Disturbed nitric oxide and homocysteine production are involved in the increased risk of cardiovascular diseases in the F1 offspring of maternal obesity and malnutrition

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Abstract
Purpose The present study aimed to evaluate the changes in levels of different independent risk factors for vascular diseases in the rat offspring of maternal obesity and malnutrition as maternal health disturbances are thought to have direct consequences on the offspring health. The effect of postnatal diet on the offspring was also assessed.
Methods Three groups of female Wistar rats were used (control, obese and malnourished). After the pregnancy and delivery, the offspring were weaned to control diet or high-caloric (HCD) diet and followed up for 30 weeks. Every 5 weeks postnatal, 20 pups (10 males and 10 females) of each subgroup were sacrificed after overnight fasting, the blood sample was obtained, and the rats were dissected out to obtain heart muscle. The following parameters were assessed: lipid profile, NEFA, homocysteine (Hcy), nitric oxide end product (NOx) and myocardial triglyceride content.

Results Maternal obesity and malnutrition caused significant elevation in the body weight, triglycerides, NEFA, Hcy and NOx in the F1 offspring especially those maintained under HCD. Also, the male offspring showed more prominent changes than female offspring.

Conclusions Maternal malnutrition and obesity may increase the risk of the development of cardiovascular diseases in the offspring, especially the male ones.

Keywords Diabetes · Fetal reprogramming · Malnutrition · Obesity

Introduction
The developmental origins of adult health and disease have become one of the most investigated topics worldwide. It is thought that the majority of the diseases that manifest during early childhood or even persist to adulthood are linked to the embryonic and fetal programming [1, 2].

Cardiovascular disease is one of the most prominent diseases that is linked to metabolic syndrome. The typical risk factors for cardiovascular disease include insulin resistance, disturbed blood lipid levels, hypertension and obesity. Further factors contribute to exaggerate the susceptibility to cardiovascular disease such as smoking and unhealthy diet. Moreover, some groups of subjects have more tendencies to develop cardiovascular disease such as males and the elderly. Another vital factor of cardiovascular disease that mainly causes impaired endothelial function is the disturbance in nitric oxide generation. Nitric oxide (NO) is primarily produced by endothelial cells in the cardiovascular system, and it is responsible of vasodilation and apoptosis. Moreover, NO is responsible of protection of cardiovascular system [3].
NO is synthesized by three isoforms of nitric oxide synthases (NOS), which are identified as: endothelial (e), neuronal (n) and inducible (i) NOS. The cardioprotective effect of NO is mediated by eNOS as it produces low concentrations of NO that enable the proper endothelial function, while high pathological concentrations of NO are produced by iNOS [4].

Insulin is well known for its paramount metabolic effect. It is also exerts cardioprotective properties that are mainly illustrated in its regulatory role in NO production through enhancing eNOS phosphorylation and inhibiting iNOS expression to maintain the optimum levels of NO causing vasodilatation and increased blood perfusion that subsequently provide the cardiovascular protection [5].

High levels of circulatory homocysteine (Hcy) have been proven to be related to impaired nitric oxide production and endothelial dysfunction [6]. In addition, hyperhomocysteinemia has been shown to be an independent risk factor for cardiovascular diseases [7]. Even in mild cases of hyperhomocysteinemia, the mild elevation in Hcy levels increases the risk of developing cardiovascular disease [8]. This clearly explains why homocysteine has become one of the most investigated topics that are related to either attenuating or exaggerating the risk of cardiovascular disease.

The previous work of our laboratory [9] indicated that, maternal obesity and malnutrition have a diabetogenic effect on the first-generation offspring (F1) through changing normal glucose sensing and tolerance, mitochondrial DNA copy number and the expression of genes involved in the mitochondrial biogenesis and function in skeletal muscles and adipose tissue. The postnatal feeding appears to play a central role in these effects. The male F1 offspring appears to be more sensitive for fetal diabetogenic programming than female offspring. Based on these results, we hypothesized that those offspring of maternal obesity and malnutrition may be at higher risk of developing cardiovascular diseases through mechanisms related to the observed disturbance in glucose homeostasis and that also other mechanisms may be involved. The present study aimed to evaluate the changes in levels of different independent risk factors for vascular diseases in the rat offspring of maternal obesity and malnutrition in addition to the effect of postnatal diet was also assessed.

