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Regulation mechanisms of endocrine disruptors on vasodilation and vasoconstriction: Insights from *ex vivo* models

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Abstract: Cardiovascular diseases (CVD) are one of the leading causes of death worldwide. The knowledge and understanding of CVD are based on the study of vascular physiology and how the smooth muscle cells and tissues perform their different functions. Exposure to endocrine disruptors (EDCs), such as phytoestrogens, polycyclic aromatic hydrocarbons, flame retardants, plasticizers, pesticides, and cosmetics, is an integral and fundamental part of human exposure. Humans are exposed to EDCs by multiple pathways including air, food, water, and consumer products. However, this exposure can lead to several adverse effects on human health, including on the cardiovascular (CV) system. The negative impact that EDC toxicity has on human CV health is a serious problem that must not be overlooked. In this point of view, we proposed the use of the human umbilical artery as a human model to study the direct effects of EDCs on the vascular level. Several works where these cells were directly exposed to EDC's were presented to highlight this well-established model as a great strategy to be used. In the future, we emphasize the need to continue to carry out different investigations using HUA to unveil and understand the vascular toxicity of EDCs and improve human CV health.

Introduction

Cardiovascular diseases (CVD) are one of the leading causes of death worldwide (Mc Namara *et al.*, 2019). The knowledge and understanding of CVD are based on the study of vascular physiology and how the smooth muscle cells and tissues perform their different functions.

Humans are daily exposed to endocrine disruptors (EDCs), "exogenous chemicals that interfere with hormone action" by different cellular and molecular mechanisms (see review (La Merrill *et al.*, 2020)). Exposure to EDCs, such as phytoestrogens, polycyclic aromatic hydrocarbons, flame retardants, plasticizers, pesticides, and cosmetics is an integral and fundamental part of human exposure and can occur by multiple pathways including air, food, water, and consumer products. However, this exposure led to several adverse effects on human health, including on cancer, reproductive, metabolic, and neurobiology systems, and cardiovascular disorders (e.g., coronary artery disease, hypertension, atherosclerosis, or myocardial infarction) (Zlatnik, 2016; Gore *et al.*, 2019; Papalou *et al.*, 2019; Fu *et al.*, 2020; Mariana and Cairrao, 2020; Mesquita *et al.*, 2021). Concerns about endocrine exposure have increased as the modulation of EDCs on the actions of natural hormones is discovered to involve a range of additive, synergistic, or negative biological effects (Feron *et al.*, 2002; Fowler *et al.*, 2012; Ribeiro *et al.*, 2017).

Currently, one of the main research challenges is to discover the mechanisms of action of EDCs (Satpathy, 2020) to improve human health. However, studying the toxicity of EDCs presents some challenges, namely the complex network through which EDCs can act. In this sense, La Merrill *et al.* (2020) have recently presented a suggestion to classify into 10 key characteristics EDCs according to their disruptive effects and respective hormonal actions: 1) EDCs can act by activating/agonism of hormone receptors; 2) EDCs can act by inactivating/antagonism of hormone receptors; 3) EDCs can act by altering the expression of hormone receptors; 4) EDCs can alter signaling transduction; 5) EDCs can induce epigenetic changes; 6) EDCs can change hormone synthesis; 7) EDCs can change hormone transport; 8) EDCs can change hormone distribution; 9) EDCs can alter metabolism; and 10) EDCs can cause modification in the fate of hormone-producing or reactive cells (La Merrill *et al.*, 2020).

Furthermore, it is essential not to forget that the endocrine system is itself a complex, integrative system and

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involves a series of hormonal feedback processes, and therefore very difficult to understand (Couderq *et al.*, 2020). The existence of non-monotonic responses by EDCs (in which their effects change, in an inverted U or U-shape) also makes the work of researchers very difficult, since it is necessary to be aware that a high dose of EDC is not always it is the most toxic, but it can be a lower dose—this event makes difficult to define the safe dose of a given compound (Diamanti-Kandarakis *et al.*, 2009; Vandenberg *et al.*, 2012; Couderq *et al.*, 2020). Indeed, one of the greatest challenges in toxicology is the choice of concentrations for *in vitro* testing, an issue that has been discussed recently (Leist *et al.*, 2017; Albrecht, 2020; Hengstler *et al.*, 2020). Currently, the use of relatively high concentrations (20 to 200 times higher than *in vivo* blood concentrations (C_{max}) is agreed upon, as these high concentrations lead to better accuracy results than lower concentrations. Overall, higher concentrations in the culture medium are required to observe cell damage compared to the C_{max} that is known to cause adverse effects *in vivo* (*in vitro–in vivo* scaling factor). Thus, it is advised to use a concentration range close to and above the maximum concentrations observed in human plasma (Hengstler *et al.*, 2020). Furthermore, most EDCs remain understudied, which constituted a major force of investigation but, highlight the need for more emergent investigations to discover the toxicological effects. Thus, evaluating EDCs as an integral part of the human exposome has been the current challenge of greatest interest.

