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Are the cardioprotective effects of the phytoestrogen resveratrol sex dependent?

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Abstract

Cardiovascular disease (CVD) is the number one cause of death in both men and women. Younger women have a lower risk for CVD, but their risk increases considerably after menopause when estrogen levels decrease. The cardiovascular protective properties of estrogen are mediated through decreasing vascular inflammation and progression of atherosclerosis, decreasing endothelial cell damage by preventing apoptosis and anti-hypertrophic mechanisms. Estrogen also regulates glucose and lipid levels which are two important risk factors for CVD. Resveratrol (RES), a cardioprotective polyphenolic compound is classified as a phytoestrogen due its capacity to bind to and modulate estrogen receptor signaling. Due to its estrogen like property, we speculate that the cardioprotective effects of RES treatment could be sex dependent. Based on earlier reports and more recent data from our lab presented here, we found that RES treatment may have more favourable cardiovascular outcomes in females when compared to males. This review will discuss estrogen and phytoestrogens such as RES, mediated cardioprotection with a specific focus on sex dependent effects reported in preclinical and clinical studies.

Key words: Resveratrol, Phytoestrogen, Estrogen, sex dependent cardioprotection, polyphenol, cardiovascular disease.

Introduction

Globally, cardiovascular disease (CVD) is the number one cause of mortality (WHO 2017). In 2015, 422.7 million people worldwide were diagnosed with CVD and 17.7 million people died (31% of total deaths) from it (Roth et al. 2017). Ischemic heart disease (7.4 million) followed by stroke (6.7 million) were the leading causes of CVD deaths (Roth et al. 2017). Sociodemographic analyses show that between 1990 and 2015 there was a significant decline in CVD mortality in high income countries, while the low and middle income countries continue to have a high burden of CVD mortality (Roth et al. 2017). In Canada in 2012, 2.4 million (8.5%) adults over 20 years of age were living with ischemic heart disease and ischemic heart disease was the second leading cause of death accounting for 48,000 lives (PHAC 2018). Of Canadians living with ischemic heart disease, 20% of them also suffer from heart failure (HF) (PHAC 2018). The annual economic burden for health care due to CVD in Canada was C$12 billion (2008), United States, US$ 317 billion (2011-2012) and €111 billion in Europe (AISBL 2017; Boisclair et al. 2018; CDC 2016; PHAC 2005-2008).

The prevalence, pathophysiology and mortality rates associated with CVD differ between males and females below and above 65 years of age (Kuznetsova 2018). Premenopausal women (<65 years of age) have decreased CVD prevalence and mortality as compared to males, while postmenopause (>65 years of age) the prevalence of CVD in women is similar to that of men (Kuznetsova 2018). Among the very elderly (>85 years of age), women have a higher incidence of CVD and mortality from CVD than men (Kuznetsova 2018; PHAC 2018). After –menopause, women experience a dramatic drop in estrogen levels.

Estrogen has been demonstrated to be cardioprotective (Murphy 2011). Resveratrol (RES) is a plant polyphenol also with cardioprotective properties (Bonnefont-Rousselot 2016). Many of its physiological actions have been proposed to be mediated through estrogen receptor (ER) signalling (Gehm et al. 1997). Since RES has estrogen like properties (phytoestrogen), it is important to assess if there are any sex dependent differences in in the efficacy, bioavailability, and adverse effects of RES.

In this review, we will discuss the current evidence on sex differences in the prevalence, pathophysiology, and mortality rates of CVD. We will also discuss potential cardioprotective mechanisms of estrogen and explore any reported sex dependent effects in biological effects of RES that may or may not be estrogen dependent.

CVD in males and females

Women still consider breast cancer as being the biggest threat to their health while, in Canada CVD kills 7 times more women every year than does breast cancer (Capllonch-Amer et al. 2014). In the United States, CVD affects approximately 47.8 million women while breast cancer affects only 3.3 million (Mehta et al. 2018). According to the Global Burden of Disease study CVD was the leading cause of death in women (35%) and men (32%) (Mortality and Causes of Death 2015). Women have a higher life expectancy than men and thus the majority of the elderly population (>65 years old) are women (Vehkavaara et al. 2000) . The prevalence of CVD is very high in the elderly population and hence mortality due to CVD is higher in elderly women than in men. It is known that premenopausal women aged 20-64 have less CVD risk than men, the lower risk was associated with estrogen's cardioprotective properties (Murphy 2011). Accordingly, women unlike men have lower CVD risk before 65 years of age and a higher risk

thereafter (Mosca et al. 2011). Regardless, there is a growing awareness that sex differences are important considerations in the study of CVD because, epidemiology, pathophysiology, clinical presentation, and prognosis of diseases varies between men and women (Regitz-Zagrosek 2012). Women also have sex-specific risk factors such as pregnancy and breast cancer treatment associated heart diseases (Bradshaw et al. 2016; James et al. 2005).Women also have additional factors such as their socioeconomic status and gender disparity in health care delivery that could contribute to the CVD risk (Sacco et al. 2016). The first manifestation of CVD in men and women is also different. Men will often have coronary heart disease as the first sign, whereas women have either stroke or HF as the first event (Leening et al. 2014). The incidence of CVD and HF is disproportionate in men vs. pre-menopausal women (Crandall et al. 2012). For example, among people who have had a myocardial infarction (MI) and are between 45-64 years of age, men have an 8% 5 year risk of developing HF, however, the risk for HF in women MI patient is 18% at 5 years (Crandall et al. 2012). After 65, the relative risks of HF rise for patients following MI and are 20% for men and 23% for women (Crandall et al. 2012). During age 40-54, men with HF have a 16 fold increased risk of dying within 1 year as compared to those without HF. In contrast women in the same age group with HF have a 27 fold increased risk of dying within 1 year (Crandall et al. 2012). Large differences in the incidence of CVD between men and women diminish with increasing age. We will discuss sex differences in coronary artery disease (CAD) and stroke between men and women.

