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Steap-3 gene expression profiling in ischemic and dilated cardiomyopathy rat models

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Abstract
Background: Despite the implementation of contemporary therapeutic approaches, individuals diagnosed with heart failure have a mere 50% survival rate over a five year period. In order to enhance the advancement of novel treatment approaches, it is imperative to utilize preclinical disease models that accurately replicate the complexities of the human situation. The relationship between aberrant iron regulation, myocardial function, and prognosis in heart failure is established in the scientific literature. However, there is a lack of comprehensive understanding about the expression patterns of genes associated with iron regulation, both at the tissue level of the myocardium and at the level of individual cells.
Objectives: Measuring differential gene expression of STEAP3 gene in animal models of heart failure VS. Control.
Methodology: Quantitative polymerase chain reaction (qPCR) was used to measure gene expression profiling of STEAP3 gene in blood and left ventricular tissue samples of circumflex artery ligation ischemic injury rat models and doxorubicin induced dilated cardiomyopathy rat models VS. control rats.
Results: STEAP3 gene was significantly downregulated in both rat heart failure models compared to control rats.
Conclusion: STEAP3 gene is significantly downregulated in heart failure animal models.

Keywords: Heart failure, STEAP3 gene, Gene expression, Animal models, Iron regulation

Introduction
Heart failure (HF) is a significant clinical and public health concern characterized by a range of symptoms and a high prevalence globally. Dilated cardiomyopathy (DCM) is a prominent etiological factor in heart failure (HF), distinguished by the enlargement of the left ventricle and impaired systolic function, occurring in the absence of identifiable abnormal loading circumstances or substantial coronary artery disease [1, 2]. Heart transplantation (HTx) and left ventricular assist device (LVAD) implantation continue to be the prevailing surgical interventions for managing patients with cardiomyopathy, despite the inherent challenges in addressing the advanced stage of the disease and the various concerns raised regarding their effects on quality of life and long-term results [3,4]. Hence, there is a constant exploration of novel therapeutic approaches. Iron metabolism problems, including both iron deficiency (ID) and iron overload, have become a subject of increasing interest in relation to metabolic processes linked with heart failure (HF) [6, 7, 8, 9]. The investigation of iron mechanisms implicated in heart failure at the systemic level is currently being pursued; yet, their comprehension specifically at the cardiac level remains enigmatic. Iron is a crucial micronutrient that plays a vital role in various physiological processes. These functions encompass oxygen transportation and storage, metabolism in cardiac and skeletal muscles, mitochondrial respiration, as well as protein synthesis and degradation [10, 11, 12, 13]. Simultaneously, it should be noted that iron possesses the capacity to induce toxicity when its levels above the recommended threshold. This is mostly due to its role in catalyzing the production of reactive radicals, which have the potential to inflict harm onto DNA, proteins, and lipids [14].

