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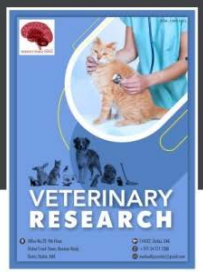

Impact of Cholesterol-Methionine Diet on Serum and Cardiovascular Oxidative Stress Parameters of Female Rabbit and Their Newborn

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Key words: Cholesterol- methionine, diet, heart, aorta, oxidative stress, pregnancy, newborn

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Abstract: The altered lipid metabolism in the placenta that is due to high maternal blood cholesterol, causes the mother and fetus deterioration. Thus, we investigated the effect of cholesterol-methionine enriched diet on serum, cardiac lipids and oxidative stress parameters of female rabbits and their offsprings. We analyze the effects of different diets administered to females 15 days before the mating and during 2 pregnancies on cardiovascular tissues and on their relative offsprings (first and second pregnancies). Thus, 3 lots are composed, a control group fed with Standard Diet (SD), one fed with cooking Oil enriched Diet (OD) and one with 1% cholesterol-0,25% Met enriched Diet (CD). Results show that CD causes newborn body weight reduction, reducing survival rate. In female plasma, total cholesterol, PL, HDL-C and LDL-C increase with CD. In plasma CD newborn, glucose and HDL-C decrease while PL and LDL-C rise. Pro-oxidant factors, MDA, AOPP, NO and anti-oxidant factors, UA, AOA, Vit C rise in CD female. Newborns subject to CD present modifications of MDA, NO, UA, AOA, Vit C, Catalase activity and Iron. In newborn cardiac tissue, OD and CD rise cholesterol and TG. The PL vary with OD. Analysis of cardiac lipids by TLC shows the involvement of PL, specialy phosphatidyl choline, lysophosphatidyl choline and sphingomyelin. In cardiac CD newborn we found a decrease of conjugated dienes, CAT, MDA and an increase of iron. Our study indicates the negative effects of CD (Chol+Met) ingested by female rabbit on serum, cardiovascular lipids and oxidative stress factors of their offspring.

INTRODUCTION

During normal pregnancy, according to the fetus's needs, the placenta transfer up to 50% of the lipids provided by maternal circulation (Coleman *et al*., 1986). In fact, cholesterol plays a major role in embryonic and fetal development. A major source may be endogenous from fetal liver or exogenous from placenta and maternal

circulation (Wadsack *et al*., 2003). The altered lipid metabolism in the placenta that is due to the high maternal blood cholesterol increases the risk of fetal vascular anomalies beguines in fetal life (Palinski *et al*., 2009). It has been proved that maternal hypercholesterolemia can increase the risk of cardiovascular diseases among children and adult lives (Napoli *et al*., 1997). Evidence from different experimental animal models showed that the exposure of embryos before implantation under disadvantageous developmental conditions as under-nutrition, over-nutrition and inflammation both *in vitro* and *in vivo* can not only hinder the embryonic quality and implantation success but also exert more subtle effects that are expressed later during intrauterine development or even during adulthood (Williams *et al*., 2011; Luzzo *et al*., 2012). A recent study has shown that exposition to hypercholesterolemia before implantation can alter embryo growing. Results indicate that maternal nutrition affects embryo gene expression in the very early stages of pregnancy (Howie *et al*., 2008; Picone *et al*., 2010). Other studies indicate that disturbing could modify the cell allocation in blastocystes (Fleming *et al*., 2004), restriction in fetal growth (Maloney and Rees, 2005) and modification in placental morphogenesis (Watkins *et al*., 2007). The research in humans and animal models is focused on specific mechanisms for in utero programming (Vuguin, 2007) existing evidence for utero programming for hypercholesterolemia (Palinski, 2001) including increased maternal oxidative stress (Liguori *et al*., 2008) and altered adaptative immune response to oxidized LDL (Yamashita *et al*., 2006). Some blood parameters can be modified in pregnancy status by homocysteinemia that can be caused by methionine diet (Hirche *et al*., 2006) that decreases during the first and second trimester and rises a little in the end of pregnancy (Walker *et al*., 1999). One of explanation is the utilization of maternal homocysteine by the fetus (Malinow *et al*., 1998). In recent years several complications have been attributed to hyperhomocysteinemia during pregnancy like repeated miscarriages, preeclampsia, neuronal tube defects, abortion placenta, intra uterine growth retardation and fetal death (Goddijn-Wessel *et al*., 1996).

Anterior works conducted with rats fed seven different types of dietary proteins suggest that the dietary content methionine influences cholesterolemia at least in part through alteration of hepatic phospholipids metabolism (Sugiyama *et al*., 1996). In our study, we report the effect of the combined high cholesterol and methionine diet on rabbit newborns tissues taking into account the prior conception and gestational maternal state.

MATERIALS AND METHODS

Animals: A total of forty local domestic female rabbits aged (6-8 months) weighted (2-2, 8 kg) are used in our study. The animals were placed in animal caging system. Temperature ambient (22-25°), light /dark cycle (12 h) and humidity was controlled. The animals had 150 g of food per day and the access to water ad libitum. All experiments were carried out in compliance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) following approval by the local Ethical Committee of the Houari Boumediene University of Sciences and Technology, Algeria.

Diet: Females are divided into 3 groups: SD group (Standard chow diet group, n = 6) fed with a standard chow (41.8% Lucerne, 28% Bran, 23% Barely, 2.7% Corn, 3.5% Soy, 1% Mineral-Vitamin complement), OD group (Oil Diet group, n = 15) fed with SD+2 mL cooking oil (55% sunflower, 40% soy, 5% olive) per day per animal, CD group (Combined Diet group, n = 19) fed with SD+1% Cholesterol (DL-cholesterol 95%, ALFA AESAR®, United Kingdom) and 0,25% methionine (DL-methionine, sigma®) per day per animal; the cholesterol was dissolved in cooking oil.

Experimental protocol: The females are submitted to an acclimatization period of 15 days, fed the standard chow. After this period and through 80 days, they received too their respective diet 15 days before mating and during 2 successive pregnancies. The females were followed by weight monitoring and blood was taken from marginal vein. At the end of experimentation, the females are weighted, sacrificed by decapitation, their blood and tissues (heart, aorta, liver) are also collected. The same procedure is performed for the newborns (0-6h. old) for 2 pregnancies of each animal group.

Blood was centrifuged and kept in (-40°C) for biochemical assays. Female and newborn left ventricular hearts, thoracic aorta and liver fragments have been: frozen in -40°C for oxidant stress assays, kept in Folch solution (1 V chloroform/2 V methanol) (Sigma-Aldrich®, Germany) for lipid determination.