Coronary artery disease

In the United States, 6.6 million women are affected by CAD every year, and women have a higher mortality rate when compared to males (Mozaffarian et al. 2015). Young men (20-39)

with ischemic heart disease have an 11 fold increased risk of dying as compared to age matched men without ischemic heart disease. Whereas, young women with ischemic heart disease have an 18 fold risk of death within 1 year as compared to controls (PHAC 2018). Generally, women are older than men when they present with CAD (Pancholy et al. 2014). In this instance, more than sex differences, age and the higher number of CVD risk factors associated with older age in women may result in higher mortality rates from CAD. On the other hand, there is pathological data supporting the increased prevalence of CAD in women. Post menopause, >50 years of age, frequency of plaque ruptures increases every decade (Falk et al. 2013). Plaque ruptures, a common cause for coronary thrombosis are less frequent in pre-menopausal women (60% of coronary thrombosis) compared to men (80% of coronary thrombosis) of the same age (Burke et al. 1998). Symptoms of CAD are also different in men and women. Typical male symptoms of CAD such pain in upper back and neck, nausea, and vomiting are often absent in women. This difference in presentation makes it harder to identify as a CAD event and delays initiation of treatment (Brewer et al. 2015; Canto et al. 2012). Chetan et al., reported that women with ST-segment elevation myocardial infarction (STEMI) received suboptimal care and worst outcomes compared to men with STEMI (Huded et al. 2018). All these factors contribute to the higher mortality rate in elderly women (>65 years of age) from CAD as compared to elderly men.

Stroke

Stroke is the third leading cause of death in men and women. 15 million strokes are reported worldwide every year and there is a stroke reported every 40 seconds in United States (Roger et al. 2011). Prevalence of stroke increases with age in both men and women. The incidence of stroke is higher in men compared to same age women until 55 years of age (Wang et al. 2013b).

This difference narrows after 55 years of age and equalizes after 65 years of age. However, >80 years of age women have a higher mortality rate from stroke than men (Appelros et al. 2009; Benjamin et al. 2018; Lofmark and Hammarstrom 2007; Rothwell et al. 2005). Risk factors for stroke includes hypertension, diabetes, smoking, overweight, CAD, atrial fibrillation, metabolic disorders, and hyperlipidemia (Boehme et al. 2017). These risk factors accumulate with age and women living longer will have a higher risk of stroke (Bradshaw et al. 2016). Overall, aging disproportionately increase risk of stroke in women than in men. Oral contraceptives, more pregnancies, eclampsia in peri- and post-partum periods are also strong risk factors for stroke in women (James et al. 2005; Lanska and Kryscio 1998, 2000). Higher testosterone levels in men are considered to be a contributing factor to increased cerebrovascular disease (Boehme et al. 2017). At the same time, higher levels of estrogen during premenopause render protection from stroke through increased cerebral blood flow, lower atherosclerosis, and vasodilatory properties (Murphy 2011). It is postulated that the postmenopause decrease in estrogen and consequent increase in androgens may contribute to the increased risk for stroke in older women. Very low and very high testosterone levels in postmenopause women were associated with an increased risk for coronary heart disease, but an association to an increased risk of stroke was inconclusive (Laughlin et al. 2010).

Mechanisms of estrogen mediated protection against CVD

Estrogen mediated protection against CVD is postulated to be primarily through protecting vasculature function in the heart and brain (Miller and Duckles 2008). Atherosclerosis in coronary arteries and cerebral arteries leads to CAD or stroke. Reduced estrogen levels have been linked to increased endothelial dysfunction leading to atherosclerosis and CAD

(Kublickiene and Luksha 2008). Inflammation is a major pathological event in the development and progression of CAD. There are increased levels of circulating inflammatory molecules in women after menopause and could be a major factor disturbing the balance of cardiometabolic parameters (Karim et al. 2010; Razmjou et al. 2018). Further, a cross sectional study has shown that inflammatory markers including Intercellular Adhesion Molecule 1 (ICAM-1), Vascular cell adhesion protein 1 (VCAM), and E- and P-selectins were significantly increased in postmenopausal women as compared with premenopausal women (Oger et al. 2001). Hormone therapy with estrogen reduced these increases in postmenopausal women suggesting an anti-inflammatory role for estrogen (Oger et al. 2001). Estrogen reduces pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα), and thereby prevents TNFα stimulated endothelial release of chemokine factors such as interleukin 8 and platelet activation factor (Chakrabarti et al. 2008). These chemokine factors are responsible for recruitment of leukocytes that initiates vascular inflammation and atherosclerosis (Chakrabarti et al. 2008). Estrogen also prevents TNFα induced activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling (Xing et al. 2012). The vasodilatory property of estrogen plays a major role in preventing CAD and stroke in women. Nitric oxide (NO) is a key gaseous signalling molecule that regulates many cardiovascular functions and has cardioprotective properties (Lundberg et al. 2015). Compared to males, basal levels of NO are higher in females. Estrogen upregulates endothelial NO production and increases endothelium dependent vasodilation. Estrogen promotes nitric oxide synthase (NOS) gene expression, increases NOS activity and NO release (Tostes et al. 2003). Estrogen mediated rapid vasorelaxation could be mediated through increasing endothelial nitric oxide synthase (eNOS) phosphorylation at the plasma membrane through src-caveolin1 mechanism (Sud et al. 2010). Another mechanism of protecting vascular function by estrogen is

its inhibition of the synthesis of the vasoconstrictor endothelin-1 in endothelial cells (Bilsel et al. 2000; Dubey et al. 2001). Estrogen also interacts with the renin angiotensin system (RAS), which functions as a vasoconstrictor and pro-inflammatory mechanism. Estrogen negatively regulates levels of renin, angiotensin converting enzyme and angiotensin 1 receptor, which collectively would reduce RAS signalling and vasoconstriction (Hinojosa-Laborde et al. 2004). Estrogen counteracts RAS mediated increase in inducible NOS, nicotinamide adenine dinucleotide phosphate oxidase, reactive oxygen species generation, and recruitment of leukocytes to prevent vascular inflammation (Alvarez et al. 2002; Gragasin et al. 2003). Endothelial damage leads to increased vascular permeability which then facilitates leukocyte migration promoting progression of inflammation in the vascular lumen (Chakrabarti et al. 2008). TNFα mediated apoptosis is one of the mechanisms of inducing endothelial damage. Estrogen protects the vascular endothelium from TNFα stimulated apoptosis through Notch-1 mediated phosphorylation of protein kinase B or AKT. This mechanism of action of estrogen was initiated through estrogen receptor-β (Fortini et al. 2017). G protein-coupled estrogen receptor (GPER) is also reported to mediate Notch-1 activation by estrogen (Rocca et al. 2018) (**Figure: 1**).

