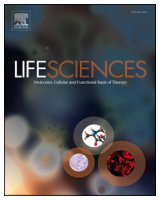
Life Sciences 345 (2024) 122594

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie



Exploring the therapeutic potential of tetrahydrobiopterin for heart failure with preserved ejection fraction: A path forward

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ARTICLE INFO

Keywords:
BH2
BH4
GCH1
HFpEF
NO

ABSTRACT

A large number of patients are affected by classical heart failure (HF) symptomatology with preserved ejection fraction (HFpEF) and multiorgan syndrome. Due to high morbidity and mortality rate, hospitalization and mortality remain serious socioeconomic problems, while the lack of effective pharmacological or device treatment means that HFpEF presents a major unmet medical need.

Evidence from clinical and basic studies demonstrates that systemic inflammation, increased oxidative stress, and impaired mitochondrial function are the common pathological mechanisms in HFpEF. Tetrahydrobiopterin (BH4), beyond being an endogenous co-factor for catalyzing the conversion of some essential biomolecules, has the capacity to prevent systemic inflammation, enhance antioxidant resistance, and modulate mitochondrial energy production. Therefore, BH4 has emerged in the last decade as a promising agent to prevent or reverse the progression of disorders such as cardiovascular disease.

In this review, we cover the clinical progress and limitations of using downstream targets of nitric oxide (NO) through NO donors, soluble guanylate cyclase activators, phosphodiesterase inhibitors, and sodium-glucose co-transporter 2 inhibitors in treating cardiovascular diseases, including HFpEF. We discuss the use of BH4 in association with HFpEF, providing new evidence for its potential use as a pharmacological option for treating HFpEF.

1. Introduction

Heart failure (HF) has become a significant socioeconomic burden in the developed world. Although HF patients have diastolic dysfunction, approximately half of HF patients have a nondilated left ventricle with preserved ejection fraction (HFpEF) [1–3].

1.1. Clinical context of HFpEF

As an independent entity, HFpEF presents a clinical dilemma. Patients with HFpEF are often older, female, with a history of metabolic disorders such as obesity, diabetes, hypertension, or atrial fibrillation [2,4–8]. To date, there is no effective therapy. Despite the preserved systolic function, the morbidity and mortality rates of HFpEF are high, whereas the survival rates of HFpEF have not been improved over

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https://doi.org/10.1016/j.lfs.2024.122594
Received 17 January 2024; Received in revised form 10 March 2024; Accepted 24 March 2024
Available online 25 March 2024

several decades, underscoring our limited knowledge of the molecular mechanisms [9,10]. The difficulties are mainly because HFpEF patients do not have a uniform syndrome of diastolic dysfunction [11] but have heterogeneous conditions consisting of various pathophysiological abnormalities, including abnormal ventricular–arterial coupling with exercise [12,13], impaired systemic vasodilator reserve [12,14], chronotropic incompetence [14,15], myocardial contractile dysfunction despite a normal EF [16,17], left atrial dysfunction [18], pulmonary hypertension with intrinsic pulmonary vascular disease [19,20], arterial stiffening [13], endothelial dysfunction [12,21], and volume overload related to extracardiac causes [22]. Thus, hypotheses about the pathogenesis of HFpEF are complex and diverse.

1.2. Accepted paradigm in HFpEF pathophysiology and molecular mechanisms

It is well accepted that noncardiac co-morbidities, such as obesity, diabetes, hypertension, and aging, cause and keep the body in a chronic inflammatory state, which triggers a signaling cascade in the coronary microvasculature [2], resulting in myocardial stiffness [23] and diastolic dysfunction [24,25]– the hallmarks of HFpEF (Fig. 1). Among various signaling pathways, disrupted nitric oxide (NO) signaling is associated with HFpEF [26,27].

At a structural level (Fig. 1), cardiac endothelial inflammation facilitates the differentiation of myocardial fibroblasts into collagen-producing myofibroblasts in the extracellular matrix (ECM), thereby causing fibrosis to increase the ECM-based stiffness of the myocardium [28,29]. At the molecular level, coronary microvascular endothelial cells produce excessive reactive oxygen species (ROS), which limits the bioavailability of NO for diffusion into adjacent cardiomyocytes, thereby disrupting the NO-mediated myocardial contraction and relaxation cycle through cyclic guanosine monophosphate (cGMP)-dependent [30] and independent pathways [27,31,32].

1.3. The cGMP-dependent and cGMP-independent pathways

The cGMP-dependent pathway (Fig. 2): Under physiological conditions, NO functions by binding to its cytosolic receptor, soluble guanylyl cyclase (sGC), followed by activation of cGMP, thereby ensuring that cGMP-dependent protein kinase G (PKG) phosphorylates proteins that are related to antihypertrophy, antifibrosis, and angiogenesis [33–35]. Under pathological conditions, however, the NO-cGMP-PKG pathway is blunted in several species that can exhibit HFpEF, including humans [36,37] mice [38], rats [36] and pigs [39]. This is due not only to