Biochemical assays: Glycemia (GLY), lipids assays from blood and tissues like Total Cholesterol (TC), Triglycerides (TG) and cholesterol in High and low Density Lipoprotein (HDL-c, LDL-c) were assayed by kits (Sprinreact®, Spain). Serum and tissue phospholipids were assayed respectively according to Folch *et al*. (1957) and Rouser *et al*. (1970). Blood and tissue oxidant and antioxidant parameters were measured as follows: uremia (Sprinreact®, Spain), Malonyldialdehyd (MDA) (Buege and Aust, 1978), Conjugated Diene (CD) (Knight and Voorhees, 1990), Nitric Oxides (NO) (modified Griess method), Advanced Oxides Protein Products (AOPP) (Witko-Sarsat *et al*., 1996) Serum and tissue Catalase Activity (CAT), respectively by Aebi *et al*. (1984) and Claiborne *et al*. (1995), Vitamin C (Vit C) by Jagota and

Dani (1982), Antioxidant Activity (AOA) by Koracevic *et al*. (2001), serum and tissue iron by Barry and Sherlock (1971).

Thin Layer Chromatography (TLC): Neutral lipids and phospholipids thin layer chromatography were performed in accordance with respectively Prabha *et al*. (2008) and Skipski *et al*. (1962).

Statistical analysis: Data are expressed as means±SD. Statistical differences among groups were determined using ANOVA test with $p<0.05$ considered statistically significant.

RESULTS

In our research three groups of female rabbits received different kind of diet, the standard diet which represents the control group diet (SD), a second group supplemented with cooking oil which constitutes an incipient for cholesterol (OD) and a third group supplemented with cholesterol and methionine, named Combined Diet (CD). The offspring of these females keep the same name.

Combined diet (Chol+Met) has an impact on the birth weight and on newborn biochemical parameters: Combined diet causes a reduction of 10% in the female body weight compared to the control (Table 1). In the newborns the weight reduction varies between 7% and 29% under oil diet and 52% under combined diet, explaining the low survival rate (30%) (Table 2). During gestation with standard diet all parameters of females, such as blood glucose, triglycerides, HDL-C remained stable while with combined diet glycemia rises by 33% (Table 1). Plasma lipids appear to have been affected by our combined diet, so, we note, comparatively to control, an increase (240%) in total cholesterol and (200%) in phospholipids (Table 1 and Fig. 1). Compared to those of standard diet, HDL-C and LDL-C rise by combined diet in 130 and 170, respectively.

Newborns show a decrease of 38 and of 72% in glycemia under oil diet and combined diet, respectively (Table 2). Glycemia of second pregnancy newborn increases slightly. Phospholipids of combined diet newborns of first and second pregnancies rise more that 115 %. Triglyceridemia offsprings seems to be essentially affected by oil diet (rise of 132%). Newborns of second pregnancy under combined diet show a rise of 89% in LDL-C and a decrease of 55% in HDL-C; this evolution explains the nature of atherogenic combined diet administered newborn and decreases (59%) in the first pregnancy combined diet newborn. The Vit C and Iron decrease in combined diet newborn of 22 and 69%, respectively.

The combined diet could affect the newborn serum oxidative stress: Plasma of pregnant rabbit show a decrease of some pro -oxidant parameters such as MDA, AOPP, NO and anti-oxidant such as UA (Table 1). These parameters rise with combined diet, the increase is 140% in MDA, 435% in AOPP, 254 % in NO and 300% in UA. Conjugated dienes and Iron seem no affected by diets, while AOA and Vit C seem much more sensitive by the diet and register an increase from 55-60%, respectively. Our study shows that diets with oil and combined (Chol+Met) appear to have an impact on the oxidative stress parameters. So, when the newborns were subject of combined diet in the first of pregnancy it led an increase in pro-oxidant such as MDA (283%) compared to the control group, this parameter decreases in the second pregnancy but still very high relative to the control value (Table 2 and Fig. 2). No. of combined diet newborn rises in 155% in first pregnancy and then decrease in the second. The other antioxidant factors such as the UA, AOA, Vit C and Iron seem to be affected by the combined diet. The total antioxidant activity increases by 64% for newborns in the first pregnancy and decreases 60% in the second pregnancy compared with the control. Catalase activity rises (74%) in oil diet.

The cardiovascular lipids and oxidative stress parameters of combined diet newborn: Even if the heart is considered the organ that has less possibility of alteration, it deals with different high fat diets compared to the liver, it appears that the combined diet and even oil diet caused variation in cardiovascular lipid metabolism, especially in offspring. In cardiac tissue, we note a rise in total cholesterol and triglycerides in female oil diet by 350 and 635%, respectively and in female combined diet by 113% (Chol) and 145% (TG) (Table 3 and Fig. 3). The addition of methionine in combined diet appears to reduce the harmful effect of cholesterol. For their part, PL increases in female combined diet by 79%. In female aorta tissue, combined diet decreases the levels of TC, TG, PL by 62, 67 and 31%, respectively (Table 3 and Fig. 3).

In newborn cardiac tissue, oil diet rises cholesterol level by 200%, while combined diet causes a rise of 99% in the first pregnancy and a rise of 400% in the second pregnancy (Table 4 and Fig. 4). Likewise, TG register more augmentation in combined diet (450%) than in oil diet (354%) compared to control. Whereas PL are subject of fluctuations in oil diet, they register a constant rise, reaching 233% in second pregnancy. Biochemical results are consistent with TLC results that show the involvement of the PL in cardiac tissue, especially those presenting choline as phosphatidyl choline, lysophosphatydil choline and sphingomyelin (Fig. 3 and 4). Oxidant and antioxidant parameters seem to have been affected by the combined diet in the cardiac tissue. So, it appears that in