Pathological stresses such as MI or hypertension result in cardiac hypertrophy (Doggrell and Brown 1998). I*n vivo* and *in vitro* models showed that estrogen supplementation decreases angiotensin II induced cardiac hypertrophy (Pedram et al. 2005). This protection was associated with activation of MCIP1 (an inhibitor of calcineurin) gene and decreased calcineurin levels. Calcineurin- Nuclear factor of activated T-cells (NFAT) signalling is an important pathway in regulating hypertrophic response in heart (Molkentin 2004).

Changes in glucose and lipid metabolism lead to diabetes and obesity, which are considered to be contributory factors to the increased CVD risk for women at menopause (Rendell et al. 2001). Studies with animal models with either estrogen receptor-alpha (ERα) deficiency or decreased estrogen levels show that there is an increase in circulating lipids (Lizcano and Guzman 2014). Estrogen was found to limit circulating lipid levels through many different mechanisms including suppressing lipogenesis, reducing cholesterol synthesis through decreasing 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase levels, promoting synthesis of bile acids and secretion of cholesterol by liver into bile acids and increasing low density lipoprotein receptor mRNA levels (De Marinis et al. 2008; Li et al. 2001; Palmisano et al. 2017). Accordingly, vascular and cardiac protective properties of estrogen involve modulating inflammation, oxidative stress, apoptosis in the endothelium, regulating hypertrophic signalling in the heart and circulating lipid levels (Figure 1).

Hormone Replacement Therapy

Given the association of estrogen to changing CVD risk in women pre and post-menopause, it is important to discuss the strategies used to mitigate the effects of decreased estrogen levels during menopause. Based on existing preclinical evidence, it was assumed that keeping the level of female hormones closer to premenopausal state would protect women from increasing CVD risk during menopause. This concept is known as hormone replacement therapy (HRT) and many observational studies suggested that HRT were associated with a reduction in heart disease and stroke (Kodama and Kodama 2001; Matthews et al. 2018; Paganini-Hill et al. 1988). Later, the Postmenopausal Estrogens Progestins Interventions (PEPI) trial showed that estrogen alone or in combination with progestin increased high-density lipoprotein and decreased cholesterol levels

in postmenopausal women (Trial 1995). In 2002, there was a practice changing clinical trial from Women's Health Initiative (WHI) trial showing an increased risk of coronary heart disease, stroke and breast cancer with estrogen plus progestin treatment (Rossouw et al. 2002). There is controversy in the interpretation of this clinical trial as some suggest that it is not appropriate to consider HRT as a harmful option in all postmenopausal women (Langer 2017). For example, estrogen alone did not show any adverse outcomes in the WHI trial (Rossouw et al. 2002). There are also consistent reports of benefits of HRT to women in early stages of postmenopause (<10 years postmenopause) (Langer 2017). However, there had been other reports (discussed below) that suggested an increased risk of stroke with HRT. In an observational study, the increase in risk of stroke was associated to the dose of estrogen, where 0.3 mg/day was found to reduce CVD while an increase in risk for stroke was observed with greater than 0.625 mg/day in combination with progestin (Grodstein et al. 2000). A meta-analysis consisting of 28 trials concluded that HRT increased the risk of ischemic stroke and that HRT cannot be used as a primary or secondary prevention against stroke (Bath and Gray 2005). The risk for stroke with HRT was found to be minimal in women <60 years old while there was significantly higher risk in older women (Bath and Gray 2005). More recently, HRT was found to increase the risk of ischemic stroke but this increased risk was limited to oral route of hormone administration, whereas the transdermal route was reported to be relatively safer in this study (Canonico et al. 2016). The Early versus Late Intervention with Estrogen (ELITE) trial reported that early intervention is the key to success of HRT because initiation of HRT 10 years after menopause did not show any benefit in reducing risk of atherosclerotic vascular disease (Hodis et al. 2016). Recently, the International Menopause society has published an updated guideline reflecting this evidence of the benefits of early HRT in postmenopausal women (Baber et al. 2016).

Estrogen, breast cancer therapy and CVD

As mentioned earlier, women have a unique risk factor for CVD in the form of breast cancer therapy. It is ironic that ER signalling which is responsible for cardioprotection in women is also responsible for causing hormone receptor-positive breast cancer in women (Lipovka and Konhilas 2016). Tamoxifen is a selective estrogen receptor modulator (SERM) and widely used as a breast cancer treatment in ER positive tumors (Kodama and Kodama 2001). Tamoxifen therapy has reported to lower density lipoprotein levels, have antioxidant and cardioprotective effects as compared to other breast cancer hormone modulating treatments like aromatase inhibitors (AI) (Wiseman 1995). AI are associated with increased vascular disease, angina, and MI in breast cancer patients (Matthews et al. 2018). Long term use of tamoxifen is associated with increased endometrial cancer (Kodama and Kodama 2001). It was reported that the efficacy of AI treatment against breast cancer might be reduced in obese postmenopausal women due to the abundance of aromatase enzyme in adipose tissue (Ioannides et al. 2014; Oberguggenberger et al. 2017). Contrary to this finding, a recent retrospective re-analyses of an earlier study showed that obesity did not adversely affect the outcome of AI treatment (Zekri et al. 2018). Fulvestrant is an estrogen receptor down regulator that blocks the effects of estrogen in breast tissue thereby preventing the progression of ER positive breast cancer (Boer 2017). Fulvestrant is not reported to have any cardiotoxic effects. Therefore, the use of breast cancer therapies is mostly based on risk to benefit ratio and needs to be personalized to optimize beneficial outcomes.