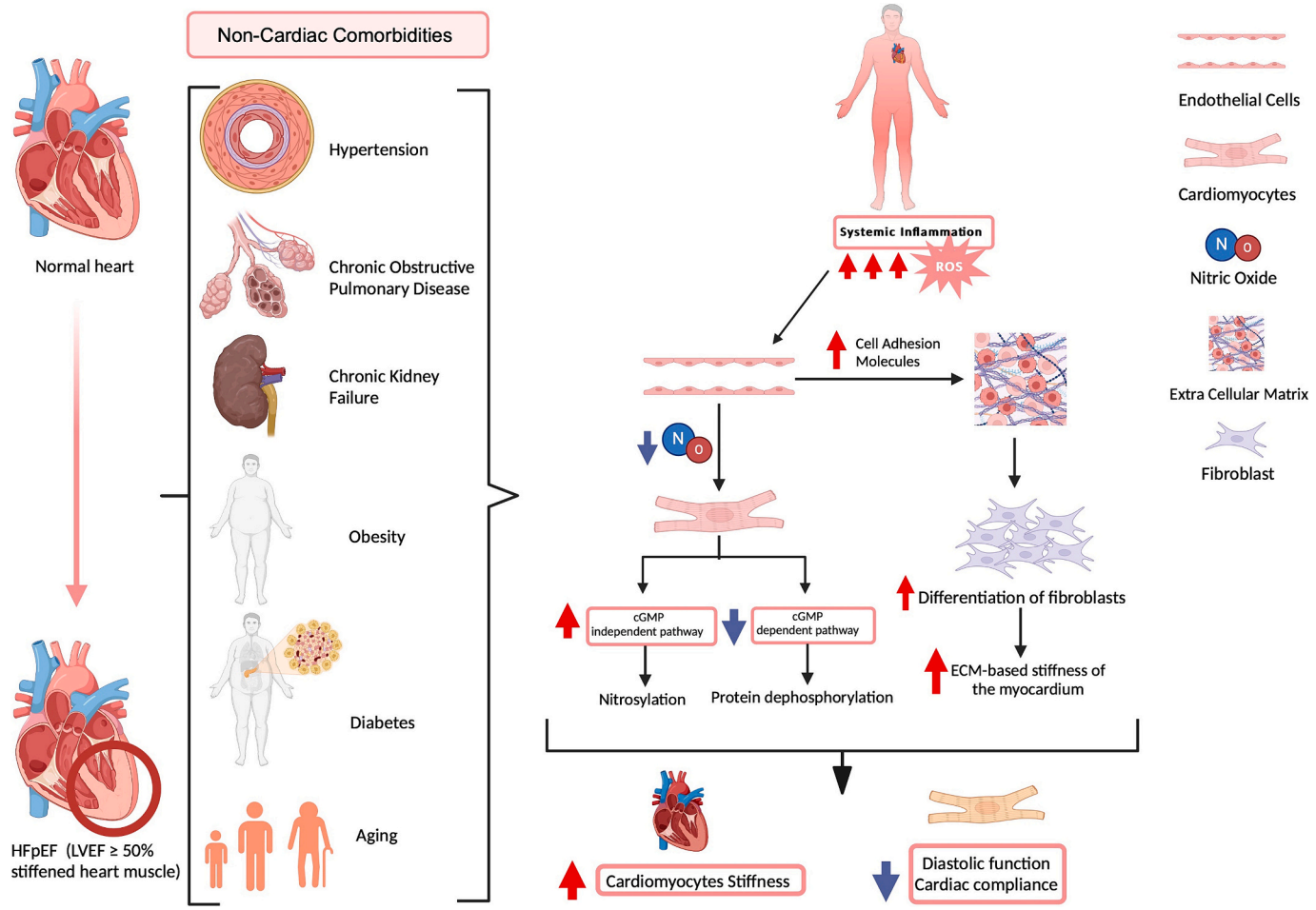

Fig. 1. Pathophysiology of heart failure with preserved ejection fraction (HFpEF). Noncardiac comorbidities such as obesity, diabetes, hypertension, and aging induce chronic inflammation within the body. This inflammation triggers a signaling cascade in the coronary microvasculature, leading to myocardial stiffness and diastolic dysfunction in patients with HFpEF. Disrupted nitric oxide (NO) signaling is a key factor associated with HFpEF, affecting both structural and molecular aspects. Cardiac endothelial inflammation promotes the differentiation of myocardial fibroblasts into collagen-producing myofibroblasts, resulting in increased myocardial stiffness due to fibrosis in the extracellular matrix (ECM). Additionally, excessive production of reactive oxygen species (ROS) by coronary microvascular endothelial cells limits the bioavailability of NO, which disrupts the NO-mediated contraction and relaxation cycle in cardiomyocytes through both cyclic guanosine monophosphate (cGMP)-dependent and independent pathways.
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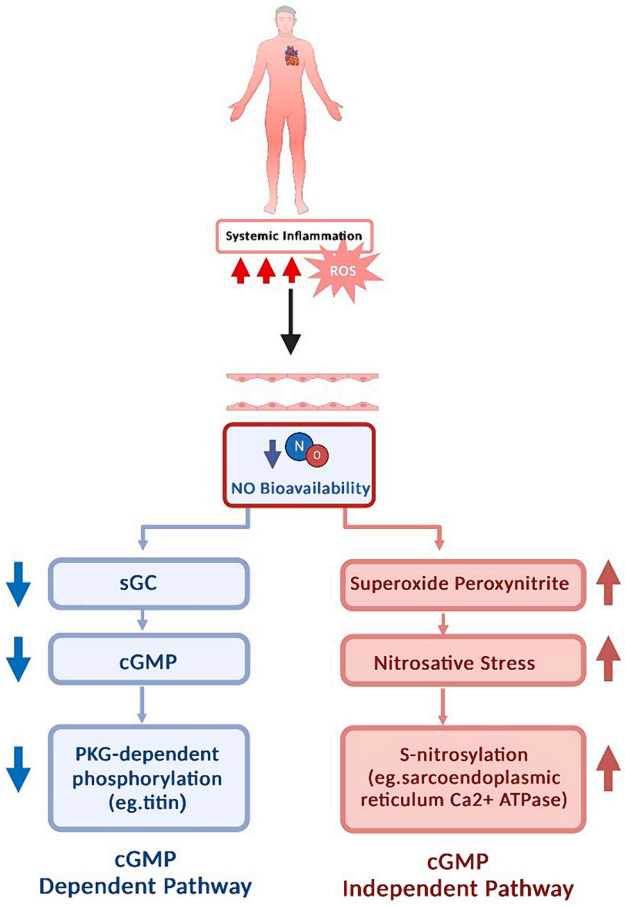

Fig. 2. The cGMP-dependent and cGMP-independent pathways. Under chronic inflammation, NO availability decreases, leading to reduced diffusion into cardiomyocytes. This causes oxidative stress-mediated sGC oxidation and inactivation of PKG in the cGMP-dependent pathway. Consequently, titin, a protein in cardiac muscle, is dephosphorylated in failing human hearts, resulting in cardiomyocyte stiffening. In the cGMP-independent pathway, NO reacts with superoxide to form peroxynitrite, an unstable oxidizing species. Nitrosative stress, characterized by peroxynitrite overexpression, is a driver of HFpEF as it induces cell death through nitrosylation of cysteine or tyrosine thiol on biomolecules such as proteins, lipids, and DNA.
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reduced diffusion of NO into cardiomyocytes [2] but also to the oxidative stress-mediated shift of sGC toward an oxidized, heme-free form that is unresponsive to endogenous and exogenous NO [40] and to inactivation of PKG. As a result, titin, a giant cytoskeletal protein that constitutes the third myofilament of cardiac muscle, is dephosphorylated in human failing hearts compared with donor hearts [23,41], resulting in cardiomyocyte stiffening [42,43].

The cGMP-independent pathway (Fig. 2): Unlike its role in buffering ROS under physiological conditions, NO reacts with superoxide under pathological conditions to form peroxynitrite, a very unstable and reactive oxidizing species [44]. Overexpression of peroxynitrite is termed nitrosative stress and has been identified as a driver of HFpEF [27] owing to its ability to induce cell death through nitrosylation of cysteine or tyrosine thiol on several types of biomolecule, such as proteins, lipids, and DNA [31,32].

Historically, neurohormonal blockade has been used for treating HFpEF. However, after dozens of disappointing trials, it has mostly been abandoned [45–48] and therefore will not be discussed here. In this review, we cover the clinical trials treating HFpEF with NO-mediated downstream targets along NO-cGMP-dependent and -independent pathways, as well as the limitations of the approach (for detailed clinical outcomes of HFpEF see recent reviews, *e.g.* [26,49,50]). We discuss the potential use of tetrahydrobiopterin (BH4), a molecule upstream of the production of NO, as a clinical or pharmaceutical strategy for the prevention or treatment of HFpEF, with a focus on clinical progress and limitations in cardiovascular research, as well as the possible strategies to enhance its efficacy as a treatment.

2. Clinical trials on HFpEF: insights into cGMP-dependent and independent pathways

Along the NO-cGMP-dependent pathway, there are two main targets: endothelial NO and myocardial cGMP (Fig. 3). Interventions with NO donors, including nitrite and nitrate in both organic (synthetic compounds) and inorganic forms [51–55], were presumed to restore myocardial NO levels for patients with HFpEF. However, clinical trials have shown that NO donors do not benefit in terms of quality of life or aerobic capacity in HFpEF [51,55]. The limitations include contribution to hypotension, nonspecific interactions with various biomolecules, lack of response following prolonged administration, and significantly increased risk of adverse cardiovascular events [56].

There are three approaches for intervening with respect to cGMP. One is to enhance its production by sGC activators or stimulators, such

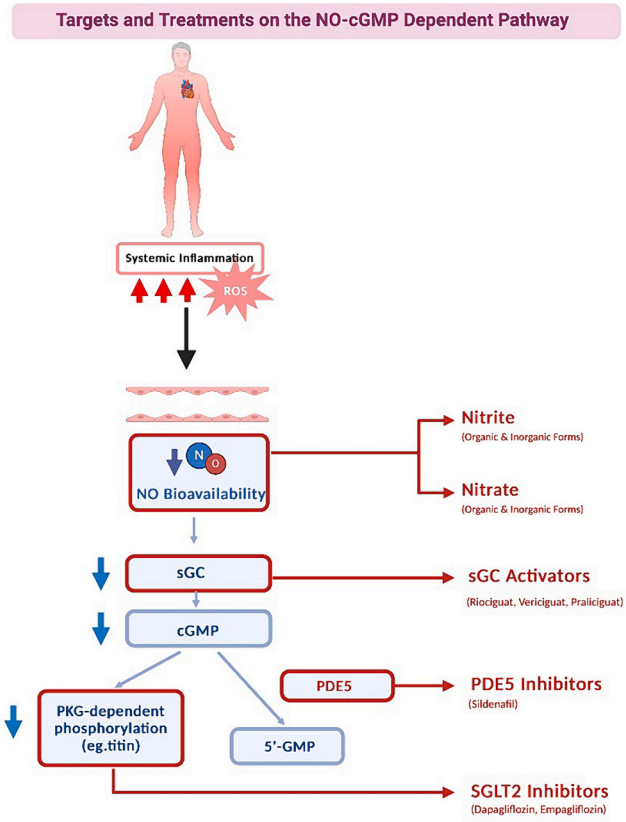

Fig. 3. Current targets and treatments for the NO-cGMP-dependent pathway. In the NO-cGMP-dependent pathway, there are two primary targets: endothelial NO and myocardial cGMP. Attempts to restore myocardial NO levels in patients with HFpEF through interventions with NO donors, including both organic and inorganic forms of nitrite and nitrate, have been unsuccessful in improving quality of life or aerobic capacity. Alternatively, cGMP production can be enhanced through sGC activators or stimulators such as riociguat, vericiguat, and praliciguat. Degradation of cGMP can be prevented by inhibiting the key enzyme phosphodiesterase type 5 (PDE5i) with sildenafil. Additionally, activating PKG downstream of cGMP is another approach. Sodium-dependent glucose transporter 2 inhibitors (SGLT2is) are proposed as activators of the NO-cGMP-PKG pathway.
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as riociguat [57], vericiguat [58–60], and praliciguat [60]. The second is to prevent its degradation by inhibiting the key enzyme phosphodiesterase type 5 (PDE5i) [61] *via* sildenafil [62–65]. The third approach aims downstream of cGMP by activating PKG. In this regard, sodium-dependent glucose transporter 2 inhibitors (SGLT2i) are proposed to activate the NO-cGMP-PKG pathway.