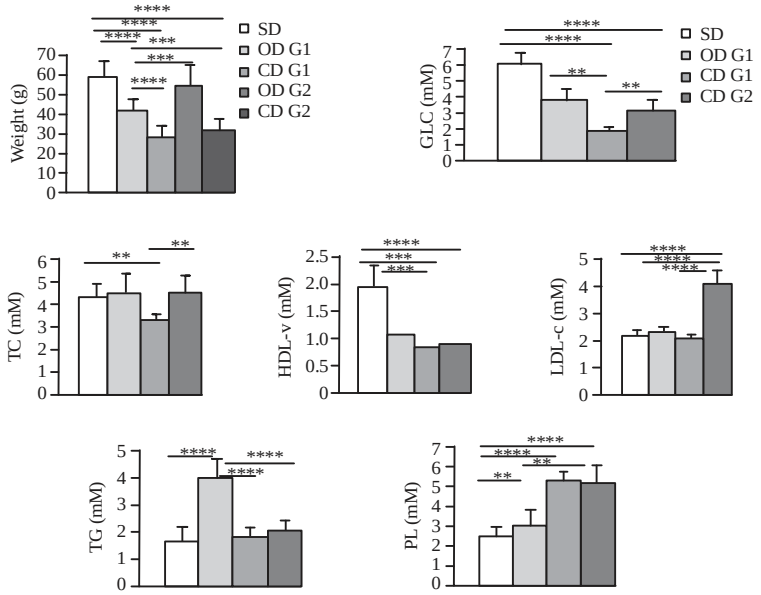

Fig. 1: Newborn serum biochemical parameters and weight (g), (mM.) GLY: Glycemia, TC: Total Cholesterol, TG: Triglycerides, PL: Phospholipids, HDL-C: Cholesterol High Density Lipoproteins, LDL-C: Cholesterol Low Density Lipoproteins: SD: Newborn from standard diet female (n = 9), OD1: newborn from first pregnancy of oil diet females (n = 6), CD1: newborn from first pregnancy of combined diet female (n = 4). CD2: Newborn from second pregnancy of combined diet female (n = 7) (*p<0.05)

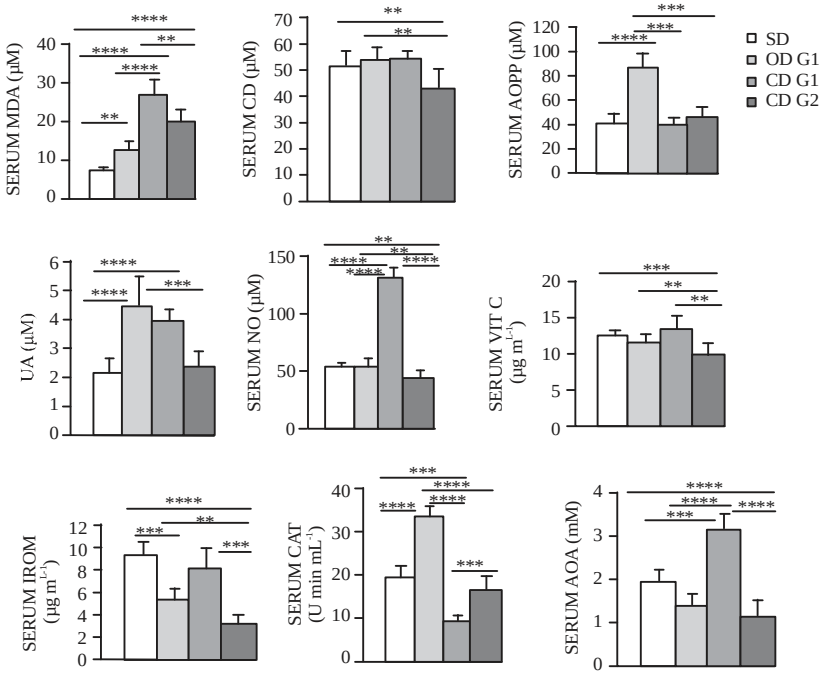

Fig. 2: Newborn serum oxidant stress parameters. (µM) MDA: Malonyl Dialdehyde, CD: Conjugated Diene, AOPP: Advanced Oxidized Protein Products, UA: Uric Acid, NO: Nitric Oxide, AOA (mM): Antioxidant Activity, CAT (U min^{-1} mL^{-1}): catalase activity, Vit C (µg mL^{-1}): vitamin C and total serum Iron (mg L^{-1}). SD: Newborn of standard diet female (n = 9), OD1: Newborn from first pregnancy of oil diet female (n = 6), CD1: Newborn from first pregnancy of combined diet female (n = 4). CD2: Newborn from second pregnancy of combined diet female (n = 7) (*p<0.05)

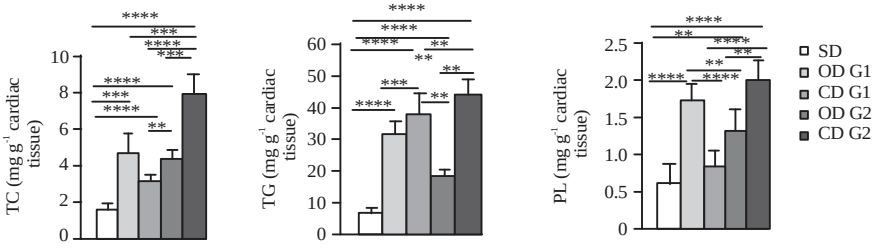

Fig. 3: Newborn cardiac lipids content (mg g^{-1}. tissue). TC: Total Cholesterol, TG: Triglycerides, PL: Phospholipids. SD: Standard Diet (n = 10), OD1: Oil Diet (n = 8), CD1: Combined Diet (n = 4). OD2 (n = 6), CD2 (n = 6). MEAN±SEM (*p< 0.05)

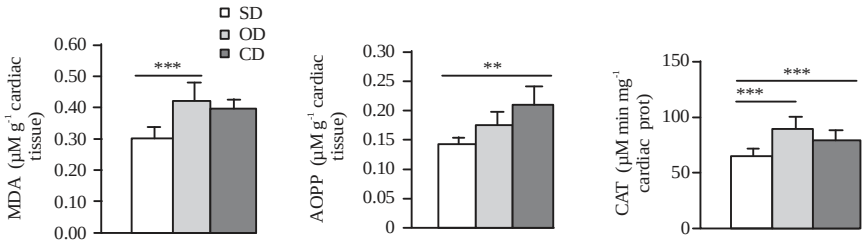

Fig. 4: Maternal cardiac oxidative stress parameters (µM g^{-1} tissue) MDA: Malonyl Dialdehyde, NO: Nitric Oxide, AOPP: Advanced Oxidized Protein Products (µM min^{-1} mg^{-1} prot.) CAT: catalase activity (mg/g tissue) total Iron. SD: standard diet (n = 5), OD: Oil diet (n = 5), CD: Combined Diet (n = 7). MEAN±SEM (*p< 0.05)

Table 1: Maternal serum parameters and weight

Maternal SERUM	Biochimical parameters							
	Weight (kg)	GLY (mM)	TC (mM)	TG (mM)	PL (mM)	HDL-C (mM)	LDL-C (mM)	AI
T0 SD	2.5±0.3	5.82±0.14	2.08±0.47	1.17±0.26	1.39±0.04	0.42±0.07	1.43±0.08	////
OD	2.5±0,3	5.59±0.36	2.11±0.36	1.03±0.23	1.31±0.16	0.44±0.09	1.46±0.36	////
CD	2.4±0.1	6.02±0.26	1.93±0. 40	1.31±0.22	1.28±0.10	0.40±0.07	1.47±0.14	////
TF SD	3.6±0.2****	5.67±0.54	1.47±0.28	0.90±0.12	1.18±0.42	0.36±0.05	0.93±0.26****	4.07
OD	2.9±0.3**	4.30±1.33	2.59±0.13°°	1.13±0.20	1.33±0.20	0.80±0.11****°°°°	1.56±0. 16°°	3.24
CD	2.9±0.3****	7.59±1.10 ****°°°ΔΔΔ	5.02±0.70 ******°°°ΔΔΔΔ	1.06±0.37 ***°°°° ΔΔΔΔ	3.53±0.33 ****°°°	0.83±0.10 3.97±0.64 ***°°°ΔΔΔ		6.03

