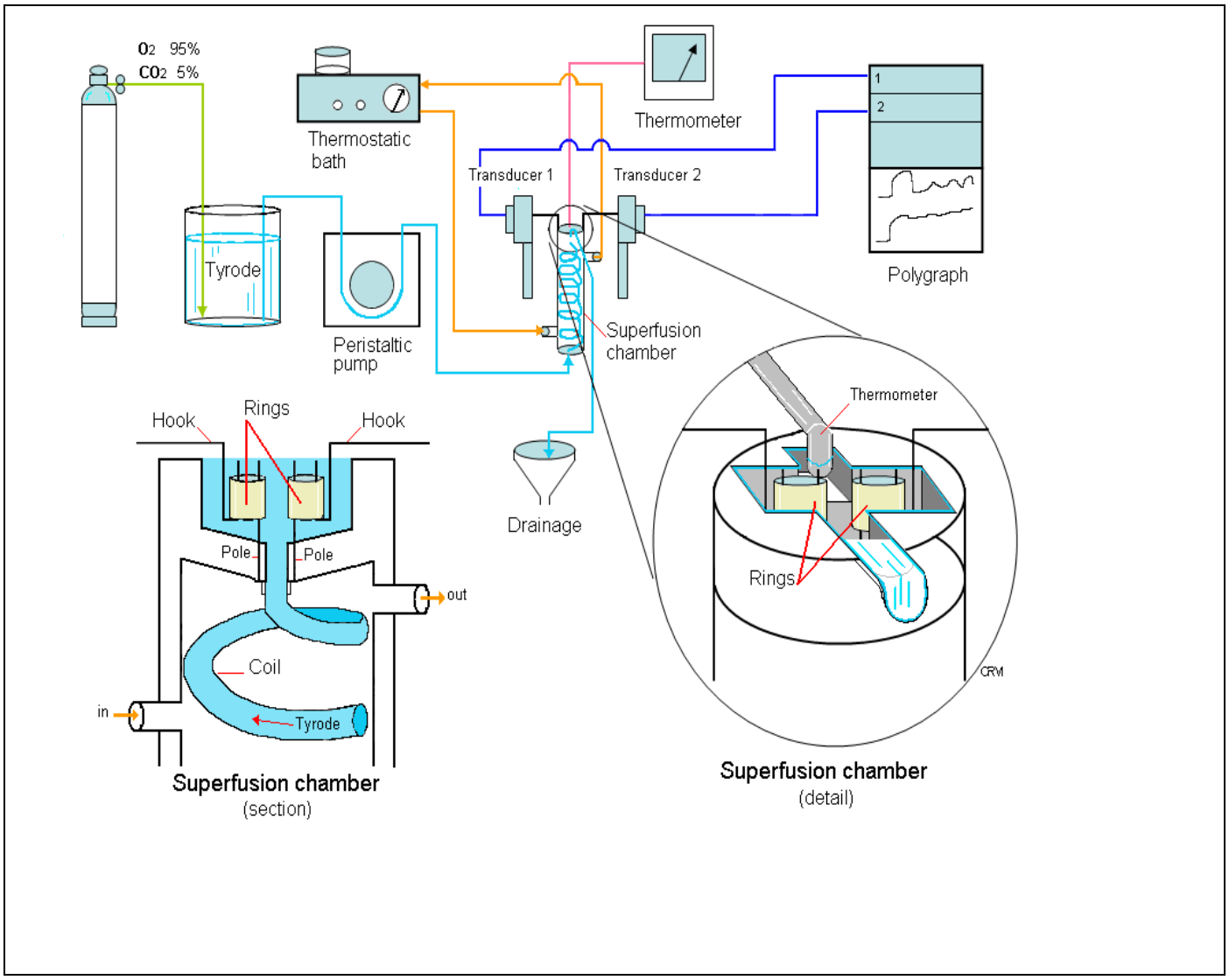
The basic experimental protocol used by us to test the effects of a given substance on the vasoreactivity of aortic rings is as follows: after confirming the functional integrity of the pair of ring (as described above) we induce a contracture with a solution containing 10^{-5} M phenylephrine (maximal tension development or T max), after stabilization of this contracture (approximately 10 min) we start a concentration response curve to the test compound by switching the solution to solutions containing in addition to phenylephrine successively increasing concentrations of the test compound. The optimal concentration determined by the procedure is then used to compare the concentration-response curves to phenylephrine (10^{-9} to 10^{-5} M) in the presence or absence of the test compound. In order to analyze the plausible involvement of nitric oxide and/or cyclooxigenase metabolites in the observed effects we repeat, thereafter, the procedures in the presence of L-NAME or indomethacin respectively.
Obviously, this protocol can be adapted to the use of a great variety agonists, antagonists or enzymatic inhibitors

Data analysis.