In this point of view, we proposed the use of the human umbilical artery as a human model to study the direct effects of EDCs on the vascular level. Several works where these cells were directly exposed to EDC's are presented to emphasize this well-established model as a great strategy to be used.

The Role of Smooth Muscle Cells

The main function of vascular smooth muscle is to regulate vasodilation and vasoconstriction of vessels. Thus, the vascular tone depends on the mechanisms that control the intracellular cytosolic Ca^{2+} concentration: vasoconstriction is due to the increase of Ca^{2+} levels, while vasodilation occur by decreasing them (Lorigo *et al.*, 2018). The main mechanisms involved in the vasoconstriction are 1) a cell membrane depolarization and 2) an agonist stimulation. In the 1^{st} mechanism, voltage-operated Ca^{2+} channels are activated causing a Ca^{2+} influx. In the 2^{nd} mechanism, there is an activation of a G-protein, which induces Ca^{2+} release by intracellular reservoirs of the cell, such as the sarcoplasmic reticulum. On the other hand, the main mechanisms involved in vasorelaxation are the cyclic nucleotides and K^+ channels activation. In this sense, the smooth muscle cells regulate the contractile properties of this highly specialized structure (Morgado *et al.*, 2012; Manoury *et al.*, 2020)—the human umbilical artery—through responses to a series of hormonal and hemodynamic stimuli (Owens *et al.*, 2004), but also due to the expression of several contractile proteins, functional ion channels and signaling molecules (Owens *et al.*, 1996; Kudryavtseva *et al.*, 2013; Wang *et al.*, 2015).

The functions of smooth muscle cells result from a multiplicity of phenotypes (contractile to the synthetic phenotypes range), with well-defined structural characteristics (Rensen *et al.*, 2007). As smooth muscle cells are very plastic, in pathophysiological response, such as exposure to EDCs, they may alter their contractile state and signaling mechanisms (Owens *et al.*, 2004; Gloria *et al.*, 2018; Lorigo *et al.*, 2018), including the cyclic nucleotides compartmentalization (Feiteiro *et al.*, 2016). This phenotypic modulation, as it impairs vascular tone (comprise the vasoconstriction and vasorelaxation responses), is associated with vascular lesions (Huang *et al.*, 2016) and may be an inductor of cardiovascular disorders, as atherosclerosis. As these underlying molecular mechanisms are not yet clear, the use of smooth muscle cells is crucial to elucidate them and thus, to understand the development of some vascular diseases. However, the obtaining of smooth muscle cells is limited by the difficulty of acquiring human tissue for isolation and cell culture.

In this sense, the human umbilical artery (HUA) is an excellent source of vascular smooth muscle cells (Cairrao *et al.*, 2009) and could be a good model for studying the effects of endocrine disruptors on the vascular system (Gloria *et al.*, 2018; Lorigo *et al.*, 2018). Easily isolated from the tunica media of the vessels (Meyer *et al.*, 1978; Rockelein and Schneider, 1992), human umbilical artery smooth muscle cells (HUASMC) play a critical role in vascular physiology and pathophysiology (Santos-Silva *et al.*, 2008; Morgado *et al.*, 2012; Lorigo *et al.*, 2018; Lorigo *et al.*, 2020) (Fig. 1).

Why Study the Human Umbilical Artery?