Oxidative stress parameters	SD		OD		CD	
	T0	TF	T0	TF	T0	TF
MDA (µM)	2.24±0.53	1.35±0.31***	2.13±0.46	2.80±0.49°°	2.35±0.61	3.25±0.72°°°
CD (µM)	62.78±10.20	99.97±18.01****	61.09±9.41	127.91±15.66****	66.04±13.44	128.97±17.71***
AOPP (µM)	35.65±8.16	14.79±2.40****	33.87±10.91	15.09±4.46**	37.17±6.00	79.15±18.99 ****°°°°ΔΔΔΔ
UA (µM)	147.36±20.01	29.53±4.79****	//////////////	73.74±13.78******°°°	//////////////	119.28±11.76 °°°°ΔΔΔ
NO (µM)	47.11±4.35	42.32±5.58****	55.00±10.59	70.12±16.70°°	54.29±3.30	149.35±22.86 ****°°°°ΔΔΔ
AOA (mM)	1.23±0.27	1.55±0.33	1.04±0.21	1.03±0.22°°	1.42±0.30	2.41±0.27 ****°°°ΔΔΔΔ
CAT (U/min/ml)	19.57±2.17	20.37±3.04	18.14±1.55	39.14±5.20****°°°°	20.35±1.94	22.12±2.98 ΔΔΔΔ
VITC (µg/ml)	21.49±3.11	18.93±2.72	19.73±3.20	25.90±1.59**°°	23.60±1.05	30.54±1.46 ****°°°°ΔΔΔ
IRON (mg/l)	1.67±0.24	1.74±0.21	1.74±0.22	1.65±0.40	1.59±0.24	1.65±0.37

Weight (kg, (mM) GLY: Glycemia, TC: Total Cholesterol, TG: Triglycerides, PL: Phospholipids, HDL-C: Cholesterol High Density Lipoproteins, LDL-C: Cholesterol Low Density Lipoproteins. AI: Atherogenicity Index (AI = 4,.5).MDA: Malonyldialdehyde, CD: Conjugated Diene, AOPP: Advanced Oxides Protein Products, UA: Uric Acid, NO: Nitric Oxide, AOA (mM): Antioxidant Activity, CAT (U min^{-1} mL^{-1}): catalase activity , Vit C (µg mL^{-1}): vitamin C and total serum iron (mg L^{-1}). SD: Standard Diet (n = 6), OD: Oil Diet (n = 7), CD: Combined Diet (n = 8). MEAN±SEM.; (p<0.05) (*TF/T0) (°/TF/SD) (Δ TF/OD)

female rabbit, AOPP and CAT increase by 50 and 26%, respectively, compared to the control diet (Table 5, Fig. 5) whereas in newborns it's noted a decrease in CDs, CAT, specifically MDA which decreases by 33% at the first gestation and 41% to the second (Table 6 and Fig. 6). Finally, iron level in cardiac tissue increases in combined diet newborn by 300% in the first pregnancy and reduces slightly in the second pregnancy.

Table 2: Newborn serum parameters and weight

	Biochimichal parameters				
Serum newborns	SD	OD G1	CD G1	OD G2	CD G2
Survival rate (%)	100%	97%	15%	67%	39%
SEXE ♂%	62.5%	53.3%	30.0%	61.5%	35.0%
♀%	37.5%	46.7%	70.0%	38.5%	65.0%
Weight (g)	57.9±8.3	40.8±6.3	27.7±6.1	53.8±10.5	30.4±6.9
GLY (mM)	6.03±0.59	3.73±0.78	1.68±0.24	////	3.12±0.76
TC (mM)	4.17±0.60	4.38±0.88	3.20±0.28	////	4.35±0.81
TG (mM)	1.69±0.42	3.93±0.63	1.72±0.35	////	1.96±0.38
PL (mM)	2.45±0.53	3.00±0.79	5.31±0.41	////	5.15±0.91
HDL-C (mM)	1.91±0.41	1.05±0.37	0.82±0.17	////	0.85±0.23
LDL-C (mM)	2.12±0.20	2.23±0.19	2.00±0.11	////	4.01±0.47
AI	2.19	4.17	3.92	////	5.12
	Oxidative stress parameters				
	SD	OD 1	CD 1	CD 2	
MDA (µM)	6.85±0.98	12.24±2.23	26.39±4.11	19.79±3.07	
CD(µM)	51.32±5.65	53.54±4.70	53.87±3.11	42.24±7.59	
AOPP (µM)	39.44±7.97	86.03±12.16	38.55±4.50	45.47±9.32	
UA (µM)	119.86±28.32	257.11±47.81	233.75±21.25	138.43±27.00	
NO (µM)	51.14±5. 37	52.96±7.58	130.51±9.83	40.48±5.88	
AOA (mM)	1.89±0.31	1.39±0.26	3.11±0.37	1.14±0.35	
CAT (U/min/ml)	19.15±2.96	33.04±2.63	8.97±1.53	16.20±3.31	
VIT C (µg/ml)	12.18±0.91	11.23±1.45	13.33±1.78	9.48±1.74	
IRON (mg/l)	9.14±1.29	5.38±0.89	7.98±1.83	2.97±0.99	

Weight (g), newborns survival rate (%) and rate of sexes between diet groups (%) ,(mMGLY: Glycemia, TC: Total Cholesterol, TG: Triglycerides, PL: Phospholipids, HDL-C: Cholesterol High Density Lipoproteins, LDL-C: Cholesterol Low Density Lipoproteins. AI: Atherogenicity Index (AI = 4,85). (µM) MDA: Malonyldialdehyde, CD: Conjugated Diene, AOPP: Advanced Oxides Protein Products, UA: Uric Acid, NO: Nitric Oxide, AOA(mM): Antioxidant Activity, CAT (U min mL^{-1}): catalase activity , Vit C (µg mL^{-1}): Vitamin C and total serum Iron (mg L^{-1}) SD: Newborns from Standard Diet female (n = 9), OD1: newborns from first pregnancy of oil diet female (n = 6), OD2: newborns from second pregnancy of oil diet female (n = 8), CD1: Newborns from first pregnancy of combined diet female (n = 4); CD2 : Newborns from second pregnancy (n = 7); MEAN±SEM

