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"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

This study aimed to evaluate the effects of estrogen deficiency on cardiac remodeling and to establish its association with advanced inflammatory markers and telomere length using a 4-vinylcyclohexene diepoxide (VCD)-induced chemical ovarian failure model in adult female albino rats. The animals were divided into two experimental groups (Control and VCD-treated). Ovarian failure was induced by daily intraperitoneal (IP) injections of VCD (80 mg/kg BW) for 15 consecutive days. Blood and cardiac tissue samples were collected 6 to 8 weeks post-injection for biochemical, molecular, and histopathological analyses.

The results showed a significant decrease in the serum estrogen level in the VCD group indicating successful induction of ovarian failure. There was also a marked rise in systemic inflammatory markers (Interleukin-6 (IL-6) and C Reactive Protein (CRP)) and elevated cardiac injury markers (cTnI and CK-MB). There was significant shortening of the telomeres (T/S ratio) in cardiac tissue, as was determined by molecular analysis using quantitative real-time PCR (qPCR), which is suggestive of cellular senescence. In addition, the ER gene expression (*ERα* and *ERβ*) was found to be down-regulated, while the level of malondialdehyde (MDA) and peroxynitrite ($ONOO^-$) was found to be increased, along with reduction of reduced glutathione (GSH) and nitric oxide (NO) concentration. The results of the histopathological examination revealed myocardial fiber degeneration, inflammatory cells infiltration, tissue fibrosis and structural changes in thoracic aorta. Finally, cardiac remodeling after estrogen deficiency is a complex phenomenon involving systemic inflammation, oxidative stress, and accelerated telomere shortening, further suggesting telomere length and inflammatory cytokines as molecular targets and biomarkers for cardiovascular disease in the setting of ovarian failure.

Keywords: Estrogen deficiency, Ovarian failure, VCD model, Cardiac remodeling, Telomere length, Oxidative stress.

1 INTRODUCTION

Estrogen has direct cardioprotective activity through its regulation of genes associated with cell survival and its anti-fibrotic properties, and therefore is an important regulator of pathological cardiac remodeling (Mendelsohn, 2002; Murphy & Steenbergen, 2021). On the other hand, other factors such as menopause or premenopause disrupt the balance between pro-oxidant and anti-oxidant, leading to a chronic low-grade inflammation that precedes clinical "cardiovascular diseases (Thurston et al., 2021; Maas et al., 2019). This systemic modification is closely associated with

elevated circulating inflammatory markers, particularly Interleukin-6 (IL-6) and C-reactive protein (CRP), which impair the structural and functional capacity of the heart (Ridker, 2016; Libby et al., 2018).

The ovarian failure (OF) model induced by 4-vinylcyclohexene diepoxide (VCD) is a biochemical approach that is highly effective and closely mimics the progressive hormonal and cellular changes during the natural menopausal transition in humans without directly affecting other non-reproductive organs (Koebele & Bimonte-Nelson, 2020; Brooks et al., 2022).

Previous investigations utilizing the VCD model have documented that estrogen loss precipitates myocardial fiber degeneration and interstitial fibrosis alongside heightened oxidative stress and inflammatory signaling (Hoyer & Keating, 2014).

Traditional research has shown associations between hypoestrogenism and cardiovascular dysfunction, but the mechanistic understanding of cellular ageing is lacking based on traditional biomarkers. The length of the telomeres (TL) is a specific marker of biological ageing and sensitive to chronic low-grade inflammation and oxidative damage (Blackburn et al., 2015; Martens & Nawrot, 2021). Increased rates of telomere shortening affect the efficiency of mechanisms that repair damaged cells and lead to premature senescence of the cardiomyocytes and vascular tissue, thus increasing their vulnerability to structural alterations (Haycock et al., 2014; Zhao et al., 2022). This study aims to analyze the interaction between inflammatory markers (IL-6, CRP), oxidative stress and telomere shortening to gain improved mechanistic understanding of cardiac remodeling induced by VCD-mediated estrogen deficiency.