Androgens and CVD risk in men and women

Androgens play an important role in women as testosterone is a precursor and converted to estrogen by the aromatase enzyme (Montalcini et al. 2012). Androgens are positively associated with increased bone and muscle mass, improvement in cognitive function in postmenopausal women (Davis et al. 2011; Davis and Worsley 2014). Androgen replacement therapy was recommended by the International Menopause Society in women with adrenal failure and to improve quality of sex life postmenopause (Sturdee et al. 2011). Hyper-androgenism was thought to increase CVD risk and metabolic disorders in women because it was assumed that higher risk of CVD in middle aged men was due to the increased presence of androgens. However, many clinical studies have concluded that androgen levels and CVD risk are not associated. Moreover, testosterone therapies are now considered to be beneficial in older (>50yrs of age) men and women (Rosano et al. 2017). Testosterone therapy reduced QT interval in men with hypogonadal and HF conditions (Charbit et al. 2009; Malkin et al. 2003). Testosterone therapy also reduced angina pectoris in elderly men with ischemic heart disease (Wu and Weng 1993). In women with advanced chronic HF, testosterone therapy improved functional capacity, insulin resistance and muscle strength without any adverse effect on heart (Iellamo et al. 2010; Spoletini et al. 2013). Overall, there was no direct evidence of reduction in CVD risk with androgen therapy in healthy older men and women.

Resveratrol

Food and nutrients play a vital role in maintaining the health of an individual, and a healthy diet can help delay and/or prevent many disease complications. The cardiovascular benefits of food have been largely credited to components such as fatty acids, phytosterols, polysaccharides, vitamins and polyphenols (Eilat-Adar et al. 2013). RES is a polyphenol which is produced by

plants in response to environmental stresses, such as extreme temperatures, infections and ultraviolet radiation (Harikumar and Aggarwal 2008; Mullin 2011; Pirola and Frojdo 2008). RES is found in a wide variety of dietary sources including grapes, berries, plums and peanuts (Harikumar and Aggarwal 2008; Mullin 2011; Pirola and Frojdo 2008). RES research gained attention when it was detected in red wine (Siemann EH 1992) and was subsequently associated with an observation termed the "French paradox". This is a phenomenon wherein a lower incidence of CVD is found in the French population despite a high-fat diet intake (Lippi et al. 2010). Moderate, but regular consumption of wine, particularly red wine, has been postulated as the basis for this cardioprotection (Chawla 2004; Kopp 1998) and partly attributed to RES (Baur and Sinclair 2006; Li et al. 2012; Saiko et al. 2008; Wu and Hsieh 2011). Numerous studies have also reported positive effects of RES in various other diseases such as cancer and neurodegenerative disorders (Baur and Sinclair 2006; Saiko et al. 2008). Over the past 13 years, our laboratories have been researching on cardioprotective properties of RES and published multiple reports showing that RES can prevent the development of abnormalities in cardiac structure and function in three different animal models of heart disease (Juric et al. 2007; Louis et al. 2012; Thandapilly et al. 2011; Wojciechowski et al. 2010). Most recently, we reported (Raj et al. 2016) that RES: was as effective as perindopril, an angiotensin converting enzyme inhibitor, in preventing cardiac dysfunction and inhibited mechanisms contributing to HF such as fibrosis, oxidative stress, and inflammation. This report was the first to compare the efficacy and potency of RES to a front-line HF drug. Cardiovascular benefits of resveratrol have been well documented in detail earlier and therefore not elaborated here (Bonnefont-Rousselot 2016; Raj et al. 2016; Raj et al. 2014; Sung and Dyck 2015; Wang et al. 2012).

Phytoestrogen resveratrol and sex dependent cardioprotective effects

RES is considered to be a phytoestrogen, which are a class of plant derived compounds that have pro-estrogenic effects, however partial-estrogenic or anti-estrogenic effects have also been reported for RES (Gehm et al. 1997; Jenkins et al. 2012; Sinha et al. 2016). The endocrine modulatory actions of RES have been demonstrated in isolated primary human cells and in human clinical trials (Banaszewska et al. 2016; Di Liberto et al. 2012; Dubey et al. 2010; Evans et al. 2017; Saluzzo et al. 2016). Several different points in the estrogen signaling pathway may be regulated by RES as it has been shown both to bind to ERα and to inhibit aromatase, an enzyme that converts testosterone to estrogen (Baravalle et al. 2017; Chakraborty et al. 2013). RES has been shown to function via ERα in neural and vascular smooth muscle cells (Di Liberto et al. 2012; Dubey et al. 2010). Interestingly, these two studies demonstrated opposite effects for RES on estrogen function, which suggests that cell type and disease model may be important modifiers of its control of estrogen signaling. RES competes with estrogen for ERα binding; however it was found that in certain conditions RES displays either weak estrogenic or weak anti-estrogenic properties (Ashby et al. 1999; Bhat et al. 2001). This confirms that RES is a SERM like tamoxifen and not a true agonist. RES has been shown to have anti-cancer properties in preclinical models of breast cancer (Shindikar et al. 2016). RES and tamoxifen can work synergistically as RES sensitizes tamoxifen resistant breast cancer cells to tamoxifen and induces apoptosis (Shi et al. 2013). ERα exhibits ligand specific conformational changes that could determine DNA binding sites and transcription of genes (Brzozowski et al. 1997). There is only a single RES ligand binding site in ERα (Chakraborty et al. 2013)but RES binding could result in 2 ERα conformations, one is an agonist conformation while, the other is an antagonist confirmation. The partial agonist property of RES is also a resultant of these conformations and interaction with co-activators of ER (Chakraborty et al. 2013). Therefore RES will have varying