Table 3: Maternal cardiac and aortic lipids

Maternal tissues lipids			
Heart	TC (mg g^{-1} tissue)	TG (mg g^{-1} tissue)	PL (mg g^{-1} tissue)
SD	1.53±0.45	2.56±0.58	0.113±0.014
OD	6.93±1.62°°°°	18.83±2.76°°°	0.136±0.024
CD	3.27±1.16°°°ΔΔΔ	6.29±2.33°°ΔΔΔΔ	0.203±0.033°°
AORTA			
SD	43.16±1.21	117.91±21.71	0.837±0.070
OD	21.64±4.06°°°°	49.92±15.90°°°	0.715±0.184
CD	14.14±3.52°°°°ΔΔ	44.70±12.54°°°	0.575±0.176

TC: Total Cholesterol, TG: Triglycerides, PL: Phospholipids. SD: Standard Diet (n = 5), OD: Oil Diet (n=5), CD: Combined Diet (n = 7). MEAN± SEM ($p<0.05$) (°/SD)(Δ/OD)

Table 4: Newborn cardiac parameters

Newborns cardiac lipids	SD	OD G1	CD G1	OD G2	CD G2
Weight (Heart/body) (%)	0.73±0.08	0.73±0.08	0.75±0.06	0.99±0.09	0.85±0.13
TC (mg g^{-1} tissue)	1.54±0.38	4.63±1.06	3.07±0.36	4.28±0.42	7.76±1.11
TG (mg g^{-1} tissue)	6.99±0.99	31.78±4.28	38.42±6.74	18.24±2.56	44.95±4.62
PL (mg g^{-1} tissue)	0.603±0.255	1.736±0.202	1.303±0.306	0.860±0.181	2.000±0.265

Relative weight (%) and lipids content (mg g^{-1}. tissue). TC: Total Cholesterol, TG: Triglycerides, PL: Phospholipids. SD: Standard Diet (n = 10), OD1: Oil Diet (n = 8), CD1: Combined Diet (n = 4). OD2 (n = 6), CD2 (n = 6); MEAN±SEM

Table 5: Maternal cardiac oxidative stress parameters

Maternal cardiac parameters	SD	OD	CD
MDA (µm g^{-1} tissue)	0.30±0.03	0.42±0.06	0.40±0.03
CD (µm g^{-1} tissue)	0. 44±0. 07	0. 48±0. 07	0. 52±0. 08
NO (µm g^{-1} tissue)	10.43±1.00	9.53±0.49	9.51±0.58
AOPP (µm g^{-1} tissue)	0.14±0.017	0.17±0.026	0.21±0.036
CAT (µm min mg^{-1} prot)	60.98±9.72	87.17±12.87	77.01±10.33
IRON (mg g^{-1} tissue)	0.025±0.004	0.029±0.003	0.028±0.004

MDA: Malonyl Dialdehyde ,CD: Conjugated Diene, NO: Nitric Oxide, AOPP: Advanced Oxides Protein Products.(µm min mg^{-1} prot) CAT: catalase activity,(mg g^{-1} tissue) total iron..SD: Standard Diet (n = 5), OD: Oil diet (n =5), CD: Combined Diet (n = 7). MEAN±SEM

Table 6: Newborn cardiac oxidative stress parameters

Newborns cardiac parameters	SD	OD G1	CD G1	ODG2	CD G2
MDA (µm g^{-1} tissue)	0.24±0.04	0.26±0.04	0.16±0.05	0.22±0.03	0.14±0.03
CD (µm g^{-1} tissue)	0.47±0.05	0.60±0.06	0.29±0.04	0.44±0.07	0.35±0.05
NO (µm g^{-1} tissue)	9.34±1.16	9.58±0.77	10.60±0.56	8.04±0.84	9.18±0.49
CAT (µm min mg^{-1} prot)	58.33±8.15	63.33±9.01	61.21±8.11	43.50±4.98	43.56±8.97
IRON (mg g^{-1} tissue)	0.06±0.01	0.10±0.02	0.25±0.03	0.15±0.03	0.09±0.01

MDA: Malonyl Dialdehyde; CD: Conjugated Diene; NO: Nitric Oxide; (µm min mg^{-1} prot) CAT: Catalase Activity, (mg g^{-1} tissue total iron; SD: Standard Diet (n = 10), OD1: Oil Diet (n = 8), CD1: Combined Diet (n = 4). OD2 (n = 6), CD2 (n = 6); MEAN±SEM

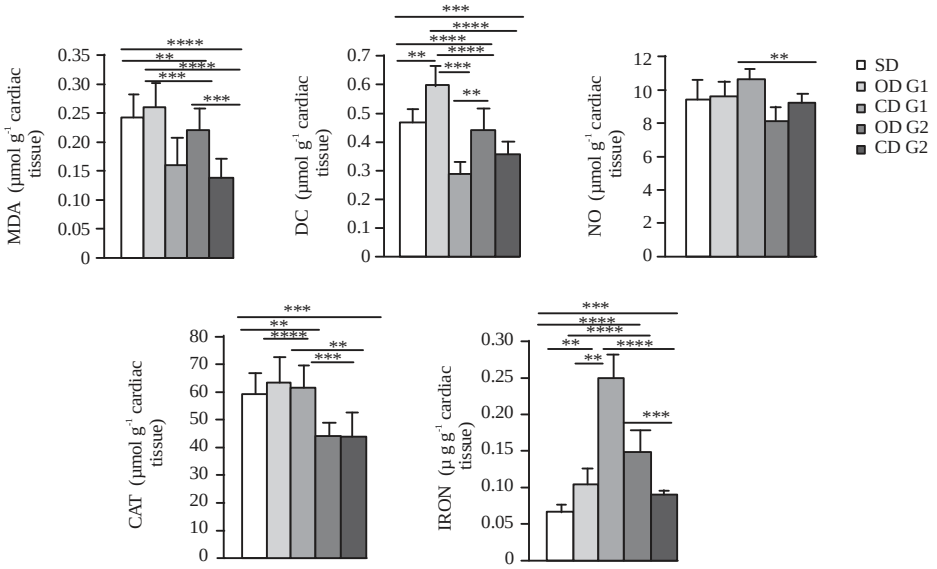

Fig. 5: Newborn cardiac oxidative stress parameters (µM g^{-1} tissue) MDA: Malonyldialdehyde, CD: Conjugated diene, NO: Nitric Oxide, (µM min^{-1} mg^{-1} prot) CAT: Catalase Activity, (mg g $^{-1}$ tissue), total iron. SD: Standard Diet (n = 10), OD1: Oil Diet first pregnancy (n = 8), CD1: Combined Diet first pregnancy (n = 4). OD2 (n = 6), CD2 (n = 6); MEAN±SEM; (*p<0.05)

