# Grape Seed Proanthocyanidin Extract Alleviates Cold Stress-Induced Hyperlipidemia and Lipid Peroxidation in the Aging Rat Heart

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**Abstract:** *Hyperlipidemia under cold stress can increase the risk of atherosclerosis and oxidative stress in the heart. <u>Objectives</u>: We investigated two models of cold stress on serum corticosterone (CORT), plasma total cholesterol (TC), low density lipoprotein (LDL), very low density lipoprotein (VLDL), triglycerides (TG), high density lipoprotein (HDL), and total antioxidant capacity (TAC), along with blood glucose (BG) and blood lactate (BLa) in male Wistar rats aged 3 (adult), 12 (late-adult), and 24 (old) months. <u>Methods</u>: Rats were grouped into: control (CON) at 25±2°C, supplemented control (CON+PA), chronic cold exposure (CCS) that lasted 60 minutes daily at 10°C for 14 days, acute cold exposure (ACS) with single bout of cold stress at 10°C and animals supplemented with grape seed proanthocyanidin extract (GSPE) and exposed to CCS (CCS+PA) and ACS (ACS+PA). <u>Results</u>: CORT, BG, and BLa levels increased for all age groups exposed to either form of cold stress, with the highest increases found in the ACS group. Further, increases in TC, LDL, VLDL, and TG and plasma atherogenic index accompanied decreases in TAC. These changes were greatest under ACS than CCS. Also, old showed more stress to cold exposure than adults or late-adults. <u>Conclusions</u>: Age was significantly correlated with serum corticosterone and cardiac malondialdehyde, BG, and BLa. GSPE alleviate cold stress-induced hyperlipidemia, implying its antioxidant property, which may reduce the possible risk of atherosclerosis, especially in old rats.*

**Keywords:** Age, Atherogenic index, Cold Stress, Grape Seed Proanthocyanidin Extract, Heart

## 1. Introduction

Exposure to cold stress and hypothermia are important stressors that can initiate many responses. The measurement of blood cortisol levels in humans and serum corticosterone (CORT) levels in animals before and after exposure to stressors indicates their responses to stress. The adrenocorticotropic hormone secreted by the pituitary gland regulates the synthesis of corticosteroid in the adrenal gland [1]. Acute and chronic stress elevate plasma CORT concentration in adult rats [2] and mice [3], while also increasing plasma lipids [4]. During the increase in metabolic stress caused by cold exposure, the hypothalamic pituitary adrenal axis stimulates the hypophysis to secrete more adrenocorticotropic hormone [5]. Additionally, the glucocorticoids from the adrenal cortex of stressed mice induce increased blood glucose levels [6], which reflect the pivotal role of glucose in regulating energy. These changes are concomitant with alterations in plasma lipids, such as total cholesterol, low density lipoprotein, triglycerides, and high density lipoprotein, all of which are known to increase the risk of cardiovascular diseases (CVDs). The responses of CORT to cold stress are a function of the duration and intensity of the stress [7]. Cold stress also elevates metabolic rate in mammals [8].

Polyphenols are chemical substances which are found in plants and which have an aromatic ring with hydroxyl groups, including esters and glycosides [9]. There has been growing interest in polyphenols' potential to effectively alleviating CVDs [10] and neurodegenerative [11] disorders. Grape seed proanthocyanidin extract (GSPE), which contains flavonoids, has potential anti-oxidative properties [12],[13],[14],[15].

The present study had four objectives. The first was to determine age-related changes in plasma CORT levels and examine changes in plasma lipids in rats subjected to chronic cold stress (CCS) and acute cold stress (ACS). The second was to examine the vital risk factor of CVDs, the plasma atherogenic index, and plasma lipid profile. The third was to describe the correlation (if one exists) between plasma CORT and lipid peroxidation in the ventricles of the ageing heart. Finally, the fourth was to test whether grape seed proanthocyanidin, an effective natural antioxidant, affects the plasma and tissue changes in the above mentioned parameters.

## 2. Materials and Methods

Animal procedures were approved by the Institutional Animal Ethics Committee (IAEC), Bangalore University, Bangalore.

### 2.1 Animal maintenance and experimental design

Ninety adult (3-month-old) male albino Wistar rats of equal body mass were procured from the Central Animal Facility, IISc, Bangalore, and 30 rats each were maintained until they were 12 months old and 24 months old. They were housed at 25±2°C, with a relative humidity of 72±1°C. Rats had free access to water and standard feed (Amruth Feeds, India). After being acclimated for five weeks, they were assigned to six groups of five rats each: animals at housing room temperature (RT, 25°C, CON), animals supplemented with GSPE (CON+PA), animals exposed to CCS at 10°C (CCS) for 14 days, animals exposed to a single bout of cold stress at 10°C (ACS), animals supplemented and exposed to CCS

(CCS+PA), and animals supplemented and exposed to ACS (ACS+PA). The supplement was a daily oral gavage of 200 mg of GSPE/kg body weight.

## 2.2. Feeding efficiency

Feeding efficiency was calculated by the method described in an earlier study [16].

## 2.3. Biochemical assays

### 2.3.1. Blood sampling
At the end of the cold exposure period, rats were sacrificed by $CO_2$ asphyxiation, and blood was collected through a cardiac puncture and stored at 4°C in different vials for plasma and serum separation.

### 2.3.1.1. Blood glucose
Blood glucose was estimated by the method of Nelson [17]. Glucose was expressed as mg /dl.

### 2.3.1.2. Blood lactate
Blood lactate was estimated by the method of Barker and Summerson [18]. Blood lactate was expressed as mg lactic acid/dl.

