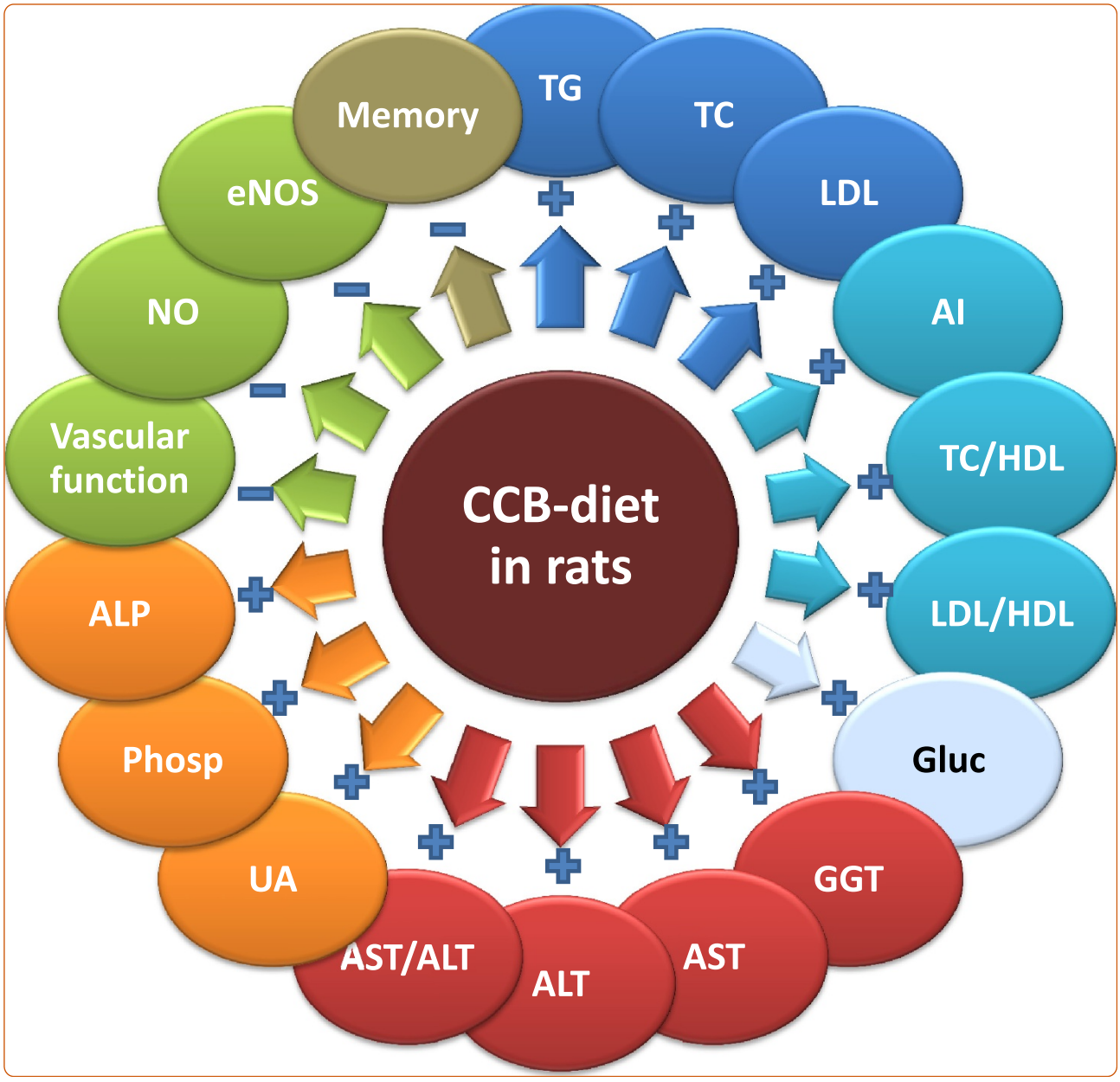
LIPIDS IN HEALTH AND DISEASE



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Cholesterol-cholate-butterfat diet offers multi-organ dysfunction in rats

Humaira Jamshed[1], Jamshed Arslan[1] and Anwar-ul-Hassan Gilani[1,2*]

Abstract

Background: Comparable to commercial expensive high-fat diets, cholesterol-cholate-butterfat (CCB) diet has also been used to induce hyperlipidemia in rats. Our objective was to explore its influence on multiple organs. Consequence of fasting was also analysed.

Methods: Rats in groups 1 and 2 received normal diet (ND) whereas groups 3 and 4 received CCB-diet. Food was withdrawn daily for two hours from groups 2 (ND-F) and 4 (CCB-F). Blood was collected at fourth and sixth week for biochemical estimation; Morris water maze was done in the sixth week for learning ability and memory; after which aortae were isolated for vascular reactivity.

Results: Apart from hyperlipidemia, CCB also induced hyperglycemia with marked increase in hepatic enzymes: gamma-glutamyl transferase (GGT), alanine and aspartate aminotransferase (ALT and AST); and vascular biomarkers: uric acid (UA), phosphorus and alkaline phosphatase (ALP). Isolated aortae, pre-contracted with phenylephrine, were less responsive to acetylcholine indicating endothelial dysfunction – serum nitric oxide (NO) production was limited with subsequent inhibition of endothelial NO synthase. CCB diet also compromised learning ability. CCB-coupled fasting potentiated hyperlipidemia but prevented memory-loss.

Conclusion: We introduce CCB-diet for multi-organ dysfunction in rats, and propose its use for research on cardiovascular diseases and associated manifestations involving immense interplay of integrated pathways.