Normal umbilical vascular reactivity is critical to maintaining the correct exchange of gases and nutrients between the fetus and the mother. As HUA does not have innervation (Santos-Silva *et al.*, 2009; Provitera *et al.*, 2019), unlike other arteries, its specific physiological regulatory control depends entirely on local mediators (such as serotonin, 5-HT, and histamine, His). On the other hand, also catecholamines (such as

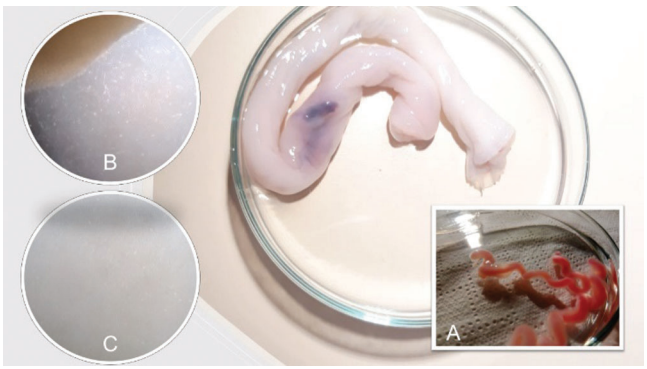

FIGURE 1. The human umbilical artery (HUA) can be easily obtained from the human umbilical cord (background) allowing the performance of human umbilical artery smooth muscle cells (HUASMC) cultures. (A) The HUA isolated. (B and C) HUASMC migrating from the smooth muscle layer and in a confluent state, respectively.

adrenaline, noradrenaline, and dopamine) play an important role in this regulation. However, it has been shown that the vascular response of HUA may differ from other vascular systems (Yoshikawa and Chiba, 1991), namely concerning the adrenergic system. This difference is mainly due to the portion of the cord that is collected because as shown by Kawano and Mori (1990), adrenergic nerve fibers are present only in HUA at the fetal end of the cord (Kawano and Mori, 1990). The main *ex vivo* methods used in HUA to study the vascular response are the organ bath and the human vessel perfusion system. Both systems allow to achievement of a physiological blood vessel environment. The main advantage of the perfusion system is that the vessel segments are maintained as 3D structures with an intact native endothelial lining (if isolated and mounted correctly), allowing quantification of nanoparticles accumulation and cellular response (Lysyy *et al.*, 2020). Regarding the organ bath, the main advantage is allowing to isolate pharmacological responses, drug testing, and easy repeatable (Jespersen *et al.*, 2015). Thus, we can conclude that HUA is a good model to evaluate the effects of EDCs on the local mediators and even to study adrenergic response in the portion proximal to the newborn.

Exposure to some EDCs can alter the hormone levels of sex steroids (Sathyanarayana *et al.*, 2014; Johns *et al.*, 2015). For example, it has been shown that perchlorate inhibits thyroid hormone synthesis (Wolff, 1998), while phthalates decrease the synthesis of testosterone (Parks, 2000; Mylchreest *et al.*, 2002). On the other hand, the estradiol levels seem to increase by exposure to herbicide atrazine (Jin *et al.*, 2013). In this sense, some studies have reported that HUA is more sensitive to the effects of estradiol than the human umbilical vein (Fausett *et al.*, 1999) and that this artery allows the short-term and long-term effects of testosterone to be studied (Cairrao *et al.*, 2008; Cairrao *et al.*, 2010; Saldanha *et al.*, 2013). Thus, the HUA can be considered an excellent tool/model for the study of genomic and non-genomic alterations that EDCs can induce at the vascular level, resulting from alterations in hormonal levels.

On the other hand, it is known that EDCs also impair thyroid hormone production (Ghassabian and Trasande, 2018; Vancamp *et al.*, 2019; Street and Bernasconi, 2020), compromising CV homeostasis and consequently promoting or increasing the risk of developing CVD (Jain *et al.*, 2013). The effects of EDCs on vascular contractility relating them to thyroid homeostasis is practically unexplored. However, a recent study showed that EDC octylmethoxycinnamate alters the contractility patterns of the HUA of pregnant women with hypothyroidism and competes with the natural hormone T3 for binding to the thyroid hormone receptor alpha active center. Although these computational simulations by docking molecular cannot clarify with certainty the absolute mode of action of an EDC, they are an asset in understanding them (Lorigo *et al.*, 2021a), even supporting the different studies of contractility. Therefore, HUA may be also a good model for the vascular study of different pathologies, as is the case of thyroid pathologies, so closely related to the CV system.

