



Original article

Efficacy of choline and DHA supplements or enriched environment exposure during early adult obesity in mitigating its adverse impact through aging in rats



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ABSTRACT

Introduction: The aim of this study was to assess the efficacy of choline and DHA or exposure to environmental enrichment in obese adult and aging rats on alterations in body mass index, serum lipid profile and arterial wall changes, despite stopping high fat diet consumption and interventions during adulthood.

Methods: 21 day old male Sprague Dawley rats were assigned as Experiment-1 & 2 - PND rats were divided into 4 groups with interventions for 7 months (n = 8/group). NC- Normal control fed normal chow diet; OB- Obese group, fed high fat diet; OB + CHO + DHA- fed high fat diet and oral supplementation of choline, DHA. OB + EE- fed high fat diet along with exposure to enriched environment. Experiment-2 had similar groups and interventions as experiment 1 but for next 5 months were fed normal chow diet without any interventions. Body mass index was assessed and blood was analyzed for serum lipid profile. Common Carotid Artery (CCA) was processed for Haematoxylin and eosin, Verhoeff Vangeison stains. Images of tissue sections were analyzed and quantified using image J and tissue quant software.

Results: In experiment.1, mean body mass index (p < 0.001), serum lipid profile (p < 0.01), thickness of tunica intima (p < 0.05), tunica media (p < 0.01) and percentage of collagen fibers (p < 0.01) of CCA were significantly increased in OB compared to NC. These were significantly attenuated in OB + CHO + DHA and OB + EE compared to OB. In experiment.2, mean body mass index (p < 0.01), serum lipid profile (p < 0.05) and thickness of tunica media of CCA (p < 0.01) were significantly increased in OB compared to NC. In OB + CHO + DHA and OB + EE, significant attenuation was observed in mean body mass index and mean thickness of tunica media compared to same in OB.

Conclusion: Adult obesity has negative impact on body mass index, serum lipid profile and arterial wall structure that persists through aging. Supplementation of choline and DHA or exposure to enriched environment during obesity attenuates these negative impacts through aging.

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Abbreviations: CCA, Common carotid artery; n, number; DHA, Docosahexaenoic acid; PND, Post natal day; HDL, High density lipoprotein; LDL, Low density lipoprotein.

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1. Introduction

In present scenario, prevalence of obesity is increasing at an alarming rate (KD et al., 2018). According to World Health Organization (WHO), incidence of childhood obesity has increased to five fold in 2016 (Sheet, World Health Organization .Fact sheets, 2018). Obesity during childhood can have a negative impact on health in adulthood (Llewellyn et al., 2016). One of the main parameters that gets affected in obesity is body mass index. Increase in body mass index leads to cardiovascular risks in adulthood. This increase in body mass index is related to exposure to high fat diet from childhood (Katzmarzyk et al., 2019). Altered body mass index due to high fat diet is also associated with increase in levels of serum cholesterol, triglyceride, low density lipoprotein and decreased levels of high density lipoproteins (DiNicolantonio and O'Keefe, 2018). Increase in lipid levels leads to increased cardiovascular risks. Low density lipoprotein cholesterol (LDL-C) is the predominant cholesterol-carrying lipoprotein, and is considered to be the main atherogenic lipoprotein. Along with this, triglycerides and cholesterol also play a direct role in causing atherogenic changes (Orozco-Beltran et al., 2017). Fatty streaks deposition is characterized by accumulation of non-foamy and foamy macrophages in arterial wall in later stages. Further, this leads to disturbances in amino acid and lipid metabolism in hypercholesterolemia (Misra et al., 2019). An altered lipid profile can cause structural changes in arterial wall leading to initial stages of atherosclerosis. Atherosclerosis is a type of arteriosclerosis which mainly affects the medium sized artery. In atherosclerosis, the tunica media of the arterial wall shows alterations due to plaque formation reducing the arterial lumen. Common carotid artery is one of the medium sized arteries frequently involved in changes in wall thickness due to atherosclerosis further increasing the risks for development of strokes (Gardener et al., 2014)

Various nutrients are available to reduce the arterial wall injury. Choline and docosahexaenoic acid (DHA) are essential nutrients which help in growth and development of cell membranes. Choline maintains cell membrane integrity, lipid metabolism and methylation. It also decreases circulating levels of free fatty acids and triglycerides. Choline also improves lipid metabolism by decreasing nicotinamide adenine dinucleotide phosphate (NADPH) generation and improving fatty acid oxidation (Li et al., 2018). Choline metabolism involves phosphatidylethanolamine N-methyltransferase (Pemt), which converts phosphatidylethanolamine to phosphatidylcholine. This is required for lipoprotein synthesis, and methyl-group metabolism (Al Rajabi et al., 2013). DHA is an omega -3 fatty acid, found in cell membranes. It helps in reducing total body fat and hepatic deposition of lipids (Shang et al., 2017). DHA also modulates inflammatory pathways and it is observed to have anti-atherogenic properties (Gladine et al., 2014). Synergistic supplementation of choline and DHA is observed to maintain growth and development (Bernhard et al., 2020).

Alternately, studies have also shown beneficial effects of social environmental enrichment in modulating physiological and disease risk and progression. Environment enrichment also maintains body weight by reducing adipocyte deposition. Long term exposure to environmental enrichment enhances and maintains the serum leptin levels thus improving lipid metabolism (McMurphy et al., 2018). Environmental enrichment reduces the incidence of stroke by its beneficial neurovascular effects preventing carotid artery stenosis. Continuous exposure to enriched environment appears as a safe and effective interventional strategy against cerebrovascular diseases (Hase et al., 2019).

But there are no studies elucidating the efficacy of choline, DHA supplements or environmental enrichment exposure on high fat diet-induced obesity during adulthood and old age, in attenuating

alterations in body mass index, serum lipid profile and carotid arterial wall structure.

Therefore, the present study aims to assess the long-term effects of high fat diet-induced obesity during adulthood and old age, with or without choline and DHA supplements or exposure to enriched environment on body mass index, serum lipid profile and carotid arterial wall structure.

2. Materials and Methods

2.1. Animals

Male Sprague Dawley rats were bred locally in the Central Animal House of Manipal Academy of Higher Education (MAHE), Manipal, India. All animals were maintained in 12:12 h day: night cycle. Each cage size is 40 cm × 24 cm with three rats per cage. Rats were maintained with standard animal feed provided *ad libitum* except for the groups that had specified interventions for periods as explained in experimental design.

2.2. Ethical approval

The experiments were conducted according to the ethical norms approved by Institutional animal ethics committee (IAEC), Manipal Academy of Higher Education (MAHE), Manipal with clearance obtained for the study (IAEC/KMC/09/2015). This is governed under the Ministry of Social Justices and Empowerment, Government of India and Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA)

2.3. Experimental design

Postnatal rat pups (n = 64) weighing 45 ± 2 gm were divided into 4 experimental groups (n = 8/group) per experimental study. Normal control -NC, Obese-OB, Obese rats supplemented with dietary choline and DHA -OB + CHO + DHA and Obese group exposed to environmental enrichment -OB + EE. A total of 32 animals in each experiment were used in this study. Postnatal obesity was induced by supplementing the animals with high fat diet (Fig. 1)

2.4. Animal dietary protocol

NC group - Standard animal feed (VRK Nutritional Solutions, VRK's "Scientist's Choice" Laboratory Animal Diets, Pune, India) used was normal pellet chow containing 21.8% of protein, 4.8% of fat, 3.1% of fiber, 1% of calcium, 65% of carbohydrates and 5.1% of phosphorus (Thomas Rajarethnem et al., 2017).

OB group - The composition of high fat diet was designed, and standardized in-house containing lard- 60%, Normal pellet (powdered) - 10%, Casein -3%, Sucrose - 1%, Cholesterol - 20%, Vitamin - 5%, dl methionine - 1% (Durga laboratory and chemicals, Mangalore, India) (Prabhu, G. S., K. G. Rao, M. and Rai, K. S. 2020).

OB + CHO + DHA group -High fat diet along with oral supplementation of choline and DHA was provided. Choline chloride 98% extra pure (Loba Chemicals Laboratory Reagents- India) was dissolved with distilled water 5 mmol/kg/day and DHA - was 150 mg/kg/ day (Nouveau Medicament Private Ltd. India) (Thomas et al., 2018).

OB + EE group - High fat diet along with environmental enrichment (EE) was provided for one hour per day for 90 days. Enrichment was provided in a cage of larger dimension than the regular cage.