Keywords: Fasting, Liver function, Vascular function, Memory

Background

Human diseases are complex – representing interplay of synchronized abnormalities in multiple organs. Cardiovascular diseases (CVDs) are the largest death burden globally [1]. They involve not only heart and vessels, but liver [2], kidneys [3] and even the nervous system [4]. As a result, common co-morbidities of CVDs are non-alcoholic fatty liver disease [5], chronic kidney disease [6] and Alzheimer's disease etc. [7,8]. Consequently, research on novel therapeutic interventions, also mandates a holistic approach, such that safety and efficacy is assessed on multiple systems simultaneously.

Animal models are excellent tools for such research, and aid in pathophysiological understanding of human ailments [9]. Genetically manipulated animals – although preferable for being precise [10,11] – may not truly represent disorders as complex as CVDs. Alternatively, there are modified diets, inducing human-like pathologies in laboratory animals [12]. Food markedly impacts health. It influences disease status of humans [13] and animals [14]. Ingredients like fats are known to increase CVD risk factors in species like rabbits [15], hamsters [16], rats [14] and mice [17]. In laboratory animals, the alterations convened by high-fat diets (HFDs) are fairly similar to human [18]. Literature reports that commercial HFDs cause hyperlipidemia [19], which consequence in lipids' deposition in tissues (both adipose and non-adipose). Eventually lipid build-up leads to cellular dysfunction of heart, vessels and liver [20].

Experimental manipulation of these pre-formed commercial diets could be challenging. In contrast, a simple modifiable diet containing cholesterol, cholate and butterfat (CCB) as fat sources, has also been used to induce hyperlipidemia [21]. We aim to inspect the possible influence of this

* Correspondence: anwar.gilani@aku.edu
[1]Natural Product Research Unit, Department of Biological and Biomedical Sciences, Aga Khan University, Karachi 74800, Pakistan
[2]College of Health Sciences, Mekelle University, P. O. Box 1871, Mekelle, Ethiopia

CCB-diet on hepatic and vascular function along with learning ability in rats. These aspects have not yet been explored for CCB-diet. In addition, since dietary restriction is known to induce adaptive changes in intermediary metabolism [22], we also inquired the consequence of daily two-hour fasting.

Results

Lipid profile

CCB-diet caused pronounced hyperlipidemia at fourth and sixth week. Serum concentrations of TG, TC and LDL were drastically increased at fourth week, from 68 ± 5, 128 ± 12 and 51 ± 5 in ND to 117 ± 11, 430 ± 113 and 173 ± 29 mg/dl in CCB group (Figure 1A). Also at sixth week, as shown in figure 1B, the serum concentrations of TG, TC and LDL were markedly increase from 72 ± 4, 139 ± 19 and 78 ± 14 mg/dl in ND to 112 ± 10, 583 ± 119 and 327 ± 55 mg/dl in CCB respectively.

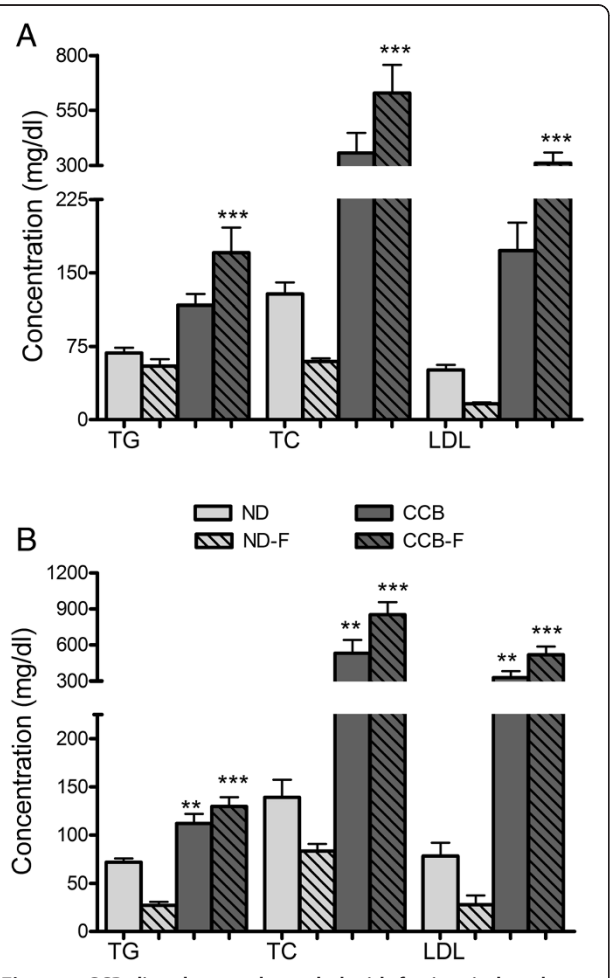

Figure 1 CCB-diet alone and coupled with fasting, induced hyperlipidemia in rats. A) Fourth week; **B)** Sixth week; TG: triglyceride; TC: total cholesterol; LDL: low-density lipoprotein; ND: normal diet; ND-F normal diet with two-hour fasting; CCB: cholesterol-cholate-butterfat diet; CCB-F: cholesterol-cholate-butterfat diet with two-hour fasting. All values are represented as mean ± standard error of mean (SEM) (n = 7 per group). This figure only shows the comparison of means using one-way ANOVA followed by Tuckey's post-test (**p < 0.01 and ***p < 0.001).

CCB coupled with fasting potentiated hyperlipidemia at fourth and sixth week, as evident from Figure 1A and B respectively. The resultant serum concentrations of TG, TC and LDL were respectively 170.6 ± 26, 629 ± 129 and 311 ± 49 mg/dl at fourth week and 130 ± 10, 853 ± 104 and 518 ± 68 mg/dl at sixth week.

For the effect of fasting *per se,* normal diet-fed rats were also fasted. Reduction in TC and LDL was observed at fourth and sixth week; TG was reduced at sixth week. Resultant concentrations of TC and LDL were respectively 59.7 ± 3.2 and 16.3 ± 1.3 mg/dl at fourth week and 83 ± 7.5 and 28 ± 9.8 mg/dl at sixth week; with TG concentration of 27 ± 3.4 mg/dl at sixth week.