Therefore, it is imperative to maintain strict regulation of both systemic and cellular iron homeostasis. The majority of bodily iron, over 70%, is found in the form of heme within hemoglobin (or myoglobin) in growing erythroblasts and mature erythrocytes. This iron is utilized for the purpose of oxygen binding and transportation to various tissues [13, 15]. Iron that is in circulation is primarily bound to transferrin (TF) and is transported to red blood cells and other cells in the body through particular absorption mechanisms [13]. Transferrin-bound iron interacts with its corresponding receptor, known as transferrin receptor protein 1 (TfR1), resulting in the ingestion of iron through the process of endocytosis. The non-transferrin-bound form of the substance is internalized by the ubiquitously expressed divalent metal transporter 1 (DMT1) located on the cellular surface. Furthermore, it has been observed that in cases of iron overload, the transportation of non-transferrin-bound iron can occur through voltage-gated calcium channels found in cardiomyocytes [16]. Within the endosome, the process of ferric iron (Fe3+) reduction to ferrous iron (Fe2+) is facilitated by a group of metalloreductases known as the six-transmembrane epithelial antigen of the prostate (STEAP) family. Subsequently, the ferrous iron is transported into the cytoplasm by the action of DMT1 [10, 13]. After entering the cytoplasm, Fe2+ is employed by erythroid 5-aminolevulinic acid synthase (ALAS2) for heme synthesis. It can also be stored in iron-ferritin complexes, transported into mitochondria for the production of heme and iron-sulfur (Fe-S) clusters, or expelled from the cell through ferroportin [9]. The primary regulators of systemic iron homeostasis are hepcidin and ferroportin, which collaborate to control the movement of iron from cells into the systemic circulation. Hepcidin, a peptide hormone of tiny molecular size, is mostly generated by hepatocytes. It engages in a negative feedback mechanism by interacting with ferroportin on target cells. Specifically, it inhibits the absorption of iron in the intestines and sequesters iron within enterocytes, hepatocytes, and macrophages [8, 17]. The heart exhibits a substantial degree of hepcidin expression, ranking second in terms of expression levels. Hepcidin serves as an autocrine protein, playing a crucial role in the regulation of iron levels specifically inside cardiomyocytes [9, 18]. In addition, the regulation of cellular iron metabolism after translation occurs through the utilization of iron response elements (IREs) and iron regulatory proteins (IRPs). In the context of iron shortage, the contact between IRE/IRPs plays a role in facilitating the absorption of iron. This is achieved by stabilizing the mRNAs of transferrin receptor (TFRC) and DMT1, thereby limiting their degradation and reducing their sequestration and efflux. Additionally, this relationship inhibits the translation of ferritin, ferroportin, and ALAS2 [19]. Systemic inflammatory disease (SID) is an often occurring comorbidity in patients diagnosed with heart failure (HF), with a reported frequency as high as 58% [20]. The predictive significance of systemic ID is significant, regardless of the existence of anemia, and is independent of the normal cardiac risk factors [21, 22]. Over the past ten years, various clinical trials have provided evidence of the advantageous effects of iron supplementation using ferric carboxymaltose in individuals diagnosed with iron deficiency (ID) and heart failure (HF) [23]. These findings underscore the significant involvement of iron metabolism in numerous systemic processes that contribute to the progression of HF [22, 24]. Previous studies have demonstrated that the administration of intravenous iron supplementation has a positive impact on the quality of life and exercise ability of patients with heart failure and iron deficiency, leading to a reduction in re-hospitalization rates [23, 25]. However, there is a lack of research on myocardial ID (MID) in HF, despite its clinical association with significant unfavorable changes such as interstitial fibrosis and cardiac hypertrophy [26]. The relationship between MID and biomarkers of systemic iron homeostasis is weak, making it unclear whether MID is a contributing factor to systemic iron deficiency or a result of it [27]. Iron overload, albeit less common than systemic infectious diseases, can result in the development of cardiomyopathy and ultimately heart failure through the process of oxidative damage [28]. In recent studies, researchers have shown a connection between iron excess and cardiovascular illness, specifically attributing this relationship to the process of ferroptosis [29, 30]. Ferroptosis is a type of planned cell death that relies on the presence of iron and is characterized by the buildup of lipid hydroperoxides [31]. There is substantial evidence from both *in-vitro* and *in-vivo* studies that supports the pathophysiological involvement of ferroptosis in several types of cardiovascular dysfunctions [32]. Numerous facets of iron metabolism, encompassing its uptake, storage, and utilization, play crucial roles in the regulation of ferroptosis.

Although the molecular mechanisms of iron have been extensively examined in several physiological and pathological processes, there has been limited research conducted on genes specifically at the cardiac cell level, resulting in a lack of complete assessment of dilated cardiomyopathy (DCM) [30]. The STEAP family, which consists of four multipass membrane proteins, was initially identified as regulators of iron homeostasis. Subsequently, its involvement in other cellular and molecular processes, such as cell fate determination and inflammatory response, has been established.The age range of individuals being referred to is between 8 and 11 years old. STEAP3, also known as Six-Transmembrane Epithelial Antigen of Prostate 3, plays a significant role in various biological processes. These processes include iron homeostasis, exosome assembly and secretion, apoptosis, proliferation, and inflammation [31-35]. For example, the role of STEAP3 in regulating cellular destiny has been elucidated by its involvement in promoting exosomal protein release via the p53-dependent secretory pathway [36, 37]. Furthermore, the absence of STEAP3 has been observed to attenuate the inflammatory responses mediated by TLR4 in macrophages [32]. In addition, it was observed that mice lacking STEAP3 exhibited protective benefits during ischemia-reperfusion injury through the inhibition of TAK1 activation, which is associated with the transforming growth factor-β signaling pathway [38].

The main goal of the current study is to measure differential expression profiling of STEAP3 gene in blood and heart ventricles of ischemic cardiomyopathy animal models.