Clinically, vericiguat has shown positive effects on quality of life in the context of HFpEF [59] but did not show improvement in physical capacity [60], as did praliciguat in the CAPACITY-HFpEF trial [66]. Sildenafil has been reported both to improve [62] and worsen [64,65] myocardial function, while consistently failing to improve quality of life [63–65]. These results suggest that the current sGC stimulators and PED5i are not suitable for treating HFpEF. In contrast, the benefits of SGLT2i include improved health status and quality of life in patients with HFpEF [67–69]. Due to their excellent performance in treating HFrEF (reserved ejection fraction) patients [70–72], both empagliflozin and dapagliflozin were evaluated in clinical trials with HFpEF patients [67,68,73,74]. While specific mechanisms have not been identified, evidence suggests that SGLT2i-mediated activation of the NO-cGMP-PKG pathway increases the phosphorylation of myofilament regulatory proteins such as titin and troponin I, thereby reducing the passive stiffness of cardiomyocytes [75,76]. Additionally, anti-inflammation effect of SGLT2i has been recently identified through single-cell RNA sequencing in a mouse model of diastolic dysfunction driven by hyperlipidemia, namely the apolipoprotein E knockout mice that fed a Western diet to recapitulate many features of HFpEF [77]. Through this newly described model of HFpEF, the authors demonstrated that myocardial inflammation and cardiac dysfunction can be ameliorated through the administration of the SGLT2i dapagliflozin [77]. In general, the limitations of SGLT2i in treating HFpEF are associated with lack of both specificity and efficacy, as well as adverse events. Dapagliflozin is the first SGLT2i shown to reduce the risk for cardiovascular death and hospitalization in patients with HF [78,79]. However, patients with chronic HF who used dapagliflozin exhibited polycythemia, a severe adverse event [80].

Regarding the cGMP-independent pathway, to date, there is no clinical trial related to a specific agent targeting this pathway. However, S-nitrosylation of endonuclease inositol-requiring protein 1α is a crucial mechanism of cardiomyocyte dysfunction in HFpEF [27]. Meanwhile, S-nitrosylation of histone deacetylase 2 at cystine 262/274 is identified as one of the prerequisites for diastolic dysfunction and is suggested to be a possible target for the development of HFpEF [81]. In addition, S-nitrosylation of intracellular Ca^{2+} regulatory proteins (L-type Ca^{2+} channel and sarco-endoplasmic reticulum Ca^{2+} ATPase [82], ryanodine receptor Ca^{2+} release channel [83] and cardiac troponin C [82]) is associated with altered excitation–contraction coupling [84–86]. S-nitrosoglutathione reductase is the primary enzyme that denitrosylates intracellular proteins in mammalian cells [87]. The activity is higher in female mouse hearts relative to age-matched males and is associated with reduced myocardial injury from ischemia–reperfusion [88]. Given that S-nitrosylation is a prominent event in HFpEF, targets of nitrosative stress should be carefully investigated.

Collectively, most of the therapeutic strategies focused on NO-downstream effectors targeting the NO-cGMP-PKG pathway have failed to show meaningful clinical benefit in patients with HFpEF [51,55,60,63,64,66,89], except empagliflozin, implying that modulating NO-upstream effectors rather than the downstream cGMP-dependent pathway may be appropriate in treating HFpEF.

3. The impact of increasing BH4 levels on cardiovascular diseases

3.1. Tetrahydrobiopterin, an upstream molecule of NO

NO is mainly produced in vascular endothelial cells from L-arginine and oxygen through the nitric oxide synthase (NOS) family (*i.e.* the three NOS isoforms: neuronal, inducible, and endothelial) [90–92]. All NOS isoforms are activated in homomeric form, which is strictly dependent on BH4 [93]. Structurally, BH4 maintains the catalytic activity of NOS by facilitating the dimerization of NOS, while BH4 is also an electron donor ensuring that L-arginine is coupled with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce NO and L-citrulline, a process termed “NOS coupling” [94] (Fig. 4). Apart from being the necessary co-factor, BH4 is a naturally occurring antioxidant capable of scavenging ROS, such as superoxide and peroxynitrite [95,96].

BH4 is widely distributed throughout most organs, including the vascular system. The biosynthesis of BH4 involves a *de novo* pathway and a salvage pathway (Fig. 4). The *de novo* pathway starts from the substrate guanosine triphosphate with the sequential actions of three enzymes: guanosine triphosphate cyclohydrolase1 (GCH1), 6-pyruvoyl tetrahydropterin synthase, and sepiapterin reductase. The salvage pathway refers to the process of converting sepiapterin as a substrate to dihydrobiopterin (BH2), followed by the reduction of BH2 to BH4 through dihydrofolate reductase (DHFR) [97,98]. Thus, the cellular availability of endogenous BH4 is mainly controlled by the relative contributions between the *de novo* and salvage pathways [97,99]. Advances in understanding the role of NO signaling have identified the bioavailability of BH4, rather than a lack of NOS *per se* [100,101], as a critical determinant of pathogenesis in HF [102]. Apart from NO-related vascular regulation, BH4 has been implicated as playing important roles in cellular energy metabolism [103,104], given that depletion of BH4 is sufficient to cause cardiac dysfunction and reduce mitochondrial biogenesis [105].

Clinically, genetic variants in GCH1 have been associated with alterations in markers of myocardial function and cardiovascular risk [106,107]. Pathologically, low levels of BH4 are associated with a broad range of cardiovascular diseases, including atherosclerosis [108], hypertension [109,110], diabetes [111], LV pressure overload [112,113], ischemic heart disease [114,115], hyperglycemia [116], and atrial fibrillation [117], in all of which impaired endothelium-dependent NO-mediated vasodilation is evident. Thus, two strategies, through either endogenous modulation or exogenous supplementation, have been used to enhance cellular BH4 levels.

3.2. Positive effect of increasing endogenous BH4 on cardiovascular diseases (CVD) and heart function

The concept of regulating endogenous BH4 is based on the findings that protein expression of GCH1 is reduced not only in vascular endothelium during diabetes and hyperglycemia [118] but also in both arterial hypertension [119] and the diabetic heart [120]. Therefore, genetically-modified murine models include mice with overexpression of human GCH1, either cardiomyocyte-specific (mGCH1Tg) [116,120–124] or endothelial-specific (Tie2-GCH1Tg) [111,125,126] can increase endogenous BH4 level.

Under physiological conditions, in comparison with wild-type mice, mGCH1Tg mice exhibit normal cardiac function but a lower BH4/BH2 ratio despite increased myocardial BH4 levels [123]. In the absence of diabetes, abnormalities in myocardial morphology and function induced by transverse aortic constriction (TAC) were similar in mGCH1Tg mice relative to wild-type mice [122]. Supplementation with BH4 prevented TAC-induced cardiac remodeling in both mGCH1Tg mice and wild-type mice, namely through suppression of inflammatory signaling and myocardial macrophage infiltration *via* a NOS-independent pathway [122]. In the presence of diabetes, mGCH1Tg mice are protected against diabetic cardiomyopathy [120] and ischemic injury during hyperglycemia [116] due to a profound effect on myocardial relaxation [123] and remodeling [120,124]. More importantly, LV dysfunction in streptozotocin-induced diabetic mice was prevented in mGCH1Tg mice [121] through a NO-sGC-PKG-dependent mechanism to increase glucose uptake and preserve myocardial energetics [121].