Lipid ratios

Results for the calculated lipid parameters show a noticeable increase ($p < 0.01$) in LDL/HDL ratio at fourth week, whereas, atherogenic index (AI) and TC/HDL ratios were elevated ($p < 0.01$) after six weeks (Figure 2A and B). Value of LDL/HDL was 1.06 ± 0.09 in ND and 2.78 ± 0.59 in CCB; atherogenic index and TC/HDL ratios at sixth week were respectively 1.94 ± 0.18 and 2.94 ± 0.18 in ND with 4.99 ± 0.88 and 5.99 ± 0.88 in CCB.

Fasting with CCB-diet potentiated the elevation of these ratios. The resultant values of AI, TC/HDL and LDL/HDL ratios were 7.4 ± 0.9, 8.4 ± 0.9 and 4.2 ± 0.4 at fourth week, and 7.8 ± 0.9, 8.8 ± 0.9 and 5 ± 0.5 at sixth week respectively (Figure 2A and B). In contrast, fasting with normal diet reduced LDL/HDL ratio (at fourth and sixth week) and AI and TC/HDL (at sixth week).

Glucose and GGT

Our data presented in Figure 3 show a prominent elevation ($p < 0.001$), by CCB-diet, in serum glucose (only at fourth week) and GGT (at fourth and sixth week). Consequent concentrations of glucose were 80 ± 5 mg/dl in ND and 129 ± 3 mg/dl in CCB. Serum GGT concentrations were 28 ± 0.7 u/l in ND and 41 ± 3 u/l in CCB (at fourth week) and 25.8 ± 33 u/l in ND and 43 ± 58 in CCB (at sixth week).

Glucose and GGT responded similarly when fasting was coupled with CCB, but when coupled with normal diet, fasting reduced serum glucose (to 69 ± 87 mg/dl), at sixth week.

Hepatic function biomarkers

Other than GGT, indicators of hepatic function e.g. aminotransferases (AST and ALT) were also distinctly elevated by CCB-diet at fourth and sixth week as presented in Figure 4A and B respectively. At fourth week increase

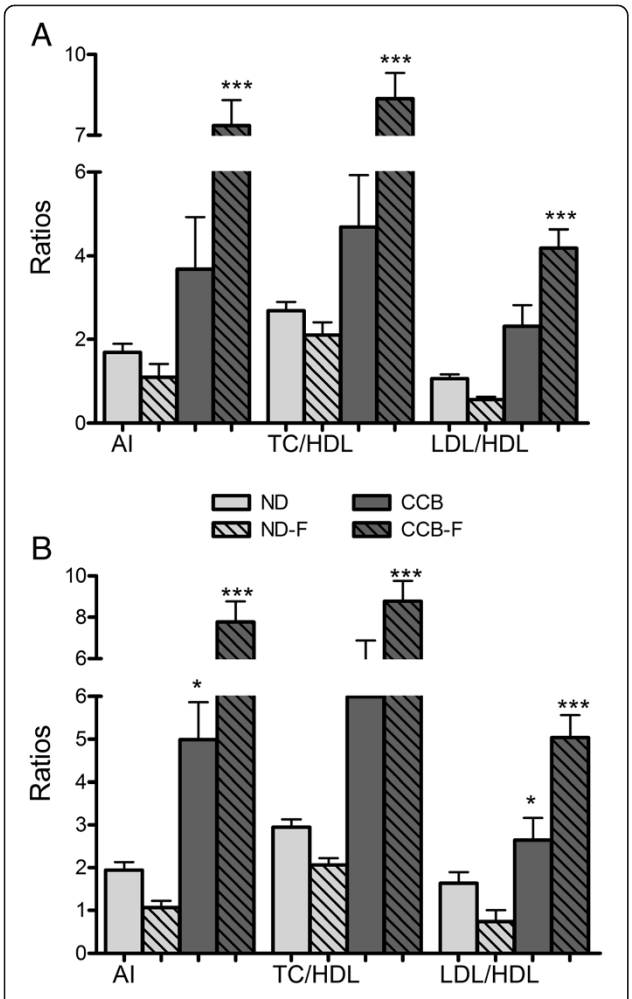

Figure 2 CCB-diet alone and coupled with fasting, increased atherogenic index and lipid ratios. A) Fourth week; **B)** Sixth week; AI: atherogenic index; ND: normal diet; ND-F normal diet with two-hour fasting; CCB: cholesterol-cholate-butterfat diet; CCB-F: cholesterol-cholate-butterfat diet with two-hour fasting. All values are represented as mean ± standard error of mean (SEM) (n = 7 per group). This figure only shows the comparison of means using one-way ANOVA followed by Tuckey's post-test (*p < 0.05 and ***p < 0.001).