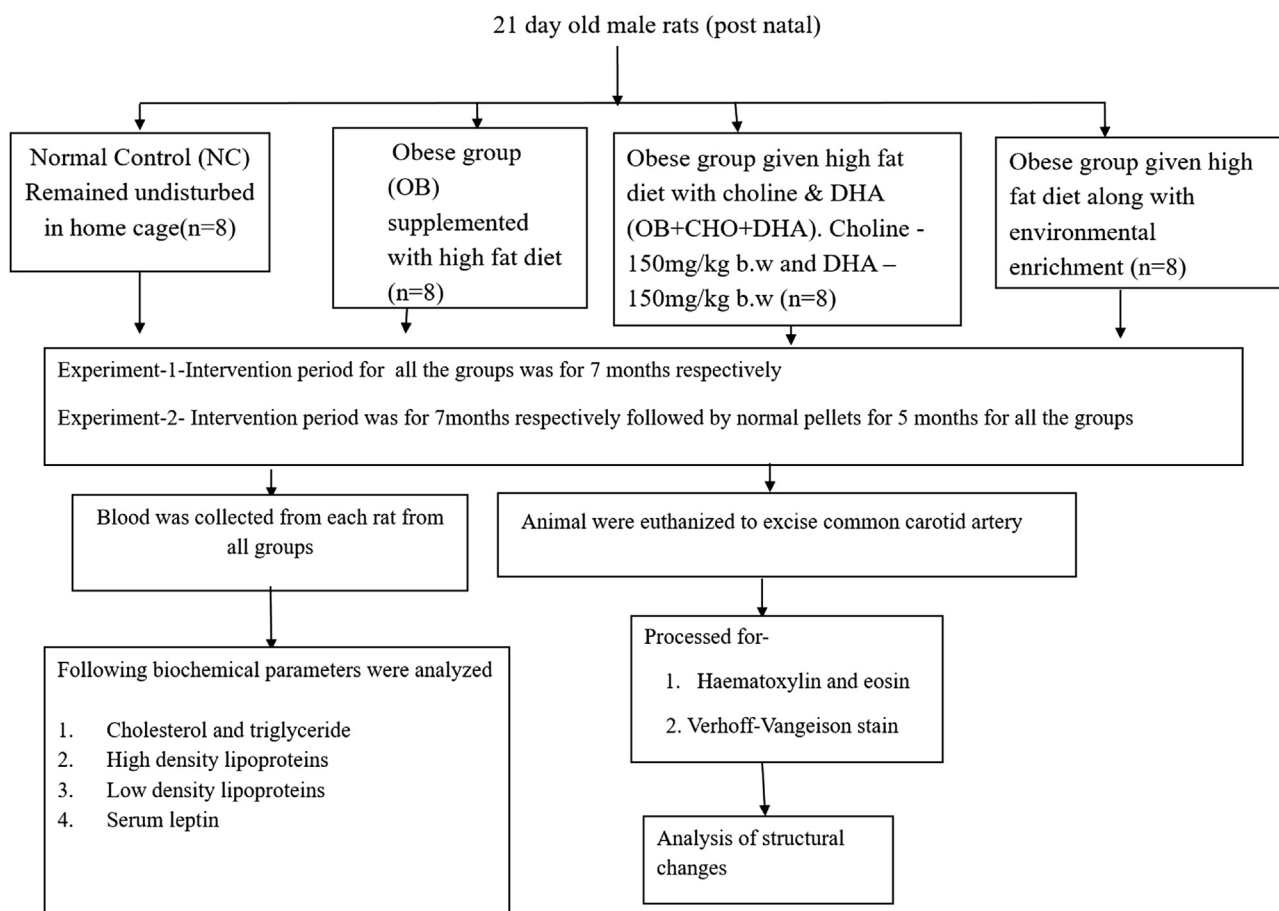


Fig. 1. Schematic representation of Experiment 1 and 2.

2.5. Environmental enrichment

Animals were housed in sterile enriched environment (EE) cage with dimensions of 52 cm × 32 cm along with husked bedding. In each EE cage, there were different objects like tubes, running wheel, ladder, and cubes. Three rats were placed in the cage at a time and allowed to explore the enriched environment for one hour per day (Duarte et al., 2012). (Fig. 2)

2.6. Body mass index

Changes in weight (g) and head to tail length (cm) for each rat from all the groups were recorded for every week. Body mass index was calculated by dividing the body weight (g) by square of body length (cm). Rats with body mass index more than 0.6 were considered as obese rats (Novelli Filho et al., 2007)

2.7. Serum lipid profile

Blood was collected from all groups of rats by *retro*-orbital vein perforation after the intervention period for all the groups. 2 ml blood was collected and serum was separated by using centrifugation procedure with 3000 rpm for 10 min. Biochemical parameters for cholesterol, triglyceride, LDL were estimated according to manufacturer's standard protocols provided in the respective kits which are based on GPO-POD method. Serum leptin was analyzed using standard ELISA kit.

2.8. Histo-pathological analyses for atherosclerotic changes in wall of common carotid artery

Animals were euthanized using high dose of ketamine anesthesia. Perfusion was performed transcardially with saline and 10% formalin was injected to clear the blood clots in CCA lumen. CCA was dissected and all the samples were washed in phosphate buffer saline (PBS) until the lumen is free of blood clots and fixed with



Fig. 2. Enriched cage.

4% paraformaldehyde for 24 h. The tissues are allowed to remain at 50%, 70%, 90% alcohol, followed by absolute alcohol. After dehydration, tissues were given two changes of xylol and infiltrated with paraffin. The labeled paraffin blocks were fixed on a rotary microtome and transverse sections with a thickness of 5μ were taken. The transverse sections were stained with Haematoxylin and Eosin and Verhoff–Vangeison stains (Tulis D, 2007, Reddy, S., Kumar, P. and Prasad, K. 2011). Light microscope at 20 and 400x magnifications was used to observe the sections and analyze the structural changes in the CCA. The transverse sections were imaged (every section was imaged at 5 regions) using image processing program “Image J” version 6.0. The thickness of tunica media and tunica intima were separately analyzed from five randomly selected images of CCA from each rat, by drawing a mark longitudinally from the internal elastic lamina to the outer layer of tunica media and from endothelium up to the sub-endothelial layer respectively. The ratio of tunica media to tunica intima of CCA was calculated from the values of the thickness obtained. The arterial lumen was analyzed by measuring the cross-lumen diameter at 5 different points around the lumen. Quantification of collagen and elastic fibers in tunica media of CCA were analyzed in Verhoff–Vangeison stained sections using Tissue Quant (version 1.0). The principle of Tissue Quant software is on the number of pixels assigned to the shade of the color analyzed for collagen fibers. The stained sections are obtained with means of color score by the software and the image is measured in terms of pixels. Total number of pixels corresponding to the color of interest is then converted to percentage value. The results obtained from all 3 images of each section were aggregated for further statistical evaluation (Kumar et al., 2014). The values for each of the parameters analyzed from the 5 selected regions were taken in terms of pixels and the mean value obtained was later converted to micrometer scale using unit.

2.9. Statistical analysis

Statistical program SPSS 16.0 was used to analyze the mean significant changes among the groups. Data were expressed in terms of Mean \pm SEM. One way ANOVA was performed and Bonnerferon's multiple comparison test was conducted to compare the means among the groups. P-value < 0.05 was considered significant. Pearson's correlation by using the mean values of each group was used to analyze the relationship (association) among the groups.

3. Results

All results obtained from the two experimental studies are represented graphically and histo-morphologically in a combined form to express the persistent effects at old age.

3.1. Changes in body mass index

Mean body mass index increased slightly in normal controls. OB rats fed with high fat diet for 7 months, showed a significant (** $p < 0.01$) increase in body mass index which persistently remained significantly higher (\wedge $p < 0.01$) in 12 month old OB rats despite being fed only normal chow diet for subsequent 5 months as compared to same in age-matched NC rats (Fig. 3)

Alternately, 7 month old OB + CHO + DHA and OB + EE rats showed significant decrease ($\#$ $p < 0.05$, $@@$ $p < 0.01$ respectively) in mean body mass index as compared to the same in age-matched OB rats although it still remained higher as compared to age-matched NC rats which persisted in 12 month old OB + CHO + DHA and not in OB + EE rats (Fig. 3)

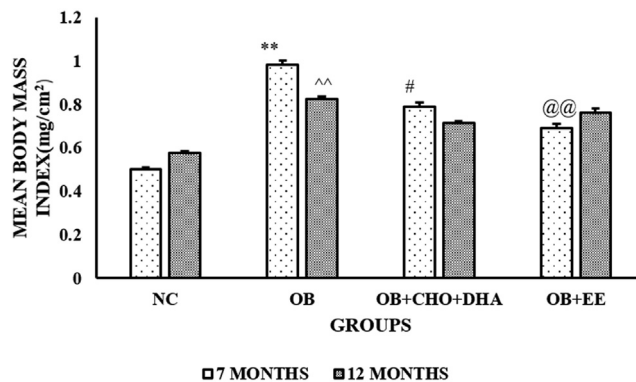


Fig. 3. Data is expressed as Mean \pm SEM. ** $p < 0.01$ in NC vs OB of 7 month & 12 month old rats respectively; $^{\wedge}$ $p < 0.05$ in OB vs OB + CHO + DHA and $^{@@}$ $p < 0.01$ OB vs OB + EE of 7 month old rats.

3.2. Metabolic and serum lipid profile changes

Significant increase in mean serum triglyceride (** $p < 0.01, 0.05$ respectively), cholesterol ($^{\wedge}$ $^{\#}$ $p < 0.05, 0.01$ respectively), low density lipoprotein ($^{\#}$ $^{\wedge}$ $p < 0.001$) and leptin ($^{@@}$, $^{\&\&}$ $p < 0.001, 0.01$ respectively) as well as a significant decrease ($^{\&}$ $p < 0.05$) in high density lipoprotein was observed at both 7 months and 12 months in OB group as compared to the same in age-matched NC group of rats (Table 1, 2).