2 MATERIAL AND METHODS

2.1 Experimental Animals and Grouping

"Albino rats, adult female, were used in this study. Animals were kept under standard laboratory conditions (22 ±2°C, 12 hours light/dark cycle) and given free access to standard chow and water. Rats were randomly divided into two groups of 8 after the one week acclimatization period: "

- **"Control Group:** Received daily intraperitoneal (IP) injections of vehicle (sesame oil) for 15 consecutive days.
- **"VCD Group:** 4-vinylcyclohexene diepoxide (VCD) was administered daily at 80 mg/kg body weight (BW) for 15 consecutive days to cause ovarian failure (Hoyer & Keating, 2014, Koebele & Bimonte-Nelson, 2020). "

The sample size (n = 8 rats/group) was confirmed by the resource equation method (Iglewicz & Hoaglin, 1993; Brooks et al., 2022), which suggests that an error degree of freedom (E) ranging from 10 to 20 ensures that the study has enough statistical power to identify significant biological differences for inflammatory biomarkers, oxidative stress parameters, and cardiac gene expression ($P < 0.05$).

2.2 Confirmation of Ovarian Failure

Daily vaginal smearing was performed to evaluate estrous cyclicity. Post-injection ovarian failure was confirmed 6–8 weeks after injection by examining the presence of the diestrus phase and by measuring serum 17 β -estradiol and progesterone (Brooks et al., 2022).

2.3 Electrocardiographic (ECG) Recording

Animals were then anaesthetised at the end of the experimental period, and Lead II ECG parameters were collected on a PowerLab data acquisition system (ADInstruments) and analysed using LabChart software (version 8.0). Other parameters measured were heart rate, P wave duration, QRS voltage, and QRS duration.

2.4 Sample Collection and Anesthesia

Rats were deeply anaesthetized by an IP injection containing ketamine (90 mg/kg) and xylazine (10 mg/kg). Cardiac punctures were made and serum was isolated for biochemical examination. Hearts were quickly removed, washed with ice cold saline and cut for molecular and histological analysis.

2.5 Biochemical Assays

Commercial ELISA kits (Abcam, UK) were used to measure the levels of Interleukin-6 (IL-6), C-reactive protein (CRP), cardiac troponin I (cTnI) and creatine kinase-MB (CK-MB) in serum, according to manufacturer's instructions (Ridker, 2016; Libby et al., 2018). Moreover, cardiac tissue homogenates were used to determine the levels of malondialdehyde (MDA), peroxynitrite (ONOO -), reduced glutathione (GSH) and nitric oxide (NO) (Maas et al., 2019).

2.6 Quantitative Real-Time PCR (qPCR) & Telomere Length Measurement

Total genomic DNA and RNA were isolated from cardiac tissues using DNeasy and RNeasy Kits (QIAGEN, Germany), respectively. Real-time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems).

- **Relative Telomere Length:** Relative telomere length was assessed by calculating the ratio of telomere repeat copy number to single-copy gene (36B4) copy number (T/S ratio) using the $2^{-\Delta\Delta C_t}$ method (Blackburn et al., 2015; Zhao et al., 2022). Specific primer sequences used were:
  - *Telomere Forward:* 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'
  - *Telomere Reverse:* 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'
- **Gene Expression:** Expression levels of estrogen receptors (*Er*α and *ERβ*) were quantified relative to the housekeeping gene *GAPDH* (Mendelsohn, 2002; Murphy & Steenbergen, 2021).

2.7 Statistical Analysis

Data are expressed as Mean ± Standard Deviation (SD). Statistical analyses were performed using IBM SPSS Statistics (Version 26.0, Armonk, NY, USA). Differences between experimental groups were analyzed using Student's t-test, with statistical significance established at $P < 0.05$.

3 RESULTS

Administering 4-vinylcyclohexene diepoxide (VCD) as a chemical inducer of ovarian failure in adult female albino rats resulted in several significant physiological, biochemical, molecular, electrophysiological, and histopathological alterations compared to the control group.

3.1 Physiological, Hormonal, and Inflammatory Baseline Changes

Chemical induction of ovarian failure via VCD resulted in significant physiological and biochemical alterations. Serum 17β-estradiol was significantly reduced (10.22 ± 0.41 pg/ml) compared to controls (17.40 ±1.25 pg/ml, $P < 0.05$), accompanied by body weight and heart weight increases ($P < 0.05$). Concurrently, systemic inflammatory markers (IL-6 and CRP) and myocardial damage indicators (cTnI and CK-MB) showed significant elevations ($P < 0.05$), as detailed in Table 1.