### 2.3.2. Serum corticosterone
Serum corticosterone was quantified using an ELISA kit (Biovendor Research and Diagnostics Products) and read in a multimode plate reader (TECAN, Infinite 200 Pro, Austria GmbH). Values were expressed in ng/ml serum.

### 2.3.3. Plasma
Blood was drawn into EDTA-coated tubes and centrifuged at $1000 \times g$ using a refrigerated centrifuge for 10 min at 5°C. The isolated plasma was aliquoted and stored at 4°C until the analysis was conducted.

### 2.3.3.1. Plasma lipid profile
The plasma lipid profile was determined using commercially available kits (Span Diagnostics Ltd, Surat, Gujarat, India). total cholesterol, high density lipoprotein, triglycerides, low density lipoprotein, and very low density lipoprotein were measured using Friedwald's method [19].

### 2.3.3.2. Total antioxidant capacity
The total antioxidant capacity of the plasma was measured based on the ferric reducing ability of plasma according to the method of Benzie and Strain [20].Total antioxidant capacity was expressed as µmol Fe(II)/L of plasma.

### 2.3.3.3. Atherogenic index of plasma
Plasma atherogenic index was calculated as (total cholesterol - HDL cholesterol)/HDL cholesterol as per the method of Yang et al. [21].

## 2.4 Tissue

After the cold exposure procedures, rats were subjected to fasting overnight and were then asphyxiated. The hearts of the rats were quickly harvested and placed in ice-cold saline to remove blood clots. The left and right ventricles were isolated, weighed, and homogenised in 50 mM of a potassium phosphate buffer (pH 7.4).

### 2.4.1. Lipid peroxidation
Lipid peroxidation was measured in terms of thiobarbituric acid-reactive substances (TBARS) using the method of Ohkawa et al. [22]. TBARS was expressed as nmol malondialdehyde/mg protein.

### 2.4.2. Protein assay
The protein levels of the ventricles were measured using the method of Lowry et al.[23] which involves the use of a bovine serum albumin as the standard.

## 2.5 Statistical Analyses

All data are presented as means ± SEM. The data was subjected Levene's test of homogeneity of variances (IBM SPSS® version 20.0 Inc., Chicago, Illinois, USA) at the beginning of the analysis so that the proper type of analysis of variance (ANOVA) for this study could be determined. Data was subsequently subjected to statistical analysis using the two-way ANOVA (GraphPad PRISM® version 6.01 Software Inc., San Diego, California, USA). Differences among the group means were evaluated by Tukey's multiple comparison post-hoc tests. The statistical significance threshold was set at $P<0.05$.

## 3. Results

### 3.1.1. Body mass
A two-way ANOVA revealed the effects of age [$F_{(2,70)}= 105.6$, $p < 0.0001$], intervention [$F_{(5,70)}=26.82$, $p < 0.0001$], and the age × intervention interaction [$F_{(10,70)}= 2.306$, $p = 0.0209$] on body mass gain.

Changes in body mass as a function of cold stress and GSPE supplementation are represented in Table 1. Body weight gain in ageing rats exposed to CCS and ACS exposure are shown in Table 2. Body weight gain varied significantly with age, decreasing from an average of $47.2\pm21$ g in the 4-month-old rats to $35.6\pm1.2$ g in the 12-month-old rats and $24.0\pm0.9$ g in the 24-month-old rats in the CCS group (Table 2).

### 3.1.2. Feeding efficiency
The two-way ANOVA revealed the effects of age [$F_{(2,70)}=17.66,p<0.0001$],intervention $F_{(5,70)}=6.184$, $p<0001$], and the age × intervention interaction [$F_{(10,70)}=0.4666$, $p = 0.9060$] in feeding efficiency.

Compared to the controls, rats exposed to CCS showed increased feeding efficiency, with the least efficiency exhibited in the old rats. Supplementation did not result in changes in feeding efficiency after cold exposure (Table 2).

## 3.3. Biochemical assays

### 3.3.1. Blood glucose and lactate

### 3.3.1.1. Glucose
A two-way ANOVA revealed the effects of age [$F_{(2,70)}=65.73$, $p<0.0001$), intervention [$F_{(5,70)}=117.2$,

$p<0.0001$), and the age × intervention interaction [$F_{(10,70)}$=1.156, $p<0.3349$) in blood glucose levels.

Glucose concentrations were significantly (p<0.0001) higher in the CCS adults (9%), late-adults (8%), and old rats (7.1%) than in their respective control groups. The increases were greater in the ACS group than in the CCS group for adults (13.6%), late-adults (14%), and old rats (10.4%) when compared to their respective controls. The decreases observed in GSPE supplemented CCS rats when compared to their unsupplemented counterparts were 4.6% (p<0.05) in the adult rats, 5.8% (p<0.001) in the late-adult rats, and 5.3% (p<0.01) in the old rats (Table 3).

### 3.3.1.2. Blood lactate
A two-way ANOVA revealed the effects of age [$F_{(2,70)}$= 172.9, $p<0.0001$, intervention [$F_{(10,70)}$ = 2.644, $p<0.0001$], and the age × intervention interaction [$F_{(10,70)}$ = 2.644, $p=0.0086$) in BLa levels.

Among rats in the CCS group, BLa significantly increased in the adults (14%) (p<0.05), late-adults (17%, p<0.05), and old rats (37%, p<0.0001). In the ACS group, the increases were also significant in the adults (22.6%, p<0.01), late-adults (30.5%, p<0.001), and old rats (45.4%, p<0.001) when compared to controls. In the GSPE supplemented CCS rats, BLa levels decreased by 18.3% (p<0.01) in adult rats, 12.1% (p<0.05) in late-adult rats, and 15.6% (p<0.001) in old rats when compared to their unsupplemented counterparts (Table 3).