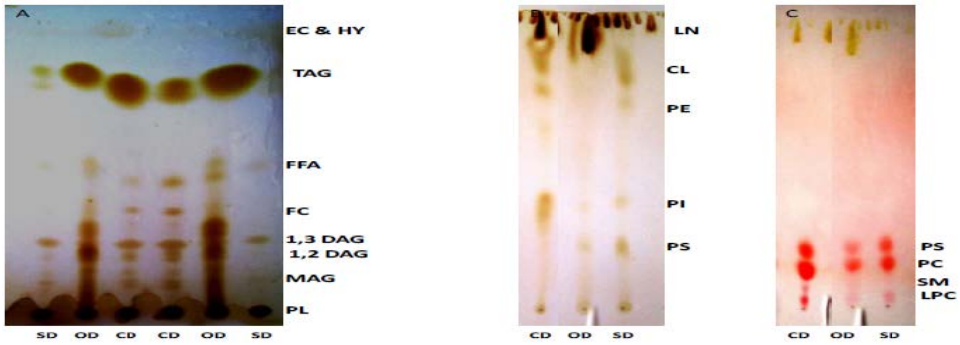

Fig. 6: Maternal cardiac lipids separated by thin layer chromatography; A) Neutral lipids detection by iode vaporization, PL: Phospholipids, MAG: Mono-Acylglycerol, (1,2) DAG,: 1, 2 Di-Acylglycerol, (1, 3) DAG1,3 Di-Acylglycerol, FC: Free Cholesterol, FFA: Free Fatty Acid, TAG: Triacylglycerol, EC&HY: Esterified Cholesterol and Hydrocarbures; B) Phospholipids detection by iode vaporization. PI: Phosphatidyl Inositol, PS: Phosphatidyl Serine and C) Phospholipids detection by ninhydrine. PC: Phosphatidyl Choline, SM: Sphingomyeline, LPC: Lysophsphatidyl Choline. SD: Standard Diet, OD: Oil Diet, CD: Combined Diet

DISCUSSION

The combined diet (Chol+Met) has an impact on the birth weight and newborn biochemical parameters: Our results demonstrate an increase in body weight of the female during pregnancy as found by anterior works from our laboratory. This increase has been also found in a female submitted to combined diet (Chol+Met). This can be for a specificity of the local breed. The weight of combined diet newborn reduces according to Montoudis *et al*. (2003) and Picone *et al*. (2010) results. This decrease was associated with an excessive accumulation of lipids in the placenta, suggesting possible interference with nutriments transport to the fetus (Montoudis *et al*., 2007). While other authors (Palinski *et al*., 2002) suggested no influence of maternal diet on newborn.

Anterior study showed that blood glucose, TG, HDL-C and LDL-C remain stable and are not affected and their value is found normal in late pregnancy (Picone *et al*., 2011). This is confirmed by our results of control parameters which did not change on the pregnancy.

In normal pregnancy glycemia homeostasis is maintained by insulin synthesis (Di Cianni *et al*., 2003) but could be affected at the end of pregnancy by high cholesterol diet (Hirche *et al*., 2006). Results confirmed in female rabbit under combined diet (chol+Met.) while newborn from this same diet showed hypoglycemia. Fetal glycemia is bound to maternal glycemia (fetal hepatic immaturity), observed by Schneider *et al*. (1981). Taylor *et al*. (2004) showed abnormal glucose metabolism in rat pregnant offsprings fed with a high fat diet. In fact, maternal hypercholesterolemia affect placental glucose facilitated transport and mediates for active transport (Kevorkova *et al*., 2006).

Cholesterol uptake can be useful for the synthesis of steroid hormones during pregnancy like progesterone synthesis (Kriesten and Murawski, 1988) but lipid accumulation during pregnancy may suppress trophoblast invasion and placental development, lipid metabolism and transport in the fetus (Jarvie *et al*., 2010). Females submitted to the combined diet show an increase in serum total cholesterol. It has been suggested by anterior studies that a diet enriched with cholesterol induces an increase in the level of total cholesterol in rabbits (Huang *et al*., 2012; Thangaraj *et al*., 2013) and humans (Liu *et al*., 2006). It has been explained by the modification in the hepatic and placental enzymes involved in the cholesterol metabolism (i.e., HMG-COA, ACAT, cholesterol 7a hydroxylase) (Montoudis *et al*., 2003).

It has been noted that offsprings from high cholesterol diet pregnant express a high level of serum cholesterol and triglycerides (Montoudis *et al*., 2003; Picone *et al*., 2010). Another study showed its decrease (Aliev *et al*., 1993). Moreover, another studies working about high cholesterol diet doesn't demonstrate a modification in offsprings plasmatic level (Palinski *et al*., 2007). According to our study it neither reveals an increase of this parameter in plasma of oil diet newborn nor in female combined diet.

The triglycerides which constitute the major storage of lipid intermediates are usually employed as indicators of intracellular lipid accumulation (Di Cianni *et al*., 2004; Khan, 2007). It has been shown that with 1% high cholesterol diet, female rabbit triglycerides remained stable (Montoudis *et al*., 2003) as obtained by our results in the combined diet females. The last days of rabbit pregnancy, lipolysis is stimulated (Liu *et al*., 2006). Another study suggests the implication of LPL (Martinez *et al*., 2004) and notes an increase of triglycerides in hypercholesterolemia (Frantz *et al*., 2011; Huang *et al*., 2012). Also an hyperlipidic diet study, done with WHHZ rabbit newborn, the results are in agreement with ours, these ones suggest the increase of TG in oil diet female (Aliev *et al*., 2005).

In maternal cardiac tissue, our results showed an increase in TG and total cholesterol under oil diet and not with the combined diet compared to the control, meanwhile it's seems that phospholipids are involved in the combined diet. Whereas in the maternal aortic tissue, lipid decrease in each combined diet compared to the oil diet and compared to the control. Our results concord with a previous study which proved that rabbit males where subject to 1% of cholesterol diet showed a decrease in the aortic total cholesterol.