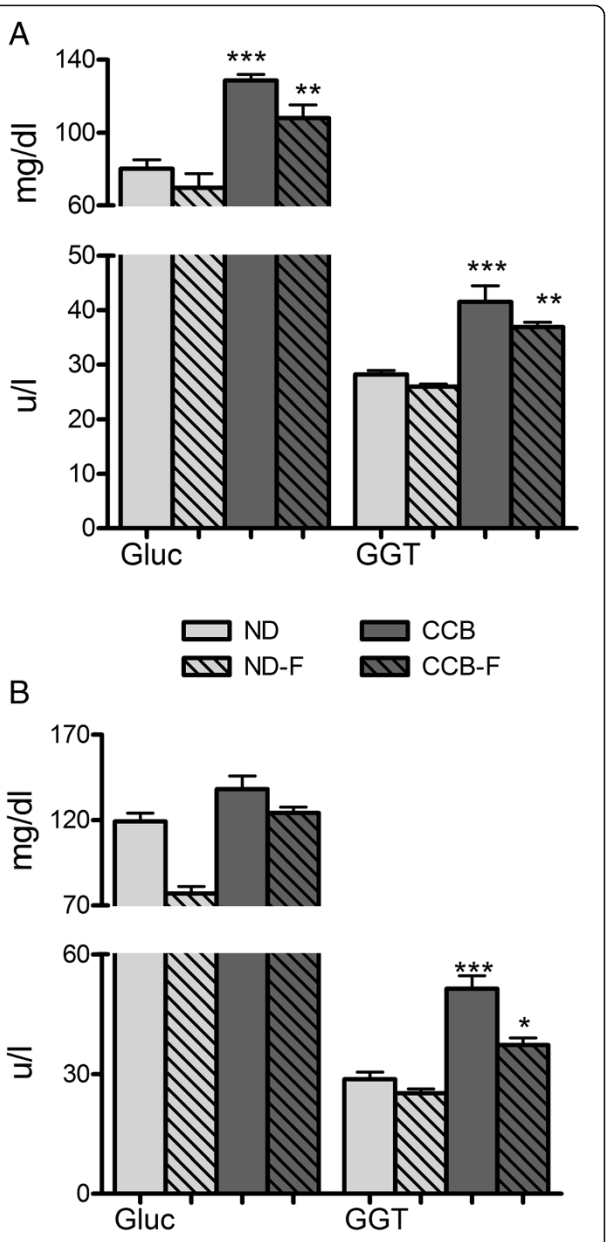

Figure 3 CCB-diet alone or coupled with fasting, increased serum glucose and GGT in rats. A) Fourth week; **B)** Sixth week; Gluc: glucose (mg/dl); GGT: gamma-glutamyl transferase (u/l); ND: normal diet; ND-F normal diet with two-hour fasting; CCB: cholesterol-cholate-butterfat diet; CCB-F: cholesterol-cholate-butterfat diet with two-hour fasting. All values are represented as mean ± standard error of mean (SEM) (n = 7 per group). This figure only shows the comparison of means using one-way ANOVA followed by Tuckey's post-test (*p < 0.05, **p < 0.01 and ***p < 0.001).

in AST and ALT were respectively 1.55 and 1.8 folds whereas at sixth week the concentrations were 2.56 and 1.7 folds higher in CCB compared to ND.

Two-hour fasting with CCB led to almost equivalent increase in these aminotransferases ($p > 0.05$) as CCB alone did, whereas fasting with normal diet decreased AST at fourth week. The calculated AST/ALT ratio remained unchanged in all groups throughout the experiment duration.

Vascular function

Vascular function was studied at three levels. Firstly, some bio-molecules associated with endothelial dysfunction (UA, phosphorus and ALP) were found to be elevated profoundly ($p < 0.001$) by CCB-diet. Fasting coupled with normal diet had no effect on these parameters ($p > 0.05$). Serum concentrations of UA were 1.4 ± 0.1, 2.4 ± 0.1 and 2.37 ± 0.08 mg/dl in ND, CCB and CCB-fasting respectively at fourth week and 1.7 ± 0.09, 3.29 ± 0.2 and 3.18 ±

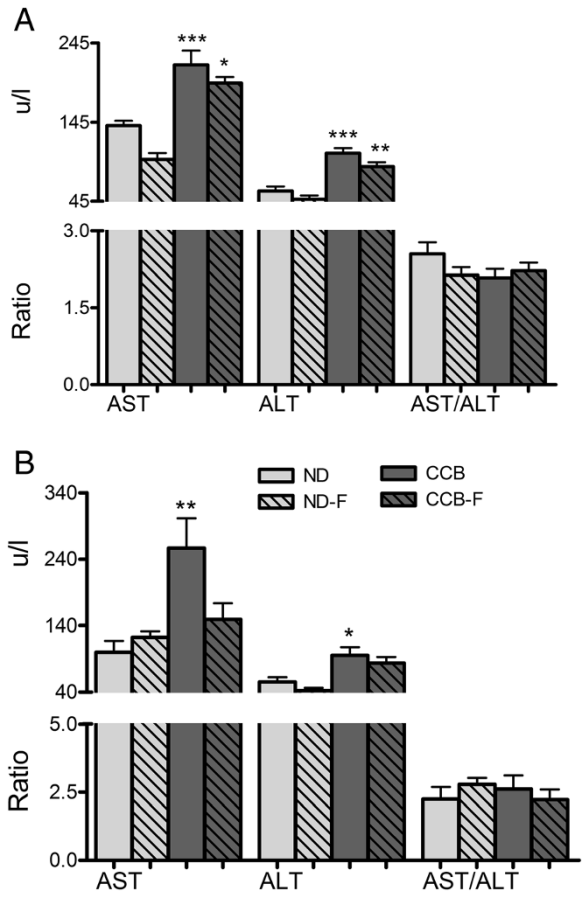

Figure 4 CCB-diet alone or coupled with fasting, induced mild hepatic injury raising serum biomarkers. A) Fourth week; **B)** Sixth week; AST: aspartate aminotransferase (u/l); ALT: alanine aminotransferase (u/l); ND: normal diet; ND-F normal diet with two-hour fasting; CCB: cholesterol-cholate-butterfat diet; CCB-F: cholesterol-cholate-butterfat diet with two-hour fasting. All values are represented as mean ± standard error of mean (SEM) (n = 7 per group). This figure only shows the comparison of means using one-way ANOVA followed by Tuckey's post-test (*p < 0.05, **p < 0.01 and ***p < 0.001).