In 7 month old rats, OB + CHO + DHA and OB + EE groups when compared to OB group of rats the levels of serum triglyceride, low density lipoprotein, ($^{\#}$, $^{\&}$ $p < 0.05$ respectively) has been significantly decreased and serum high density lipoprotein has been significantly ($^{\wedge}$, $^{\&}$ $p < 0.05$) increased. (Table 1)

In 12 month old rats, OB + CHO + DHA and OB + EE groups when compared to OB group of rats the Serum levels of triglyceride, low density lipoprotein and leptin ($^{\#}$, $^{\wedge}$, $^{\&}$, $^{\&}$ $p < 0.05$ respectively) in OB vs OB + CHO + DHA and OB + EE has been significantly decreased (Table 2)

3.3. Common carotid arterial wall changes

3.3.1. Tunica intima:

Mean thickness of tunica intima of the CCA in NC rats was observed to be similar at 7 and 12 months of age. Whereas, mean thickness of tunica intima in 7 month old OB rats was observed to be significantly high (** $p < 0.01$) compared to the same in age-matched NC rats. But in 12 month old OB rats mean thickness of tunica intima was reduced compared to 7 month old OB rats although it was not significant when compared to the same in age-matched NC rats. No significant decrease in mean thickness of tunica intima was observed in OB + CHO + DHA and OB + EE groups of rats compared to OB group of rats (Fig. 4).

3.3.2. Tunica Media:

Mean thickness of tunica media of the CCA in NC rats was observed to be similar at 7 and 12 months of age. But 7 month old and 12 month OB group of rats showed a significant increase (** $p < 0.01$; $^{\wedge}$ $p < 0.01$ respectively) in mean thickness of tunica media as compared to age-matched NC group of rats. In both 7 and 12 month old OB + CHO + DHA groups showed significant attenuation ($^{\#}$, $^{\&}$ $p < 0.05$) as compared to OB group of age-matched rats. But in 7 and 12 month old OB + EE rats, a significant ($^{\&}$, $^{\$}$ $p < 0.05$) increase in mean thickness of tunica media was observed when compared to the same in age-matched NC rats

Table 1

Data expressed in Mean ± SEM. Serum levels of triglyceride, cholesterol, low density lipoprotein, and leptin (**, ^, ##, @@@ p < 0.01, 0.05, 0.01, 0.001) respectively in NC vs OB, serum high density lipoprotein (& p < 0.05) in NC vs OB. Serum levels of triglyceride, low density lipoprotein *, #, @, \$ p < 0.05 respectively in OB vs OB + CHO + DHA and OB + EE. Serum high density lipoprotein ^, & p < 0.05 in OB vs OB + CHO + DHA and OB + EE.

Groups	Serum triglyceride (mg/dl)	Serum total cholesterol (mg/dl)	Serum high density lipoprotein (mg/dl)	Serum low density lipoprotein (mg/dl)	Serum leptin (pg /dl)
NC	63.3 ± 3.98	98.77 ± 3.17	51.4 ± 2.11	20.13 ± 1.46	413.8 ± 36.5
OB	99.1 ± 3.38 **	113.57 ± 3.18 ^	33.2 ± 1.87 &	54.4 ± 3.9 ##	504.4 ± 71.4 @@@
OB + CHO + DHA	86.0 ± 2.11 *	101.38 ± 2.70	46.5 ± 2.68 ^	36.1 ± 2.93 #	499.6 ± 66.4
OB + EE	84.8 ± 3.39 @	100.94 ± 2.02	45.8 ± 2.68 &	38.5 ± 2.62 \$	496 ± 33.1

Table 2

Data expressed in Mean ± SEM. Serum levels of triglyceride, cholesterol, low density lipoprotein, and leptin (*, #, #, ^, ^, & p < 0.05, 0.01, 0.01, 0.01) respectively in NC vs OB. Serum levels of triglyceride, low density lipoprotein and leptin (*, #, #, ^, @, \$ p < 0.05 respectively) in OB vs OB + CHO + DHA and OB + EE.

Groups	Serum triglycerides (mg/dl)	Serum total cholesterol (mg/dl)	Serum high-density lipoprotein (mg/dl)	Serum low-density lipoprotein (mg/dl)	Serum leptin (pg /dl)
NC	54.9 ± 3.98	95.77 ± 2.17	36.4 ± 2.17	42.33 ± 1.34	509.6 ± 36.5
OB	68.3 ± 3.38 *	116.97 ± 5.18 ##	31.2 ± 2.87	59.4 ± 4.6 ^^	561.4 ± 52.2 &&
OB + CHO + DHA	56.0 ± 2.11 *	101.88 ± 3.65	35.1 ± 2.58	49.2 ± 1.90 #	527.6 ± 43.4 ^
OB + EE	58.1 ± 3.39 &	100.84 ± 2.02	35.5 ± 1.38	52.5 ± 1.92 @	539 ± 41.3 \$

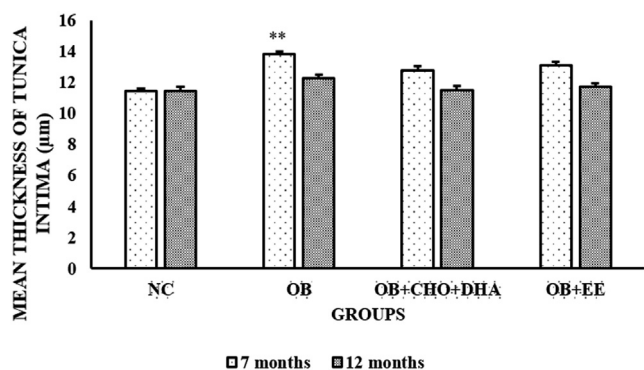


Fig. 4. Data is expressed as Mean ± SEM. Significant increase in mean thickness of tunica intima in 7 month old OB rats as compared to same in age-matched NC group of rats ** p < 0.01 NC vs OB.

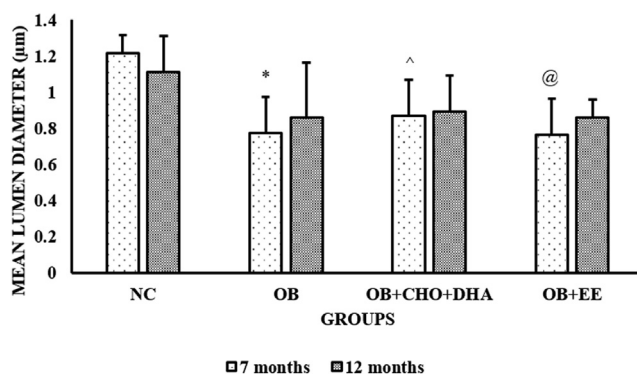


Fig. 6. Data is expressed as Mean ± SEM. Mean lumen diameter (µm) in 7 month old. * p < 0.05 NC vs OB, ^ p < 0.05 NC vs OB + CHO + DHA, @ p < 0.05 NC vs OB + EE. No significant change among the groups in 12 month old rats.

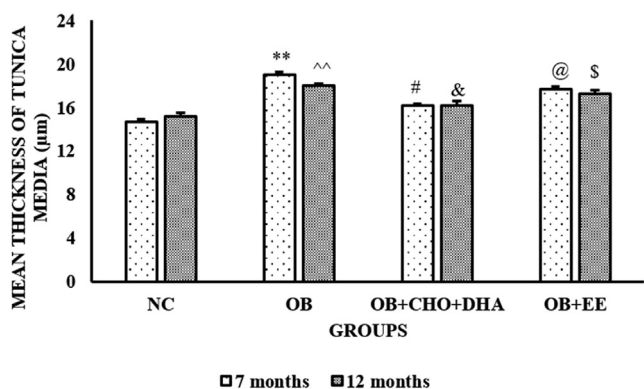


Fig. 5. Data is expressed as Mean ± SEM. Mean thickness of tunica media in 7 and 12 month old rats. **, ^^ p < 0.01 in NC vs OB respectively, #, & p < 0.05 in OB vs OB + CHO + DHA respectively, @, \$ p < 0.05 in NC vs OB + EE respectively.

and no significant attenuation was observed when compared to the same in age-matched OB rats (Fig. 5).

3.3.3. Arterial lumen diameter:

The mean arterial lumen diameter of CCA in 7 vs 12 month NC group of rats showed slight decrease with aging. 7 month old OB group rats showed a significant decrease (*p < 0.05) in mean arte-

rial lumen diameter as compared to the same in age-matched NC rats. Moreover, in both OB + CHO + DHA and OB + EE group the CCA lumen diameter showed a significant decrease (^, @ p < 0.05) as compared to the same in age-matched NC rats but not with OB rats. In 12 month old OB + CHO + DHA and OB + EE groups of rats the mean CCA lumen diameter was not significantly changed as compared to same in age-matched OB rats although it was decreased compared to same in NC rats (Figs. 6–8).

3.3.4. Percentage of collagen and elastic fibers in tunica media

Verhoff Vangeison stained sections of CCA were analyzed using tissue quant software.

Mean percentage of collagen fibers in tunica media of CCA was slightly increased in 12 month NC group rats compared to same in 7 month NC group rats. But mean percentage of collagen fibers in tunica media of OB rats was observed to be significantly higher (** p < 0.01) as compared to age-matched NC rats which did not persist till 12 month in OB rats. Moreover in 7 and 12 month old OB + CHO + DHA and OB + EE rat groups, there was no significant increase in percentage of collagen fibers as compared to same in age-matched NC and OB group of rats (Fig. 9).