effects on different sexes and even different organs of the same sex. There is a common belief by the public that plant derived products are safe, however, it may not be always true (Bode and Dong 2015). Soy isoflavones and phytoestrogens genistein and daidzein were shown to reduce contractile dysfunction of cardiomyocytes induced by glucose toxicity, inhibit cardiovascular collagen accumulation, and reduce ischemic cardiomyopathy (Hintz and Ren 2004; Li and Zhang 2017; Mizushige et al. 2007). Soy isoflavones also have anti-cancer properties as shown by reduced prevalence of cancer in populations regularly consuming soy rich diet possibly through early stimulation of cell differentiation (Applegate et al. 2018). A recent report on safety evaluation of isoflavones based on *in vivo* studies to date found that overall, the evidence is inconclusive regarding adverse effects of long term isoflavone consumption (Huser et al. 2018). However, this report points out that isoflavone administration needs to be carefully monitored in certain risk groups of people such as those with iodine deficiency and hypothyroidism, which is common in post-menopausal women (Huser et al. 2018). There are also potential adverse cardiac outcomes from administering soy isoflavone genistein with other drugs, such as a tyrosine kinase inhibitor due to inhibition of signalling required for normal functioning of cardiomyocytes (Harvey and Leinwand 2015).

Historically, many researchers have preferred using males for their animal studies to avoid any interference from the cyclical hormonal fluctuations in female. This bias towards using male animals or male cells has been raised as a concern by the research community (Maric-Bilkan et al. 2016). The preclinical studies of RES are also the same; most of the animal studies with resveratrol were done in males alone. Thus the ER agonist activity of RES is still inconclusive and may not be universal through both sexes. However, there are reports of beneficial effects of RES in female animal models. RES has been shown to help prevent cardiovascular dysfunction

following acute myocardial ischemia (4 weeks) in female rats that have been ovariectomized (Meng et al. 2016). Due to its ER targeting property, RES could interfere with normal estrogen signalling required for maintaining the physiological balance in female. There are studies that also reported adverse effects of RES on certain physiological aspects of female sexuality. RES administration was found to disrupt estrous cycle, reduce body weight and induce ovarian hypertrophy in female rats (Henry and Witt 2002). There are other studies that have reported sex dependent activity of RES. We will discuss some of the major findings here.

RES has also shown to have sex dependent vascular benefits. RES was found to increase aortic relaxation in response to estrogen in male rats significantly more than female rats (Soylemez et al. 2008). However, this difference could be because the female rat aorta was preconditioned to higher levels of estrogen. Although sex specific effects of RES have been observed in other models, no study have reported sex differences in RES mediated changes in cardiac structure and function (Wang et al. 2013a; Zendulka et al. 2009). The metabolism of RES is sex dependent as well. Glucuronidation and RES metabolism was more efficient in female liver microsomes, when compared to male (Dellinger et al. 2014). This is important since the RES metabolites glucuronides and sulfates could have health benefits. In contrast to the above reports, there are also studies that have reported that RES is equally beneficial in males and females (Pektas et al. 2015; Pektas et al. 2017). In a model of prenatal hypoxia in high fat fed rats, RES improved cardiac recovery power after ischemia reperfusion injury in both male and female offspring (Shah et al. 2017). Accordingly current evidence suggests cardioprotective effects of RES are independent of sex. However, since it binds to ER there could be sex dependent off target effects of RES which needs to be thoroughly examined. In total, these findings point to the importance of proper design of clinical trials to examine sex dependent effects of RES.

There is a concern that the concentration of RES and its metabolites maybe too low to have any biological effects. Bioavailability data for RES in different sexes is not currently available and sex dependent differences in bioavailability could modulate RES function. A recently published study in male mice showed that after a single oral dose of RES 10 mg/kg, trans-resveratrol (the active form) was detected in higher levels than the glucuronides and sulfates in heart tissues (Bohmdorfer et al. 2017). Earlier, another study in mice using 150 mg/kg, single and sustained (3 months) administration showed similar results where trans-RES was detected in heart but was found at very low concentrations (2.42 nmol/g of tissue) (Menet et al. 2017). It is still unclear how RES is able to sustain the beneficial effects on heart structure and function as reported in numerous animal models. β-glucuronidases and sulfatases are present in most tissues of the body and may perhaps convert RES metabolites back to native RES as needed in target tissues. This hypothesis has yet to be proven experimentally.

Further, RES has cardioprotective and anti-cancer properties, hence, would RES potentially be a better agent for HRT? (Bonnefont-Rousselot 2016; Shi et al. 2013). Currently there is lack of evidence that shows any CVD risk benefits of RES as an HRT. There was one study in postmenopausal women that showed improved cerebrovascular function with resveratrol (150 mg daily) (Evans et al. 2017). There is no current evidence suggesting that RES could affect the levels of estrogen in women. However, there are some other beneficial effects reported with use of RES including increasing estrogen metabolism, sex steroid hormone binding globulin, improvement in mood, cognitive function and decrease in pain experienced by postmenopausal women (Chow et al. 2014; Evans et al. 2017; Wong et al. 2017). More studies are necessary to measure the risk to benefit ratio of using RES as an HRT agent with a particular focus on CVD risk factors and risk of breast cancer.

