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Original Reserach Article

The Relationship between Advanced Inflammatory Markers and Telomere Length in Cardiac Remodeling Induced by VCD-Induced Ovarian Failure in Female Rats

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Abstract: Based on the influence of estrogen deficiency, inflammatory markers related to heart remodeling were determined and corresponding telomere lengths compared with those in the chemically induced ovarian failure model (4-vinylcyclohexene diepoxide (VCD)) in female rats. Estrogen deficiency induces cardiovascular dysfunction by increased inflammation, oxidative stress and cellular senescence. Adult female albino rats were used in both groups: (a) controls (untreated) and VCD-treated (undergoing ovarian failure). Blood and cardiac tissue were obtained 6–8 weeks after 15 days of VCD (80 mg/kg) intraperitoneal administration to induce ovarian failure for biochemical, molecular and histopathological analyses. Serum levels of interleukin-6 (IL-6) and C-reactive protein (CRP), as well as telomere length, using quantitative real-time PCR. Moreover, oxidative stress markers were assessed and the expression of estrogen receptor genes were investigated. The results demonstrated a high and significant decrease in serum estrogen levels (indicating ovarian failure) in the sera of the VCD-treated group, which was confirmed by increased serum levels of IL-6 and CRP only. The length of telomeres was significantly reduced in cardiac tissues, which prompted to an enhanced cellular senescence. In addition, levels of oxidative stress were achieved higher due to elevated malondialdehyde (MDA) levels followed by reduced glutathione and nitric oxide. Histopathological examination showed signs of degeneration, inflammatory infiltration and fibrosis in the myocardium**.** In conclusion, estrogen deficiency-related cardiac remodeling involves a complex web of pathways (such as inflammation, oxidative stress, and telomere shortening) that can be integrated into molecular targets indicating their feasibility to serve as potential predictive biomarkers for cardiovascular disease.

Keywords: Estrogen Deficiency, Ovarian Failure, VCD Model, Cardiac Remodeling, Inflammation, IL-6, C-Reactive Protein (CRP), Telomere Length, Oxidative Stress, Female Rats.

INTRODUCTION

Endothelium-dependent vasodilation using nitric oxide, estrogen is known to play an important role in cardiovascular health regarding the endothelial cells, as well as beneficial effects on oxidative stress and anti-inflammatory effects (El Khoudary *et al.,* 2020; Murphy *et al.,* 2021). Exert direct cardioprotective effects through modulation of genes with a role in cell survival; and prevent fibrosis within the heart, establishing it as one of the central regulators that obstructs pathological remodelling (Mendelsohn, 2019).

By contrast, in the absence of estrogens for example after menopause or ovarian insufficiency there exists a state in which oxidants surpass antioxidants, resulting in an inflammatory state prior to clinical manifestation with persistent low-grade inflammation and elevated susceptibility to develop cardiovascular disease (Thurston *et al*., 2021; Maas *et al*., 2019). Moreover, this modifications have also been related to increased inflammation marker such as interleukin-6 (IL-6) and C-reactive protein (CRP) that regulate the structural and functional capacity of the hear (Ridker, 2022; Libby *et al*., 2021).

ConclusionThe 4-vinylcyclohexene diepoxide (VCD)-induced ovarian failure model is a biochemical tool that efficiently induces age-related cellular and hormonal physiological changes in the ovary while selectively sparing non-reproductive organs, allowing for an experimental investigation of non-pathological adaptations to natural menopause-like decline (Koebele *et al*., 2020; Brooks *et al*., 2022). Through this model, cardiovascular histopathology researchers identified myocardial fibre degeneration and fibrosis along with biomarkers of oxidative stress and inflammation (Hoyer & Keating, 2014).

Although past research has increased our understanding of how lower estrogen levels relate to heart disease, most investigations have focused only on conventional biomarker evidence. How this relationship between ageing and inflammation at the cellular level could be exploited for therapeutic purposes we need to assess other types of high-level evidence that show us how cellular ageing is associated with inflammation. Telomere length is one of the most distinctive advanced biomarkers which relates with biological cellular age and it is confirmed to be significantly influenced by oxidative stress and chronic inflammation (Blackburn *et al*., 2019; Martens *et al.*, 2021). Low TL – An association of shorter telomeres with an increased.

The risk of heart disease stems from reduced ability to repair cells and increased inflammatory response (Haycock *et al*., 2017).