Mean percentage of elastic fibers in tunica media of CCA was slightly higher at 12 month NC group rats compared to same in 7 month NC group rats. Moreover the mean percentage of elastic fibers in OB rats as well as OB + CHO + DHA and OB + EE rats at both

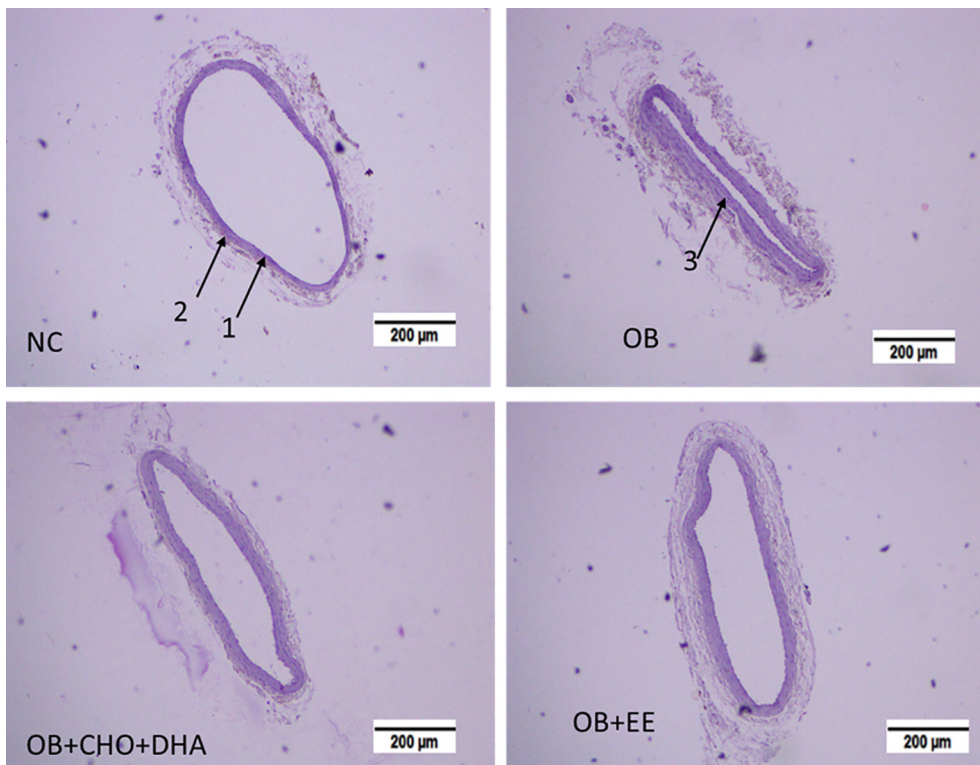


Fig. 7. Representative Photomicrograph (20 & 400x) of common carotid artery of 7 month old rats showing histo-morphological changes in tunica intima and tunica media of common carotid artery. 1- Tunica intima of NC group, 2- Tunica media of NC group, 3- increase in thickness of tunica media in OB group.

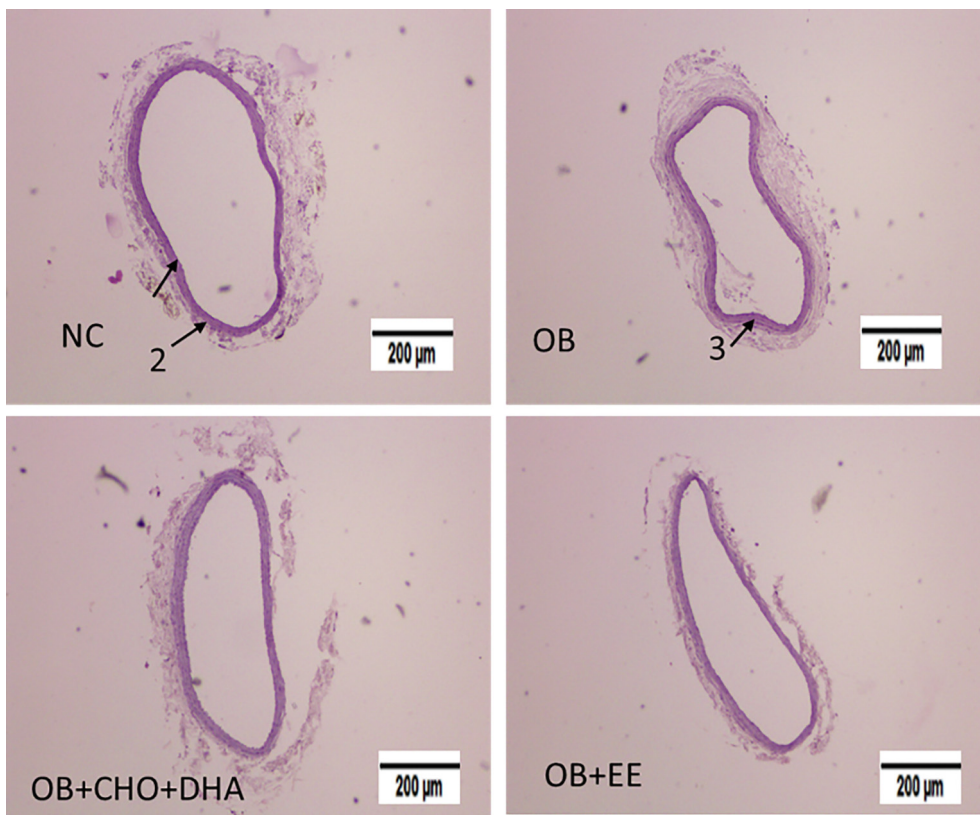


Fig. 8. Representative Photomicrograph (20 & 400x) of common carotid artery of 12 month old rats showing histo-morphological changes in tunica intima and tunica media of common carotid artery. 1- Tunica intima of NC group, 2- Tunica media of NC group, 3- increase in thickness of tunica media in OB group.

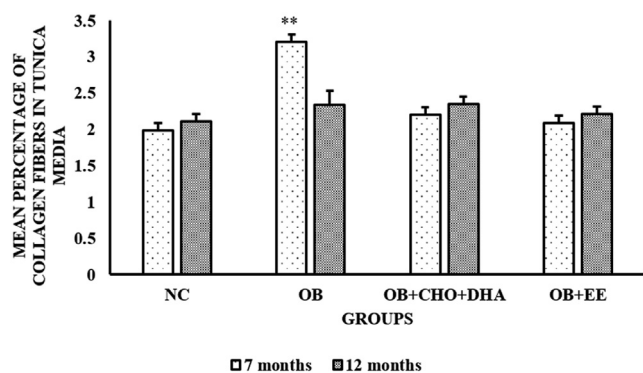


Fig. 9. Data is expressed as Mean \pm SEM. Represents mean percentage of collagen fibers in tunica media of common carotid artery in 7 month old rats. * $p < 0.01$ NC vs OB.

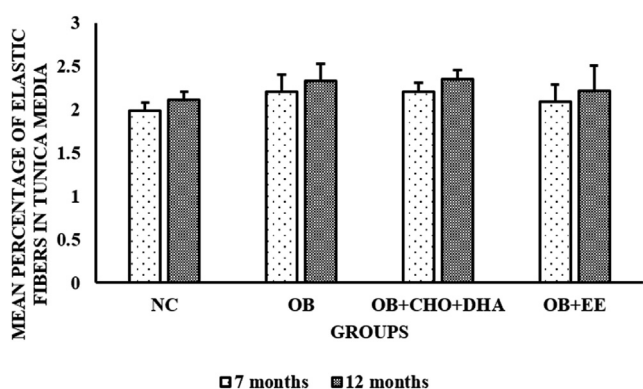


Fig. 10. Data is expressed as Mean \pm SEM. Represents mean percentage of elastic fibers in tunica media of common carotid artery in 7 and 12 month old rats.

7 and 12 months of age did not show any significant increase compared to the same in age-matched NC group of rats (Fig. 10). Figs. 11. and 12.

3.4. Correlations-

The mean thickness of tunica media was correlated to serum lipid profile. A positive linear correlation of thickness of tunica

media was observed in relation to increased levels of serum cholesterol, triglyceride, low density lipoprotein and leptin in age-matched (7 months) OB group of rats compared to NC (Fig. 13 a, b, c, d).

Moreover, the mean thickness of tunica media was significantly correlated to serum lipid profile levels in OB vs OB + CHO + DHA groups. A significant positive linear correlation of mean thickness of tunica media to serum triglycerides, low density lipoproteins and leptin levels was observed in OB group when compared to OB + CHO + DHA groups (Fig. 14 a, b, c, d).

Additionally, the mean thickness of tunica media was observed to be correlated to serum lipid profile in OB vs OB + EE group. As the serum lipid profile level is decreased in OB + EE group, the mean thickness of tunica media was observed to be lesser as compared to the same in age matched OB group of rats (Fig. 15.a, b, c, d)

4. Discussion

4.1. Changes in body mass index

In the present study, NC group of rats showed slight increase in body mass index between the age of 7 and 12 month indicating age-related increase of BMI. This is seen because aging is associated with increase in free fatty mass deposition and further related to physiological and pathological alterations in fatty mass deposition (Pappas and Nagy, 2019). Whereas 7 month old OB rats, fed with high fat diet, a significant increase in body mass index was observed compared to same in age-matched NC rats. Interestingly, 12 month old OB rats which did not consume high fat diet for subsequent 5 months were observed to have persistent high BMI compared to the same in NC rats. Studies show that, most importantly, rapid increase in body mass index from an early age is a significant predictor for future obesity and cardiovascular risks (Mumena et al., 2018). Long duration of feeding with high fat diet induces hypothalamic oxidative stress leading to obesity at increasing rate (Cavaliere et al., 2018). Importantly, exposure to high fat diet from a juvenile age or during adulthood can also have an impairment on endocrine metabolism and evokes leptin resistance by hyperleptinemia which is associated with deficits in hippocampal-dependent behaviors (Del Olmo and Ruiz-Gayo, 2018). With the period of aging, the body mass index shows varied changes with an increase in cardiovascular and cerebrovascular risks due to influence of body weight and fat deposition (Espinoza, 2019).