Moreover, the HUA can also be used for the study of hypertensive disorders of pregnancy (HDP), such as pre-eclampsia or gestational hypertension (Naderi *et al.*, 2017). Additionally, in HDP and specifically in pre-eclampsia, it would be interesting also use placenta or placental chorionic plaque arteries, using the *ex vivo* placental perfusion model as described by Hitzerd *et al.* (2019), as its pathophysiology is believed to be mainly originate in the placenta. On the other hand, the use of more peripheral placental arteries should also be considered as a model depending on the study to be performed. Overall, understanding the regulation of vascular reactivity and the remodeling of blood vessels in the umbilical cord is essential to understand the pathophysiology of HDP and to investigate the best therapeutic treatment strategies for HDP.

As mentioned before, HUA is mainly regulated by local mediators (such as serotonin, 5-HT, and histamine, His) (Santos-Silva *et al.*, 2009; Provitera *et al.*, 2019). Changes in 5-HT and His receptors increase HUA sensitivity and reactivity to these mediators, which causes an increase in vascular resistance (Bolte *et al.*, 2001; Brew and Sullivan, 2006). Consequently, this may promote the development of gestational hypertension and pre-eclampsia (Bolte *et al.*, 2001; Brew and Sullivan, 2006; Feinberg, 2006; Gupta *et al.*, 2006; Lorigo *et al.*, 2018). Different EDCs have already been shown to interfere with the HUA 5-HT and His receptors (Gloria *et al.*, 2018), which highlights a possible role of these EDCs in promoting the development of HDP.

Furthermore, ion channels also play a key role in HDP (Kuo *et al.*, 2011). The HUA was also used to demonstrate the role of EDCs tributyltin (Gloria *et al.*, 2018), and octylmethoxycinnamate (Lorigo *et al.*, 2019; Lorigo *et al.*, 2021a; Lorigo *et al.*, 2021b) in the blockade of L-type Ca^{2+} channels of HUA. Moreover, this effect in EDCs di-(2-ethylhexyl) phthalate and bisphenol A is also proved by patch-clamp in rat vascular smooth muscle cells (Feiteiro *et al.*, 2018; Mariana *et al.*, 2018). The EDC octylmethoxycinnamate also appears to have effects on K^+ channel activation by cGMP-dependent protein kinase activation (Lorigo *et al.*, 2021b), which can compromise vascular homeostasis and induce hypertension (Cox *et al.*, 2001; Cox, 2005).

In summary, and according to the suggested for other authors (Kelley *et al.*, 2019; Street and Bernasconi, 2020; Tang *et al.*, 2020), the exposure to EDCs seems to be related to the promotion and development of some cardiovascular disorders, such as atherosclerosis, hypertension, or pre-eclampsia. Therefore, changes in HUA reactivity induced by EDCs exposure are an asset to understanding the risk of developing CVD, highlighting the clinical importance of this artery.

Cultures of HUASMC and Their Vascular Importance

In addition to being used in different studies of arterial contractility, the HUA is also an important model to perform isolation of HUASMC (as reviewed by Lorigo *et al.* (2018)). These cells can be directly exposed to different EDCs and used to perform cell contractility, gene expression, or cell cytotoxicity studies. As demonstrated by Cairrao *et al.* (2009) these cells express functional ion channels (Cairrao *et al.*, 2009), enabling the successful realization of electrophysiological studies by Patch-Clamp,