Methodology

Animals purchasing and housing

In the current study, Twenty male Sprague- Dawley (SD) rats, with an average weight ranging from 250 to 350 grams, were procured from Qingdao Drug Administration in China. The rats were kept in a designated pathogen-free environment, characterized by a controlled temperature of 24° ± 2°C and humidity of 55 ± 10%. They were provided

with unrestricted access to standard rat food and water within the experimental facility located at the Veterinary of Medicine, University of Mosul**.** Ten SD rats were treated as control groups, five rats underwent circumflex artery ligation procedure and five rats underwent doxorubicin induced dilated cardiomyopathy procedure.

Preparation of rat ischemic injury model by circumflex artery ligation

The Sprague-Dawley rats underwent anesthesia with intraperitoneal injection of 7% chloral hydrate, sourced from Aoxin in China. A surgical procedure involving a left thoracotomy and pericardiotomy was carried out. The dissection of the left coronary artery occurred above the initial diagonal branch, followed by ligation in close proximity to the origin of the left circumflex artery using silk thread. The occurrence of blockage caused by Slipknot persisted for a duration of 30 minutes. Immediate observation of R wave amplification and ST segment depression was noted in lead II of the provided ECG. The myocardium located distal to the ligation line had a darker appearance, which is indicative of myocardial ischemia. Following a 30-minute period of myocardial ischemia (MI), proceeded to release the slipknot for a duration of 120 minutes, thereby facilitating reperfusion (R) [39, 40].

Preparation of doxorubicin-induced dilated cardiomyopathy rat model

A group of five Sprague-Dawley rats were administered six intraperitoneal injections of DXR at a dosage of 2.5 mg/kg per dose, over a span of two weeks. This resulted in a cumulative dose of 15 mg/kg of body weight. The evaluation of cardiac function was conducted before and after DXR therapy using 2D echocardiography, following the methodology outlined in a previous study [41]. Following the administration of DXR injection, the animals that remained alive were subsequently assessed for left ventricular (LV) function at specific time intervals as determined by previously published research employing short-term evaluations [42]. The control animals were assessed concurrently with the animal model group.

The study utilized M-mode echocardiography to perform two-dimensional short-axis imaging of the left ventricle (LV) at the papillary muscle level. This imaging technique was employed to measure the internal diameters of the left ventricles during systole and diastole. These measurements were then used to calculate the fractional shortening (%FS), which serves as an indicator of systolic function. Additionally, the left ventricular ejection fraction (LVEF) was determined using the Teichholz method [43]. Furthermore, measurements of the posterior and septal wall thickness of the left ventricle (LV) were conducted and afterwards utilized in the calculation of LV mass. All measurements were conducted by a sole investigator. All measurements were examined by a second scientist who possessed expertise in the study of mouse hearts using echocardiography. The measurement of the LV end-diastolic dimension (LVDd) was conducted at the point of maximal diastolic dimension, whereas the measurement of the LV end-systolic dimension (LVDs) was taken at the point of maximal anterior motion of the posterior wall. The measurements were obtained by calculating the average of five consecutive cardiac cycles, while maintaining a consistent transducer location and angle inside the same stop picture frame. The temporal duration separating two successive cardiac cycles was quantified for the purpose of determining heart rate.

Blood and heart sampling of the animal models:

Blood samples were obtained subsequent to echocardiography by cardiac puncture and thereafter subjected to centrifugation at a force of 2000 g for a duration of 10 minutes. The serum and plasma samples were kept at a temperature of -80°C in order to facilitate subsequent analysis. The rats were euthanized by means of direct intraventricular injection of KCl, as described by Gao *et al.* [40]. The hearts were extracted and subsequently washed using a solution of ice-cold phosphate-buffered saline. The ventricular tissue was rapidly frozen using liquid nitrogen and thereafter preserved at a temperature of -80°C.

q RT-PCR

Forty samples (five ischemic cardiomyopathy rat model heart samples VS. five control heart samples, five ischemic cardiomyopathy animal model blood samples VS. five control blood samples, five dilated cardiomyopathy animal models heart samples VS. five control heart samples and five dilated cardiomyopathy animal models blood samples VS. five control blood samples) were processed using qRT-PCR analysis for the STEAP 3 gene. The RT-PCR primers are [44].