The contractile responses induced by phenylephrine are expressed as tension increment in grams above the basal tension (imposed on the vessel throughout the experiment). Carbachol-induced relaxations are expressed either as the percent reduction in tension relative to maximal tension developed in response to phenylephrine (10^{-5} M) or as the percentage of that maximal tension.

All data are expressed as means ± S.D. PD_2 (-Log of the mean molar concentration of agonist producing 50% of the maximal response) is determined with the software package Graph Pad Prism (San Diego, CA., USA).
Comparisons of means are made by One Way Analysis of Variance (ANOVA) and differences among groups are evaluated using Student-Newman-Keuls Method (Sigma Stat software; St. Louis, MO., USA). A P value of 0.05 or less is considered significant.

Figure 3



A schematic diagram showing the different components of the system used for recording aortic rings mechanical activity.

DISCUSSION

The mechanical response i.e. tension increase or decrease, of "*in vitro*" superfused vascular segments to natural products or their extracts is widely used to analyze their presumptive vasoactive actions. Must such studies use a vascular ring (generally an arterial rings) suspended in a rather large organ bath (volume 10 ml or more) with no continuous flow. Substances to be tested are either added as a bolus or by bulk solution exchange, and washout of the substance is achieved by repeated bulk solution exchange. Since bulk solution exchange cause mechanical artifacts continuous tension recordings are thus not possible which precludes the detection of plausible short lasting transient responses.

To our knowledge, in none of the studies performed to test whether or not the endothelium is involved in the response to a given substance, the experiments were performed with a pair of rings (one with and one without a functional endothelium) suspended in the same bath and, hence, simultaneously exposed to the test substance. It is, obviously, a valid approach to perform the experiments on rings superfused separately but we consider that the

simultaneous exposure of a pair of rings gives more direct evidence regarding the role of the endothelium in theobserved responses.

The rather high flow rate (2 ml/min) combined with the small bath volume (0.5 ml) guarantees a rapidsolution change which allows the detection of early plausible brief transient responses and on the other hand, avoids uncontrolled alterations of the extracellular medium (by either accumulation or depletion of molecules) which mayinterfere with the effect produced by a given challenge.

Our experience with this model, in testing the effects of synthetic estrogens, *Spirulina* extracts, *Rutachalepensis* infusion, *psithacantus callyculatus* extract, thrombin analogues, endo- and synthetic cannabinoids,Ranolazine and some of its derivatives and different types of tea, has been always successful.

Aorta model in phytochemical evaluation

As Furchgott described (Furchgott and Zawadzki, 1980), aorta model as biomarker is dependent of endothelium functionality. In 1982, helical strips were used to demonstrate the effect induced by extract of *Ginkgo biloba*. *Ginkgo biloba* provoked a concentration-dependent contraction of spirally cut rabbit aortic strips (Auguet et al., 1982). However, after precontracting the strips with phenylephrine, *Ginkgo biloba* also induced relaxation, effect partially endothelium-dependent (Delaflotte *et al*., 1984).

The popularity of the isolated aorta as *in vitro* model is closely associated to endothelial function. As we know, endothelium plays a key role on vascular relaxation and its dysfunction is characterized by a shift of the actions of the endothelium toward reduced vasodilation, a proinflammatory state, and prothrombic properties (Endemann and Schiffrin, 2004; Félétou and Vanhoutte, 2006). Dysfunction of the endothelium has been implicated in the pathophysiology of different forms of cardiovascular disease, including hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure (Endemann and Schiffrin, 2004; Félétou and Vanhoutte, 2006).

On the other hands, there is evidence indicating that many phytochemical from different natural sources may protect the endothelium. On this basis, many extracts and isolated phytochemicals have been tested in this model (see Table 1). Among phytochemicals, polyphenols have a great importance since their total dietary intake could be as high as 1 g/d, which is much higher than that of all other classes of phytochemicals and known dietary antioxidants. Their main dietary sources are fruits, vegetables, cereals and plant-derived beverages such as fruit juices, tea, coffee and red wine (Scalbert *et al*., 2005).

Polyphenolic compounds derived from grapes have been evaluated in isolated aortic rings showing relaxing properties (Fitzpatrick *et al*., 1993; Andriambeloson *et al*., 1998) which is endothelium-dependent. On the other hand, aqueous extracts of a variety of vegetables, fruits, teas, nuts, herbs, and spices show endothelium-dependent and independent relaxing ability *in vitro*. These results may support that herbal medicines and plant foods contain compounds that, if absorbed intact and in sufficient quantities, could conceivably be beneficial in prevention of cardiovascular disease (Fitzpatrick *et al*., 1995).