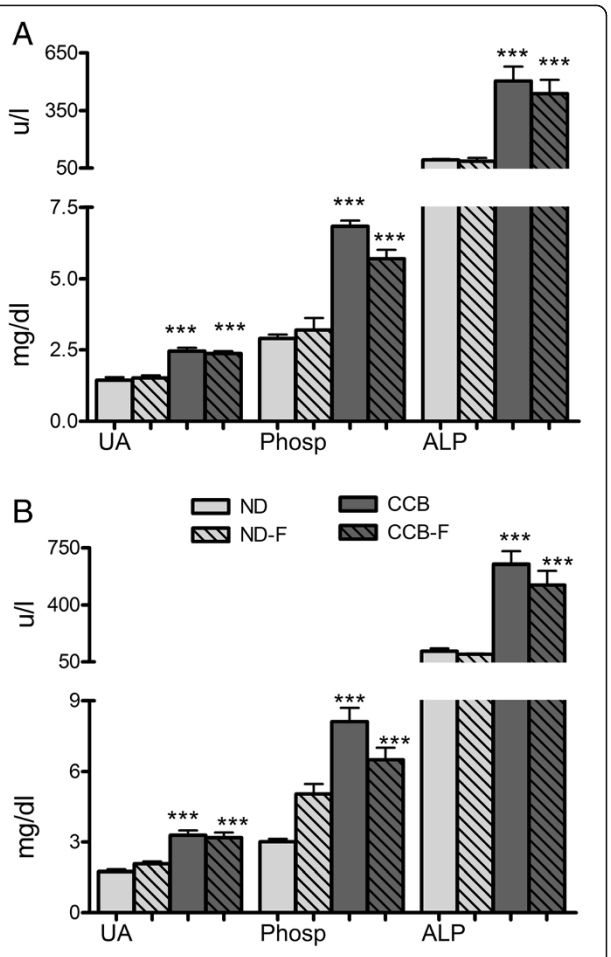

Figure 5 CCB-diet alone and coupled with fasting, elevated vascular biomarkers in rats. A) Fourth week; **B)** Sixth week; UA: uric acid (mg/dl); Phosp: phosphorus (mg/dl); ALP: alkaline phosphatase (u/l); ND: normal diet; ND-F normal diet with two-hour fasting; CCB: cholesterol-cholate-butterfat diet; CCB-F: cholesterol-cholate-butterfat diet with two-hour fasting. All values are represented as mean ± standard error of mean (SEM) (n = 7 per group). This figure only shows the comparison of means using one-way ANOVA followed by Tuckey's post-test (***p < 0.001).

0.2 mg/dl respectively at sixth week (Figure 5A and B). Phosphorus and ALP levels, at fourth week, were 2.9 ± 0.1, 6.8 ± 0.2 and 5.7 ± 0.3 mg/dl in ND, CCB and CCB-F, and 93 ± 4.7, 504 ± 76 and 437 ± 72 u/l respectively (Figure 5A). Whereas at sixth week, phosphorus was 3 ± 0.1, 8 ± 0.6 and 6.5 ± 0.5 mg/dl and ALP were 116 ± 17, 650 ± 79 and 522 ± 87 u/l respectively (Figure 5B).

In the second step, vascular function of isolated thoracic aortae was analysed on isolated tissue bath assembly. Concentration-response curves of acetylcholine (ACh: 0.01 μM to 100 μM) were prepared after pre-contracting the aortae with phenylephrine (1×10^{-6} mol/L). As shown in figure 6A, acetylcholine (at concentration of 1 μM and above) inhibited the phenylephrine-induced contractions of aortic rings from normal controls in a concentration-dependent manner. However, in aortae from CCB-fed rats, this response was inhibited ($p < 0.001$) at high concentrations of ACh (i.e. 3 μM and above), indicating endothelial dysfunction. Fasting coupled with CCB partly prevented this impairment, evident in Figure 6A by the partial inhibition of PE-induced contraction by ACh at high concentrations (10 μM and above).

Thirdly, we explored the probable downstream events contributing to endothelial dysfunction. Endothelial nitric oxide synthase (eNOS) activity was assayed in aorta, and total nitric oxide (NO) concentration was measured in serum. As elaborated in Figure 6B, production of serum NO was diminished perhaps due to inactivation of eNOS by the CCB-diet. Enzyme activity (represented

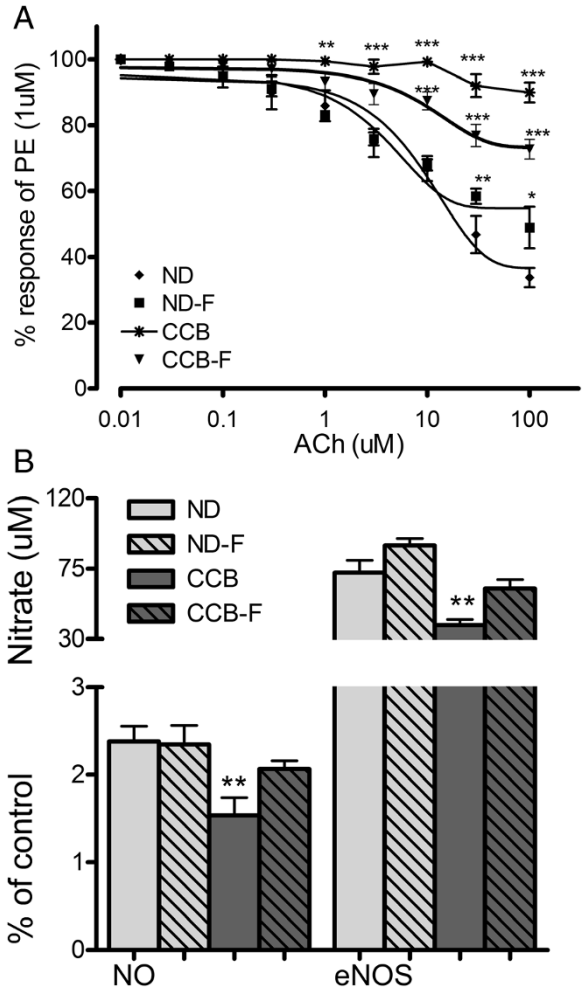

Figure 6 CCB-diet impaired vascular reactivity of thoracic aortae by amending nitric oxide pathway. A) Isolated aorta experiment; PE: phenylephrine; Ach: acetyl choline, Two-way ANOVA followed by Bonferroni's post-test is applied. **B)** Nitric oxide pathway; NO: nitric oxide (uM); eNOS: endothelial nitric oxide synthase; ND: normal diet; ND-F normal diet with two-hour fasting; CCB: cholesterol-cholate-butterfat diet; CCB-F: cholesterol-cholate-butterfat diet with two-hour fasting. All values are represented as mean ± standard error of mean (SEM) (n = 7 per group). This figure only shows the comparison of means using one-way ANOVA followed by Tuckey's post-test (*p < 0.05, **p < 0.01 and ***p < 0.001).