### 3.3.2. Serum corticosterone
A two-way ANOVA revealed the effects of age [$F_{(2,36)}$ =147.0, $p<0.0001$], intervention [$F_{(5,36)}$ = 2134, $p<0.0001$], and the age × intervention interaction [$F_{(10,36)}$=12.06, $p<0.0001$] on serum corticosterone levels.

Corticosterone levels in the CCS group were significantly higher (p<0.0001) in the adults (311%), late-adults (187%), and old animals (168%) than in the control groups. In the ACS groups, the levels also significantly increased (p<0.0001), by 542% (adults), 294% (late-adults), and 333% (old rats) when compared with their respective controls (Fig.1). GSPE reduced the corticosterone levels in the CCS group to a similar extent in the and late-adults (20%, p<0.001), while the reduction was smaller in the old rats (10%, p<0.01) (**Fig.1**).

### 3.3.3. Plasma lipid profile
### 3.3.3.1. Total cholesterol
A two-way ANOVA revealed the effects of age [$F_{(2,76)}$=3325,p<0.0001],intervention $F_{(5,76)}$=132.3, p < 0.0001], and the age × intervention interaction [$F_{(10,76)}$=27.78, p < 0.0001] on plasma TC levels.

The TC of the CCS group significantly increased (p<0.0001) in the adults (17.3%), late-adults (29.4%), and old animals (10%). In the ACS group, the increase was significant among the late-adults (38.8%, p<0.0001) and old rats (4.5%, p<0.05) when compared to their respective controls (Fig. 2A). GSPE reduced the plasma total cholesterol in the CCS groups of adult (9.5%, p<0.01) and late-adult rats (8.7%, p<0.0001) and, to a lesser extent, in the old rats (6%,

p<0.001) when compared with their unsupplemented counterparts.

### 3.3.3.2. Low density lipoprotein
A two-way ANOVA revealed the effects of age [$F_{(2,76)}$= 2574, p < 0.0001], intervention [$F_{(5,76)}$= 163.7, p<0.0001], and the age × intervention interaction [$F_{(10,76)}$ = 46.05, p<0.0001] on the LDL.

LDL in the CCS groups was significantly higher (p<0.0001) in the adults (11%), late-adults (57%), and old rats (18 %) rats than in the control group. Meanwhile, ACS increased LDL by 9.3% in adults, 65.8% (p<0.0001) in late-adults, and 9.6% (p<0.01) in old rats. GSPE supplementation significantly (p<0.0001) reduced the plasma LDL of the adults (46.5%) and late-adult rats (16.7%) in the CCS groups. Old rats exposed to CCS also showed reductions in LDL, albeit to a lesser extent than in the other two age groups (8.7%, p<0.001). Among the ACS groups, LDL significantly (p<0.0001) decreased by 64.5% in the adults, 14% (p<0.0001) in the late-adults, and 2.7% in the old rats (Fig. 2B).

### 3.3.3.3. Very low density lipoprotein cholesterol
A two-way ANOVA revealed the effects of age [$F_{(2,76)}$= 3374, $p<0.0001$], intervention [$F_{(5,76)}$= 183.5, $p < 0.0001$], and the age × intervention interaction [$F_{(10,76)}$= 25.93, $p < 0.0001$] on VLDL.

The VLDL of CCS groups increased significantly (p<0.0001) in adult rats by 39%, in late-adult rats by 5.7%, and in old rats by 19.4%. Meanwhile, in the ACS group, VLDL increased by 13.3% (p<0.01) in the adults, 18% in late-adults, and 18% in old rats (p<0.0001 compared to their respective controls. GSPE was effective in reducing the plasma VLDL in the CCS group for adults (13%, p<0.0001), late-adults (6%, p<0.05), and old rats (20%, p<0.0001). In the ACS group, subtle decreases were seen in adults (5.2%), late-adults (5.6%, p<0.05), and old animals (7.7%, p<0.0001) when compared to their unsupplemented counterparts (Fig. 2C).

### 3.3.3.4. Triglycerides
A two-way ANOVA revealed age [$F_{(2,76)}$= 3370, $p < 0.0001$], intervention [$F_{(5,76)}$= 181.0, $p < 0.0001$], and the age × intervention interaction [$F_{(10,76)}$= 25.93, $p < 0.0001$] on TG levels.

The TG levels of CCS groups increased by 39% in the adults (p<0.0001), 5.7% in the late-adults, and 18.6% in the old rats (p<0.0001). Meanwhile, the ACS group experienced increases of 13.4% in adults (p<0.01), 19% in late-adults (p<0.0001), and old rats (p<0.0001). GSPE reduced the plasma TG in adults (13%, p<0.0001), late-adults (6%, p<0.05), and old rats (19.6%, p<0.0001) exposed to CCS. Triglyceride also decreased in the ACS group, though to a lesser extent than in the CCS group; in the ACS group, TG decreased by 5.7% in adults, 5% in late-adults (p<0.05), and 8% in old animals (p<0.0001) (Fig. 2D).

### 3.3.3.5. High density lipoprotein cholesterol
The two-way ANOVA revealed the effects of age [$F_{(2,76)}$= 610.5, p < 0.0001], intervention [$F_{(5,76)}$= 204.6, p < 0.0001],

and the age × intervention interaction [$F_{(10,76)}$= 16.99, p < 0.0001] on HDL.