Furthermore, recent studies indicate a complicated relationship between chronic inflammation and telomere shortening. Continuous presence of high levels of inflammatory cytokines hastens Telomere attrition, which results in cell senescence owing to depletion of telomeres and increased vulnerability to tissue injury (including cardiac tissue) (Zhao *et al*., 2022; D'Mello *et al*., 2018). This relationship between telomere attrition, cellular senescence and tissue damage suggests that the integrative assessment of these 2 biomarkers may be valuable tools in providing further insight into the mechanisms underlying cardiac remodeling in states of estrogen deficiency.

Armed with this information, the current study aims to determine whether inflammatory markers such as CRP and IL-6 can predict structural and functional changes in heart of female rats with chemically induced ovarian failure. These findings may inform more accurate testing and treatment, specifically for prevention of cardiovascular risk caused by estrogen deficiency.

MATERIALS AND METHODS

Experimental Animals and Grouping

The adult female albino rats were used in this investigation. The rats were maintained under controlled conditions (22 ± 2°C, 12-hour light/dark photoperiod with free access to standard chow and water). Subject methods each rat was randomly placed into one of the following two experimental groups after one week acclimatization:Control Group (N = 8): Daily intraperitoneal (IP) injections of vehicle (sesame oil) for 15 days.Vinyl cyclohexene diepoxide group (N = 8): were given daily intraperitoneal (IP) injections of 4-vinylcyclohexene diepoxide (VCD) in a dose of 80 mg/kg body weight for 15 days consecutively to establish a gradual ovarian failure model.

Confirmation and Induction of Ovarian Failure

Serum 17β-estradiol and progesterone levels were analyzed around 6–8 weeks based on the daily assessment of vaginal smear data, following the last injection of vehicle control or VCD to reveal evidence of ovarian failure. Induction success was assessed either by measuring serum estrogen (E2) or monitoring physiological changes (e.g., increased body weight).

Electrocardiographic (ECG) Recording

During this experiment ECG were recorded at the end on a PowerLab data acquisition system (ADInstruments). Rats were anaesthetised and lead II electrodes implanted sub-cutaneously. LabChart (version 8.0) was used to analyze measurements (heart rate, P wave duration and the QRS complex for time/voltage).

Sample Collection and Anesthesi

Anesthetization to a deep degree was performed with ketamine (90 mg/kg) and xylazine (10 mg/kg), both via intraperitoneal injection. Blood samples were obtained and serum separated by cardiac puncture. After almost all of the animal is dead, hearts are elevated and rinsed with ice-cold saline before being sliced into slices for molecular biology or histology.

Biochemical Analysis

Peripheral blood samples were collected to measure serum levels of IL-6 (pg/ml), CRP (ng/ml), cTnI (ng/ml) and CK-MB (ug/L) by ELISA with kits purchased from Abcam (UK) according to the manufacturer's protocols. Malondialdehyde (MDA), reduced glutathione (GSH) and nitric oxide (NO) levels were also investigated in cardiac tissue homogenates.

Molecular Assessment Using Quantitative PCR Methods (qPCR)

DNA extracted using DNeasy Kit and RNA extracted using RNeasy Kit by QIAGEN, Germany. Real-time PCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR System.Telomere Length: Relative telomere length was determined using the telomere-to-single-copy gene (36B4) ratio ($T/S\ ratio$) according to $the\ 2-\Delta\Delta Ct$ method.

Gene Expression:

The expression of ERα and ERβ was quantified relative to the GAPDH housekeeping geneThe primer sequences used were:

Telomere:

Forward: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'

Reverse: 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'

Statistical Analysis

Data are expressed as mean ± SD. Statistical analyses were performed using the software version 26.0 from SPSS Statistics (IBM, Armonk, NY USA). Independent sample t-test for statistical significance between groups Based on the resource equation method, a sample size of 8 rats per group was justified (Iglewicz and Hoaglin 1993), based on standard practice in mechanistic and exploratory animal studies regardless of whether the expected effect size can be adequately estimated. Based on the method of resource equation, error degree of freedom (E) was calculated as follows: E = Total number of animals — Total number of groups. In this case, E = 16 animals – 2 groups = 14 animals A guideline based on the 3Rs principle (replacement, reduction, and refinement) indicates that E values between 10–20 can be considered statistically sufficient to reveal biological differences comparing two or more groups while minimizing the number of animals used. In addition, several prior experimental studies using a VCD-induced ovariectomy model of ovarian failure have reported that a sample size equal to or greater than 6−8 rats per group is adequate in providing reasonable statistical power for detecting significant differences in reduced/increased inflammatory biomarker levels, oxidative stress parameters and cardiac gene expression ($p < 0.05$ being statistically significant).