In newborn heart (left ventricle) we observed an increase in triglycerides, total cholesterol and phospholipids with oil and combined (Chol+Met) diets. Our study shows a decrease of HDL-C and LDL-C in normal pregnancy, this result concord with previous studies (Montoudis *et al*., 2003; Marseille-Trembley *et al*., 2007). It has been reported that LDL-C decrease could be related to the increase of estrogen observed in the end of pregnancy, since, it induces LDL-R expression (Kriesten and Murawski, 1988). Therefore, it has been proven that with high fat and high cholesterol diet both lipoproteins increase (Picone *et al*., 2010; Frantz *et al*., 2011; Huang *et al*., 2012) as observed in our study in combined diet females. Some studies suggested that the increase in LDL concentration in the maternal plasma induces the regulation of the LDL receptor expression in the whole placenta (Etheir-Chiasson *et al*., 2007). Whence, the importance of prenatal control of lipoproteins level as a preventive factor against the early development of atherosclerosis (Frantz *et al*., 2011). However, it has supported that the atherogenic particle in the cholesterol fed rabbits is not simply LDL but predominantly B VLDL and IDL (Craeyveld *et al*., 2010). It depends also to the topographic site and correlation of

the extent of atherosclerosis between different sites is dependent on the type of hyperlipidemia (Craeyveld *et al*., 2011). Maternal HDL-C maintains sterols balance in extra-embryonic tissues that helps to fetal growth and development (Mc Conihay *et al*., 2001). In newborn of diet combined female, we noted that HDL-C decreases while LDL-C increases, according to a previous study (Montoudis *et al*., 2002) that showed an increase in LDL.

The combined diet could affect the newborn serum oxidative stress parameters: The plasma free radicals trapping and antioxidant potential are able to counteract oxidative stress in normal pregnancy through enzymatic induction and activity (i.e., catalase, SOD), through a non enzymatic free radical protectors and scavengers also (i.e., Vit C and E, uric acid, protein thiol) (Myatt and Midovnik, 1999; Kharb *et al*., 2002). Our results obtained in the female control group involved the decrease of serum MDA, AOPP and UA in normal pregnancy. These results concord with a previous study, that indicate a decrease of serum MDA in normal pregnancy of NWZ rabbit (Napoli *et al*., 2000). Whereas, in the maternal hyper-cholesterolemia end products could directly or indirectly increase lipid peroxidation products in fetal plasma and fatty streak formation in the fetal aorta (Napoli *et al*., 2000). Our study mentioned an increase of MDA and AOPP, like observed by Palinski *et al*. (2007) in hypercholestrolemic NWZ females and in hypercholesterolemic women (Liu *et al*., 2006) after breeding. The source of MDA was not yet determined, one way speculated is the role of oxides lipoproteins (Ferdinandy *et al*., 2002) in addition to that, it has been shown that the high level of cholesterol and oxides LDL impair endothelial function. The conjugated diene increase registered in each experimental group of rabbit female proved their importance and implication in the maternal oxidative stress after breeding.

In the other hand, serum NO, AOA, Vit C increased in oil diet females. A recent study showed that exposure to excess fat diet during pregnancy and suckling reduces endothelial NO dependant vaso-relaxation through unbalance production of reactive species scavengers in the offprings (Torrens *et al*., 2012). In fact, some data suggest that enhanced activities of antioxidant enzymes with gestational ages may act protective mechanism against oxidative stress during early human and sheep placental growth and development (Al Gulbary *et al*., 2010). The serum total iron stay unchanged in each groups of our study, it agrees with the results of Araujo *et al*. (1995) which mentioned no plasmatic change of iron with any type of diet.

The measuring of oxidative stress markers lipids peroxidation and total antioxidant activity has been found to have a good correlation between the oxidative status of the mother and the neonates (Arguelles *et al*., 2006). So, the increase of oxidative stress indicators in the maternal serum conducts to the increase in the newborns. Thus, our study showed enhanced serum MDA, NO, UA, AOA in the combined diet newborn of the first pregnancy. While, conjugated diene, NO, AOA, CAT, Vit C, iron decreased in the second pregnancy. We can suggest the implication of the repetitive pregnancies on the oxidative status in hypercholesterolemic rabbit. Whereas, the AOPP, UA and CAT shown a positive response in oil diet newborns. Thus, the excess free fatty acid or lipotoxicity may also produce an unfavorable effect on embryonic metabolism and growth (Jungheim *et al*., 2011). This lipotoxicity may activate a number of cell stress cascades stress to exacerbate insulin resistance at later stage of post natal life (Boden *et al*., 2005). Moreover, this could stimulate the release of PAF which increases inflammatory cytokine synthesis known to stimulates the oxidative stress product by the nuclear leucocytes (Martinet *et al*., 2007).

It has been proposed that the ascorbic acid is a strong antioxidant capable of scavenging a wide variety of reactive oxygen and nitrogen species (Halliwell, 1996) and a protector against oxidative degradation of collagen, cyt P450 mediated lipid peroxidation and oxidation of protein. Our results show this importance in hypercholesterolemia by the significant increase in the serum of combined diet females and their newborns. Also, a direct relationship between the concentration as well as, the total content of iron storage in the fetal liver with the maternal plasma has been shown (Gambling *et al*., 2011). Previous data suggest that cholesterol diet altered metabolism of iron (Turbino-Ribeiro *et al*., 2013) like observed in our enhanced serum total iron in newborn. Thus, it has been proposed that the iron can also induce LDL peroxidation and increase their atherogenic potential (Dabbagh *et al*., 1997). Some studies proved that the iron could induce aggregation (thromboxane B2 and PKC) in a dose-dependent manner and may also acts as a NO scavenger to induce endothelial dysfunction (Day *et al*., 2003).

The cardiovascular lipids and oxidative stress parameters of combine diet newborns: Our results have shown that the combined diet (Chol+Met) acts negatively on the cardiac system. Firstly, in maternal cardiac tissue, triglycerides and total cholesterol increase in oil diet and not in the combined diet (compared to the control) while phospholipids seem to be involved in the combined diet.

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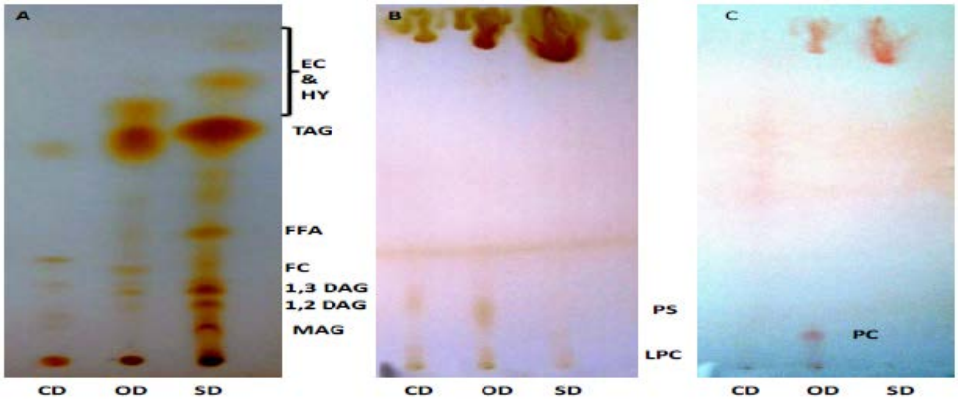