Comparison of resveratrol and estrogen signalling in CVD

There are a few common mechanisms used by RES and estrogen to mediate cardioprotection. Firstly, both molecules bind to the same receptor. Despite the differences in ER confirmation after RES and estrogen binding that result in a different downstream signalling, there are similarities in the end results (Chakraborty et al. 2013). Vasodilation mediated by NO is a common downstream target of RES and estrogen signaling. Estrogen binding to ERα stimulates rapid NO production through phosphorylation of eNOS or through the genomic pathway wherein, ERα migrates into the nucleus and initiates transcription of eNOS gene which in turn increases NO production (Boisclair et al. 2018). RES binding to ERα activates downstream targets AMPK and SIRT1. SIRT1 migrates into nucleus and initiates transcription of eNOS gene expression, while AMPK increases NO production through upregulating eNOS phosphorylation (Bonnefont-Rousselot 2016). The vasodilatory properties of RES were associated with a decrease in insulin resistance by improving blood circulation in skeletal muscles (Wong and Howe 2018). Although, loss of ERα resulted in obesity and insulin resistance, evidence for restoring insulin sensitivity with estrogen treatment was inconclusive (Bryzgalova et al. 2006; Heine et al. 2000). Estrogen may indirectly improve insulin sensitivity through upregulation of adiponectin and mitochondrial biogenesis (Capllonch-Amer et al. 2014). Estrogen failed to improve insulin sensitivity in healthy postmenopausal women (Vehkavaara et al. 2000). RES was shown to improve insulin sensitivity in older adults with impaired glucose tolerance (Crandall et al. 2012). Therefore, reduction in insulin resistance secondary to diabetes could be a potential mechanism of RES mediated cardioprotection. Estrogen and RES mediated anti-inflammatory mechanisms are similar and involve inhibiting TNFα induced translocation of NFκB and transcription of interleukin-6 (Csiszar et al. 2006; Xing et al. 2012) (**Figure: 1**). RES

is also known for its antioxidant properties mediated through increased expression of Mn-superoxide dismutase, increased glutathione levels, decreased nicotinamide adenine dinucleotide phosphate oxidase levels and upregulation of nuclear factor (erythroid-derived 2)-like 2 (Bonnefont-Rousselot 2016).

The main difference in RES and estrogen is at the level of ER binding. RES binding to ERα results in a RES-specific ERα confirmation which is different from estrogen bound ERα (Nwachukwu et al. 2014). RES specific ERα has a different co-regulator binding surface than estrogen which means RES and estrogen might interact with separate set of downstream targets (Nwachukwu et al. 2014). Although, there are reported anti-estrogenic role of RES, none have been reported in the cardiovascular system.

***In vivo* evidence from our recent animal study**

Most of our earlier results on cardioprotective effects of resveratrol were on male rat models (Louis et al. 2012; Raj et al. 2016). Recently, we have conducted an animal study wherein, male and female rats with surgically induced MI were treated with RES for 8 weeks.

Methods and materials:

This *in vivo* study protocol (F-17-003) was approved by the University of Manitoba Office of Research Ethics & Compliance and Animal Care Committee. Animal care procedures were based on guidelines described in the Canadian Council for Animal Care and Use of Experimental Animals (vol. 1, 2nd ed., 1993).

5 week old male and female rats underwent coronary artery ligation surgery to induce MI. Sham surgery rats were used as controls. RES (2.5 mg/kg/d) was dissolved in 50% ethanol and administered by gavage starting in the morning before the surgery and was continued for 8 weeks post-surgery. Sham rats received a similar volume of 50% ethanol as that administered to the RES group. Echocardiographic analyses were done at the end of 8 weeks as described earlier (Raj et al. 2017).

Statistics:

One Way ANOVA was used to compare changes between the same sex groups. Tukey post hoc analyses were used to measure significance. Two tailed paired t-test was used to compare changes between male and female rats in the same group. $P<0.05$ was considered significant.

Results and discussion:

Consistent with earlier reports our results clearly show sex differences in the presentation of MI associated structural and functional changes in the heart. There was no sex difference in the cardiac hypertrophy marker interventricular septal wall dimension at systole (IVSDs) in untreated MI rats. IVSDs was significantly improved in females, but not in male rats treated with RES (**Figure: 2A**). Cardiac dilatation marker left ventricular internal dimension at systole (LVIDs) was significantly higher in male (72% higher vs male shams) untreated MI rats when compared to females (70% higher vs female shams). However, there was also a significant difference between male and female sham rats; males had a 25% larger LVIDs than female sham rats. RES treatment significantly decreased LVIDs in female (27% decrease vs female MI group) and male (12.5% decrease vs female MI group) rats, but did not normalise to control levels

(**Figure: 2B**). However, it has to be taken into consideration that MI induced changes in LVIDs with MI in females were significantly smaller than male rats. Better outcome with RES treatment in female could be due to the less severe MI in those rats. An increase in internal dimension of left ventricle is often accompanied by an increase in end systolic volume (ESV) which is the amount of the blood left in the left ventricle after systole. In this study ESV was significantly increased in both male and female rats with MI. Similar to LVIDs data, the increase in ESV was significantly smaller in female compared to male rats. Treatment with RES normalised ESV in female rats, but not in male MI rats (**Figure: 3A**). Again, this disparity in effect of RES between male and female could be due to a smaller pathological change in female rats. Ejection fraction (EF) is an important determinant of cardiac function and MI reduces EF significantly in animal models as well as in humans. Decrease in EF was also significantly smaller in female (32% decrease vs sham) compared to male MI rats (42% decrease vs sham). Treatment with RES significantly improved EF in male, but not in female rats (**Figure: 3B**). This could be a sex dependent effect of RES. Fractional shortening (FS) is a marker for left ventricular dysfunction. FS was significantly lower in both female (44% decrease vs Sham) and male (55% decrease vs sham) MI rats. RES treatment induced increase FS was not statistically significant between male and female rats. However, there was a larger (26% increase vs female MI rats) improvement in FS with RES treatment in females when compared to male rats (20% increase vs male MI rats). In RES treated MI group females had significantly higher FS than female rats. Prolongation of diastolic function parameter isovolumic relaxation time (IVRT) is a marker for diastolic dysfunction. IVRT was significantly increased in both sexes in MI group (21.6% in females and 26% in males vs corresponding shams), RES treatment significantly decreased IVRT in both female (27.4% decrease vs MI) and male (12.2% decrease vs MI) rats. The decrease in females

was larger compared to male rats. IVRT was normalised to control levels in females but not in male rats (**Figure: 4**). Although, sex differences were observed in the effect of RES on IVSDs, ESV, EF, FS and IVRT, the effect on IVRT is more likely to be sex dependent, because the magnitude of the observed increases in IVRT in male and female MI rats were comparable. Changes in all other parameters were smaller in untreated females compared to male MI rats. Therefore, the differences in IVRT observed in RES treated females vs male MI rats is purely sex dependent. Accordingly, this study reports for first time that RES has a sex dependent cardioprotective effect.