STEAP3-F CCC GTC CAT TGC TAATTC CCT and STEAP3-R CAG AAA AGA GAC CCGAAC CCA

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as an internal control in this study. The extraction of total RNA from the samples was performed using QIAamp RNA Mini kits (QIAGEN, Hilden, Germany) in accordance with the guidelines provided by the manufacturer. The concentration and purity of RNA were assessed using a spectrophotometer (Nanodrop, Therom Fisher, Walthm, USA). The RNA was extracted and afterwards subjected to reverse transcription, followed by quantification of its concentration. The calculation of relative gene expression was performed using the 2^-ΔΔCt formula, and the resulting values were further converted using the log2FC formula. All gene symbols were annotated using the Human Gene Nomenclature Committee (HGNC) nomenclature.

Results

Table 1: q RT-PCR of Ischemic cardiomyopathy rats heart samples VS. Control heart samples

Relative STEAP3 gene expression in ICM animal models heart samples VS. control heart samples										
Sample no.	**S1**	**S2**	**S3**	**S4**	**S5**	**C 1**	**C2**	**C 3**	**Cl4**	**C5**
Steap3gene CT	24	25	25	23	25	22.1	22	22.2	22.3	22
Housekeeper gene CT	22	21.9	22	22	22.1	21.9	22	22.1	22	22
ΔCt	2.4					0.12				
2^-ΔΔCt	0.81									

Log2FC	0.24

As explained in table 1, STEAP3 gene expression profiling was down regulated in heart samples of ICM rat models relative to heart samples of control rats.

Table 2: q RT-PCR of Ischemic cardiomyopathy rats blood samples VS. control blood samples

Relative STEAP3 gene expression in ICM animal models blood samples VS. control blood samples										
Sample no.	**S 1**	**S 2**	**S 3**	**S 4**	**S 5**	**C 1**	**C2**	**C3**	**C4**	**C5**
Steap3gene CT	23	25	25	23	22	22	22	22.1	22.3	22
Housekeeper gene CT	21.9	22.3	22	22	21	21.8	22	22	22.1	22.1
ΔCt	1.76					0.08				
2^-ΔΔCt	0.90									
Log2FC	0.27									

As explained in table 1, STEAP3 gene expression profiling was down regulated in blood samples of ICM rat models relative to blood samples of control rats.

Table 3: q RT-PCR of Dilated cardiomyopathy rats Heart samples VS. control heart samples

Relative STEAP3 gene expression in DCM animal models Heart samples VS. control Heart samples										
Sample no.	**S 1**	**S 2**	**S 3**	**S 4**	**S 5**	**C 1**	**C2**	**C3**	**C4**	**C5**
Steap3gene CT	22	23	24	22	22	22	22	22.1	22.3	22
Housekeeper gene CT	21.8	22.3	22	22	21	21	22	21.7	22.2	22.1
ΔCt	0.78					0.28				
2^-ΔΔCt	0.85									
Log2FC	0.25									

As explained in table 3, STEAP3 gene expression profiling was down regulated in heart samples of DCM rat models relative to heart samples of control rats.

Table 4: q RT-PCR of Dilated cardiomyopathy rats blood samples VS. Control blood samples

Relative STEAP3 gene expression in DCM animal models Heart samples VS. control Heart samples										
Sample no.	**S 1**	**S 2**	**S 3**	**S 4**	**S 5**	**C 1**	**C2**	**C3**	**Cl4**	**C5**
Steap3gene CT	22	23	24	22	22	22	22	22.1	22.3	22
Housekeeper gene CT	21.9	22.9	23.9	22	21	21	22	21.9	22.1	22.2
ΔCt	0.26					0.24				
2^-ΔΔCt	0.957									
Log2FC	0.28									

As explained in table 4, STEAP3 gene expression profiling was down regulated in blood samples of DCM rat models relative to blood samples of control rats.

