ORIGINAL ARTICLE

Hormone replacement therapy reduces lipid oxidation directly at the arterial wall: A possible link to estrogens' cardioprotective effect through atherosclerosis prevention

Carlos Gómez Escalante[1,2], Silvia Quesada Mora[2], Laura Navarro Bolaños[2]

[1]Department of Gynecology, San Juan de Dios Hospital, Costa Rica, [2]Department of Biochemistry, Faculty of Medicine, School of Biochemistry, University of Costa Rica, Washington, USA

ABSTRACT

Background: The first step in atherosclerosis formation is the ingurgitation of an oxidized low-density lipid (LDL) molecule by a macrophage which then turns into a foam cell within the vascular wall and initiates a cascade of inflammatory responses. Could it be that the potential cardioprotective effect observed in women receiving hormone replacement therapy (HRT) is modulated by estrogen's capacity to decrease LDL oxidation in the vascular wall and thus decrease atherosclerotic foam cells?
Materials and Methods: Thirty-four adult female Wistar rats were divided into three groups. All were double oophorectomized. After recovery, Group 1 received Estradiol Valerate subcutaneous (SC) (2.5 mg/kg/week), Group 2 Estradiol Valerate SC (2.5 mg/kg/week) + Progesterone SC (10 mg/kg/48 h), and Group 3 Placebo SC. After 10 weeks, all rats were sacrificed and a vascular dissection performed. Malondialdehyde (MDA) was measured directly on the vascular extract to determine lipid oxidative levels and HRTs' effect. Renal and hepatic tissue was also studied. Total antioxidant status (TAS) was measured to determine overall oxidative behavior.
Results: Vascular MDA levels for Group 1 = 80.80 (±16.8) μmol/ml/g, Group 2 = 107.69 (±24.9) μmol/ml/g, and Group 3 = 140.96 (±32.4) μmol/ml/g. ANOVA ($P < 0.05$), with a *post hoc* Bonferroni corrective *t*-test, showed that both Group 1 and 2 have statistically significant lower levels of MDA than Group 3. Renal tissue showed less oxidative damage in the HRT groups, while hepatic tissue showed an inverse behavior with less lipid oxidation in the placebo group. TAS decreased with oophorectomy in all groups but decreased less in both groups that received HRT compared to placebo ($P < 0.05$).
Conclusion: HRT significantly reduces lipid oxidation directly in the arterial wall.

Key Words: Atherosclerosis, hormone replacement therapy, oxidative stress

INTRODUCTION

Cardiovascular disease (CVD), and it is complications, continues to be, by far, the main cause of death in postmenopausal women in Europe and the United States.[1] Some developing countries such as Costa Rica are also showing this trend of increased cardiovascular mortality, and it has become a main topic of public health.[2]

Address for Correspondence: Dr. Carlos Gómez Escalante, Oficina #314, Distrito Cuatro, Guachipelín de Escazú, San José, Costa Rica.
E-mail: dr.escalante@gmail.com

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Atherosclerotic lesions have been directly linked to being the cause of vascular occlusion leading to acute myocardial infarction and ischemic stroke.[3] The physiopathological process leading up to these vascular lesions begins with the migration of monocytes to the intima layer of the arteries where they differentiate into macrophages. Once these macrophages come in contact with the fatty streak, oxidized low-density lipid (ox-LDL) molecules are presented to them through CD-34, these macrophages ingurgitate the ox-LDL and become foam cells.[4,5] It is these foam cells that initiate a chemotactic process that includes the liberation of radical oxygen species, metalloproteinase, myeloperoxidase, and interleukin 1, which in turn attract more macrophages to the site and also cause oxidative damage to the LDL deposited in the fatty streak, therefore, promoting a loop which favors the formation of more atherosclerotic plaque. It is this plaque that progressively reduces the lumen of the arteries inducing shear stress which causes turbulent blood flow at the site and favors the rupture of the plaque, giving way to possibly catastrophic outcomes.[6] The key point in this pathophysiological process is understanding that the LDL molecule has to be oxidized in order for the macrophage to ingurgitate it, if the LDL is not oxidized the macrophage will not ingurgitate it, and thus, not become a foam cell.[4-6]

Estrogens, *per se*, can act as free radical scavengers due to their phenol ring. Many previous authors have published *in vitro* studies that show how estrogens can reduce oxidative damage to molecules such as LDL, $CuSO_4$, and DNA.[7,8] Estrogens can also induce the formation of other protective enzymatic antioxidants such as superoxide dismutase (SOD).[9] In addition, in a previous study, our group was able to show that estrogens have the capacity to directly decrease lipid oxidation in postmenopausal women who used hormone replacement therapy (HRT).[10] It is through this mechanism of action that allows us to link estrogens to a possible cardioprotective effect.