The HDL of CCS groups significantly decreased (p<0.0001) in the adults (18.3%), late-adults (43.3%), and old animals (19.4%). In the ACS groups, smaller decreases (p<0.0001) were seen in the adults (14%), late-adults (40%), and old animals (19.3%). Supplementation with GSPE increased the plasma HDL in the CCS group for adults (11%, p<0.01), late-adults (23.5%, p<0.001), and old animals (19.5%, p<0.0001) compared to their unsupplemented counterparts (Fig. 2E).

### 3.3.4. Total antioxidant capacity
A two-way ANOVA revealed the effects of age [$F_{(2,70)}$= 3096, *p<0.0001*], intervention [$F_{(5,70)}$= 543.3, *p<0.0001*], and the age × intervention interaction [$F_{(10,70)}$=21.01, *p<0.0001*] on plasma TAC levels. The TAC levels of the CCS group were significantly increased (p<0.0001) in adults (9.1%), late-adults (6.4%), and old animals (5.6%). Moreover, the TAC levels of the ACS group increased for adults (9.3%), late-adults (4.6%), and old rats (5.5%). GSPE supplementation improved TAC levels significantly (p<0.0001) in adult (4.78%), late-adult (3.4%), and old CCS groups (2.6%, p<0.001) when compared to their respective controls (Fig. 2F).

### 3.3.5. Plasma atherogenic index
A two-way ANOVA revealed the effects of age [$F_{(2,70)}$= 1667, *p<0.0001*], intervention [$F_{(5,70)}$= 268.6, *p<0.0001*], and the age × intervention interaction [$F_{(10,70)}$= 52.16, *p<0.0001*] on AIP.

The AIP was significantly (p<0.0001) higher in the CCS group for adults (110.3%), late-adults (198.4%), and old rats (47.8%). In the ACS groups, increases were also seen in adults (54.4%), late-adults (66.4%), and old rats (41.8%) compared to their respective controls. Conversely, the AIP of the GSPE supplemented CCS group decreased for the adults (31.5%, p<0.05), late-adults (30.6%, p<0.0001), and old rats (25.5%, p<0.0001). The ACS group also experienced significant (p<0.0001) decreases in the AIP for adults (3.8%), late-adults (20%), and old rats (14.8%, p<0.01) compared to their respective controls (Table 4).

### 3.4. Tissue lipid peroxidation

### 3.4.1. Left Ventricle
A two-way ANOVA revealed the effects of age [$F_{(2,70)}$= 1160, *p<0.0001*], intervention [$F_{(5,70)}$= 436.4, *p<0.0001*], and the age × intervention interaction [$F_{(10,70)}$= 3.331, *p=0.0014*] on MDA levels in the LV.

The MDA levels of the CCS group were significantly increased (p<0.0001) for adults (44.2%), late-adults (27.5%), and old animals (23%). These increases were found to be higher in the ACS group than in the CCS group for adults (63.7%), late-adults (36.5%), and old animals (31.2%) when compared to their respective controls. GSPE supplementation reduced malondialdehyde levels in the CCS groups significantly (p<0.0001) for adults (15.1%), late-adults (10.3%), and old rats (9%). In the ACS group, MDA

reduction was almost 50% that of the CCS groups for adults (3.6%) late-adults (6.7% p<0.01), and old rats (5.7%, p<0.01) (Fig. 3A).

### 3.4.2. Right Ventricle
A two-way ANOVA revealed the effects of age [$F_{(2,70)}$=1726, *p<0.0001*], intervention [$F_{(5,70)}$= 496.7, *p<0.0001*], and the age × intervention interaction [$F_{(10,70)}$=3.267, *p=0.0016*] on malondialdehyde levels in the RV.

The MDA levels of the CCS group were significantly increased (p<0.0001) for adults (49%), late-adults (39.6%), and old animals (26.4%) when compared to their respective controls. Similar to the LV, results for the RV showed that MDA levels increased significantly (p<0.0001) and to a greater extent in the ACS group than in the CCS group for adults (80.6%), late-adults (47.7%), and old animals (33.6%). GSPE reduced MDA levels in the CCS group significantly (p<0.0001) for adults (12.7%), late-adults (10%), and old animals (9%). In the ACS group, MDA reduction was seen in adults (9.7%, p<0.001), late-adults (7%, p<0.01), and old rats (6 %, p<0.01) (Fig. 3B).

## 4. Discussion

We chose intermittent cold exposure (ICE) over continuous cold exposure as a model of cold stress because ICE is a well-known metabolic stressor and, under natural working environments, is encountered more often than continuous exposure. In this study, we investigated the effects of ICE of the CCS and ACS on the plasma lipid profile and blood substrates, lactate, and glucose, and we determined the extent of oxidative stress in the left and right ventricles of ageing rats.

The increase in body weight in the CCS group is in accordance with the findings reported by Sahin and Gumuslu [24] and Retena-Marques et al.[25]. However, the increase was higher in the adults than in the late-adults and old animals. Our data on increased feeding efficiency in the CCS animals in the adult and late-adult animals (and, to a lesser extent, in the old rats) is not surprising, as enhanced food intake is linked with the energy expenditure necessary for heat production. Following 14 days of cold exposure at 10°C, the efficiency increased irrespective of GSPE supplementation.