RESULTS

VCD, as a chemical ovarian failure inducer, caused several adverse changes in the bodies of female albino rats compared with controls. These included physiology, biochemistry, molecular genetics and histopathology.

Physiological Findings

Body mass was significantly greater, and heart mass greater in the VCD-treatment group compared to controls ($P < 0.05$). Moreover, the blood estrogen levels were also significantly lower in the VCD-treated subjects verifying effective ovarian failure induction. Investigation of vaginal smears showed that VCD-treated animals had irregular cycles and prolonged diestrus. Table (1) summarizes the physiological data in addition to serum estrogen levels from both groups. Compared with control, estrogen levels were significantly lower in the VCD-treated group and heart weight was also significantly higher in the VCD-treated group compared to that of control animals.

Table 1: Physiological parameters and serum estrogen levels

Parameter	Control (n=8)	VCD (n=8)	P-value
Body weight (g)	191 ± 6.47	217.8 ± 4.46	P < 0.05
Heart weight (g)	1.21 ± 0.07	1.64 ± 0.11*	P < 0.05
Serum estrogen (pg/ml)	17.40 ± 1.25*	10.22 ± 0.41	P < 0.05

Inflammatory Biomarkers

Table (2) shows the levels of inflammatory and cardiac injury biomarkers in both control and VCD-treated groups. The results demonstrated a significant increase in IL-6 and CRP levels in the VCD group, indicating activation of systemic inflammation. In addition, cardiac injury markers, including cTnI and CK-MB, were significantly elevated in the treated group compared with the control group ($P < 0.05$), reflecting myocardial damage associated with estrogen deficiency.

Table 2: Inflammatory and cardiac injury biomarkers in control and VCD-treated groups

Marker	Control (n=8)	VCD (n=8)	P-value
IL-6 (pg/ml)	62.4 ± 5.1	148.7 ± 12.3	P < 0.05
CRP (mg/L)	1.25 ± 0.15	3.82 ± 0.44	P < 0.05
cTnI (pg/ml)	24.60 ± 0.5	30.56 ± 1.44	P < 0.05
CK-MB (ng/ml)	1.51 ± 0.07	2.33 ± 0.12	P < 0.05

Telomere Length Analysis

Quantitative real-time PCR analysis demonstrated that relative telomere length was significantly shorter ($P < 0.05$) in cardiac tissue of the treated rats than in untreated rats. This shortening is suggestive of the rats treated with VCD undergoing premature aging of their cells and consequently being more sensitive to oxidative/inflammatory damage of their cardiomyocytes.

Gene Expression Findings

A large number of genes were identified in the cardiac tissue; a possible mechanism of action for VCD is suppression of estrogen receptors ERα & ERβ in response to treatment but significantly higher expression of the inflammatory pathway (& related pathways, such as oxidative stress) && VCD treated overall than untreated.

Table 3: Molecular changes and Relative Telomere Length

Marker	Control (n=8)	VCD (n=8)	Effect
Telomere length (T/S ratio)	1.00 ± 0.08	0.62 ± 0.06	Shortened
ERα (Relative Expression)	1.00 ± 0.05	0.28 ± 0.03	Downregulated
ERβ (Relative Expression)	1.00 ± 0.07	0.42 ± 0.05*	Downregulated

Oxidative Stress Parameters

Cardiac tissue analysis revealed a significant increase in malondialdehyde (MDA) levels in the VCD-treated group, indicating enhanced lipid peroxidation. Reduced glutathione (GSH) and nitric oxide (NO) levels were significantly decreased compared with controls, reflecting impaired antioxidant defense mechanisms.