Tie2-GCH1Tg mice produce significantly increased BH4 in cardiac

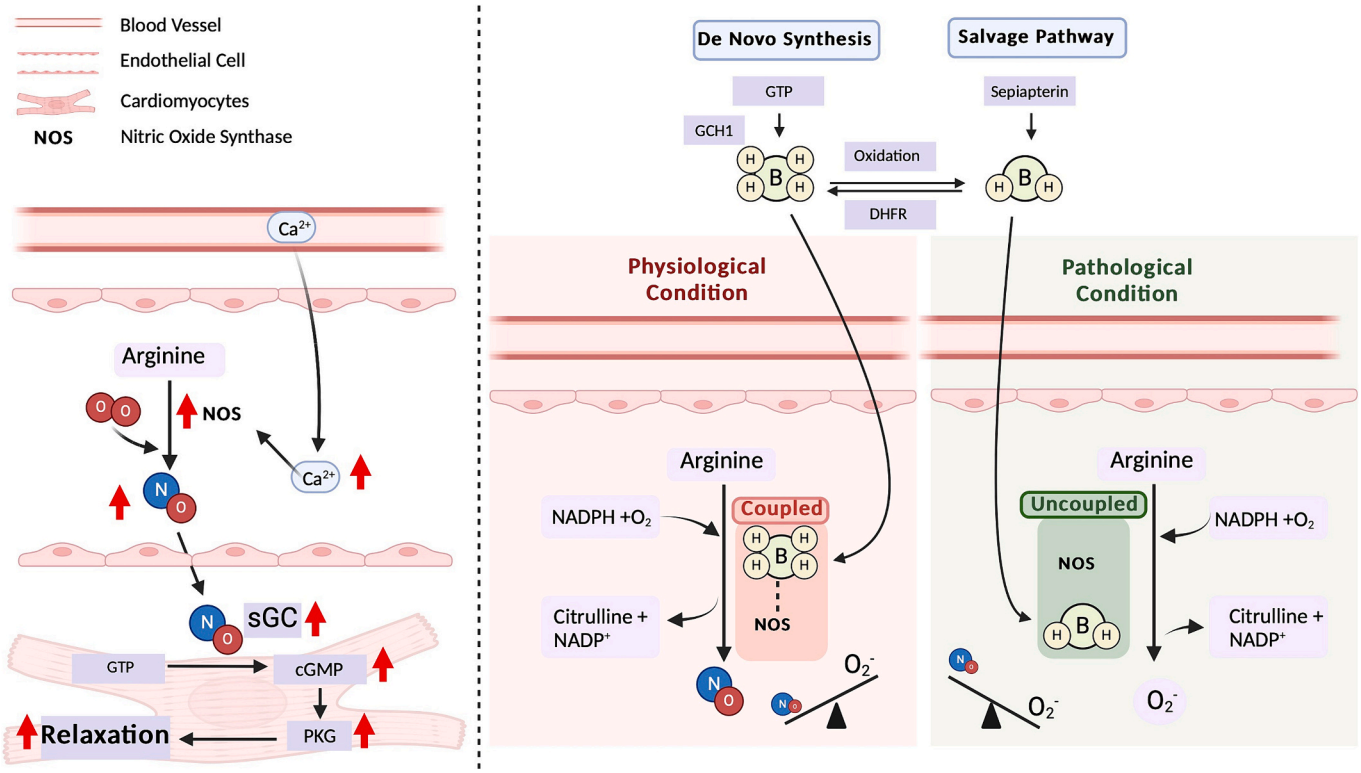

Fig. 4. The effects of BH4 in HFpEF. BH4 is a widely-distributed compound found in various organs, including the vascular system. Its biosynthesis involves two pathways: *de novo* and salvage. The *de novo* pathway is initiated from guanosine triphosphate, which undergoes sequential enzymatic reactions involving guanosine triphosphate cyclohydrolase I (GCH1), 6-pyruvoyl tetrahydropterin synthase, and sepiapterin reductase. The salvage pathway converts sepiapterin into dihydrobiopterin (BH2), which is subsequently reduced to BH4 through the action of dihydrofolate reductase. The cellular availability of endogenous BH4 is primarily regulated by the interplay between the *de novo* and salvage pathways. Under normal physiological conditions, the reaction between NADPH and L-arginine results in the production of nitric oxide (NO) and L-citrulline, along with the generation of superoxide (O_2^-). However, under pathological conditions, the activity of dihydrofolate reductase is significantly reduced, leading to increased oxidation of BH4 and consequently a lower BH4/BH2 ratio. This imbalance in the BH4/BH2 ratio promotes the production of O_2^-.
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endothelial cells but not in cardiomyocytes. More interestingly, lower production of myocardial superoxide was observed when Tie2-GCH1Tg mice were subjected to pressure overload [126]. Therefore, Tie2-GCH1Tg mice were protected against atherosclerosis by reducing vascular inflammation [127] and preserving endothelial-dependent vascular relaxations [125] and blood pressure progression [119]. However, these mice were not protected against TAC-induced myocardial remodeling unless exogenous BH4 was supplied [126]. Together, these results add weight to the concept that maintaining endothelial BH4 levels is sufficient to correct endothelial dysfunction [128,129] and reduce progression in atherosclerosis [125,127,130,131], but it is not as powerful as BH4 in cardiomyocytes to prevent the heart from hypertrophic remodeling [126].

To date, there has been no clinical trial related to cardiovascular disease in which endogenous BH4 is modulated through GCH1.

3.3. *Positive effect of increasing exogenous BH4 on CVD and heart function*

Both acute and chronic effects of increasing exogenous BH4 have been observed in clinical studies. The main clinical finding with acute BH4 treatment (Table 1) is the improvement of endothelial dysfunction-related symptoms such as vasodilation, as reflected by enhanced vascular relaxation and blood flow. This occurred in patients with hypertension [132], coronary artery disease [133], hypercholesterolemia [134], diabetes [135], aging [136], long-term smokers [137,138], and post-menopausal women [139,140]. One of the limitations of acute BH4 treatment is related to its specificity. For example, infusion of BH4, dosing from 50 μg/min to 500 μg/min, increased plasma BH4 levels to 50 or 100 μmol/L within 30 min [141]. This is >1000-fold higher than the normal physiological plasma BH4 concentration of 10–50 nmol/L [142,143], raising the question as to whether improved vasodilation is driven by the nonspecific effects of BH4 on the vessel wall.

Concerning clinical trials with chronic BH4 treatment (Table 1): (1) Safety and efficacy: Due to the instability of BH4, sapropterin dihydrochloride (6R-BH4), the active form of BH4, has also been used in clinical studies in patients with hypertension [144], hyperlipidemia [145], and pulmonary hypertension [146] (Table 1) with good tolerability [147,148]. Patients with pulmonary hypertension tolerated 6R-BH4 at doses ranging from 2.5 to 20 mg/kg for over eight weeks without causing systemic hypotension [146]; a dose of 5 mg/kg most significantly improved the six-minute walk distance [146]. Small clinical trials have supported the benefits of BH4 treatment in patients with vascular disorders for improving symptoms along with ameliorating endothelial function and vascular oxidative stress [145]. (2) Limitations: Apart from the limited number of enrolled participants, suboptimal dosing should be introduced based on the degree of oxidative stress in patients. This is important, based on the concept that variability of redox conditions could produce a therapeutic window that varies between patients. To measure the degree of oxidative stress, F2-isoprostanes (F2-isoPs) are measured as biomarkers for oxidative stress in humans [149,150]. Of these studies, elevated levels are associated with diabetes [151], cardiometabolic disease [152–155], and increased mortality from coronary heart disease or stroke in post-menopausal women [156]. Compared to other biological fluids, elevated urinary levels of F2-isoPs provide the most reliable quantitative index of oxidative stress *in vivo* [150]. In

Table 1
Clinical observations of BH4 treatment in patients with cardiovascular diseases.

Acute effect of BH4					
Pathological conditions	Nr	Age	Dosing, duration	Effect on endothelial function	Refs
Hypertension (essential)	8	37–59	500 μg/min, 15 min	Improved (endothelium-dependent vasodilation)	[132]
	6	38–54	500 μg/min, 15 min	Improved (endothelium-dependent vasodilation)	[132]
Coronary artery disease	19	56 ± 10	10^{-2} M (1 mL/min), 4 min	Improved (mild coronary vasodilation)	[133]
Hypercholesterolemia	13	32–36	10; 100; 500; 1000 (μg/min, 5 min each)	Improved (NO-dependent vasodilation)	[134]
Diabetes (Type II)	23	52 ± 2	500 μg/min, 3 min	Improved (endothelium-dependent vasodilation)	[135]
Aging	37	41 ± 18	500 μg/min, 15 min	Improved (endothelium-dependent vasodilation)	[136]
Long-term smoker	10	28–32	2 mg/kg (one dose), 1 day	Improved (brachial artery flow-mediated dilation/endothelium-dependent vasodilation)	[137]
	13	52–54	500 μg/min, 10 min	Improved (basal/stimulated NO-mediated vasodilation)	[138]
	17	52–54	500 μg/min, 30 min	Improved (basal/stimulated NO-mediated vasodilation, increased Acetylcholine-induced vasodilation)	[138]
	15	52–54	50 μmol/L, 2 days	Augmented Acetylcholine-induced vasodilation	[138]
Post-menopausal women	24	57 ± 1	BH4 10 mg/kg, 3 h	Increased endothelial dependent vasodilatation	[139]
	19	58 ± 5	BH4 10 mg/kg, 3 h	Increased brachial artery flow-mediated dilation, but did not reach the levels in premenopausal women	[140]