Premenopausal women have relatively low incidence of CVD compared to men, but once women reach menopause and their estrogen levels decrease, it is quickly observed how CVD increases and how it soon surpasses the incidence observed in men.[11,12] The "window of opportunity" tells us that if we initiate HRT early in postmenopausal women, we will observe a predominately cardioprotective effect.[13] Observational studies enrolling only early postmenopausal women showed an average of 30%–50% decrease of risk for coronary heart disease with HRT.[14-16] Furthermore, an open-label prospective study such as The Danish Osteoporosis Study has confirmed this data and one of its composite endpoints showed a 49% reduction in risk for coronary heart disease over a 16 year period in users of HRT.[17] The 11-year cumulative follow-up of the estrogen-only arm with regards to coronary disease of the Women's Health Initiative showed a heart rate of 0.96.[18] A meta-analysis done by Salpeter *et al*., which included only early postmenopausal women, showed a 39% reduction in total mortality.[19] We propose that, during the reproductive period, estrogens physiologically modify the oxidative status of the arterial wall, inhibiting or at least delaying the oxidation of LDL molecules and therefore the initiation of the atherosclerotic plaque. Once women enter menopause, and thus, lose the anti-oxidative effect offered by estrogens, LDL oxidation rate is increased, and the progression of the atherosclerotic plaque is unopposed. We set out to show that estrogens have an anti-oxidative effect on vascular lipids and that lipid oxidation would be decreased at this level with the use of estrogen-based HRT. The inclusion of other key organs such as kidney and liver may also shed new information in this field.

MATERIALS AND METHODS

Animals

Due to the nature of the biological tissue required for analysis, an animal model was selected to carry out our protocol. Thirty-four adult female Wistar rats were obtained from the University of Costa Rica's Center for Animal Research. All weighed between 240 and 315 g at the beginning of the study. They were kept in a low-stress environment with a controlled temperature between 21 and 25°C. 12 h day–12 h night cycles were maintained during the greater part of the study, and all rats were kept in individual cages and received unrestricted water and daily feeding. The protocol was approved by the University's Bioethical Committee and was in accordance to international standards for animal research.

Bilateral oophorectomy

All the rats had a double oophorectomy performed to induce a menopausal model. Antiseptic solution using topic 3% chlorhexidine was applied on all of the abdomen. Anesthesia was carried out with an intraperitoneal injection of a mixture of ketamine at a dose of 90 mg/kg and xylazine at a dose of 6 mg/kg.[20,21] Great care was carried out to avoid any kind of pain during and after the procedure. Local, subcutaneous (SC) lidocaine was applied in the area where the incision was going to be carried out. Preoperative metamizol was applied subcutaneously at a dose of 200 mg/kg.[21] A low, midline incision was carried out, with careful dissection the ovaries were identified and removed bilaterally. Closure was carried out with individual 4-0 nylon sutures. Twenty-four hours after oophorectomy, all the rats received one more dose of metamizol for pain control.

Groups and medication

After allowing a week of recovery, the rats were divided into three groups. Group 1 (#12 rats) received Estradiol Valerate by SC injection (2.5 mg/kg/week), Group 2 (#12 rats) Estradiol Valerate SC (2.5 mg/kg/week) + Progesterone SC (10 mg/kg/48 h), and Group 3 (#10 rats) Placebo SC. Based on previous studies using ovariectomized rats, intermediate doses were used to try to mimic "normal" hormonal levels and thus better represent menopausal hormone therapy in women.[22-28] Since sesame seed oil was used to dilute the progesterone in Group 2, it was also used as the placebo in Group 3.

Samples and measurements

Serum was obtained from whole blood extracted at baseline (preoophorectomy) and then again at the time of sacrifice to evaluate total antioxidant status (TAS). TAS[29] was measured using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) which is a stable purple colored free radical. Once an antioxidant is added to DPPH, it is reduced and its' color changes to yellow. This change in color is proportional to the antioxidants' capacity of the added substance and can be measured spectrophotometrically at 517 nm.

After 10 weeks of medication, the rats were sacrificed by guillotine. Immediately after, a complete midline incision was performed to expose the rat's circulatory system. Resection of the aortic arch, the renal arteries and the bifurcation of the iliac arteries all the way to the femoral arteries was carried out. The liver and kidneys were also removed.