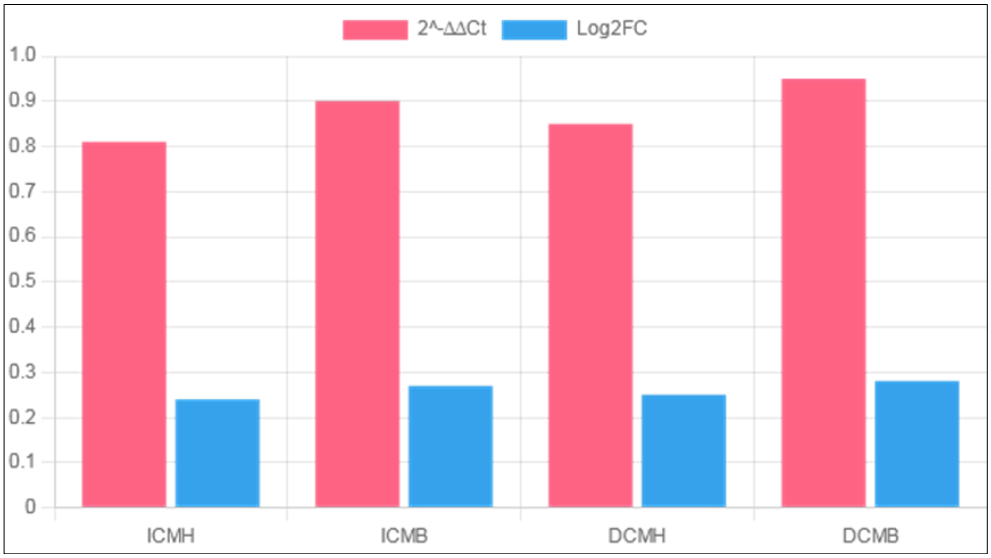

Fig 1. Relative gene expression profiling of STEAP3 gene in ICMH: Ischemic cardiomyopathy heart, ICMB: Ischemic cardiomyopathy blood, DCMH: Dilated cardiomyopathy heart and DCMB: Dilated cardiomyopathy blood

Discussion

In the current study, we measured STEAP3 gene expression profiling in blood and heart samples of circumflex artery ligation ischemic injury rat model and doxorubicin-induced rat dilated cardiomyopathy model. We observed down regulation of steap3 gene in blood and heart samples of both models relative to control rats.

The presence of cardiac fibrosis is a common characteristic observed in cases of pathological cardiac hypertrophy, when there is an abnormal increase in the size of the heart. This condition is distinguished by the excessive buildup of collagen within the cardiac tissue. Nevertheless, the existing literature does not provide any research findings regarding the impact of STEAP3 on cardiac fibrosis. The findings of Li *et al*, investigation suggest that the absence of STEAP3 exacerbates cardiac fibrosis and enhances the expression of fibrotic markers in the presence of pressure overload-induced ventricular hypertrophy [45]. Conversely, the overexpression of STEAP3 exhibits a contrasting phenotype. Therefore, it can be inferred that STEAP3 has the potential to alleviate cardiac fibrosis in cases of ventricular hypertrophy generated by pressure overload.

Over the past few years, numerous randomized studies have extensively proven the advantageous effects of iron supplementation in patients with heart failure (HF). However, a comprehensive understanding of the basic pathophysiological mechanisms that underlie these benefits remains to be established. It is worth mentioning that the effectiveness of iron supplementation has primarily been observed with intravenous administration of ferric carboxymaltose at elevated dosages [46].

The primary iron irregularities observed in DCM cardiomyocytes, as illustrated in Figure 3, result in a notable decrease in the availability of intracellular Fe2+ and the accumulation of heme. SCARA5 serves as a significant regulatory locus for maintaining iron homeostasis due to its capacity to bind and internalize ferritin. This interaction facilitates the entry of Fe3+ and its subsequent transportation to the endosome. In this context, the Fe3+ ion is liberated and then undergoes reduction to Fe2+ by the action of STEAP3 [47]. It is worth mentioning that a prior study demonstrated a reduction in SCARA5 expression inside cardiomyocytes, resulting in iron deficit, in the context of failing human hearts [48]. The STEAP3 gene, which plays a crucial role in maintaining iron balance, regulating cell death, and controlling inflammation, has been identified as a significant inhibitory factor in the development of cardiac hypertrophy [49]. In a recent analysis [50], Massaiu *et al* observed a down-regulation of both the ferritin receptor SCARA5 and the ferrireductase STEAP3, which provides further evidence of dysregulation in iron metabolism within the context of dilated cardiomyopathy (DCM).

According to the results of the current study, STEAP3 gene, one of the most important responsible for iron homeostasis in cardiomyocytes, was found to be significantly downregulated in ischemic and dilated cardiomyopathy animal models.

Conclusion

STEAP3 gene is down regulated in blood and heart of ischemic cardiomyopathy and dilated cardiomyopathy rat models.

Study limitations

1. Small sample size
2. Small number of studied genes
3. We did not study the mechanism of STEAP3 gene as a contributing pathological factor in pathological mechanisms of ischemic and dilated cardiomyopathy

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