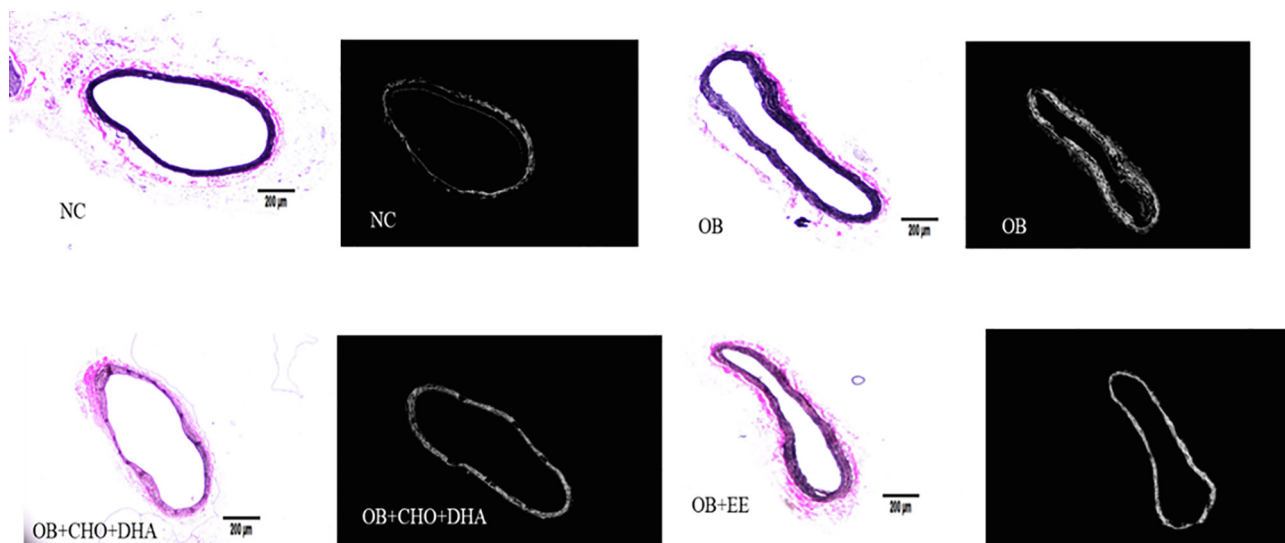


Fig. 11. Representative Photomicrograph (20 & 400x) of collagen fibers in tunica media of common carotid artery using Verhoff-VanGieson and tissue quant software images for 7 month old rats of -NC, OB, OB + CHO + DHA and OB + EE groups.

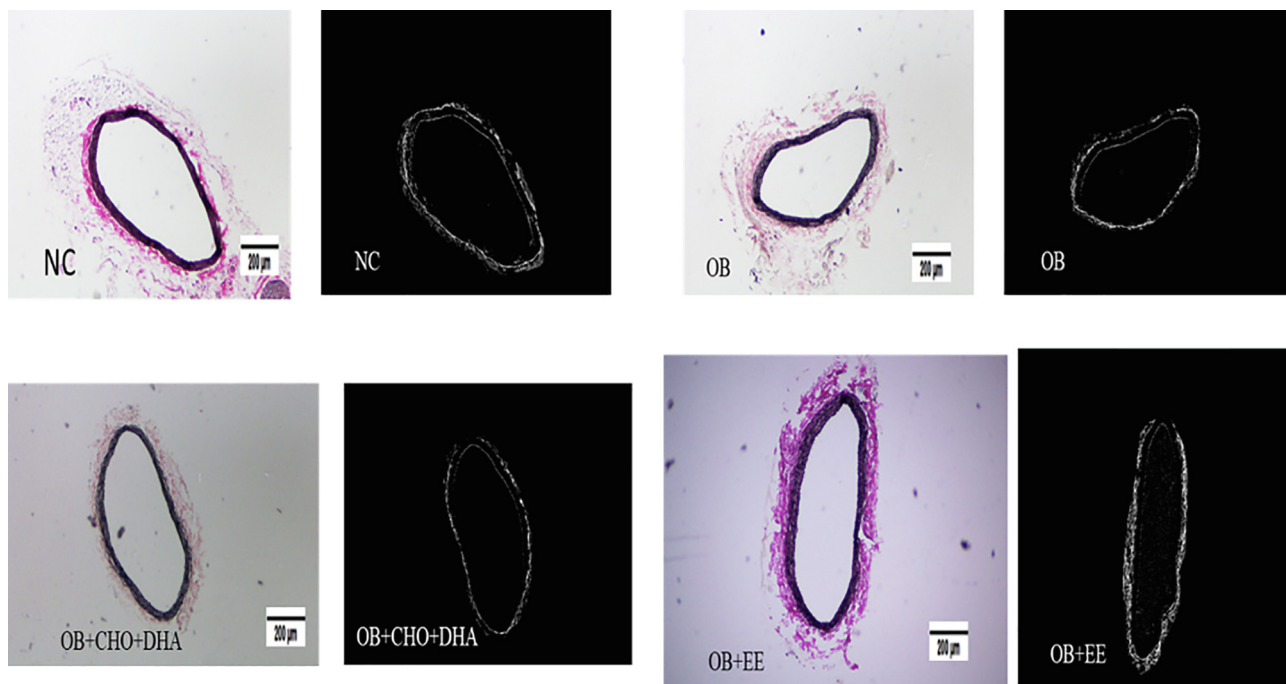


Fig. 12. Representative Photomicrograph (20 & 400x) of collagen fibers in tunica media of common carotid artery using Verhoff-Vangeison and tissue quant software images for 12 month old rats of NC, OB, OB + CHO + DHA and OB + EE groups.

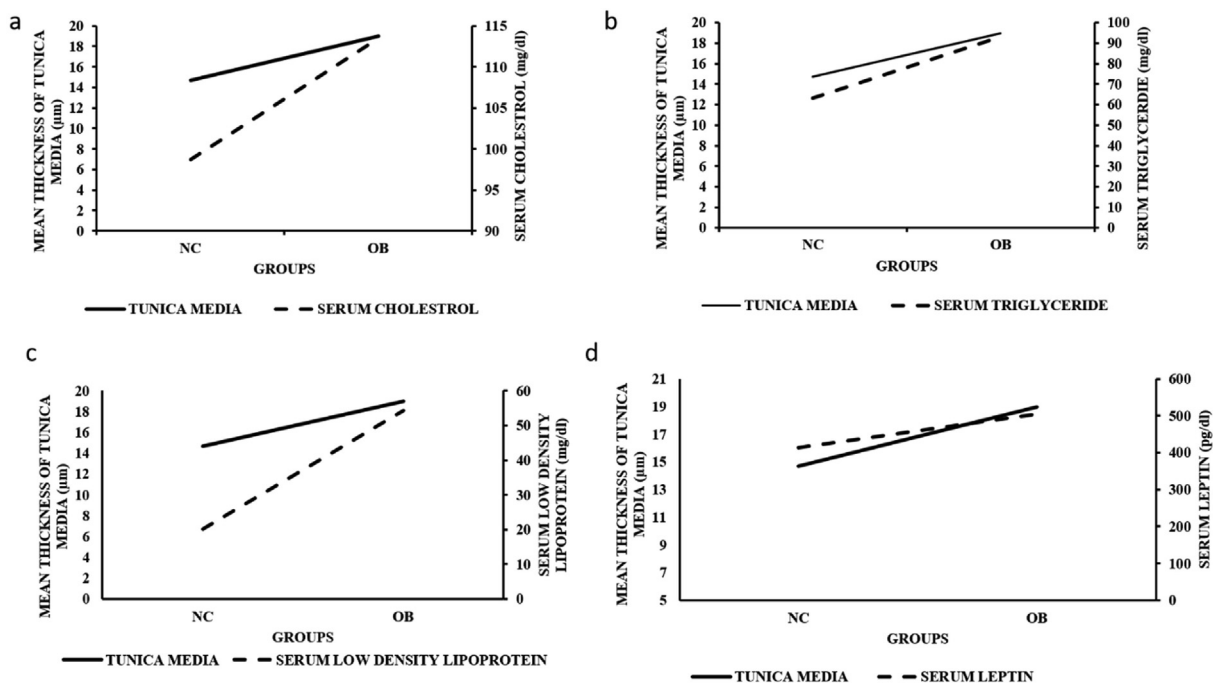


Fig. 13. Correlations between mean thickness of tunica media (μm) vs serum cholesterol (mg/dl) a; vs serum triglyceride (mg/dl) b; vs serum low density lipoprotein (mg/dl) c; vs serum leptin levels (pg/dl) d; in 7 month old NC and OB group of rats.