Vascular tissue was rinsed first with saline and then with buffer solution, after which they were minced with a scalpel and homogenized during 1 min with an Ultra-Turrax. Tubes were then centrifuged at 4000 rpm for 10 min at 4°C. The supernante fraction was removed and analyzed. The whole liver and both kidneys were also minced separately and processed in a similar fashion.

Lipid peroxidation was measured in the tissues using a standardized TBARS Assay Kit (Cayman Chemicals #10009055). The measurement of thiobarbituric acid (TBA) reactive substances is the most widely employed assay used to determine lipid peroxidation by measuring malonaldehyde, which is a naturally occurring by-product of lipid peroxidation.[30,31] The malondialdehyde (MDA)-TBA adduct formed by the reaction is measured colorimetrically at 532 nm. All tests were done in duplicates.

RESULTS

All rats completed the study protocol, and there was no evidence of complications from the surgical procedure which would modify the oxidative status of the animals. Weight and behavioral patterns were normal throughout the study period.

TAS measured at baseline showed no difference among the three groups reflecting similar antioxidant status among them. After oophorectomy, all three groups showed a statistically significant decrease in their TAS compared to baseline. The placebo group suffered the greatest decline in their antioxidant status and was significantly lower than the two groups that received HRT [Table 1].

Regarding lipid oxidation, Table 2 shows that Group 3, which received placebo, had much higher levels of MDA compared to Groups 1 and 2, which received HRT, ($P \leq 0.05$), indicating that the groups that did receive hormone replacement had lower levels of lipid oxidation in their vascular extracts. When comparing Group 1 with Group 2, there seems to be a lower level of lipid oxidation in the estrogen-only group compared to the estrogen/progesterone group; nevertheless, this difference was not statistically significant, ANNOVA ($P < 0.05$), with a *post hoc* Bonferroni corrective *t*-test. Renal MDA levels showed less lipid oxidation in both groups that received HRT ($P < 0.05$). Hepatic tissue showed an inverse behavior, expressing a lower level of lipid oxidation in the group which received placebo, being significantly lower than the E2V/Pro and E2V groups ($P < 0.05$).

Table 1: Serum total antioxidant status at baseline before oophorectomy and 8 weeks after with hormone replacement according to group

	TAS DPPH (reduction percentage)	
	Baseline	8 weeks postoophorectomy
E2V/Pro ($n=12$)	51.34 (±9.8)	33.98 (±5.8)*
E2 ($n=12$)	57.90 (±6.6)	23.82 (±4.4)*
Placebo ($n=10$)	52.01 (±6.9)	18.20 (±4.0)*,**

*$P \leq 0.05$ with regard to baseline, **$P \leq 0.05$ with regard to other groups. TAS: Total antioxidant status, DPPH: 1,1-diphenyl-2-picrylhydrazyl

Table 2: Determination of lipid oxidation in rat vascular, renal, and hepatic extracts through malondialdehyde levels according to hormone replacement status

	Malondialdehyde (μmol/ml/g)		
	E2V/Pro ($n=12$)	E2V ($n=12$)	Placebo ($n=10$)
Vascular tissue	107.69 (±24.9)	80.80 (±16.8)	140.96 (±32.4)*
Renal tissue	108.44 (±28.3)	126.73 (±14.1)	156.44 (±27.7)*
Hepatic tissue	16.37 (±1.6)	16.18 (±1.3)	12.32 (±1.2)*

*$P \leq 0.05$ with regard to other groups. E2V: Estradiol valerate, Pro: Progesterone

DISCUSSION

TAS has been proven to decrease after menopause. A review of the literature has shown that this may be due to either an increase of oxidative stress or a decrease of antioxidant enzymes in the body.[32,33] It has also been proven that HRT improves this antioxidant status in postmenopausal women.[34] TAS of our laboratory animals showed a similar behavior, with a decrease of TAS after oophorectomy. The greatest decrease in TAS was observed in the group receiving placebo, and an overall better antioxidant status was maintained in both the groups that received hormone replacement. Nevertheless, this is a marker which shows the overall status of an organism and is the sum of all the individual processes that are occurring, and thus the importance of analyzing tissues on an individual basis.

Recent clinical evidence has been able to reinforce the existence of the timing hypothesis related to estrogens' administration in early menopause.[17] This evidence shows that HRT may have a cardio-protective effect if given early compared to late in postmenopausal women.[13] Although there is a growing amount of clinical data in this regard, there is little information on the complete physiopathological mechanism on how estrogen-based HRT would achieve the proposed cardioprotective effects.