This result is comparable to those of our earlier studies on combined vitamin E and C supplementation in which old rats showed similar behaviour during ICE at 5°C [16]. Our findings on alleviated levels of corticosterone in GSPE supplemented rats exposed to CCS explain that oxidative stress is enhanced through the elevated generation of free radicals that signal sympathetic nerves, alter sympatho-adrenomedullary activity in cold stressed rats [26], and impact metabolic pathways involved in the secretion of glucocorticoids in unsupplemented cold stressed rats. However, studies have shown that the timing of both the adrenocorticotropic hormone and corticosterone responses are dependent on stressor modality and intensity [27]. Further, our results on corticosterone are comparable to the findings of studies on chronic stress induced in rats through

the exogenous supplementation of adrenocorticotropic hormone with resveratrol from grape seeds [28]. The possible decline in corticosterone, therefore, suggests that antioxidant activity is affected by GSPE during cold stress. Old ACS animals exhibited greater declines in corticosterone levels than their CCS counterparts in response to GSPE supplementation.

The findings on increased BG concentrations as a response to cold exposure correlate strongly with previous findings reported by Adan et al [29] in rats exposed to temperatures of 4°C. This phenomenon may be caused by the reduced ability of the animals to oxidise carbohydrates (especially for old rats) and the heightened glucagon levels in the plasma, which results in elevated hepatic gluconeogenesis [30]. Interestingly, the elevated BLa that accompanied cold exposure was consistent with the increased BG, a mechanism that could result in reduced glucose flux and its oxidation via the TCA cycle [31]. Remarkably, old rats, when compared to adults, experienced higher BLa levels, which is suggestive of a greater diversion of glucose towards non-oxidative pathways in response to cold stress.

The present result on the increased availability of glucose and lactate (apart from increased calculated oxygen consumption rate under cold irrespective of age) may explain a possible alleviation of thermogenesis through non-oxidative pathways and other physiological factors, such as norepinephrine, that reduce blood flow to the muscles in the old rats owing to its vasoconstrictive property [32].

In addition, the major GSPE intervention effects in cold stressed rats – including increases in the plasma TAC, the alleviation of TC, LDL and VLDL, and increased HDL – can be related to the concentrations of monomeric catechin, epicatechin, and gallic acid in the plasma [15]. Razavi et al.[33] have reported an improvement in the lipid profile of mildly hyperlipidemic humans supplemented with red grape seed extract for eight weeks.

Because cold stress-related increases in serum corticosterone levels were accompanied by increases in LDL and VLDL most strongly in the old rats, we analysed the AIP. Our results on the marked improvement in the AIP under cold stress may be due to the remarkable antioxidant effects of proanthocyanidin, which accumulates in the blood vessel walls and scavenges the reactive oxygen species in the interstitial fluid of vessel walls, thus preventing the onset of plaque formation in humans [34] and cholesterol-loaded rabbits [35]. Grape seed extract has also been shown to prevent LDL oxidation in hyperlipidemic patients [36].

Lipid peroxidation, which results from free radicals interacting with lipids, has end products measured as thiobarbituric acid-reactive substances. Our findings on increased thiobarbituric acid-reactive substances (MDA) in the hearts of cold stressed rats are in accordance with similar findings related to the liver, brain [37], and heart [38], all of which could be caused by a shift in the prooxidant-antioxidant balance of the organism [39]. However, GSPE attenuated oxidative stress occurred in both ventricles under cold stress, thus protecting the heart against cardiac tissue injury.

# 5. Conclusions

Overall, the present study demonstrates that intermittent CCS and ACS are accompanied by increased serum corticosterone levels and the induction of hyperlipidemia in adult, late-adult, and old animals. However, the changes were greater in old rats than in adults. Moreover, interestingly, ACS evoked a stronger response than CCS. In addition, although BG and BLa increased in all age groups exposed to cold stress, the old rats were more sensitive to cold stress than the other age groups. This implies that carbohydrates were partially oxidized for thermogenesis. Further, cold stress exacerbated lipid peroxidation in the LV and RV of the heart more in the old rats than in the other age groups. This exacerbation of lipid peroxidation led to oxidative stress and increased AIP. However, GSPE, as a natural antioxidant, partially attenuated oxidative stress in the heart, increased serum corticosterone levels, and possibly reduced the oxidation of LDL under cold stress.

# 6. Acknowledgements

# 7. Disclosure

The authors have no conflict of interest related to the manuscript.

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## Author Profile

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**Table 1:** Changes in body mass as a function of function of age and GSPE supplementation in cold stressed rats

| Age (months) | Days | Groups | | | | | |
|---|---|---|---|---|---|---|---|
| | | CON | CON+PA | CCS | CCS+PA | ACS | ACS+PA |
| **4[!]** | D1 | 229.4 ± 3.3 | 236.0 ± 2.7 | 242.4 ± 2.7 | 233.8 ± 2.9 | 237.4 ± 1.4 | 231.4 ± 2.5 |
| | D7 | 246.2 ± 3.5 | 248.8 ± 2.5 | 262.8 ± 2.7 | 250.4 ± 2.3 | NA | NA |
| | D14 | 261.6 ± 3.9 | 259.8 ± 2.2 | 289.6 ± 4.9 | 277.6 ± 1.7 | 270.8 ± 1.9 | 262.6 ± 3.0 |
| **12[!]** | D1 | 382.2 ± 12.0 | 382.8 ± 9.0 | 405.8 ± 5.2 | 391.2 ± 6.0 | 386.4 ± 14.3 | 384.6 ± 12.6 |
| | D7 | 384.25 ± 9.0 | 392.8 ± 9.3 | 420.8 ± 4.6 | 403.6 ± 5.8 | NA | NA |
| | D14 | 408.2 ± 11.7 | 403.2 ± 9.3 | 441.4 ± 4.4 | 421.8 ± 5.5 | 411.8 ± 14.4 | 409.6 ± 12.9 |
| **24[!]** | D1 | 418.8 ± 6.8 | 419.0 ± 7.2 | 433.0 ± 8.7 | 430.6 ± 12.9 | 419.0 ± 3.7 | 417.6 ± 9.5 |
| | D7 | 427.8 ± 6.7 | 425.6 ± 7.5 | 443.6 ± 8.7 | 439.6 ± 13.0 | NA | NA |
| | D14 | 437.60 ± 6.6 | 433.2 ± 7.8 | 457.0 ± 9.0 | 451.8 ± 12.9 | 438.4 ± 4.8 | 436.0 ± 9.2 |