as % of control) and nitrate concentrations in ND were 72.5 ± 7.9% of control and 2.4 ± 0.17 uM respectively. In CCB, eNOS and nitrate were 38.7 ± 3.7% of control and 1.5 ± 0.2 uM respectively ($p < 0.01$). In fasting with CCB, the concentrations of eNOS and NO were 62 ± 5.9% of control and 2 ± 0.9 uM respectively. Fasting almost completely protected the CCB-induced endothelial dysfunction such that eNOS activity and NO concentration were similar to normal controls ($p > 0.05$).

Learning/memory

The CCB-diet slowed the learning process and/or impaired memory. This was evident on day six and day seven of Moris water maze (MWM), when CCB-fed rats took longer time ($p < 0.01$) to find the hidden platform (escape latency), compared to normal controls. On the first five days of MWM trials, there was no significant difference in the escape latency of rats from different groups, as evident in Figure 7. Escape latency on day six and seven were 2.05 and 2.24 times higher in CCB compared to ND (Figure 7). Fasting had no significant effect on normal controls, but almost completely prevented CCB-induced memory loss, as response was similar to normal controls.

Discussion

Our study presents CCB-diet model as a candidate for research on cardiovascular diseases with associated complications, as this diet ensures the multi-organ dysfunction induced in rats. In addition to serum lipid profile and glucose, interestingly, this CCB-diet also elevated biomarkers of hepatic and vascular function. CCB impaired the vascular reactivity of isolated aorta, by restraining eNOS activity, thereby limiting nitric oxide production. Besides, this simple high-fat diet containing cholesterol, cholate and butterfat, also affected the nervous system by lessening the learning capabilities of experimental rats.

Comparable to other commercial HFDs [20], CCB-diet increased triglyceride, cholesterol and LDL, with little effect on HDL. The lipid profile is considered as a good indicator of cardiovascular health status. Nevertheless, it is suitable mainly for extreme high and extreme low risk individuals and not the majority at medium risk [23]. Conversely, TC/HDL and LDL/HDL ratios have been shown to be better markers than LDL and HDL levels per se [24-26]. TC/HDL is known to be a better predictor of ischemic heart disease than LDL/HDL ratio [27] since it involves

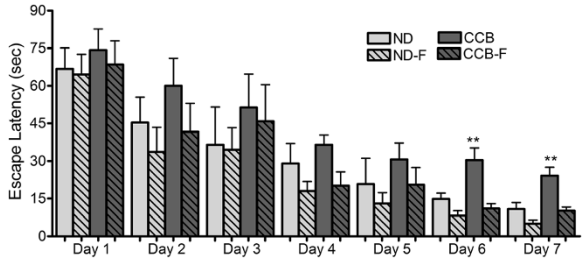

Figure 7 CCB-diet impaired learning ability in rats. ND: normal diet; ND-F normal diet with two-hour fasting; CCB: cholesterol-cholate-butterfat diet; CCB-F: cholesterol-cholate-butterfat diet with two-hour fasting. All values are represented as mean ± standard error of mean (SEM) (n = 7 per group). This figure only shows the comparison of means using one-way ANOVA followed by Tuckey's post-test (**p < 0.01).

very-low-density-lipoprotein (VLDL) and intermediate-density-lipoprotein, in addition to LDL and HDL. Although the significance of LDL/HDL ratio is believed to be compromised in hypertriglyceridemias (where majority of the serum cholesterol resides in VLDL); yet it is an excellent indicator of drug response [28] as it simultaneously represents atherogenic and anti-atherogenic lipids. This is why we calculated and presented the results of these ratios.

The hypertriglyceridemia and LDL elevation we obtained could be attributable to the cholesterol in CCB-diet: it accelerates glycolysis in liver [29], producing fatty acids which are esterified to triglycerides and cholesterol-esters [30]. These esters integrate in LDL, and release in blood [31], along with the excess triglycerides [32]. Dietary cholesterol also blocks the receptor-mediated LDL uptake sustaining increased plasma LDL [33]. While explaining CCB-diet induced hypercholesterolemia, however, we do realize that humans and rats resist dietary cholesterol-induced hypercholesterolemia [34] in contrast to rabbits and hamsters [35,36]. Whereby, cholesterol blocks the de-novo synthesis by inhibiting HMG Co-A reductase [37], facilitates the catabolic conversion of cholesterol to bile acids [38] and accelerates biliary cholesterol excretion [39]. This prevents the rise in serum cholesterol, where bile overproduction ensures that excess cholesterol is eliminated.

The butterfat in CCB-diet contains palmitic, oleic and stearic acids. It counteracts by enhancing de-novo cholesterol synthesis via activation of HMG Co-A reductase [40,41]. Our CCB-diet is also supplemented with cholate, which ensures greater intestinal cholesterol absorption [42] in addition to promoting cholesterol synthesis [43]. The probable synergism of butterfat and cholate could justify hypercholesterolemia in the CCB-diet model.