Our group, as well as other authors, have shown that HRT can decrease overall lipid oxidation in women who receive this therapy.[10] Since lipids have a vast distribution in the body, a systemic decrease of lipid oxidation may not be reflecting what is happening precisely at the vascular level where the atherosclerotic plaque is being formed. The fact that we were able to isolate vascular tissue and extract the lipids allowed us to eliminate any confounding factors which would modify this analysis. In Table 2, we see how vascular lipid oxidation levels were lower in both groups which received HRT compared to placebo. Under our controlled conditions, we can say that this decrease in lipid oxidation is due to the administration of HRT. Wistar rats conserve 99% of human genes and physiologically resemble humans very well.[35] This 10-week time laps in rats represent a 7-year period in a human lifespan,[35,36] thus suggesting that lipid oxidation at a vascular level may effectively be reduced using HRT during the early postmenopausal period. This allows us to hypothesize that if there is less lipid oxidation directly at the vascular site, and this is a key step in foam cell formation, then there will be less foam cell formation, leading up to less atherosclerotic plaque and thus a possible cardioprotective effect.

Regarding the lower, but not statistically significant, levels of lipid oxidation observed in the estrogen-only group, there is yet no clear explanation for this phenomenon. Previous authors have shown that estrogens have both a direct and indirect antioxidative effect. They not only act directly as an electron donor, but they also induce the formation of some of the most potent natural antioxidants enzymes available, such as SOD.[7-9,37,38] If estrogen is solely acting as an antioxidant molecule, as suggested by the work of Simpkins *et al*. and his group,[39] one would not expect the progestin to make much difference. On the other hand, if estrogen is acting to increase biochemical antioxidant defenses via hormone receptors, then the progesterone might well attenuate this effect by causing opposing changes in cell antioxidant metabolism. Wassmann[40] published findings where they found that progesterone decreases estrogen-induced synthesis of SOD in vascular smooth muscle. Progesterones' role in modifying estrogens' antioxidative role *in vivo* is yet to be defined, but this is a very important topic since most of women reach menopause with their uterus and will require the addition of a progestin to their HRT.

HRT exhibited a protective effect on lipid oxidation in renal tissue. These results are in accordance with a hormone induced renal protection previously documented by multiple authors and involves modulation of STAT3-dependant oxidative responses,[41] AGTR2[42] and estrogen receptor-α[43] expression. Renal physiology is a key determinant of cardiovascular function and may also be contributing to an improved vascular oxidative status.

Regarding the analysis of the hepatic tissue we observed a significant increase in lipid oxidation in both groups which received HRT, thus the placebo group had less oxidative damage at this level. Estrogen has been known to be a potent inductor of certain liver enzymes, but when it comes to the oxidative mechanisms involved, there has been some mixed results. While Can[44] recently concluded that 17 β-estradiol was not very effective in preventing oxidative-induced liver damage, Sobocanec *et al*.[45] proposed that 17 β-estradiol may actually have a protective effect on age-related liver oxidative damage through Nrf2-Keap1 pathways. Compared to other organs, the liver may have the most complex metabolic pathways of all and may respond in a grand variety of ways to specific metabolites. There is very little information regarding specific lipid oxidative pathways at the hepatic level, but we at least infer that estrogen might actually increase an oxidative path or decrease an antioxidative mechanism at this level.

To best of our knowledge, this is the first study that analyzes estrogens' antioxidative capacity to decrease lipid oxidation directly at the vascular wall as part of the atherosclerotic process. Physiopathology of CVD is a very complex topic but this protective effect shown by estrogens

may shed some light on the real gender induced difference shown in humans and how the approach to prevention and treatment may be differ in women.

CONCLUSION

Estrogen-based HRT reduces lipid oxidation directly at the vascular level, a process which is key to the formation of the foam cell. If foam cell formation is being reduced, and thus there is less formation of atherosclerosis, this antioxidative mechanism may be one of the keys to understanding estrogens' cardio-protective effect when given early in menopause. Vascular rat models have previously been used to study human atherosclerotic and stroke physiopathology, and thus this model might give us an insight to what is happening in the menopausal woman, allowing us to explore new options. Kidney oxidative stress is reduced by HRT. Hormone therapy increased oxidative damage in liver lipids and is a result of a complex metabolic system yet to be understood.

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Conflicts of interest

There are no conflicts of interest.

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