Table 1: Physiological parameters, serum estrogen, systemic inflammatory, and cardiac injury biomarkers in Control and VCD-treated groups

"Category	Parameter	Control (n=8)	VCD (n=8)	P-value
Physiological	Final Body Weight (g)	191 ±6.47	217.8 ±4.46	P < 0.05
	Absolute Heart Weight (g)	1.21 ±0.07	1.64 ±0.11	P < 0.05
Hormonal	Serum Estrogen (pg/ml)	17.40 ±1.25	10.22 ±0.41	P < 0.05
Inflammatory	Interleukin-6 (IL-6, pg/ml)	62.4 ±5.1	148.7±12.3	P < 0.05
	C-Reactive Protein (CRP, mg/L)	1.25 ±0.15	3.82 ±0.44	P < 0.05
Cardiac Injury	Cardiac Troponin I (cTnI, pg/ml)	24.60 ±0.50	30.56 ±1.44	P < 0.05
	Creatine Kinase-MB (CK-MB, ng/ml)	1.51 ±0.07	2.33 ±0.12	P < 0.05"

3.2 Molecular, Oxidative Stress, and Electrophysiological Alterations

Cardiac telomere shortening (T/S ratio: 0.62 ±0.06 vs. 1.00 ±0.08, $P < 0.05$) showed by quantitative PCR analysis further suggested accelerated cellular senescence of the cardiomyocytes. Results from cardiac gene expression analysis showed a strong decrease of ER α (0.28-fold) and ER β (0.42-fold) expression compared to the controls.

Oxidative stress assessment of cardiac homogenates showed a significant increase ($P < 0.05$) in malondialdehyde (MDA) and peroxynitrite ($ONOO^-$) and a significant decrease of endogenous antioxidants, such as reduced glutathione (GSH) and nitric oxide (NO) ($P < 0.05$). In addition, the electrophysiological changes observed between the ECG recordings of rats treated with VCD and control rats were significant, including heart rate, P wave duration, QRS voltage ($P < 0.05$), and QRS duration ($P < 0.001$). The results of all the molecular, redox and electrophysiological parameters are summarized in Table 2.

Table 2: Molecular markers, telomere length, oxidative stress, and electrocardiographic (ECG) parameters

Category	Parameter / Marker	Control (n=8)	VCD (n=8)	P-value/Effect
"**Molecular**	Relative Telomere Length (T/S ratio)	1.00 ±0.08	0.62 ±0.06	Shortened ($P < 0.05$)
	ERα Relative Expression	1.00 ±0.05	0.28 ±0.03	Downregulated ($P < 0.05$)
	ERβ Relative Expression	1.00 ±0.07	0.42 ±0.05	Downregulated ($P < 0.05$)
Oxidative Stress	Malondialdehyde (MDA, ng/ml)	27.90 ±0.99	33.83 ±0.70	$P < 0.05$
	Peroxynitrite ($ONOO^-$, pg/ml)	14.97 ±0.63	19.94 ±1.00	$P < 0.05$
	Reduced Glutathione (GSH, ng/l)	11.42 ±0.89	7.17 ±0.97	$P < 0.05$
	Nitric Oxide (NO, μmol/l)	3.36 ±0.13	2.69 ±0.18	$P < 0.05$
ECG	Heart Rate (bpm)	258.2 ±17.91	435.8 ±16.58	$P < 0.01$
	P wave duration (s)	0.02 ±0.001	0.052 ±0.002	$P < 0.05$
	QRS voltage (mV)	0.024 ±0.002	0.040 ±0.004	$P < 0.05$
	QRS duration (ms)	16.56 ±1.20	6.22 ±0.38	$P < 0.001$"

3.3 Histopathological Findings and Correlation Analysis

Histological structure of the cardiac tissue of the control rats showed no abnormal changes and the myocardial fibres were arranged in an organised manner, and the nuclei were intact. However, the heart sections in VCD-treated rats had marked pathological changes, such as myocardial fiber degeneration, vacuolation of the cytoplasm, focal necrosis, infiltration of inflammatory cells, and interstitial oedema. Masson's trichrome staining resulted in an excessive collagen deposition (interstitial fibrosis) between myocardial fibers. Thoracic aorta from VCD rats treated with VCD had thickened vascular walls, disrupted elastic lamellae and fibrotic remodeling of the vessel wall as determined by histopathological analysis.

There were significant negative correlations between serum levels of estrogen and inflammatory markers (IL-6 and CRP) by correlation analysis. In addition, there was a positive correlation between relative telomere length and GSH and NO levels, but a negative correlation with MDA concentration and histopathological lesion score, suggesting that hypoestrogenism-induced oxidative stress and inflammation are directly responsible for cardiac telomere attrition and tissue remodeling.