Positive effect from relevant cardiovascular clinical trials with BH4-analogs					
Pathological conditions	Nr	Age	BH4 dosing, duration	Effects	Refs
Hypertension	8	18–75	1, 5 or 10 mg kg/day of BH4, 8 weeks	Reduced systolic pressure and improved endothelial dysfunction and oxidative stress	[144]
Pulmonary arterial hypertension	18	18–75	2.5 mg/kg to 20 mg/kg of sapropterin dihydrochloride, 8 weeks.	Improvement in 6-minute walk distance	[146]
Hypercholesterolaemia	22	Mean 57	400 mg/twice daily, 4 weeks	Reversal of endothelial dysfunction and oxidative stress	[145]
Negative effect from relevant cardiovascular clinical trials with BH4-analogs					
Hypertension	84	18–75	5 mg/kg of oral sapropterin dihydrochloride twice daily, 8 weeks.	No significant effect on systolic blood pressure	[177]
Endothelial dysfunction	52	18–75	5 mg/kg of oral sapropterin dihydrochloride twice daily, 2 weeks.	Did not affect endothelium-dependent vasodilation in patients	[178]
Coronary artery disease	49	>18	400 or 700 mg/day sapropterin dihydrochloride, 2–6 weeks.	No net effect on improving vascular redox state or endothelial function	[175]

addition, clinical studies have an uneven distribution of sex, which should be avoided in future studies, as evidence has shown that, in general, stress levels are higher in females than males [150], indicative of sex-related differences in oxidative stress, which can potentially affect the efficiency of BH4 [154].

Regarding the animal models used in chronic BH4 supplementation, the genetic mouse models with reduced endogenous BH4 bioavailability have included GCH1 knockout mice [157,158], sepiapterin reductase knockout mice ($Spr^{-/-}$) [104,159], and DHFR heterozygous knockout mice (DHFR KO) [160], in all of which hypertension and mitochondrial dysfunction are common [158,160,161]. Pathological animal models with BH4 supplementation (Table 2) have included hypertension (mice [109,113], rats [162,163]), atherosclerosis [108,125,164], cardiac hypertrophy and HF induced by pressure overload through transverse aortic constriction [126,165], and diabetes [121].

Apart from the genetic murine models which have shown proof of the concept that BH4 can alter cardiovascular pathogenesis [104,159], pathological models with chronic BH4 treatment benefitted from improved endothelial function [108,109,121,126,127]. More importantly, BH4 supplementation reduced myocardial infarction and improved contractility by reducing superoxide while increasing NO production in ischemic hearts [166]. Another striking finding is that in the absence of diabetes, BH4 has the capacity to suppress myocardial remodeling and dysfunction in the presence of ongoing pressure overload through PKG-independent mechanisms [126]. The anti-hypertrophic effect in the TAC mouse heart is dependent on BH4 in cardiomyocytes but not in endothelial cells [126]. In the presence of diabetes, BH4 also preserved cardiac function in the TAC mouse heart, albeit through cGMP-dependent-PKG mechanisms [121].

However, there are limitations in the use of these animal models in relation to HFpEF as each of them only emphasizes part of the features of HFpEF in humans. For example, EF was only preserved in 8–11-week-old Dahl salt-sensitive hypertensive rats, while progressively deteriorated hemodynamics were evident after 13 weeks and looked more like HFrEF [167]; however, this is rare in humans [168]. Therefore, continued use of these models should be discouraged in future studies for HFpEF with BH4.

In recent years, additional HFpEF models have been developed, including the two-hit model in C57BL/6 mice with a high-fat diet plus chronic inhibition of NOS with *N*-nitroarginine methyl ester [27] or angiotensin II [169], and the three-hit mouse models that combine the two-hit model with additional risk factors such as aging [170], cardiomyocyte-specific expression of the l-type Ca^{2+} channel β2a-subunit [171], or intraperitoneal injection with deoxycorticosterone pivalate to accentuate hypertension and systemic inflammation [172]. The three-hit models induced a more profound HFpEF phenotype than the two-hit [27,170–172]. Importantly, two-hit HFpEF mice exhibit reduced NO bioavailability not only in circulation but also in the heart [173]. Furthermore, therapy combining sodium nitrite with hydralazine (an sGC stimulator and peroxynitrite scavenger) [174] significantly attenuated the severity of HFpEF while reducing systemic inflammation and oxidative and nitrosative stress [173]. However, the beneficial effect did not translate into enhanced exercise capacity, as assessed by treadmill running. The two-hit and three-hit murine models are the best-characterized for HFpEF so far, despite limitations such as reduced efficacy in producing HFpEF in female mice; notably, this is not the case in

Table 2
Animal models used for BH4 supplementation and its cardiac effect.

Pathological conditions	Species	Treatment/ duration	Cardiovascular effect	Refs
Hypertension (Angiotensin-II induced)	Rats	20 mg/kg/ day, 7 days	Prevented the development of hypertension and myocardial hypertrophy	[162]
Hypertension (Spontaneously)	Rats	10 mg/kg/ day, 16 weeks	Improved vascular responses to acetylcholine and suppressed the development of hypertension	[163]
Hypertension (Angiotensin-II induced)	Mice	5 mg/kg/ day, 12–14 days	Improved diastolic function	[113]
Hypertension (Deoxycorticosterone acetate-salt induced)	Mice	5 mg/kg/ day, 10 days	Blunts the increase in blood pressure	[109]
Diabetes (Streptozotocin-induced)	Mice	200 mg/kg/ day, 6 weeks	Prevents diabetes-induced myocardial dysfunction	[121]
Atherosclerosis (Apolipoprotein E-knockout)	Mice	10 mg/kg per day, 5 weeks	Improved endothelial dysfunction, attenuated inflammatory factors in the aortas	[108]
Atherosclerosis (Apolipoprotein E-knockout)	Mice	10 mg/kg/ day, 12 weeks	Prevents diabetes-induced Left ventricular dysfunction	[127]
Heart failure (Pressure-overload induced)	Mice	12, 36, 200 or 400 mg/ kg/day, 5 weeks	Prevents the development of hypertension and myocardial hypertrophy	[165]
Heart failure (Pressure-overload induced)	Mice	200 mg/kg/ day, 9 weeks	Improved diastolic dysfunction	[126]

humans with HFpEF [27].

3.4. Negative association studies

Several clinical studies have failed to achieve a beneficial effect on CVD with BH4 (Table 1), as indicated by its limited efficacy in improving vascular hemodynamics in patients with coronary artery disease [175–177] or in reducing high blood pressure in hypertensive patients [144]. One important observation is that chronic BH4 supplementation in patients with coronary artery disease significantly increased BH4 levels in plasma and in the saphenous vein along with an increased level of BH2. In addition, BH4 was able to improve systolic and mean blood pressure at a lower dose of 5–10 mg/kg/day or a higher dose of 400 mg/kg/day, but not at 200 mg/kg/day [144].

3.5. Potential mechanisms underlying the failure of BH4 supplementation on CVD and strategies for improvement

The most common use of 6R-BH4 in the clinic to date is for treating a subset of patients with *L*-phenylketonuria, an inherited disorder that increases the blood levels of phenylalanine [178]. Compared with patients with coronary artery disease or hypertension, one would propose that the differential efficacy of oral BH4 may due to differences in the degree of oxidative stress, increase of which would cause more oxidation of BH4 compared with patients with *L*-phenylketonuria [175]. Thus, the low efficacy of BH4 in such cases may be attributable to increased oxidation and reduced recycling of BH4. At the same time, inadequate cellular uptake may be an additional reason for the mixed clinical results.

3.5.1. Increased oxidation of BH4

The conventional view is that orally-supplemented BH4 is available in its reduced form for intracellular transportation. However, due to its reductive nature, BH4 is rapidly oxidized by peroxynitrite or other ROS to its inactive forms, such as oxidized biopterin or partially reduced BH2, under oxidative conditions [179–181]. BH2 and BH4 are structurally alike, with similar binding affinities to NOS [182], enabling them to compete for NOS binding [183]. Unlike BH2, BH4 binding to NOS results in the formation of NO [95,184], while BH2 binding produces peroxide [181,185] which, in turn, creates a vicious cycle of vascular oxidative stress [181,186], as seen in diabetes, atherosclerosis and hypertension [109,187,188].