Supplementation of choline and DHA or exposure to enriched environment during 7 month period in OB rats causes a significant decrease in body mass index in both OB + CHO + DHA group as well as OB + EE rats compared to same in age-matched OB rats. This indicates an important finding that, supplementation of choline and DHA or early exposure to environmental enrichment along with high fat diet has a positive influence on preventing body weight gain. The body mass index in 12 month old OB + CHO + D

HA group as well as OB + EE rats was not significantly attenuated when compared to the same in age-matched OB rats but remained slightly higher compared to same in NC rats. Interestingly, 12 month old OB rats which did not consume high fat diet nor supplementation of choline and DHA or exposed to enriched environment for subsequent 5 months were observed to have persistent high BMI compared to the same in NC rats. Choline helps in maintaining body weight by preventing deposition of lipoproteins by

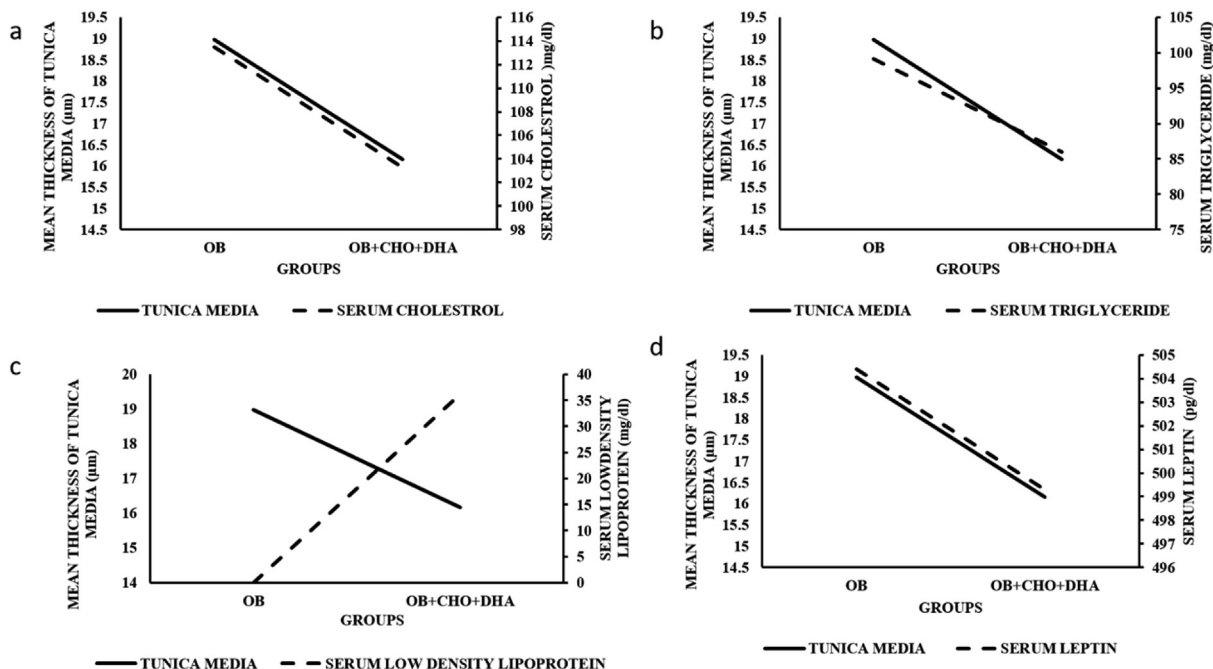


Fig. 14. Correlations between mean thickness of tunica media (µm) vs serum cholesterol (mg/dl) a; vs serum triglyceride (mg/dl) b; vs serum low density lipoprotein (mg/dl) c; vs serum leptin levels (pg/dl) d; in 7 month old OB and OB + CHO + DHA group of rats.

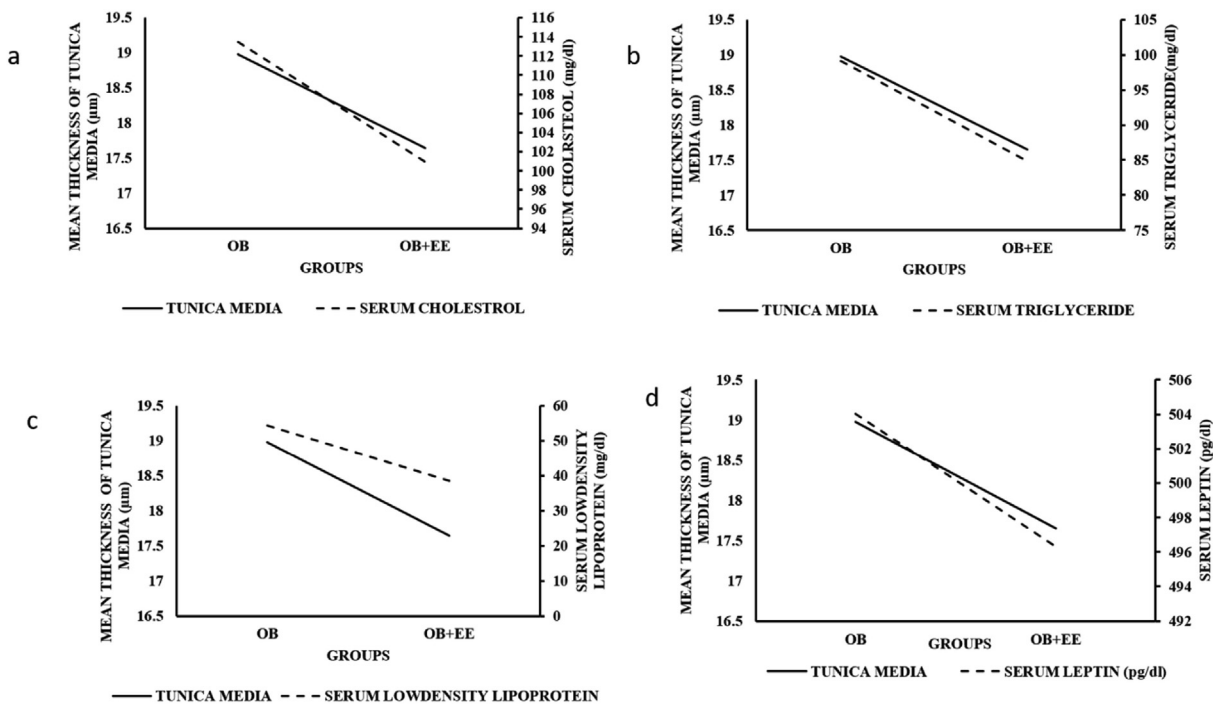


Fig. 15. Correlations between mean thickness of tunica media (µm) vs serum cholesterol (mg/dl) a; vs serum triglyceride (mg/dl) b; vs serum low density lipoprotein (mg/dl) c; vs serum leptin levels (pg/dl) d; in 7 month old OB and OB + EE group of rats.

promoting fatty acid beta oxidation and thus shows a positive correlation with body mass index (Gao et al., 2016). DHA directly influences the lipid metabolism in liver and maintains the inflammatory damage and body weight gain thus maintaining the body mass index (Shang et al., 2017).

Apart from nutrient supplements, studies have shown that exposure to environmental enrichment helps in maintaining body weight and metabolism (Rodríguez-Ortega et al., 2019). Although studies have shown that environmental enrichment can have var-

ied changes depending on the duration of the enrichment provided, the precise mechanism of this change remains unclear (Tsai et al., 2002).

4.2. Metabolic and serum lipid profile changes

Serum lipid profile in NC group of rats did not show any significant variations among the 7 and 12 month old rats. But the 7 month old OB group of rats showed a significant increase in

serum triglycerides, cholesterol, low density lipoproteins and leptin levels as compared to the same in age-matched NC group of rats indicating metabolic disturbances due to the early exposure to high fat diet. But in 12 month old OB rats despite stopping high fat diet consumption, serum triglycerides, cholesterol, low density lipoproteins and leptin showed a persistently significant increase as compared to same in age-matched NC group of rats. These changes are observed because of early exposure to high fat diet influencing and altering metabolism causing metabolic and functional changes during aging. Studies show that, when high fat diet is fed for a longer period, hepatic lipid peroxidation is observed which can directly contribute to metabolic changes (Nunes-Souza et al., 2016). Changes in body composition with age increases body fat mass resulting in lesser energy expenditure and increased metabolism which results in more fat storage. This fat storage alters the lipid parameters leading to metabolic disturbances and inflammation (Cavaliere et al., 2018). With aging, changes in cellular metabolism of adipose tissue and metabolic parameters are linked to adipose tissue function (Miller et al., 2017). Fatty diets have an increased tendency for hyperlipidemia further leading to cardiovascular risks (DiNicolantonio and O'Keefe, 2018). Obesity produced due to high fat diet has shown to have dyslipidemia in which there is an increase in serum lipids, including leptin and reduction in levels of high density lipoproteins (Bonomini, Rodella and Rezzani, 2015). Increase in circulating triglyceride can cause major lipid abnormalities because there is a delayed clearance of the TG-rich lipoproteins and increase in formation of small dense LDL. This increase in triglyceride and LDL in turn reduces the levels of HDL leading to alterations in lipid metabolism (Klop, Elte and Cabezas, 2013). Intake of lard in diet leads to loss of leptin action on hypothalamic neurons that is involved in maintaining body weight regulation, leading to progression of obesity (Viggiano et al., 2016). Resistance to circulating leptin levels in diet-induced obesity often seen with absent sensitivity to food intake suppresses leptin receptors in arcuate nucleus of hypothalamus (de Git et al., 2018). These studies support the findings of the present study.

An important observation in this study is that supplementation of choline and DHA have attenuated the levels of serum cholesterol, triglyceride, low density lipoproteins and leptin in OB + CH O + DHA group as compared to the same age matched OB group of rats. This study marks an important relation of combined supplementation of choline and DHA in attenuating high fat diet induced changes in lipids when supplemented from an early age. Other important findings in this study is that the lipid levels except HDL in 12 month old OB + CHO + DHA group is lesser as compared to same in aged matched OB group of rats. Supplementation of both choline and DHA reduces the risks of hyperlipidemia (Takkella et al., 2018). Choline supplementation increases lipid metabolism by reducing the levels of free fatty acids and triglycerides. This in turn, improves hepatic lipid metabolism by decreasing nicotinamide adenine dinucleotide phosphate (NADPH) generation, improving fatty acid oxidation (Li et al., 2018). DHA reduces the risks for liver damage and maintains the lipid metabolism (Shang et al., 2017). Combined supplementation of choline and DHA maintain body metabolism and metabolic functions (Bernhard et al., 2020). These studies support the findings of the present study, indicating that combined supplementation of choline and DHA can help in maintaining the serum lipid levels and attenuating the metabolic disturbances through aging, despite high fat diet consumption and obesity.