Values are mean ± SEM (n=4-5). Statistical significance was analyzed by Two-way ANOVA with Tukey's multiple comparison tests.CON, control; CON+PA, control supplemented with GSPE; CCS, chronically cold stressed; CCS+PA, chronically cold stressed and supplemented with; ACS, acutely cold stressed; ACS+PA, acutely cold stressed and supplemented with GSPE. Values are statistically significant at !p< 0.05 as compared to control.

**Table 2:** Body weight gain, food intake and feeding efficiency as a function of age and GSPE supplementation in cold stressed rats

| Parameters | Age (months) | | |
| --- | --- | --- | --- |
| | Groups | 4* | 12* | 24* |
| Weight gain (g) | CON | $32.2 \pm 3.1$ | $26.0 \pm 1.3$ | $18.8 \pm 1.3$ |
| | CON+PA | $23.8 \pm 1.0^!$ | $20.4 \pm 0.7$ | $14.2 \pm 1.0$ |
| | CCS | $47.2 \pm 3.7^*$ | $35.6 \pm 1.2^!$ | $24.0 \pm 0.9^!$ |
| | CCS+PA | $43.8 \pm 3.2^{\$}$ | $30.6 \pm 1.1^!$ | $21.2 \pm 1.6$ |
| | ACS | $33.4 \pm 2.7$ | $25.4 \pm 0.4$ | $19.4 \pm 2.0$ |
| | ACS+PA | $31.2 \pm 1.0$ | $25.0 \pm 0.7$ | $18.4 \pm 1.1$ |
| Food intake (g) | CON | $37.2 \pm 1.1$ | $33.0 \pm 1.1$ | $28.0 \pm 1.0$ |
| | CON+PA | $35.6 \pm 1.4$ | $26.8 \pm 0.9^!$ | $23.8 \pm 0.7$ |
| | CCS | $47.0 \pm 1.4^*$ | $38.8 \pm 2.8^!$ | $31.2 \pm 1.3^!$ |
| | CCS+PA | $46.8 \pm 1.7^*$ | $34.6 \pm 1.3^!$ | $29.0 \pm 1.2^!$ |
| | ACS | $38.0 \pm 0.6$ | $32.4 \pm 1.0$ | $28.8 \pm 0.7$ |
| | ACS+PA | $36.6 \pm 0.8$ | $32.2 \pm 0.7$ | $28.2 \pm 1.0$ |
| Feeding efficiency (%) | CON | $86.4 \pm 7.3$ | $78.8 \pm 3.2$ | $67.2 \pm 4.3$ |
| | CON+PA | $67.0 \pm 2.3$ | $76.9 \pm 5.1$ | $59.7 \pm 3.7$ |
| | CCS | $99.9 \pm 6.1^!$ | $93.9 \pm 6.9^!$ | $78.7 \pm 6.0^!$ |
| | CCS+PA | $94.1 \pm 7.8^!$ | $88.6 \pm 2.3^!$ | $72.9 \pm 4.5^!$ |
| | ACS | $87.9 \pm 7.2$ | $79.0 \pm 3.8$ | $67.9 \pm 7.8$ |
| | ACS+PA | $85.5 \pm 1.0$ | $78.0 \pm 3.5$ | $66.3 \pm 6.4$ |

Values are mean ± SEM (n=4-5). CON, control; CON+PA, control supplemented with GSPE; CCS, chronically cold stressed; CCS+PA, chronically cold stressed supplemented with GSPE; ACS, acutely cold stressed; ACS+PA, acutely cold stressed supplemented with GSPE. Statistical significance was analyzed by two-way ANOVA with Tukey's multiple comparison tests and are significant at * $p < 0.0001$; \$ $p < 0.001$; # $p < 0.01$; ! $p < 0.05$ compared to control.

**Table 3:** Blood glucose and lactate as a function of age and GSPE supplementation in cold stressed rat

| Blood Glucose (mg/dL) | Age (months) | | |
| --- | --- | --- | --- |
| Groups | 4$^{\$*}$ | 12$^{\$}$ | 24* |
| CON | $110.08 \pm 0.68$ | $113.54 \pm 0.94$ | $119.17 \pm 1.10$ |
| CON+PA | $105.00 \pm 0.84^!$ | $110.42 \pm 1.06$ | $115.83 \pm 0.99$ |
| CCS | $120.17 \pm 1.33^*$ | $122.50 \pm 1.59^*$ | $126.92 \pm 0.54^*$ |
| CCS+PA | $115.17 \pm 0.77^!$ | $115.42 \pm 1.09$ | $120.25 \pm 1.20$ |
| ACS | $125.83 \pm 1.23^*$ | $128.85 \pm 1.66^*$ | $131.33 \pm 0.77^*$ |
| ACS+PA | $122.08 \pm 1.01^*$ | $125.83 \pm 1.10^*$ | $129.92 \pm 0.15^*$ |
| Blood Lactate mg/dL) | | | |
| Groups | 4* | 12* | 24* |
| CON | $18.63 \pm 1.19$ | $21.4 \pm 1.08$ | $22.14 \pm 0.58$ |
| CON+PA | $13.77 \pm 0.39^*$ | $19.53 \pm 0.72$ | $21.85 \pm 0.51$ |
| CCS | $21.25 \pm 0.85$ | $25.04 \pm 0.71^!$ | $30.29 \pm 0.80^*$ |
| CCS+PA | $17.37 \pm 0.53$ | $22.00 \pm 0.48$ | $25.55 \pm 0.33^!$ |
| ACS | $22.84 \pm 1.00^{\#}$ | $27.93 \pm 0.68^*$ | $32.19 \pm 0.84^*$ |
| ACS+PA | $21.56 \pm 0.50^!$ | $25.51 \pm 0.61^*$ | $29.44 \pm 0.49^*$ |