To avoid BH4 oxidation, NADPH oxidase may be a candidate target because when NOS is coupled, NADPH reacts with L-arginine to produce NO and L-citrulline (Fig. 4), whereas when uncoupling occurs, NADPH oxidase actively catalyzes the NADPH reaction with O_2 to produce superoxide. In this regard, ascorbic acid or *N*-acetyl cystine effectively protected the diabetic mouse heart from NOS uncoupling and ischemic injury by preventing the oxidation of BH4 to BH2 [189].

3.5.2. Reduced recycling of BH4

Exogenous BH4 is first oxidized to BH2 in the plasma before entering the cell, where DHFR converts BH2 back to BH4 [190]; notably, this process is impaired under pathological conditions due to low expression of DHFR [191]. Patients with calcific aortic valve disease, a common CVD, express a significantly lower level of DHFR in calcified aortic valve leaflets compared with normal ones, along with reduced plasma and aortic valve BH4 concentrations and thereby a reduced BH4/BH2 ratio [192].

To enhance the recycling of BH4, folic acid, a water-soluble B-vitamin and widely-used food supplement, is effective in increasing DHFR expression and activity [98], as seen in patients with diabetic cardiovascular complications [193] and mice with hyperchloremia [192]. The beneficial effects of folic acid include improved NO-dependent vasodilation, increased BH4 levels and BH4/BH2 ratio along with attenuated calcification and fibrosis in the aortic valve [192], and also prevention of ischemia-mediated functional decline in mice [194]. In addition to modulating DHFR, preventing the degradation of GCH1 is another approach to enhance the bioavailability of BH4. In this context, elevated activity of the 26S proteasome, a ubiquitous enzyme, is associated with degradation of myocardial GCH1 [118,120,195,196]. This degradation, however, is prevented by MG-132, an inhibitor of the 26S proteasome [120], with an improvement in LV wall thickness, mitral E/A ratio, and myocardial function in diabetes [120].

3.5.3. Inadequate cellular uptake or accelerated urinary elimination

Dose-dependent effects of BH4 were investigated in a mouse TAC heart [165], in which a dose of 36 mg/kg/day was the peak effective dose for improving cardiac function, attenuating hypertrophy and fibrosis, whereas doses lower than 12 mg/kg/day and higher than 400 mg/kg/day conferred minimal to no significant benefit [165]. Likewise, the dose of BH4 used in most clinical studies was 5–10 mg/kg/day, which was able to increase plasma biopterin levels by ~50-fold and improve systolic and mean blood pressure in hypertensive patients. However, the anti-hypertensive effect was not observed with 200 mg/kg/day. Clinical studies with BH4 treatment in patients with coronary artery atherosclerosis demonstrated that high plasma levels of BH4 were not evident in endothelium [197]. These findings suggest that, in human, BH4 is not freely diffusible [198].

Another interesting finding is that intestinal absorption of BH4 is age dependent, with higher concentrations in younger animals. This is demonstrated by the fact that oral administration of BH4 (10 mg/kg) in two-week-old mice resulted in a five-fold greater amount of BH4

radioactivity in the brain compared with a higher dosage (100 mg/kg) in six-week-old mice [199]. Whether this is true for BH4 being taken up into the heart remains unclear. These results imply that the large variability in the effects in clinical trials of orally-administered BH4 in patients may be, in part, due to differential intestinal absorption, although the age-mediated difference cannot be ruled out. In addition, the bioavailability of BH4 to organs following oral intake is low, with 90 % renal elimination within 120 min [200] due to high-capacity transporters in the kidney [200,201]. It remains unclear whether urine elimination of BH4 differs between the individuals post-BH4 treatment; this elimination can be suppressed by cyclosporin A, an inhibitor of transporters with broad specificity in the excretion of xenobiotics and metabolic waste [201].

Collectively, it is the ratio of BH4/BH2, not the absolute levels of BH4, that maintains proper NOS function. The degree of systemic oxidative stress in patients is an important factor in determining the therapeutic effects of BH4 through alteration of BH4/BH2 status.

3.5.4. To enhance the efficacy of BH4

One approach is to develop novel BH4 analogues with oral bioavailability for longer duration and better accessibility into intracellular space, while another approach is to develop advanced delivery systems, such as encapsulation, to enhance BH4 bioavailability at the desired sites, while avoiding differential intestinal absorption and minimizing urinary exclusion.

Relative to discovering BH4 analogs, the development of polymeric nanoparticle system arose drastically in recent years, through which, cargos can be covalently crosslinked and cleaved under specific intracellular conditions, which ensures the biomolecules to be delivered into subcellular destinations. Importantly, the progress in nano-encapsulation of sepiapterin, the natural precursor of BH4, has been made by Kiplennik et al [202]. The possibility is fascinating as specific delivery of BH4 to endothelium or cardiomyocytes would reduce the off-site unfavorable effect of BH4 and diversify the therapeutic options for HFpEF patients. Yet, integrating nano-encapsulated sepiapterin into clinicals with HFpEF patients remains challenging. The authors are dedicated to finding optimized storage conditions that prolong the shelf-life of the nano-formulations of sepiapterin and assessing its effect on the pharmacokinetics [202].

3.5.5. Off-site effect of BH4

It is important to keep in mind that clinical trials have demonstrated that overactivation of BH4 is pathogenic, resulting in worsened clinical symptoms in association with mitochondrial dysfunction and impaired immune system [203,204]. In line with this, the stimulatory effect of BH4 on the biosynthesis of catecholamines is evident in some patients [205] and may adversely affect cardiovascular function. In addition, studies have shown that women with HF are better off with lower doses of the medical treatment [206,207], whereas adverse effects are more serious in women across the spectrum of HF [208], implicating the differences between males and females in relation to drug pharmacokinetics and pharmacodynamics following progress of HF that affects drug absorption, distribution, metabolism and elimination [209]. These findings raise a question as to whether or not optimal BH4 treatment should be sex-specific.

To clarify this, the prospective dose-optimization trails with BH4 are required, in which, both male and female HFpEF patients should be involved to evaluate whether adverse effect are one of the main reasons for not up-titrating BH4, and whether a sex-specific approach to dosage could alleviate this. In addition, cardiac hemodynamics, the association between BH4 dosage and all-cause mortality as well as HF hospitalizations should be assessed. A one-year follow-up to determine the long-term effect of cardioprotective strategies is recommended [210]. Furthermore, plasma reflects the status of biopterin synthesis and diffusion into tissues [211], but such samples need to be analyzed immediately to avoid the rapid oxidation of BH4 into BH2, which makes it difficult for clinical trials. In contrast, platelets possess the full enzymatic cascade for *de novo* BH4 synthesis [212], in which the level of biopterin is independent of external sources [213]. One clinical study demonstrated that peripheral endothelial function is progressively impaired with aging in healthy adults, and the changes are positively correlated with increased BH2 in platelets but not in plasma. Thus, platelets are more relevant than plasma to assess biopterin bioavailability [213].

4. Remaining questions regarding the effect of BH4 on CVD and where we need to go from here

4.1. BH4 and peak oxygen consumption

Exercise intolerance, an identified primary driver of morbidity and reduced quality of life, is common among HFpEF patients [214,215]. It remains unclear whether BH4 has the capacity to change maximum exercise oxygen consumption (VO_2) in patients with HFpEF.

Physiologic adaptations to exercise involve coordination between the heart (cardiac pump), the lung (respiratory system), and the arterial system to deliver inhaled oxygen to mitochondria in skeletal and cardiac muscle, thereby generating adenosine triphosphate (ATP) to support locomotion, ventilation, and cardiac contraction and relaxation. VO_2 measures the difference between cardiac output and arteriovenous oxygen [216] and is consistently reduced in HFpEF patients [215], indicative of impaired delivery of oxygen by the heart and reduced peripheral utilization of oxygen [216]. In this context, simultaneous assessment of cardiac output and VO_2 during exercise in HFpEF patients or animal models post-BH4 treatment will clarify whether the benefit of BH4 is on a peripheral or central hemodynamic basis as reflected by cardiac output. Thus, future clinical studies with BH4 in HFpEF patients should include evaluation not only the functional capacity of cardiac function but also the changes in VO_2. The effect of BH4 on the six-minute walk test distance and ventilatory efficiency should be determined in representative HFpEF patients who can perform symptom-limited exercise tests for VO_2 with adequate effort, as well as in frailer patients.