Moreover, when serum lipid levels of OB + EE with OB group in 7 month old rats were compared it was observed that there is a decrease in the levels of serum cholesterol, triglycerides, low density lipoproteins and leptin. However, this decrease is not statistically significant. Environmental enrichment reduces the hepatic

triglyceride levels and hepatic steatosis. It also helps in reducing excess glucose production even in aging (McMurphy et al., 2018). Although the mechanism in which environmental enrichment acting on lipid changes is still unclear. In the present study, 12 month old OB + EE group of rats did not show any significant changes in serum lipid levels as compared to OB group of same aged rats.

4.3. Common carotid arterial wall changes

The tunica intima and tunica media of CCA in 7 and 12 month old NC group of rats did not show significant variations in mean thickness. Alternately 7 month old OB rats had significant increase in mean thickness of tunica intima and tunica media of CCA as compared to same in age-matched NC group of rats. Additionally a significant decrease in arterial lumen and mean percentage of collagen fibers was observed in tunica media of 7 month old OB group of rats as compared to same in age matched NC group of rats. Further, in 12 month old OB group of rats, despite stopping consumption of high fat diet for 5 months, the tunica media persistently showed a significant increase in thickness compared to same in age-matched NC group of rats. But in 12 month old OB group of rats, the lumen diameter did not show a significant change as compared to the same in NC group of rats. In obesity, due to high fat intake, serum lipid level alterations and raise in body mass index increases the risks for vascular damage. Raise in cholesterol, triglycerides and low density lipoproteins marks the beginning of atherosclerosis affecting the arterial wall structure and causing vascular inflammation (Bonomini, Rodella and Rezzani, 2015). Metabolic disturbances due to changes in triglyceride and high density lipoprotein ratios lead to plaque formation associated with carotid atherosclerosis. The vascular permeability for low density lipoproteins increases and up regulates the expression of LOX-1, which is associated with increased endothelial permeability for oxLDL through activation of protein kinase C (PKC) and calcium influx. This further with molecular interactions reduces the desmosome cell to cell contacts that can further weaken the endothelial junctions causing damage to tunica intima. Along with intimal wall changes, the tunica media of the artery also thickened due to changes in smooth muscle cells, alterations in collagen fibers and fatty streaks formation which further increases the thickness of tunica media. With an increase in thickness of tunica intima and tunica media, the arterial lumen gets narrowed that may cause decrease in blood flow through the arterial lumen (Misra et al., 2019). Additionally, studies show that hydroxyproline; a content of collagen increases with age in addition to increased collagen and elastic fibers in tunica media of an artery with aging (Tsamis, Krawiec and Vorp, 2013). In the present study, long-term exposure to high fat diet alters the serum lipid levels that initiate atherosclerotic lesion formation damaging the arterial wall of the CCA. As described in above discussion, the arterial wall stiffness increases as the age advances, but the tunica media has shown relatively more damage when high fat diet was given for a longer period. This indicates that high fat diet supplementation from young age through adulthood linearly increases lipid profile levels and concomitant thickening of tunica media at adulthood posing increased cardiovascular risks.

In the present study, in 7 month old OB + CHO + DHA group of rats, a significant attenuation in the thickness of tunica media was observed as compared to the same in age-matched OB group of rats, although it was not similar to same in age matched NC rats. But, in 12 month old OB + CHO + DHA group of rats, despite stoppage of high fat diet and interventions at 7 month, persistent significant attenuation of thickness of tunica media was observed when compared to that of age matched OB group of rats. Choline an essential nutrient is known to lower circulating lipids and homocysteine thus reducing vascular damage (Millard et al.,

2018). Dietary choline supplementation could increase hepatic adiponectin content and expression of lipolysis pathway genes. It could also reduce the expression of lipogenesis pathway genes promoting a lipid-lowering effect and restoring lipid metabolism balance, thereby reducing the hepatic steatosis and subsequently, attenuating inflammation by modulating NF- κ B signaling molecules to suppress pro-inflammatory genes and increasing expression of anti-inflammatory genes (Kitson Alex. et al, 2016). DHA is also an essential nutrient which is known to have anti-atherogenic effect by reducing deposition of free fatty acids, thereby preventing the increase in thickness of arterial wall (Gladine et al., 2014). Dietary phospholipids directly act on lipoprotein deposition reducing the inflammatory process (Küllenberg et al., 2012). The present study shows compelling evidence regarding the beneficial effects of combined supplementation of choline and DHA in reducing the arterial wall thickening induced by exposure to high fat diet. This indicates that supplementation of combined choline and DHA during obesity in adults attenuates the negative impact of excessive lipids on tunica media thickening. Mainly, a significant positive correlation was observed with levels of serum leptin and mean thickness of tunica media. This study is the first to report the novel finding on the importance of combined choline and DHA supplements during obesity in attenuating mean thickness of tunica media and serum lipid levels.

Further, in the present study, 7 month old OB + EE group rats showed a significant attenuation in thickness of tunica media when compared to that of age-matched OB group rats although it was not similar to same in age matched NC rats. But in 12 month old OB + EE group rats, despite stoppage of high fat diet and interventions at 7 month, persistent significant attenuation of mean thickness of tunica media was observed when compared to same in age matched OB group of rats. Exposure to environmental enrichment shows beneficial effects on behavioral and cardiovascular health (Normann et al., 2018). Studies have also shown that environmental enrichment helps in reversing endothelial dysfunctions by mobilization of endothelial progenitor CD34 + cells which in turn maintain systemic vascular endothelial function (Bruno et al., 2018). In addition, environmental enrichment is also known to reduce the deposition of lipoproteins, thus maintaining the body weight and serum lipid levels (Tsai et al., 2002). Studies also show that environmental enrichment helps in healthy aging by hypothalamic-sympatho neural-adipocyte (HSA) axis that helps in inducing metabolic and behavioral adaptations which may be the underlying mechanisms for improved health when exposed to environmental enrichment (McMurphy et al., 2018). But, the molecular mechanisms involved in regulating vascular changes by environmental enrichment still remains unclear. The present study shows beneficial effects of environmental enrichment when given along with high fat diet in attenuating vascular inflammatory changes from an early period.

5. Conclusion

1. High fat diet supplementation from an early young age leads to higher body mass index, serum lipid levels and inflammatory changes in arterial wall with a negative impact that progresses with aging even after cessation of the high fat diet. These findings indicate that high fat diet consumption during early ages through middle age causes permanent changes in metabolism which persistently causes high BMI in old age despite stopping high fat diet or any intervention.
2. Combined supplementation of choline and DHA to obese individuals from an early young age significantly attenuates negative impact of high fat diet on body mass index, serum lipids and thickness of the arterial wall through aging, despite cessation of consuming high fat diet.

3. Additionally, exposure to environmental enrichment from an early young age attenuates negative impact of high fat diet on body mass index, serum lipids and thickness of the arterial wall and these positive findings persists progressively through aging.

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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.

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References

- Kitson, A.P., Metherel, A.H., Chen, C.T., Domenichiello, A.F., Trépanier, M.-O., Berger, A., Bazinet, R.P., 2016. Effect of Dietary Docosahexaenoic Acid (DHA) in Phospholipids or Triglycerides on Brain DHA Uptake and Accretion. *The Journal of Nutritional Biochemistry* 33. <https://doi.org/10.1016/j.jnutbio.2016.02.009>.
- Bernhard, W., Böckmann, K., Maas, C., et al., 2020 Mar. Combined choline and DHA supplementation: a randomized controlled trial. *Eur. J. Nutr.* 59 (2), 729–739. <https://doi.org/10.1007/s00394-019-01940-7>.
- Bonomini, F., Rodella, L.F., Rezzani, R., 2015. Metabolic syndrome, aging and involvement of oxidative stress. *Aging and Disease* 6 (2), 109–120. <https://doi.org/10.14336/AD.2014.0305>.
- Bruno, R.M. et al., 2018. Vascular function is improved after an environmental enrichment program the train the brain-mind the vessel study. *Hypertension* 71 (6), 1218–1225. <https://doi.org/10.1161/HYPERTENSIONAHA.117.10066>.
- Cavaliere, G. et al., 2018. Long feeding high-fat diet induces hypothalamic oxidative stress and inflammation, and prolonged hypothalamic AMPK activation in rat animal model. *Front. Physiol.* 9 (JUL), 1–12. <https://doi.org/10.3389/fphys.2018.00818>.
- DiNicolantonio, J.J., O'Keefe, J.H., 2018. Effects of dietary fats on blood lipids: a review of direct comparison trials. *Open Heart* 5, (2). <https://doi.org/10.1136/openhrt-2018-000871>.
- Duarte, F. et al., 2012. The effects of exercise modalities on adiposity in obese rats. *Clinics* 67 (12), 1469–1477. [https://doi.org/10.6061/clinics/2012\(12\)19](https://doi.org/10.6061/clinics/2012(12)19).
- Espinoza, E., 2019. Effect of the Mediterranean Diet on BMI in Middle-Aged Hispanic Women with Pre-Obesity and Obesity Central Washington State. *International Journal of Clinical Medicine* 10 (06), 363–378. <https://doi.org/10.4236/ijcm.2019.106029>.
- Gao, X. et al., 2016. Higher Dietary Choline and Betaine Intakes are Associated with Better Body Composition in the Adult Population of Newfoundland, Canada. *PLoS ONE* 11 (5), 1–17. <https://doi.org/10.1371/journal.pone.0155403>.
- Gardener, H. et al., 2014. Mediterranean diet and carotid atherosclerosis in the Northern Manhattan study. *Atherosclerosis* 234 (2), 303–310. <https://doi.org/10.1016/j.atherosclerosis.2014.03.011>.
- de Git, K.C.G. et al., 2018. 'Is leptin resistance the cause or the consequence of diet-induced obesity?', *International Journal of Obesity*. Springer, US 42 (8), 1445–1457. <https://doi.org/10.1038/s41366-018-0111-4>.
- Gladine, C. et al., 2014. The omega-3 fatty acid docosahexaenoic acid favorably modulates the inflammatory pathways and macrophage polarization within aorta of LDLR^{-/-} mice. *Genes Nutr.* 9 (5). <https://doi.org/10.1007/s12263-014-0424-4>.
- Hase, Y. et al., 2019. Carotid artery disease in post-stroke survivors and effects of enriched environment on stroke pathology in a mouse model of carotid artery stenosis. *Neuropathol. Appl. Neurobiol.* 44, 1–17. <https://doi.org/10.1111/nan.12550>.
- Katzmarzyk, P.T. et al., 2019. International study of childhood obesity, lifestyle and the environment (ISCOLE): Contributions to understanding the global obesity epidemic. *Nutrients* 11 (4). <https://doi.org/10.3390/nu11040848>.
- KD, M. et al. (2018) 'Health Effects of Overweight and Obesity in 195 Countries over 25 Years', *Yearbook of Paediatric Endocrinology*, pp. 13–27. doi: 10.1530/ey.15.15.2.
- Klop, B., Elte, J.W.F., Cabezas, M.C., 2013. Dyslipidemia in Obesity: Mechanisms and Potential Targets. *Nutrients* 5 (4), 1218–1240. <https://doi.org/10.3390/nu5041218>.