Table 4: Oxidative stress

Marker	Control	VCD	P-value
MDA (ng/ml)	27.90 ± 0.99	33.83 ± 0.7	P < 0.05
ONOO- (pg/ml)	14.97 ± 0.63	19.94 ± 1.00	P < 0.05
GSH (ng/l)	11.42 ± 0.89	7.17 ± 0.97	P < 0.05
NO (µmol/l)	3.36 ± 0.13	2.69 ± 0.18	P < 0.05

Histopathological Findings

Comparison of the heart tissues of the control group, subjected to microscopic examination notes normal architecture and arrangement of myocardial fibres with intact nuclei. Conversely, echocardiographic assessment and histopathology show that the cardiac tissue of VCD treated animals displays pronounced histopathological changes such as degenerative myocardial fibres, cytoplasmic vacuolation, focal necrosis, infiltration of immune cells and interstitial oedema. Masson trichrome stain is able to recognise the layer of collagen which our observed was formed with deposition of collagennature amongst these myocardial fibres. The thoracic aorta of VCD treated animals also exhibited contrasting structural alterations including vascular wall thickening, disrupted elastic lamellae and increased fibrotic changes.

Table 5: Electrocardiographic (ECG) parameters

Parameter	Control (n=8)	VCD (n=8)	P-value
Heart rate (bpm)	258.2 ± 17.91	435.8 ± 16.58	P < 0.01
P wave (s)	0.02 ± 0.001	0.052 ± 0.002	P < 0.05
QRS voltage (mV)	0.024 ± 0.002	0.04 ± 0.004	P < 0.05
QRS duration (ms)	16.56 ± 1.2	6.22 ± 0.38	P < 0.001

Correlation Analysis

Included in these were markers for the purity of blood — IL-6 as well as CRP ranges, which exhibited a solid inverse correlation together with estrogen assessment;†the more potent the estrogen, the low both strong proinflammatory markers. Overall, longer telomeres were associated with lower oxidative stress marker levels and milder histopathology scores. Overall, these observations support the notion that depletion of estrogen via VCD induces inflammation, oxidative stress, telomere shortening and structural remodeling of cardiac tissue.

DISCUSSION

Thus, VCD induced estrogen deficiency causes considerable inflammatory, oxidative and structural changes of cardiac tissue. In addition, prominent increase in serum IL-6 and CRP levels were observed to indicate the activation of systemic inflammation in both the treated groups. The above findings agree with previous studies that showed an association between the loss of estrogen / activation of systemic inflammation enhancing the production of inflammatory cytokines and consequently cardiovascular injury (El Khoudary *et al*., 2020; Ridker, 2022).

Moreover, oxidative stress markers (interleukin-6 and nitrite) were significantly altered. We found for example, that there were significantly higher MDA & ONOO- levels and decreased GSH & NO levels. Thus you had to have an indication that oestrogen was not a very efficient antioxidant defence. Maas *et al*., (2019) similarly found that oestrogen protected cardiovascular tissues from oxidative injury.

The main conclusion of the study is that VCD significantly reduces telomere length in myocardial tissue from VCD treated rats. Chronic inflammatory processes and oxidative damage results in telomere shortening which drives cardiac myocyte cellular senescence, leading to reduced cardiomyocyte cell lineage repair potential (Blackburn *et al*., 2019; Martens *et al*., 2021). Thus, telomere shortening may be a direct factor in cardiac remodelling and myocardial dysfunction.

"This established that the hearts of VCD-treated rats displayed considerable downregulation of both ERα and ERβ expression, as compared to the controls. Such a significant (0.28-fold) loss of ERα would suggest that estrogen-mediated cardioprotection after the failure of ovarian function is markedly reduced. And finally the 0.42-fold reduction of the expression level of ERβ would show that the second pathological compensatory mechanisms by estrogen (decrease secretion of IL-6 and increase release of TNF-alpha) through lack of both ERα and ERβ after ovarian failure would be restricted either. ERβ has recently been shown to play compensatory roles in some systems (e.g., those with early chronic deficiency) but this will eventually lead to depletion of both receptor subtypes and thus increased sensitivity of the heart to oxidative stress and structural remodelling. The biochemical results were confirmed by histological examination, which showed degeneration, inflammatory cells and fibrosis in the hearts of VCD-treated rats. Overall, these data suggest that the net effect of inflammation, oxidative stress, telomere attrition and alterations in signalling through the estrogen receptor converges to trigger cardiac remodelling by way of estrogen deficiency.