4.2. BH4 and cardiac energy metabolism

Although information regarding the effects of BH4 on myocardial energy metabolism is scarce, increased myocardial BH4 enhances mitochondrial function [217] and energy production [103,104], whereas depletion of BH4 by inhibiting GCH1 is sufficient to cause myocardial dysfunction and reduce mitochondrial biogenesis [105,217]. Mitochondrial proteomic analyses have demonstrated a positive association of BH4 with mitochondrial biogenesis regulator proteins, such as peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) [104]. BH4 is also associated with glucose transporter 1 (GLUT-1) *via* the NO-cGMP-PKG-dependent pathway in streptozotocin-induced diabetic mouse hearts [121]. Given that the blockade of the NO-cGMP-PKG pathway is only part of the pathological mechanism of HFpEF, whether the cGMP-independent pathway is involved in BH4-mediated mitochondrial energy metabolism remains unclear.

Regarding BH4 to PGC-1α in the heart: The specific signaling pathway of BH4 in relation to cardiac energy metabolism has not been identified, which makes drug development challenging. Pioneering studies have suggested that BH4 is an electron transport co-factor in the mitochondria that is independent of NOS [218,219]. Consistently, defects in cardiac mitochondrial phosphorylation in $Spr^{-/-}$ mice were reversed by oral BH4 supplementation but not by NO supplementation or inhibition [104], suggesting that BH4-mediated transcription of PGC1α controls myocardial energy metabolism through a NO-independent pathway. Altered myocardial energy metabolism is characteristic of the failing heart and contributes to the severity of HF [104,220,221]. As such, it is important to understand whether BH4

directly or indirectly alters mitochondrial oxidative phosphorylation [104,121].

Regarding BH4 and GLUT-1 in the heart, the human heart possesses complex machinery to produce ATP from mitochondrial oxidation of major substrates, such as fatty acids, carbohydrates, ketone bodies, and branched chain amino acids [222]. Clinical trials have shown that the failing heart undergoes a 30 % reduction in ATP levels in comparison with the healthy heart due to inadequate oxygen delivery and accumulation of metabolic waste [223]. Although reduced cardiac mitochondrial energetics are evident in HFpEF patients [224], the alterations in the usage of energy substrates remain largely unclear. Transgenic mice with mutations that prevent cardiac glucose uptake or oxidation developed LV hypertrophy and diastolic dysfunction [225]. Thus, it is reasonable to speculate that a BH4-mediated increase in GLUT-1 may enhance glucose oxidation in the diabetic heart, specifically because decreased myocardial glucose oxidation is prominent in obese and diabetic mice that develop LV hypertrophy and diastolic dysfunction [226–228]. As HF progresses, a reduction in glucose oxidation and an increase in ketone oxidation are evident in the human heart [229], which is consistent with a higher level of ketones in both the plasma and myocardium in patients with HFrEF [230]. However, lower levels of ketones and metabolites of the tricarboxylic acid cycle were observed in patients with HFpEF [230]. In contrast, circulating levels of ketone bodies were increased in patients with insulin resistance and type II diabetes [231], two comorbidities closely associated with the initiation of HFpEF. Notably, impaired ketone metabolism is an important feature in HFpEF myocardium [230]. Evidence from a three-hit murine model of HFpEF showed that proinflammatory cytokine-mediated mitochondrial dysfunction and fibrosis in HFpEF can be ameliorated by enhancing circulating levels of β-hydroxybutyrate [172], supporting the notion that ketone bodies are potent anti-inflammatory molecules [232,233]. Remarkably, plasma ketone levels were significantly increased in patients with or without diabetes post-empagliflozin treatment [234], which leads to speculation that SGLT2 inhibitors may increase circulating ketones, thereby reducing inflammation while enhancing myocardial ketone uptake to protect against HF [235,236]. Indeed, empagliflozin improves cardiac function in mice with diabetic cardiomyopathy by alleviating oxidative stress and promoting the expression of key enzymes in ketone body metabolism [237]. Of interest, Carnicer et al. have demonstrated that increasing myocardial BH4 availability can reverse cardiac dysfunction in diabetic mouse hearts [121], which open the possibility that BH4 supplementation may have beneficial effects in patients with diabetic cardiomyopathy [121]. However, the authors focused on the effect of GCH1/BH4 axis on glucose uptake and oxidation without exploring ketone utilization in the diabetic heart. Thus, the question as to whether BH4 may display an impact on ketone metabolism in HFpEF, as empagliflozin does, requires further clarification, as currently there is no experimental and clinical evidence comparing the ketogenic effects of BH4.

4.3. BH4 and cardiac inflammation cells in relation to HFpEF

Elevated circulating T cells are evident in HFpEF patients [238,239]. Smolgovsky *et al* have further clarified that the development of diastolic dysfunction and cardiomyocyte hypertrophy in 2-hit preclinical HFpEF model with obesity and hypertension is cardiac CD4 T cells dependent [240]. Of interest, the blockade of BH4 synthesis *in vivo* abrogates T-cell-mediated autoimmunity and allergic inflammation, whereas enhancing BH4 levels through GCH1 overexpression augments responses by CD4- and CD8-expressing T cells [204]. It appears that the response of BH4 as a pro- or anti-inflammatory molecule largely depends on the status of intracellular oxidative stress. Given the functional relevance of inflammation in HFpEF pathogenesis, similar to SGLT2i [77], oral administration of BH4 also displayed an anti-inflammatory effect in response to the progression of atherosclerosis and vascular inflammation through reducing vascular immune cell infiltration in ApoE KO mice [108,241]. Considering that reversible dysregulation of inositol-requiring enzyme 1α/X-box–binding protein 1 axis is the cardiac T cell signature of HFpEF [204], it is important to understand if this axis can be enhanced through enhancing BH4 bioavailability.

4.4. GCH1/BH4 axis and epigenetic regulation of HFpEF

Clinical and preclinical studies have demonstrated a key role of epigenetic-sensitive mechanisms in the regulation of transcriptional programs underlying pathogenesis of HFpEF, *via* changing gene environment through DNA methylation, histone modifications, and non-coding RNA, suggesting that epigenetic modulation could be a promising therapeutic tool to improve HFpEF [242–245].

Regarding GCH1/BH4 axis in relation to epigenetic regulation, several observations implicate a potential association under various pathological conditions. Firstly, GCH1 promoter is methylated, of which, the hypermethylation were observed not only in patients with hepatocellular carcinoma [246], but also in the plasma samples from patients with schizophrenia, a mental health disorder with a profound deficit of plasma total BH4 [247], when compared to healthy controls. As a result, GCH1 protein and mRNA expression levels were markedly down-regulated concomitant with a significantly reduced BH4 levels [246,247]. In addition, BH4- depleted T-cells displayed a defective iron-redox cycling of cytochrome *c and* mitochondrial dysfunction [204], whereas activation of GCH1/BH4 axis antagonized the defect of iron metabolism [248]. In this context, aberrant intracellular iron metabolism is closely related to the comorbidity-inflammation paradigm in HFpEF [249,250]. Given that the expression of iron metabolic enzymes can be controlled through epigenetic mechanisms [251], it is important to understand whether dysfunction of the GCH1/BH4 axis is upstream or downstream of the epigenetic modification to control transcriptional programs underlying features of HFpEF. Relevant to this point, Li *et al* demonstrated that GCH1 is a target of microRNA-133a in endothelial cells mediates endothelial dysfunction induced by multiple CVD risk factors [252]. However, the authors did not investigate if microRNA-133a antagomir-mediated up-regulation of GCH1 protein expression and BH4 content is associated with reduced methylation of GCH1 promoter.

Collectively, hypermethylation of GCH1 causes dysfunction of BH4 (Fig. 5) under different pathological conditions. Given that MicroRNA-133a regulates DNA methylation in diabetic cardiomyocytes [253], one would propose a vicious cycle between GCH1/BH4 mediated oxidative stress, microRNA and DNA methylation that may play pivotal regulatory roles in CVD. It is interesting to understand if epigenetic modifications of cardiac GCH1 promoter could potentially be a peripheral biomarker of HFpEF, and if this relationship is sex dependent. Other epigenetic modifications should also be assessed through RNA sequencing and microarrays in HFpEF animal models and in human biopsies to look for biological mechanisms that may affect GCH1 expression.