- Küllenberg, D. et al., 2012. Health effects of dietary phospholipids. *Lipids Health Dis.* 11, 1–16. <https://doi.org/10.1186/1476-511X-11-3>.
- Kumar, N. et al., 2014. Quantitative fraction evaluation of dermal collagen and elastic fibres in the skin samples obtained in two orientations from the trunk region. *Dermatol. Res. Pract.* 2014, 251254. <https://doi.org/10.1155/2014/251254>.
- Li, W. et al., 2018. Choline supplementation improves the lipid metabolism of intrauterine-growth-restricted pigs. *Asian-Australasian Journal of Animal Sciences* 31 (5), 686–695. <https://doi.org/10.5713/ajas.15.0810>.
- Llewellyn, A. et al., 2016. Childhood obesity as a predictor of morbidity in adulthood: A systematic review and meta-analysis. *Obes. Rev.* 17 (1), 56–67. <https://doi.org/10.1111/obr.12316>.
- McMurphy, T. et al., 2018. Implementation of environmental enrichment after middle age promotes healthy aging. *Aging* 10 (7), 1698–1721. <https://doi.org/10.18632/aging.101502>.
- Millard, H.R. et al., 2018. 'Dietary choline and betaine; associations with subclinical markers of cardiovascular disease risk and incidence of CVD, coronary heart disease and stroke: the Jackson Heart Study', *European Journal of Nutrition*. Springer, Berlin Heidelberg 57 (1), 51–60. <https://doi.org/10.1007/s00394-016-1296-8>.
- Miller, K.N. et al., 2017. Aging and caloric restriction impact adipose tissue, adiponectin, and circulating lipids. *Aging Cell* 16 (3), 497–507. <https://doi.org/10.1111/accel.12575>.
- Misra, B.B. et al., 2019. Analysis of serum changes in response to a high fat high cholesterol diet challenge reveals metabolic biomarkers of atherosclerosis. *PLoS ONE* 14 (4), 1–25. <https://doi.org/10.1371/journal.pone.0214487>.
- Mumena, W.A. et al., 2018. Rapid increase of overweight and obesity among primary school-aged children in the Caribbean; high initial BMI is the most significant predictor. *BMC Obesity*. *BMC Obesity* 5 (1), 1–7. <https://doi.org/10.1186/s40608-018-0182-8>.
- Normann, M.C. et al., 2018. The influence of environmental enrichment on cardiovascular and behavioral responses to social stress. *Psychosom. Med.* 80 (3), 271–277. <https://doi.org/10.1097/PSY.0000000000000558>.
- Novelli Filho, J.L.V.B. et al., 2007. Anthropometrical parameters and markers of obesity in rats. *Lab. Anim.* 41 (1), 111–119.
- Nunes-Souza, V. et al., 2016. Aging Increases Susceptibility to High Fat Diet-Induced Metabolic Syndrome in C57BL/6 Mice: Improvement in Glycemic and Lipid Profile after Antioxidant Therapy. *Oxid. Med. Cell. Longevity* 2016. <https://doi.org/10.1155/2016/1987960>.
- Del Olmo, N., Ruiz-Gayo, M., 2018. Influence of high-fat diets consumed during the juvenile period on hippocampal morphology and function. *Front. Cell. Neurosci.* 12 (November), 1–10. <https://doi.org/10.3389/fncel.2018.00439>.
- Orozco-Beltran, D. et al., 2017. Lipid profile, cardiovascular disease and mortality in a Mediterranean high-risk population: The ESCARVAL-RISK study. *PLoS ONE* 12 (10), 1–20. <https://doi.org/10.1371/journal.pone.0186196>.
- Pappas, L.E., Nagy, T.R., 2019. The translation of age-related body composition findings from rodents to humans. *Eur J Clin Nutr* 73, 172–178. <https://doi.org/10.1038/s41430-018-0324-6>.
- Prabhu, G. S., K. G. Rao, M. and Rai, K. S. (2020) 'Hippocampal neural cell degeneration and memory deficit in high-fat diet-induced postnatal obese rats—exploring the comparable benefits of choline and DHA or environmental enrichment', *International Journal of Neuroscience*.pp. 1–12. doi: 10.1080/00207454.2020.1773819.
- Al Rajabi, A. et al., 2013. Choline Supplementation Protects against Liver Damage by Normalizing Cholesterol Metabolism in Pempt/Ldlr Knockout Mice Fed a High-Fat Diet. *The Journal of Nutrition* 144 (3), 252–257. <https://doi.org/10.3945/jn.113.185389>.
- Reddy, S., Kumar, P., Prasad, K., 2011. Histomorphometric and sympathetic innervation of the human internal thoracic artery. *Clinics* 66 (1), 131–136. <https://doi.org/10.1590/S1807-59322011000100023>.
- Rodríguez-Ortega, E. et al., 2019. Environmental enrichment during adulthood reduces sucrose binge-like intake in a high drinking in the dark phenotype (HD) in C57BL/6J mice. *Front. Behav. Neurosci.* 13 (February), 1–12. <https://doi.org/10.3389/fnbeh.2019.00027>.
- Shang, T. et al., 2017. 'Protective effects of various ratios of DHA/EPA supplementation on high-fat diet-induced liver damage in mice', *Lipids in Health and Disease*. *Lipids Health Dis.* 16 (1), 1–13. <https://doi.org/10.1186/s12944-017-0461-2>.
- Sheet, World Health Organization .Fact sheets, 16 February 2018 (2018) No Title. Available at: <https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight> (Accessed: 6 June 2019).
- Takkella, N. et al., 2018. Role of choline-docosahexaenoic acid and trigonella foenum graecum seed extract on ovariectomy induced dyslipidemia and oxidative stress in rat model. *Journal of Krishna Institute of Medical Sciences University* 7 (1), 1–7.
- Thomas, R. H. et al. (2018) 'Neurocognitive developmental outcomes in early adolescent rats prenatally exposed to choline and docosahexaenoic acid', *Journal of Clinical and Diagnostic Research*, 12(1), pp. KC01-KC05. doi: 10.7860/JCDR/2018/29563.11038.
- Thomas Rajarethnem, H. et al., 2017. Combined Supplementation of Choline and Docosahexaenoic Acid during Pregnancy Enhances Neurodevelopment of Fetal Hippocampus. *Neurology Research International* 2017. <https://doi.org/10.1155/2017/8748706>.
- Tsai, P.P. et al., 2002. Impact of environmental enrichment in mice. 1: Effect of housing conditions on body weight, organ weights and haematology in different strains. *Lab. Anim.* 36 (4), 411–419. <https://doi.org/10.1258/002367702320389071>.
- Tsamis, A., Krawiec, J.T., Vorp, D.A., 2013. Elastin and collagen fibre microstructure of the human aorta in ageing and disease: A review. *J. R. Soc. Interface* 10 (83). <https://doi.org/10.1098/rsif.2012.1004>.
- Tulis, D.A., 2007. Histological and morphometric analyses for rat carotid balloon injury model. *Methods Mol. Med.* 139 (919), 31–66. https://doi.org/10.1007/978-1-59745-571-8_2.
- Viggiano, E. et al., 2016. Effects of a High-Fat Diet Enriched in Lard or in Fish Oil on the Hypothalamic Amp-Activated Protein Kinase and Inflammatory Mediators. *Front. Cell. Neurosci.* 10 (June), 1–8. <https://doi.org/10.3389/fncel.2016.00150>.