Table 1

PHYTOCHEMICAL	ANIMAL/MODEL	EFFECT	REFERENCE
Extract of *Ginkgo biloba*	Rabbit/Helical strips	Contraction	Auguet *et al*., 1982
Extract of *Ginkgo biloba*	Rabbit/Helical strips	Relaxation	Delaflotte *et al*., 1984
Vitisin C (from *Vitis* plants)	Rabbit/Aortic rings	Relaxation	Seya *et al*., 2003
ALK-1 an alkaloid from *Phyllanthus sellowianus*	Rat/Aortic rings	Relaxation	Calixto *et al*., 1984
Wine and other grape products	Rat/Aortic rings	Relaxation	Fitzpatrick *et al*., 1993
Thaliporphine (from *Neolitsea konishii K*)	Rat/Aortic rings	Contraction	Teng *et al*., 1993
Various plant extracts	Rat/Aortic rings	Relaxation	Fitzpatrick *et al*., 1995
Five Mexican medicinal plants	Rat/Aortic rings	Relaxation	Perusquía *et al*., 1995
Diacetil epitaondiol (from alga	Rat/Aortic rings	Relaxation	Martinerz *et al*., 1997

Stypopodium flabelliforme)			
Red wine polyphenolic compounds	Rat/Aortic rings	Relaxation	Andriambeloson *et al*., 1998.
Eriodictyol (isolated from *Satureja obovata Lag.*)	Rat/Aortic rings	Relaxation	Ramón Sánchez de Rojas *et al*., 1999.
Xanthorrhizol (isolated from *Lostephane heterophylla*)	Rat/Aortic rings	Relaxation	Campos *et al*., 2000
Hydroalcoholic extract of *Cissampelos sympodialis Eichl*	Rat/Aortic rings	Contraction	Freitas *et al*., 2000
Butanolic fraction of *Cuphea carthagenensis Jacq McBride*	Rat/Aortic rings	Relaxation	Schuldt *et al*., 2000
Ethanolic extract of *Spirulina maxima*	Rat/Aortic rings	Relaxation	Paredes-Carbajal *et al*., 2001
Ethyl cinnamate (from *Kaempferia galangal*)	Rat/Aortic rings	Relaxation	Othman *et al*., 2002
Extract of *Ajuga iva*	Rat/Aortic rings	Relaxation	El-Hilaly *et al*., 2004
Leaf extract of *Celtis durandii engler*	Rat/Aortic rings	Relaxation	Dimo *et al*., 2005.
Aqueous leaves extract of *Persea Americana*	Rat/Aortic rings	Relaxation	Owolabi *et al*., 2005
Biochanin A (plant-derived estrogen)	Rat/Aortic rings	Relaxation	Wang *et al*., 2005
Methanol extract of *Terminalia superb*	Rat/Aortic rings	Relaxation	Dimo *et al*., 2006
Raphanus sativus (radish) seed crude extract	Rat/Aortic rings	Relaxation	Ghayur and Gilani, 2006
Resveratrol	Rat/Aortic rings	Relaxation	Novakovic *et al*., 2006
Chloroformic crude extract of *Bupleurum fruticosum L.* roots	Rat/Aortic rings	Relaxation	Testai *et al*., 2006
Paullinia pinnata extracts rich in polyphenols	Rat/Aortic rings	Relaxation	Zamble *et al*., 2006
The aqueous-methanolic crude extract of *Andropogon muricatus*)	Rat/Aortic rings	Relaxation	Gilani *et al.,* 2007.
Friedelin (isolated from bamboo shavings)	Rat/Aortic rings	Relaxation	Jiao *et al*., 2007
Scutellarin (isolated from *Erigeron breviscapus*)	Rat/Aortic rings	Relaxation	Yang *et al*., 2007; 2009
Aqueous extract of *Melissa officinalis L. ssp. officinalis*	Rat/Aortic rings	Relaxation	Ersoy *et al*., 2008
Aqueous extract of *Cirsium japonicum*	Rat/Aortic rings	Relaxation	Kim *et al*., 2008
Aqueous extract of *Cistus ladaniferus*	Rat/Aortic rings	Relaxation	Belmokhtar *et al*., 2009
Phytoestrogens (isolated from *Curcuma comosa Roxb*)	Rat/Aortic rings	Relaxation	Intapad *et al*., 2009
Hibiscus sabdariffa crude extract	Rat/Aortic rings	Relaxation	Sarr *et al*., 2009
Beta-carboline indole alkaloids (isolated from Bark of *Neisosperma oppositifolia*)	Rat/Aortic rings	Relaxation	Ahmad *et al*., 2010
Aqueous-ethanol extract of Bauhinia candicans	Rat/Aortic rings	Relaxation	Fuentes and Alarcon, 2010
5,7-dimethoxyflavone (isolated from *Kaempferia parviflora*)	Rat/Aortic rings	Relaxation	Tep-Areenan *et al*., 2010
Chronic ingestion of an infusion of *Ruta chalepensis*	Rat/Aortic rings	Relaxation	Cartas *et al*., 2011
Resveratrol aortic contractility under oxidative stress	Rat/Aortic rings	Relaxation	Flores *et al*., 2011
Centaurium cachanlahuen extract	Rat/Aortic rings	Relaxation	Vinet *et al*., 2012*

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