The female rats used in this study were young and hence were premenopausal. The results from this study support the general scientific consensus that premenopause, females are better protected from CVD or CVD associated cardiac changes. In this study, RES treatment did significantly improve cardiac structure and function in male and female rats. However female MI rats had a more favorable response treatment with resveratrol, when compared to male rats. Some of those could be because MI induced pathological changes were less severe as compared to male rats and that could help RES induced changes.

Summary

Based on current data, compared to men, women have a lower incidence of CVD until menopause (50-60 years of age), when the cardioprotective female sex hormone estrogen decreases. Postmenopause, up to 75 years of age, the risk of CVD in women is similar to males. After 80 years, women are at a higher risk of CVD and mortality due to CVD. Compared to men, CVD risk factors disproportionately accumulate with age in women. Men have a gradual increase in CVD risk throughout the life time. Women are also uniquely affected by pregnancies

and breast cancer drugs induced increase in risk of CVD. The beneficial cardiovascular effects of HRT are higher in postmenopausal women if initiated as early as possible (<10 years) after menopause.

The plant polyphenol compound RES, which is also a phytoestrogen, is reported to have cardioprotective properties and several clinical trials are underway testing its cardioprotective properties. However, to date there was no reports on sex dependent cardiac outcomes with RES treatment. We report here for the first time that RES could have sex dependent outcomes in an animal model of cardiac disease.

Conclusion

We conclude that pathology of CVD is dependent on sex and this dependency largely results from differences in estrogen levels in premenopausal women. Estrogen activates many different cardioprotective mechanisms. RES mediated cardioprotection could be sex dependent and be modulated by estrogen signaling. Based on our preclinical model of MI we speculate that in premenopause, women may have a favourable outcome with RES treatment when compared to same age men. Further studies are required to identify the sex dependent mechanisms activated by resveratrol and whether there are any off target effects in other tissues.

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Abbreviations

CVD – cardiovascular disease

RES – resveratrol

CAD – coronary artery disease

STEMI – ST-segment elevation myocardial infarction

ICAM-1 - Intercellular Adhesion Molecule 1

VCAM - Vascular cell adhesion protein 1

TNFα - tumor necrosis factor-alpha

NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells

NO – nitric oxide

NOS - nitric oxide synthase

eNOS - endothelial nitric oxide synthase

RAS - renin angiotensin system

ER - estrogen receptor

GPER - G protein-coupled estrogen receptor

NFAT - nuclear factor of activated T-cells

HMG-CoA - 3-hydroxy-3-methyl-glutaryl-coenzyme A

HRT - hormone replacement therapy

PEPI - Postmenopausal Estrogens Progestins Interventions

WHI - Women's Health Initiative

ELITE - Early versus Late Intervention with Estrogen

SERM - selective estrogen receptor modulator

AI - aromatase inhibitors

HF – heart failure

MI –myocardial infarction

IVSDs - interventricular septal wall dimension at systole

LVIDs - left ventricular internal dimension at systole

ESV - end systolic volume

EF - ejection fraction

FS - fractional shortening

IVRT - isovolumic relaxation time

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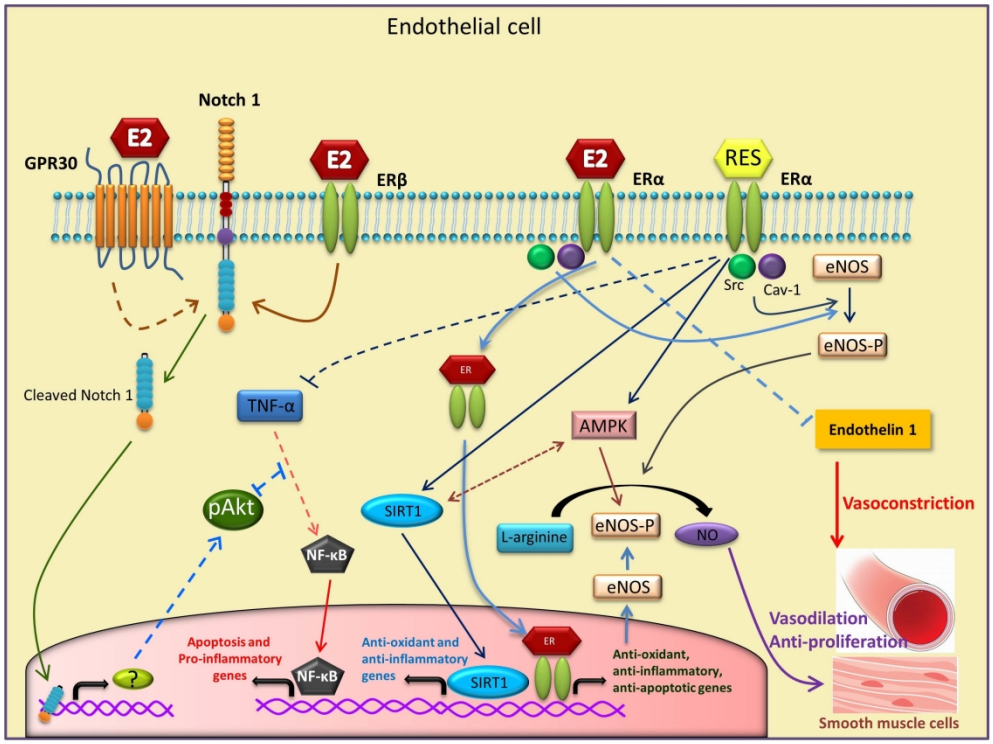
Figure legends

Figure 1: Mechanisms of estrogen (E_2) and resveratrol (RES) in endothelial cells. RES and E_2 through Src-caveolin-1 (Cav-1) increases endothelial nitric oxide synthase eNOS phosphorylation and nitric oxide (NO) production resulting in vasodilation. RES also increases NO production through 5' AMP-activated protein kinase (AMPK) mediated increase in eNOS phosphorylation and SIRT1 mediated transcription of eNOS gene. SIRT1 also mediates RES induced increase in anti-inflammatory, antioxidant and anti-apoptotic genes. RES and E_2 also inhibits tumor necrosis factor alpha (TNFα) mediated activation of nuclear factor kappa-beta (NFκB) and transcription of cytokines like interleukin-6 (IL-6) in endothelial cells. Solid lines represent direct action, while dashed lines depict indirect action. Estrogen receptor alpha (ERα); G-protein coupled receptor 30 (GPR30).