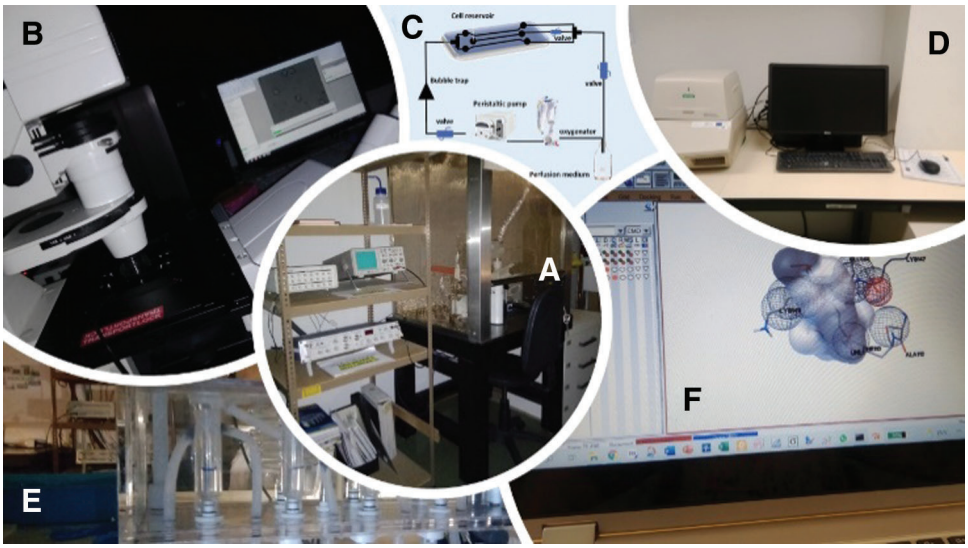

FIGURE 2. Different studies to evaluate the effects of EDCs on the human umbilical artery (HUA) and human umbilical artery smooth muscle cells (HUASMC). (A) Whole-cell patch-clamp experiments, (B) planar cell surface area technique, (C) vessel perfusion system (D) real-time polymerase chain reaction (PCR), (E) organ bath contractility, and (F) computational simulations by molecular docking.

or cell contractility studies, by planar cell surface area. These cells can also be used for cell viability studies (Gloria *et al.*, 2018), to assess the toxicity of an EDC to the CV system. Coherence between the *in vitro* results of HUASMC with those *ex vivo* (using HUA) and electrophysiological studies (Santos-Silva *et al.*, 2008; Cairrao *et al.*, 2009; Santos-Silva *et al.*, 2009; Saldanha *et al.*, 2013; Li *et al.*, 2016; Mazza *et al.*, 2016; Provitera *et al.*, 2019) and the cell contractility studies (Lorigo *et al.*, 2019; Lorigo *et al.*, 2021a) was verified. Thus, we can conclude that HUASMC can be an excellent tool for the study of EDCs *in vitro*. The use of *in silico* methods also complements the assessment of disruptive effects on the vascular system, through the computational analysis of structure-activity relationships (Molecular Docking) (Lorigo *et al.*, 2021a) (Fig. 2). Understanding how the normal physiological regulation of HUA is impaired because of exposure to EDCs is critical to understanding the development of CVD and targeting new possible treatments.

Final Remarks

In summary, the negative impact that EDC toxicity has on human cardiovascular health is a serious problem that must not be overlooked. Although several investigations are already starting to emerge in this field of research, there is still a long way to go. It should not be forgotten that exposure to EDCs is complex, often occurs through mixtures, there are non-monotonic responses, and there is also the possibility that it is influenced by various external factors (such as diet, genetics, or gender differences). Different studies on the vascular function using HUA will allow addressing this complexity: the studies can be performed in arteries from women whose births were to boys or girls, from women with different eating habits, carriers of various pathologies, whether genetic or not. However, the occurrence of pathologies, particularly CVD modulates the vascular response. This phenotypic modulation by smooth muscle cells, as it impairs vascular tone, is associated with vascular lesions, and can be studied. An example where vascular lesions have an important role to develop phenotypic modulation is atherosclerotic. In this pathological process, there is lipid accumulation (low-density lipoprotein levels increase) and mononuclear leukocyte infiltration in vascular tunica intima. Moreover, some conditions (e.g., hypertension, smoking, or diabetes) can be inductors of this disorder. In this sense, the human umbilical artery can be considered a good model to study endocrine disruption at the vascular level once allows for understanding the alterations in the regulation of contractile mechanisms controlled by smooth muscle cells. On the other hand, the possibility of non-monotonic responses induced by EDCs can be achieved by performing different techniques using the artery itself or its cells.

Therefore, we emphasize the need to continue to perform different investigations using HUA, to understand the mechanisms by which EDCs dysregulate the vascular response and lead to induce CVD in later life. In this way, it is possible to understand the vascular toxicity of EDCs and improve human cardiovascular health.

Availability of Data and Materials: Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Authors' Contribution: The authors confirm contribution to the paper as follows: study conception and design: Elisa Cairrao; data collection, analysis, and interpretation of results: Margarida Lorigo and Elisa Cairrao; draft manuscript preparation: Margarida Lorigo. All authors reviewed the results and approved the final version of the manuscript.

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