Fig. 7(A-C): Maternal aortic lipids separated by thin layer chromatography; A) Netural lipids bi iode vaporization; PL: Phospholipids; MAG: Mono-aclgycerol, (1, 2) DAG: 1, 2 Di-Acylgycerol, (1, 3) DAG 1, 3 Di-Acylglycerol; FC: Free Cholestrol; FFA: Free Fatty A,cid; TAG: Triacylgylycerol, EC&HY: Esterified Cholestrol and Hydrocarbures; B) Phospholipids detection by iode veporization; PI: Phosphatidyl Inositol; PS: Phosphatidyl Serine; LPC: Lysophosphatidyle Choline and C) Phospholipids decetion by ninhydrine; PC: Phosphatidyl choline; SD Standared Diet; OD: Oil diet; CD: Combined diet

Whereas, in the maternal aortic tissue lipids decrease in combined diet compared to the oil diet and to the control. Our results concord with the previous study on rabbit males submitted 1% of cholesterol diet it showed a decrease in the aortic total cholesterol. In our newborns we observed an increase in the cardiac lipid fractions (triglycerides, total cholesterol and phospholipids) in the oil and combined diet. It has been proved that the maternal hypercholesterolemia induces early predictors of cardiovascular diseases such as arterial gene expression, endothelial dysfunction and vascular reactivity, which accelerate atherosclerosis in the offsprings (Palinski *et al*., 2007; Palinski and Napoli, 2002). Other studies suggest that cardiac triglycerides accumulation may be accompanied by ventricular dysfunction (Christoffersen *et al*., 2003). In our results, the maternal cardiac phospholipids seem to be more affected by the combined diet. These results go hand in hand with the previous study which suggests that a significant portion of fatty acids in obese mouse heart is stored as phospholipids (Pierce *et al*., 2006). The cardiac phospholipids (TLC) has shown the implication of certain phospholipids fractions more than others such as PC, PE and SM. Thus, as known the heart tissue maintains a distinct content and composition of various phospholipids such as PE which play a crucial role during cardiomyocytes proliferation and cytokinetic process (Emoto and Umeda, 2000). The PC is more disposed to maintain the lamellar organization of membrane (Goni and Alonso, 1999) and PKC signaling (Slatar *et al*., 1996). Moreover, it has been proved that the choline plasmalogen PLPC is high in human, rabbits, dog and guinea pig myocardium (Va der Vusse *et al*., 1992). Then, the CL (cardiolipine) which is a markers of inner mitochondria membrane, increases in neonatal heart, the best characterized being its interaction with cyt C oxidase (Paradies *et al*., 2002) demonstrates the close linkage of oxidase activity in adult rat myocardium. Finally, the content of SM plays an important role as a second messenger in regulation of cell proliferation, cell cycle arrest, apoptosis and angiogenesis (Chatterjee *et al*., 2006). Secondly the implication of oxidative stress in the maternal and newborn heart have been shown on our results (Fig. 6-8).

Thus, cardiac MDA, AOPP and CAT seem to be involved in hyperlipidic maternal diet (oil and combined). MDA and CAT increase in the oil group while the AOPP increases in the combined diet. Maternal cardiac NO seems not to be affected by our diet, contrarily to the previous study on hypercholesterolemic rats which noted a decrease (Onody *et al*., 2003). We observed the decrease of MDA, CD, CAT in the newborns from the first and the second pregnancy of combined diet. The iron increases in the first pregnancy of the same diet and found its normal level at the second pregnancy. It's seems to be involved in the cardiac iron of newborns oil diet too.

Fetal exposure to maternal hypercholesterolemia induces changes in gene expression in aorta and liver, that persist in adulthood (Napoli *et al*., 2001; Goharkhay *et al*., 2008) and in cardiac tissue. It has been confirmed that maternal oxidative stress in the high cholesterol environment may adversely affect the placenta and may directly or indirectly enhance atherosclerosis lesions in the offsprings (Liguori *et al*., 2007). The supplementation of diet by methionine showed the relationship between perturbation in the mother's methionine metabolism and the fetal growth and development. So, a long exposure to methionine diet can induce hyperhomocysteinemia which can have a toxic effect on embryonic heart development, which led to congenital heart defect in the newborn by cardiomyocytes apoptosis (Lu *et al*., 2011).

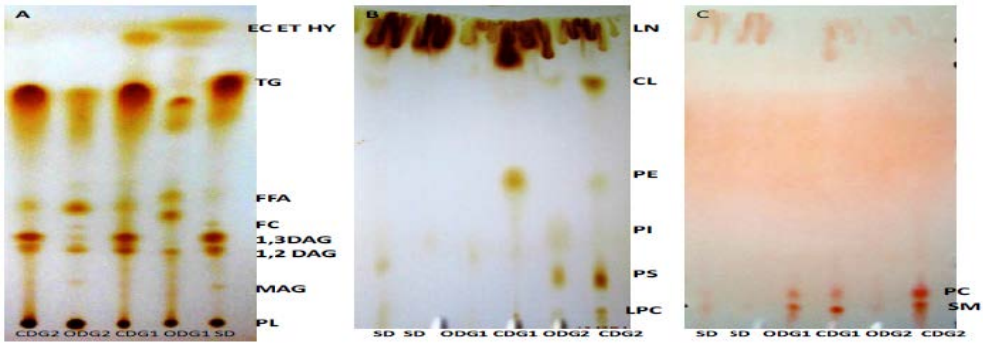

Fig. 8(A-C): Newborn cardiac lipids separated by thin layer chromatography; A) Neutral lipids detection by iode vaporization; PL: Phospholipids; MAG: Mono-Acylglycerol, (1, 2); DAG: 1, 2 Di-Acylglycerol, (1, 3); DAG: 1, 3 Di-Acylglycerol; FC: Free Cholesterol, FFA: Free Fatty Acid, TAG: Triacylglycerol, EC&HY: Esterified Cholesterol and hydrocarbures; B) Phospholipids detection by iode vaporization; CL: Cadriolipine; PE: Phosphatidyl ethanolamine; PI: Phosphatidyl inositol; PS: Phosphatidyl Serine; LPC: Lysophosphatidyl Choline and C) Phospholipids detection by ninhydrine; PC: Phosphatidyl Choline; SM: Sphingomyeline; SD: Standard Diet; OD1: Oil Diet First pregnancy; CD1: Combined Diet First pregnancy; OD2: Oil Diet Second pregnancy; CD2: Combined Diet Second pregnancy

CONCLUSION

Our results indicate that combined diet (Chol+Met), administered to pregnant female rabbit, affect more serum, cardiovascular lipids and oxidative stress factors of the offspring than hypercholesterolemic diet. The complications seem to increase with the pregnancies.

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