Values are mean ± SEM (n=4-5). All abbreviations are similar to table 1. Values are mean ± SEM (n=5). Statistical significance was analyzed by two-way ANOVA with Tukey's multiple comparison tests and significant at * $p < 0.0001$; \$ $p < 0.001$; # $p < 0.01$; ! $p < 0.05$ compared to control.

**Table 4:** Plasma atherogenic index as a function of age and GSPE supplementation in the cold stressed rat

| | Age (months) | | |
| --- | --- | --- | --- |
| Groups | 4* | 12* | 24* |
| CON | $0.68 \pm 0.05$ | $1.86 \pm 0.07$ | $3.47 \pm 0.08$ |
| CON+PA | $0.23 \pm 0.02^!$ | $1.28 \pm 0.03^{\#}$ | $3.05 \pm 0.09$ |
| CCS | $1.43 \pm 0.08^*$ | $5.55 \pm 0.24^*$ | $5.13 \pm 0.14^*$ |
| CCS+PA | $0.98 \pm 0.06$ | $3.85 \pm 0.07^*$ | $3.82 \pm 0.10$ |
| ACS | $1.05 \pm 0.05$ | $5.53 \pm 0.15^*$ | $4.92 \pm 0.17^*$ |
| ACS+PA | $1.01 \pm 0.05$ | $4.42 \pm 0.09^*$ | $4.19 \pm 0.10^*$ |

Values are mean ± SEM (n=4-5). All abbreviations are similar to table 1. Statistical significance was analyzed by Two-way ANOVA with Tukey's multiple comparison tests and values are significant at * $p < 0.0001$; # $p < 0.01$; ! $p < 0.05$ compared to control.
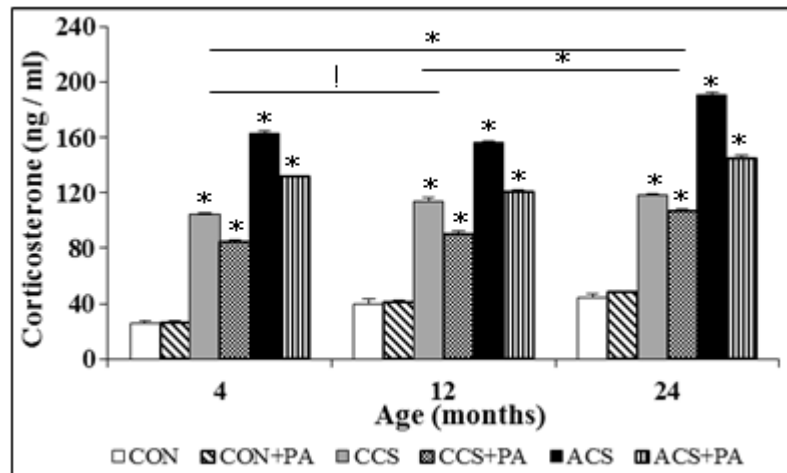
**Figure 1:** Corticosterone levels in plasma following cold stress. CON, control; CON+PA, control supplemented with GSPE; CCS; chronic cold exposure group; ACS, acute cold exposure group; CCS+PA and ACS+PA, GSPE supplemented chronic and acute cold exposure groups. GSPE, grape seed proanthocyanidin extract. Results are mean ± SE of 5 animals/group Statistical significance was analyzed by two-way ANOVA with Tukey's multiple comparison test. $^{*}p<0.0001$; $^{!}p<0.05$ compared to control.
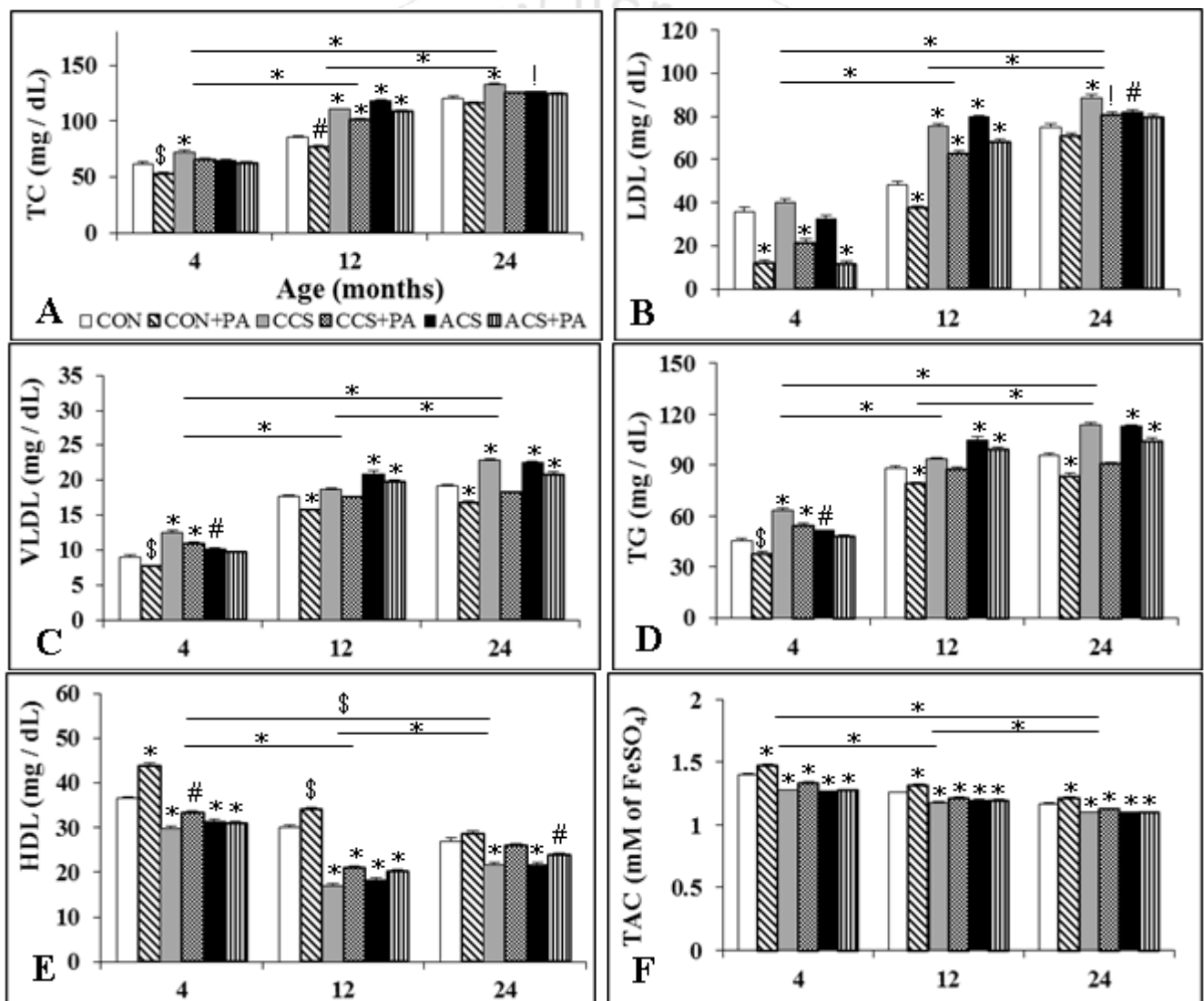