Our results showed that two-hour fasting promoted CCB-induced hyperlipidemia. Body adopt dietary amendments by altering metabolism. In well-fed state, energy is provided by carbohydrate and sugars, which steadily shifts to fats in case of fasting [44]. The fat stores (in adipocytes) disintegrate, supplying fatty acids to liver [45], increasing the cholesterol content - which secretes in blood as LDL after esterification [46]. Hepatic fatty acids should esterify causing hypertriglyceridemia [47] but we observed similar triglycerides in CCB and CCB-fasting (at sixth week). This may be because, hepatic fatty acid mainly arises from adipose stores with minor amounts synthesized from dietary sources [48]. In case of a chronic fasting (six weeks), these stores exhausted and were probably no longer accessible. Prolonged fasting also inhibits fatty acid synthesis and promotes its oxidation [49]. Fasting coupled with normal diet also reduced triglycerides. This might be because ND was not supplemented with additional fatty acids. In absence of exogenous source, depletion of endogenous stores and accelerated oxidation of fatty acids – triglycerides decreased. Extended use of CCB-diet supplemented with fatty acids (in butterfat), prevented the fall in TG.

Al-Attar (2010) has reported the combined effects of intermittent fasting and high-fat diet [22]. Where we have used cholesterol, cholate and butterfat as the source of high fat in diet, Al-Attar (2010) used 15% mutton tallow (and the remaining diet composition was not provided). This might underlie the contradicting results; they obtained similar hyperlipidemia by high-fat diet (HFD) with and without fasting. The fasting duration also varied, from two hours daily in our investigation, to 10 hours/day for five days a week in the study by Al-Attar (2010).

In addition to hyperlipidemia, we found elevation in GGT, AST and ALT by the CCB-diet. Apart from the general perception of GGT as an indicator of hepatic injury, it is now also recognized as a predictor of cardiovascular event [50]. GGT is basically a marker of oxidative stress [51] and inflammation [52], and is linked with hypertension [53,54] and hyperlipidemia [55]. Besides, it is associated with CVD [56] and reported to be present in atherosclerotic plaques [57,58], where it is anticipated to be involved in LDL oxidation [59]. Likewise, the aminotransferases (both ALT and AST) are also accepted as markers of hepatic degeneration and dysfunction, but these are also associated with diabetes mellitus and metabolic syndrome [60]. ALT is also projected as an indicator of carotid atherosclerosis [61]. Therefore, we believe that an animal model offering abnormality in these biomarkers can serve as a worthy tool for CVD research.

We found that throughout the course of six weeks, the AST/ALT ratio remained unchanged and inferred the indication of acute and mild hepatic damage. Localization of AST is not confined to liver and may also be released on injury to heart or skeletal muscles [60]. Within hepatocytes, ALT is present in the cytoplasmic space and is released even on minor hepatic damage. AST, on the other hand, resides predominantly in mitochondria, and is discharged when the destruction is severe [62]. Therefore, an increase in the AST/ALT ratio would be observed with persistent liver damage.

Among the biomarkers of vascular function, we tried looking into uric acid (UA), phosphorus and ALP. We found these to be profoundly elevated by CCB-diet. ALP is considered as a potential diagnostic marker of CVD [63]. Apart from being involved in lipid absorption [64], ALP is also recognized to regulate vascular calcification [65-67]. Both ALP and UA correlate with hypertension [68,69] and dyslipidemia [70,71]. By facilitating smooth muscle cell proliferation [72], UA induces dysfunction of vascular endothelium [73], and hence acknowledged as a risk factor for atherosclerotic diseases [74]. Phosphorus impairs endothelial function [75] by prompting vascular

calcification [76,77] analogous to ALP, and is therefore, accredited as a amendable risk factor for atherosclerosis [78]. The reason we fail to achieve hyperglycemia at sixth week could be ascribed to phosphorus which enhances glucose utilization through glycolysis [79].

Noteworthy elevations in vascular biomarkers compelled us to explore the reactivity in thoracic aortae. As anticipated, aortae isolated from CCB-fed rats were evidently less responsive to ACh, indicative of endothelial dysfunction. We opted to further inquire, among the countless possibilities, the underlying nitric oxide pathway. Since the prior experiments deduced a probable malfunctioning endothelium, we examined endothelial-NOS enzyme activity, and found it to be compromised; with a consequent reduction in nitric oxide concentration. Revealing one of the precise underlying mechanisms, we aided future research by enabling scientists to confidently select and pin-point the mechanism of novel therapeutic interventions.

Apart from hepatic and vascular dysfunction, common CVD comorbidities also include memory impairment. Different HFDs are reported to delay the learning ability in experimental animals [80]. Therefore, we considered the likely consequence of this CCB-diet on rats' memory and found consistent results, when CCB hindered the learning ability. Likewise, the fasting-induced prevention of memory-impairment that we acquired both in ND and CCB groups was also in accordance with the previous literature [81], where dietary restriction benefits learning capabilities [82].

Conclusion

This study introduced the CCB-diet for multi-organ dysfunction in rats (a brief summary presented in Additional file 1), and proposed its use for research on cardiovascular diseases and associated manifestations. Like other commercially available expensive high-fat diets, this simple and robust CCB-diet induces hyperlipidemia in rats, which we showed, can be potentiated by coupling with two-hour fasting (daily). Further, we showed that this diet also offers elevation of biomarkers indicative of hepatic damage. Vascular function was simultaneously impaired, which we demonstrated at three levels; a) elevated vascular biomarkers, b) reduced endothelial reactivity of aorta and c) inhibition of nitric oxide pathway. Interestingly, CCB-diet also presented diminished memory/learning ability in rats. Hence, we suggest that the multi-organ abnormalities obtainable by this dietary model should be opted for research while inspecting the holistic effects of pharmaceutical interventions, specifically in complex disorders like cardiovascular diseases, where there is an immense interplay of integrated pathways.