4 DISCUSSION

The major goal of this study was to investigate the novel mechanistic relationships between post-ovarian failure estrogen deficiency and advanced inflammatory signaling, oxidative injury and cardiac telomere attrition in the chemical VCD rat model. Results highlight a systemic pathological cascade of events involving systemic inflammatory activation, increased oxidative and nitrososative stress, decreased estrogen receptor expression, and cardiac cell senescence that leads to cardiac remodeling and electrophysiology changes.

The observed systemic inflammatory activation—characterized by significant elevations in serum IL-6 and CRP levels—aligns with clinical and experimental evidence highlighting estrogen as a crucial immunomodulator (Ridker, 2016; Libby et al., 2018). In physiological conditions, 17 β -estradiol inhibits the expression of pro-inflammatory cytokines by inhibiting NF-kB pathway. The absence of estrogen puts this brake off, giving rise to low-grade chronic inflammation that injures the vascular endothelial and myocardial tissue, as seen by increased serum cTnI and CK-MB levels in the VCD group (El Khoudary et al., 2020; Thurston et al., 2021).

In addition, this data shows a dramatic imbalance of pro-oxidants/antioxidants in cardiac tissue. High levels of MDA and ONOO- and severe GSH depletion with low levels of bioavailable NO confirm hypoestrogenism as disrupting the

endogenous antioxidant enzymatic systems (Maas et al, 2019). NO is also a factor that reduces vasodilation, increases systemic vascular resistance, and increases the formation of peroxynitrite, which causes protein nitration and lipid peroxidation of the cardiomyocyte membrane.

The most important finding in this investigation is the finding of significant relative telomere shortening (T/S ratio) in the myocardium of VCD-treated rats. The guanine-rich sequence architecture makes telomeres very susceptible to oxidative cleavage. Extended exposure to high levels of oxidative stress (MDA, ONOO-) and inflammatory cytokines (IL-6) can cause single strand DNA breaks within the telomeric repeats, which can in turn cause the telomerase activity to drop and lead to premature cellular senescence (Blackburn et al., 2015; Martens & Nawrot, 2021). Senescent cardiomyocytes and cardiac fibroblasts acquire a senescence-associated secretory phenotype (SASP), driving persistent local inflammation and interstitial collagen deposition (fibrosis), as confirmed by Masson's trichrome histology in our study (Haycock et al., 2014; Zhao et al., 2022).

The profound downregulation of both *ERα* (0.28-fold) and *ERβ* (0.42-fold) mRNA expression further explains the loss of intrinsic cardioprotection. While *ERα* signaling mediates anti-apoptotic and anti-fibrotic gene programs, ERβ provides crucial compensatory regulation against oxidative damage and cytokine overproduction (Mendelsohn, 2002; Murphy & Steenbergen, 2021). The loss of both receptor subtypes leaves cardiac tissue heightenedly vulnerable to structural damage and electrophysiological disturbances, as reflected by increased heart rate, prolonged P wave duration, and QRS alteration on ECG recordings.

Collectively, these integrated findings confirm that estrogen failure due to chemical ovarian damage activates a damaging pathway wherein oxidative stress and inflammatory signaling act synergistically to accelerate cardiomyocyte telomere attrition, ultimately driving fibrotic remodeling and cardiac dysfunction.

5 CONCLUSION

In conclusion, pathological cardiac remodeling secondary to VCD-induced ovarian failure is driven by a complex interplay of systemic inflammation, oxidative stress, and accelerated cardiomyocyte telomere attrition. The loss of endogenous estrogen significantly downregulates myocardial *ERα* and *ERβ* receptor expression, exacerbating oxidative injury (elevated MDA and $ONOO^-$, depleted GSH and NO) and triggering systemic inflammatory cascades (elevated IL-6 and CRP) that induce overt cardiomyocyte damage (elevated cTnI and CK-MB). Crucially, the observed shortening of cardiac telomeres highlights premature cellular senescence and diminished tissue repair capacity as central mechanisms underlying the observed histological fibrosis and electrophysiological abnormalities. Collectively, these findings demonstrate that relative telomere length, in conjunction with advanced inflammatory biomarkers, represents a promising predictive bio-target for assessing cardiovascular disease risk and guiding therapeutic strategies in estrogen-deficient conditions.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

The author declares no conflicts of interest regarding this manuscript."

Statement of ethical approval

"All experimental procedures involving animals were conducted in accordance with the institutional ethical guidelines and were approved by the Research Ethics Committee of the College of Veterinary Medicine, Tikrit University, Iraq.

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