4.5. HFpEF model in future BH4 studies

Although small rodents are undeniably the premier model system that can be used, assessing an animal model that accurately recapitulates the clinical HFpEF syndrome is undeniably challenging. To improve translational relevance, several critical points should be recognized. Firstly, HFpEF is a complex clinical syndrome, not a single disease. Transition from HFpEF to HFrEF in patients is rare [254], which, together with evidence emerging from assessments of gene expression patterns in both preclinical [27,255] and human specimens [256], has lend considerable credence to a notion that HFpEF differs mechanistically from HFrEF. Thus, models marked “temporary HFpEF” that can transit to HFrEF, or the models with the presumption that diastolic dysfunction is tantamount to HFpEF should not be used in future BH4 studies. It is critical to ensure robust validation of preserved ejection

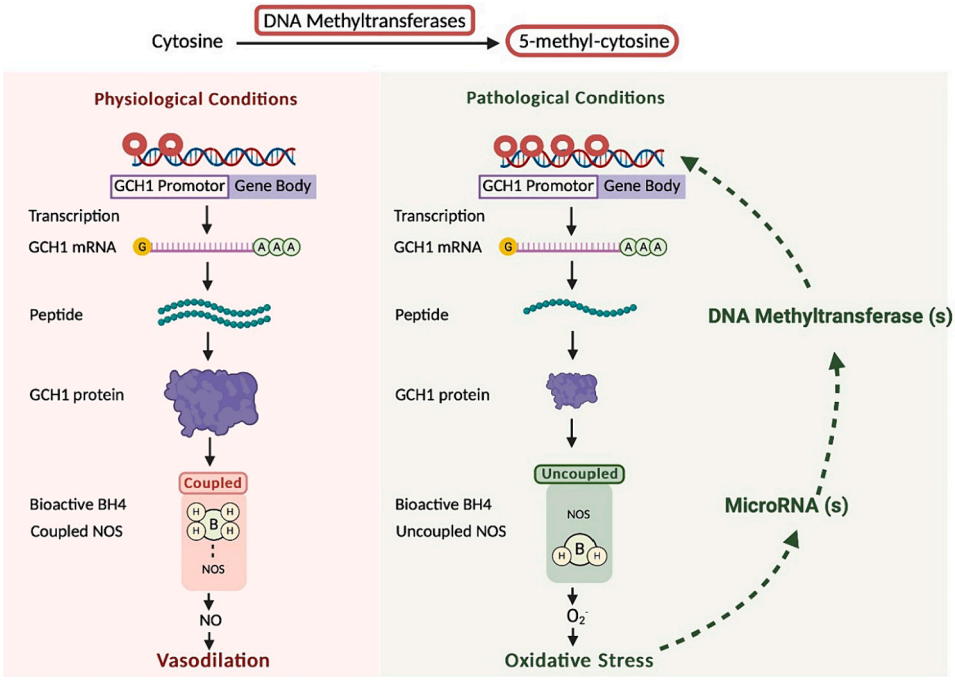

Fig. 5. Diagram showing the association of methylation of GCH1 promoter with BH4 and NO production. Under normal physiological conditions, bioactive BH4 ensures NOS coupling, thereby producing NO instead of superoxide to modulate vasodilation. In contrast, under pathological conditions, hypermethylation of GCH1 promoter causes low GCH1 protein expression thereby lowering bioactive BH4 to make uncoupling of NOS, resulting in overproduction of superoxide and oxidative stress. It remains unknown if there is a vicious cycle between GCH1/BH4 mediated oxidative stress, microRNA and DNA methylation that may play pivotal regulatory roles in CVD.
(Created with BioRender.com)

fraction in HFpEF model without a later decrement, in parallel with an impaired LV long-axis systolic function and long-axis diastolic dysfunction [257]. In addition, cardiometabolic alterations in HFpEF subjects have effects beyond the heart through crosstalk to vasculature and to organs [258]. Therefore, other signs, such as hypertension, exercise intolerance, are clinical relevant and should be evident in HFpEF animal models. Furthermore, it is unclear to what extent the cardiac and systemic impairment is sex-dependent on prognosis and drug responsiveness. In community-based studies, among HF patients, women with HFpEF were 67 % of the cases relative to 42 % of men with HFpEF [259], and post-menopausal females comprised a majority of HFpEF patients [260]. Although most studies do not investigate sex-stratified effect of modification, successful examples of sex-specific treatment are traceable, including sacubitril-valsartan in PARAGON-HF trial, that was mainly benefit in women [261], and spironolactone treatment in TOPCAT trial, showed a reduced risk of in all-cause mortality only in women [262].

To embrace these findings, models with the leading risk factors of clinical HFpEF, such as, aging, diabetes, hypertensin, et al, are required for future BH4 studies. Equal sex-distribution is required in both basic research and clinical trials investigating BH4 for HFpEF outcomes, as previously have suggested that sex may affect BH4's cardioprotective effectiveness [263] because oxidative stress is differently regulated in the vasculature by NO between male and female [264,265].

5. Future perspectives and conclusion

There are several features that make BH4 an appealing therapeutic agent for treating HFpEF from a translational perspective. (1) The ability of exogenous BH4 to reverse advanced hypertrophic remodeling and prevent the progression of cardiac dysfunction despite ongoing pressure overload is striking among existing therapies [113,126]. (2) BH4 is compartmentalized in the cytoplasm and mitochondria of the rat heart [266], as is ROS [267], thereby powerfully suppressing the resource of ROS in cardiomyocytes, whereas other antioxidants, such as tempol, are unable to replace BH4 after disease is established. This may explain why BH4 is more effective on anti-hypertrophy and antifibrosis in the failing heart than broader antioxidant scavengers [126]. (3) BH4, through NO, not only inhibits vascular smooth muscle proliferation, platelet aggregation, but also reduces expression of proinflammatory cytokines [163,268], the earlier events of HFpEF. (4) BH4 is associated with mitochondrial function and energetics during the development of HFpEF.

One of the problems leading to variable results in HFpEF studies is the lack of not only the standardized animal models, but also the cardiac biopsies from living patients because HFpEF patients rarely undergo heart transplantation [249,269]. Thus, establishment of liquid biopsy-based (such as peripheral blood) research approaches is necessary, and particularly suitable to clarify the systemic inflammatory component of HFpEF-specific comorbidities [249]. In addition, the Heart Failure Association of the European Society of Cardiology has proposed the HFA-PEFF clinical algorithms for standardizing the diagnosis of HFpEF [270], in which the HFA-PEFF score can be accurately calculated by combining the levels of the natriuretic peptide (NT-proBNP) with the functional and structural factors from echocardiographic assessments. HFpEF is diagnosed when the HFA-PEFF score is >5 and otherwise excluded if the HFA-PEFF score is <1 [171,271]. Although the current guidelines do not have a sex-specific cut-offs of plasma NT-proBNP, it is important to clarify whether the impact of BH4 on plasma NT-proBNP is sex-dependent in HFpEF. Apart from inherent sex-differences in cardiac structure and function, clinical studies with HFpEF patients have demonstrated that women have higher levels of NT-proBNP than men [272,273]. In addition, to appreciate the limitations from NT-proBNP as the biomarker of HFpEF, proteomic assessments have demonstrated that PI3-kinase and transforming growth factor-beta signaling pathways are the strongest proteomic driver of the microvascular dysfunction among women with HFpEF [274], which warrants further investigation with BH4 to elucidate the sex-specific modulations of these identified targets.

Furthermore, sex-differences in the epigenetic regulation and inflammation imply a sex-based, personalized BH4 dosing should be tailored in HFpEF.

In conclusion, the metabolism of BH4 has multiple biological roles beyond being an enzyme co-factor, which multiplicity supports its potential use as a new pharmacological option for HFpEF treatment. Efforts aimed at encapsulation to promote BH4 bioavailability, stimulating stability of DHFR, or reducing oxidative stress are important to enhance BH4 efficacy. Characterizing the dose–response window for the efficacy of exogenous BH4 in HFpEF with both sexes in all phases of clinical trials would be valuable to enhance its therapeutic benefit for clinical translation.

List of abbreviations

ATP	adenosine triphosphate
BH2	dihydrobiopterin
BH4	tetrahydrobiopterin
cGMP	cyclic guanosine monophosphate
CVD	cardiovascular diseases
DHFR	dihydrofolate reductase
DHFR	KO DHFR heterozygous knockout mice
ECM	extracellular matrix
F2-isoPs	F2-isoprostanes
GHC1	guanosine triphosphate cyclohydrolase
GLUT-1	glucose transporter 1
HF	heart failure
HFpEF	heart failure with preserved
mGCH1Tg	cardiomyocyte-specific GCH1
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
PDE5i	phosphodiesterase type 5
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1-α
PKG	protein kinase G
ROS	reactive oxygen species
sGC	soluble guanylyl cyclase
SGLT2i	sodium-dependent glucose transporter 2 inhibitors
TAC	transverse aortic constriction
Tie2-GCH1Tg	endothelial-specific GCH1
6R-BH4	sapropterin dihydrochloride

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

The authors' work was supported by the National Natural Science Foundation of China (No. 82104770, 82102306), the Guangdong Basic and Applied Basic Research Foundation (2022A1515011456, 2023A1515030239) and Sun Chieh Yeh Heart Foundation.

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