Methods and study design

Animals and diets

Adult Sprague–Dawley rats (180 to 200 grams) of either gender were housed at the animal house of The Aga Khan University maintained at 23 to 25°C. These animals were kept in plastic cages with sawdust, and had free access to food and water (except for the fasting groups). The experiments conducted were in accordance with the guidelines for care and use of laboratory animals provided by The National Research Council [83]. The study protocol was approved by the Ethical Committee for Animal Care and Use, of The Aga Khan University, Karachi, Pakistan. Four groups of seven rats each were used. Group 1 (ND) and group 2 (ND-F) were fed with normal rat diets, whereas group 3 (CCB) and group 4 (CCB-F) were provided with cholesterol-cholate-butterfat diet [84]. Contents of both theses diets are given in Table 1. From group 2 (ND-F) and group 4 (CCB-F), food was withdrawn daily for two hours, whereas group 1 (ND) and group 3 (CCB) had *ad-libitum* access to food. At the end of the fourth week, blood was drawn from rats' tail by cuff method [85]. However at the end of sixth week, blood was drawn through cardiac puncture.

Learning ability or memory

In the sixth week rat's learning ability or memory was assessed through Morris Water Maze (MWM) following the standard protocol [86] with slight modifications [87]. Briefly, in each trial, rats were allowed to swim in water and the time required to escape to the hidden platform, called escape latency, was recorded. This was continued for seven days such that the rats went through two trials on the first day and one trial per day for remaining six days.

Table 1 Contents of normal and cholesterol-cholate-butterfat (CCB) diet

Ingredients	Normal diet	CCB diet
Wheat flour	33.3%	30.9%
Bran fiber (choaker)	33%	30.6%
Fish meal	15%	13.9%
Dry skimmed milk powder	13.3%	12.3%
Cooking oil (soya)	3.3%	3.1%
Potassium metabisulphate	0.1%	0.1%
Salt	0.5%	0.5%
Nutrivet powder (bromix F-A)	0.33%	0.3%
Molasses	1%	0.9%
Cholesterol	–	2%
Cholic acid	–	0.5%
Butter fat	–	5%

Biochemical estimations and enzyme assay

From the blood obtained on fourth and sixth week, serum was separated by centrifuging at 4000 rpm and 4°C for 10 min. The concentrations of total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), glucose (Gluc), gamma-glutamyl transferase (GGT), aspartate amino-transferase (AST), alanine amino-transferase (ALT), uric acid (UA), phosphorus (phosp.) and alkaline phosphatase (ALP) were estimated on Automated Analyzer (Roche Cobas c-111) using commercially available kits. Atherogenic index (AI) was calculated as the ratio of Non-HDL and HDL [21]. TC/HDL, LDL/HDL and AST/ALT ratios were also calculated. Serum Nitric oxide concentration was estimated by Griess method [88]. At the end of experiment (sixth week), thoracic aortae were isolated and endothelial nitric oxide synthase (eNOS) enzyme activity was assayed using the Nitric Oxide Synthase Assay Kit, Colorimetric (Calbiochem, Cat. No. 482702) following the manufacturer's instructions.

Vascular reactivity

On the aortae isolated from the rats of each group, the vascular reactivity was also assayed following the protocol of Furchgott and Zawadski [89] with certain modifications [90]. Briefly, aortic rings were mounted on the tissue bath and after acclimatization, concentration-response curves of acetylcholine (ACh: 0.01 μM to 100 μM) were prepared after pre-contracting the aortae with phenylephrine (PE: 1×10^{-6} mol/L).

Statistical analysis

The data are expressed as mean ± SEM (Standard Error of Mean). For comparison between means of two groups, unpaired student's *t*-test was used. One-way analysis of variance (one-way ANOVA) was also applied when comparing the differences in means of four groups, followed by Tukey's multiple comparison test to determine the significant differences. Two-way ANOVA followed by Bonferroni's post-test was applied in vascular reactivity experiment to calculate the statistical significance. P-value less than 0.05 ($p < 0.05$) was considered as significant. Statistical analysis and plotting of graphs was done using GraphPad Prism software (version 4.0).

Additional file

Additional file 1: Graphical Abstract showing cholesterol-cholate-butterfat (CCB) diet-induced increase in triglyceride (TG); total cholesterol (TC); low-density lipoprotein (LDL); atherogenic index (AI); ratio of TC/HDL; Ratio of LDL/HDL; glucose (Gluc); gamma-glutamul transferase (GGT); aspartate aminotransferase (AST); alanine aminotransferase (ALT); Ratio of AST/ALT; uric acid (UA); phosphorus (Phosp.) and alkaline phosphatase (ALP); loss of vascular endothelial reactivity, inhibition of nitric oxide (NO) production and endothelial nitric oxide synthase (eNOS) activity and memory impairment.

Abbreviations

ACh: Acetylcholine; AI: Atherogenic index; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AST/ALT: Ratio of AST and ALT; CCB: Cholesterol-cholate-butterfat; CCB-F: Cholesterol-cholate-butterfat with fasting; CVD: Cardiovascular disease; eNOS: Endothelial nitric oxide synthase; GGT: Gamma-glutamyl transferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; LDL/HDL: Ratio of LDL and HDL; MWM: Morris water maze; ND: Normal diet; ND-F: Normal diet fasting; NO: Nitric oxide; PE: Phenylephrine; Phosp: Phosphorus; TC: Total cholesterol; TC/HDL: Ratio of TC and HDL; TG: Triglyceride; UA: Uric acid; VLDL: Very-low-density lipoprotein.

Competing interests

The authors declare no competing interests.

Authors' contributions

HJ and JA conducted research (hands-on conduct of the experiments and data collection) and analysed the data and drafted the manuscript. AHG designed research (project conception, development of overall research plan, and study oversight) and refined the manuscript. All authors have read and approved the final manuscript.

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