Figure 2: Echocardiographic measurement of cardiac structure in male and female, sham and myocardial infarction rats. A) Echocardiographic analyses of cardiac hypertrophy marker, Interventricular septal distance at systole (IVSDs); B) Echocardiographic analyses of cardiac dilation marker, Left ventricular internal dimension at systole (LVIDs). N=7-8 (sham) and 9-10 (MI). $P<0.05$ is considered significant.

Figure 3: Echocardiographic measurement of systolic function in male and female, sham and myocardial infarction rats. A) Echocardiographic measurement of end systolic volume (ESV); B) Echocardiographic measurement of ejection fraction (EF); C) Echocardiographic measurement of fractional shortening (FS). N=7-8 (sham) and 9-10 (MI). $P<0.05$ is considered significant.

Figure 4: Echocardiographic measurement of diastolic function parameter isovolumic relaxation time (IVRT) in male and female, sham and myocardial infarction rats. N=7-8 (sham) and 9-10 (MI). $P<0.05$ is considered significant.



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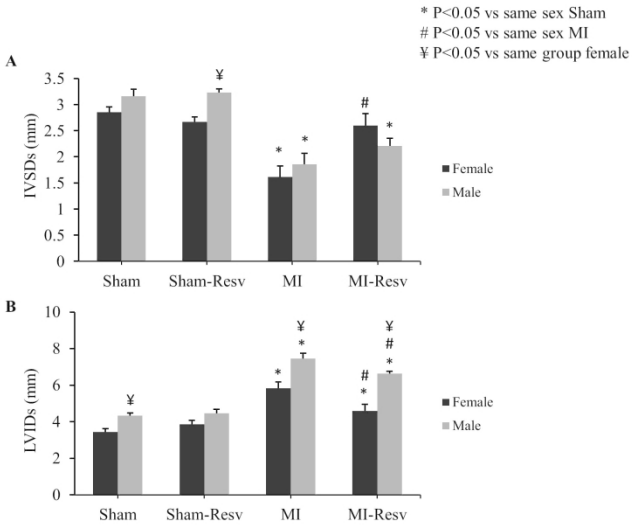
254x190mm (300 x 300 DPI)

Figure: 2



Echocardiographic measurement of cardiac structure in male and female, sham and myocardial infarction rats. A) Echocardiographic analyses of cardiac hypertrophy marker, Interventricular septal distance at systole (IVSDs); B) Echocardiographic analyses of cardiac dilation marker, Left ventricular internal dimension at systole (LVIDs). N=7-8 (sham) and 9-10 (MI). P<0.05 is considered significant.

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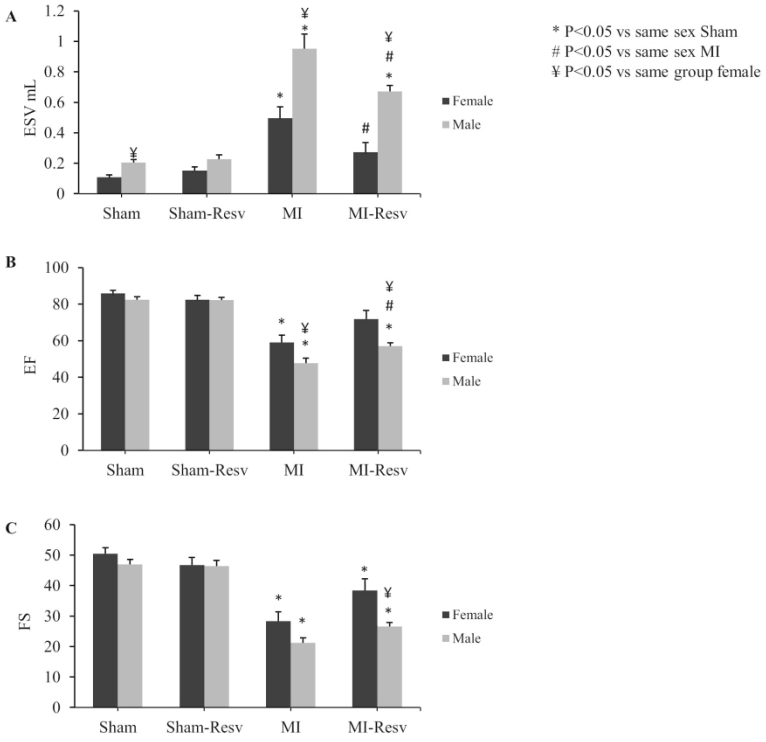

Echocardiographic measurement of systolic function in male and female, sham and myocardial infarction rats. A) Echocardiographic measurement of end systolic volume (ESV); B) Echocardiographic measurement of ejection fraction (EF); C) Echocardiographic measurement of fractional shortening (FS). N=7-8 (sham) and 9-10 (MI). P<0.05 is considered significant.

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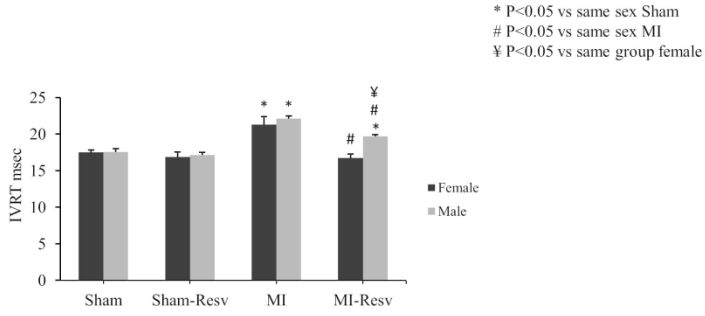

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