**Figure 2.** Plasma lipids following cold stress and GSPE supplementation. Total cholesterol, TC (**A**); low density lipoprotein, LDL (**B**); very low density lipoprotein, VLDL (**C**); triglycerides, TG (**D**); high density lipoproteins, HDL (**E**) and total antioxidant capacity, TAC (**F**) after cold stress and as a function of age. All other abbreviations are similar to Fig.1. Results are mean ± SE of 4-5 animals/group. Statistical significance was analyzed by two-way ANOVA with Tukey's multiple comparison test. $^{*}p<0.0001$; $^{\$}p<0.001$; $^{\#}p<0.001$; $^{!}p<0.05$ compared to control
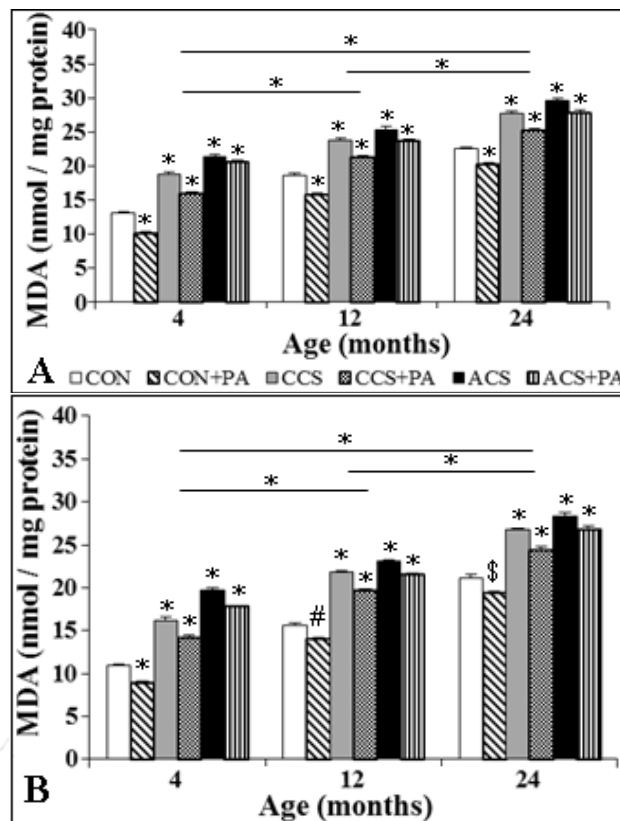
**Figure 3**: Malondialdehyde (MDA) levels in the left ventricle (**A**) and right ventricle (**B**) following cold stress and GSPE supplementation. All other abbreviations are similar to Fig.1. Results are mean ± SE of 4-5 animals/group. Statistical significance was analyzed by two-way ANOVA with Tukey's multiple comparison test. [*]$p<0.0001$; [$]$p<0.001$; [#]$p<0.01$ compared to control