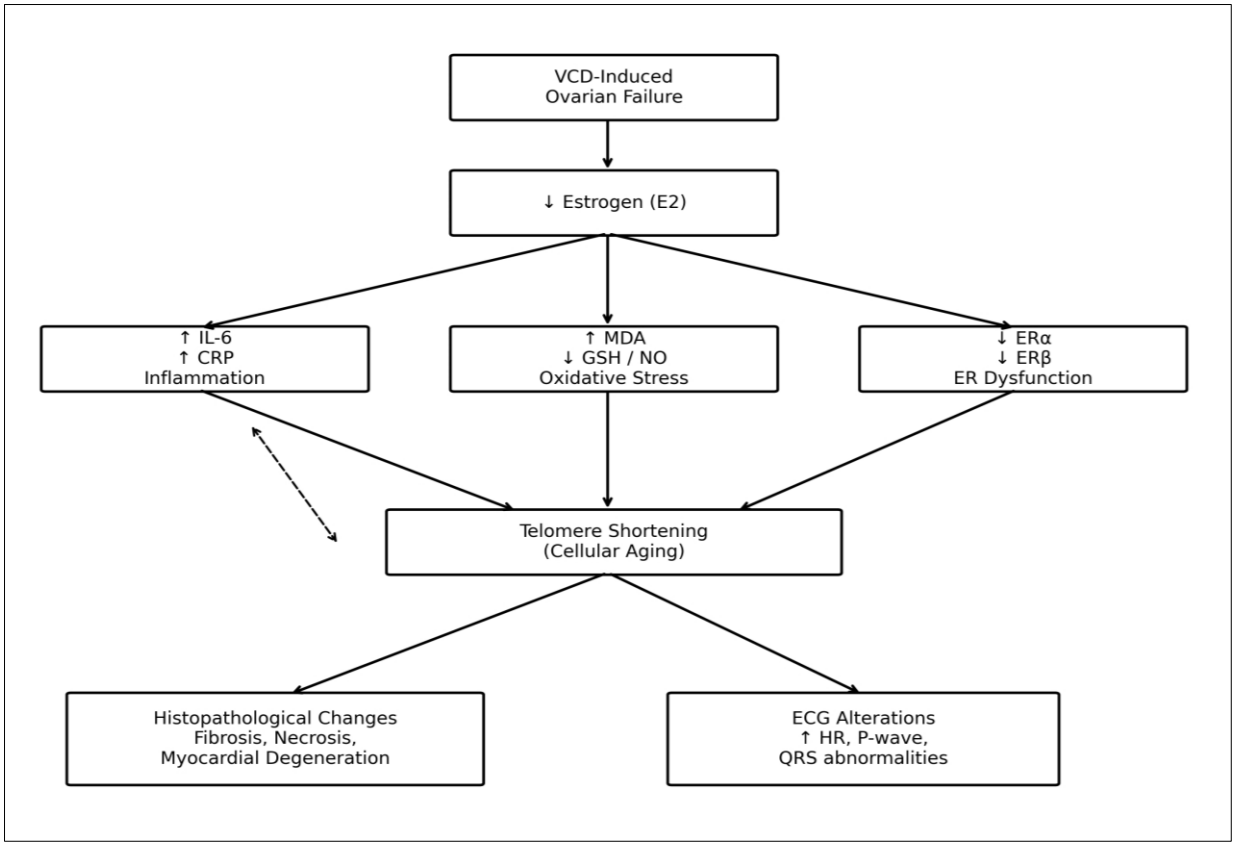

Figure 1: Proposed Mechanistic Pathway of Cardiac Remodeling in VCD-Induced Ovarian Failure

CONCLUSIONS

- Ovarian failure-induced estrogen deficiency caused pathologic cardiac remodeling via inflammation, oxidative stress and telomere erosion.
- A major telomere-shortening effect was attributable to estrogen deficiency, which led to the premature cellular senescence of viable cardiac cells and a diminished regenerative potential.
- We demonstrated that the inflammatory markers, including IL-6 and CRP, and cardiac injury markers cTnI and CK-MB were dramatically higher in DCP group than those in control group which suggested inflammation and myocardial injury.
- The loss of the antioxidant effect of estrogen elevation MDA increases and GSH and NO declines that aggravated the oxidative stress.
- Results: Reduced expression of estrogen receptors ERα and ERβ were observed, as well as histological changes including fibrotic lesions, muscle fibre degeneration and inflammatory cell infiltration.
- Our results suggested that inflammatory biomarkers and telomere length may be important predictive factors for estrogen deficiency-related cardiac remodeling and risk of cardiovascular disease.

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