Materials and methods

Animals

The animal protocol was approved by the Institutional Animal Care and Use Committee at the Medical Research Institute, Alexandria University, Alexandria, Egypt. Three groups of female Wistar rats were used (control, obese and malnourished); obesity was induced as previously described [9] by maintaining female neonates under obese-genic diet for two months period; the female rats that were 20% heavier than control rats of the same age was considered as obese rat. Malnutrition was induced by maintaining female neonates under low-protein diet (8% protein) [9] for 2 months after weaning. The female rats that were 20% lighter than the control rat of the same age were considered as malnourished.

Pregnancy was induced by mating the females with normal healthy male rats. After the pregnancy and delivery, the offspring were weaned to control diet (CD) or high-caloric diet (HCD) [9] and followed up for 30 weeks. So the resulting offspring groups were as following:

- F1 offspring of control mother under control diet (CF1/CD)
- F1 offspring of control mother under HCD (CF1/HCD)
- F1 offspring of obese mother under control diet (OF1/CD)
- F1 offspring of obese mother under HCD (OF1/HCD)
- F1 offspring of malnourished mother under control diet (MF1/HCD)
- F1 offspring of malnourished mother under HCD (MF1/HCD)

Every 5 weeks postnatal, 20 pups (10 males and 10 females) of each subgroup were sacrificed after overnight fasting, the blood sample was obtained, and the rats were dissected out to obtain heart muscle.

Methods

Routine assays

Blood glucose level, triglycerides, cholesterol, low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) levels were assayed using commercial available kits (Randox, UK). Insulin level was determined using the Enzyme Immunolinked Assay (EIA) kit (Mercodia, Sweden). The insulin resistance index (IRI) was derived using the homeostasis model assessment (HOMA) as follows: IRI = fasting insulin (μU/ml) × fasting glucose (mmol/l)/22.5. HOMA has been validated as useful method of assessing insulin resistance [10].

Determination of myocardial triglycerides content

The heart samples were homogenized in phosphate-buffered saline (PBS) and cardiac triglyceride and cholesterol contents were determined, after Folch extraction [11], with Randox kits according to the manufacturer’s instructions.
Determination of non-esterified fatty acids (NEFA)

Plasma levels of NEFA were assessed using ELISA kits (MyBioSource) according to the manufacturer’s instructions.

Determination of homocysteine

Total homocysteine level was determined using Axis Homocysteine EIA assay (Germany). Protein-bound homocysteine is reduced to free homocysteine and enzymatically converted to S-adenosyl-L-homocysteine (SAH) in a separate procedure prior to the immunoassay. The assay is based on competition between SAH in the sample and immobilized SAH bound to the walls of the microtitre plate for binding sites on a monoclonal anti-SAH antibody. After removal of unbound anti-SAH antibody, a secondary rabbit anti-mouse antibody labeled with the enzyme horse radish peroxidase (HRP) is added. The peroxidase activity is measured spectrophotometrically after addition of substrate, and the absorbance is inversely related to the concentration of total homocysteine in the sample.

Determination of nitric oxide end products (nitrite and nitrate; NOx)

The nitrite and nitrate (NOx) concentration was determined by simple Griess reaction [12]. Because the nitric oxide (NO) has a short half-life (2–30 s), it is preferable to determine nitrite, the stable product of NO which may be further oxidized to nitrate. So, the Griess reaction was supplemented with the reduction in nitrate to nitrite by NADPH-dependent nitrate reductase. The assay consists of two steps: diazotization of sulphanilic acid with nitrite ion and coupling of this product with diamine, which results in a measurable pink metabolite that measured at 540 nm.

Statistical analysis

All data are presented as mean ± SD. A one-way analysis of variance (ANOVA) was performed on each variable, and the Bonferroni statistics employed to compare the mean values from the offspring of obese and malnourished mothers and the offspring of control mothers (under control diet or HCD). t test was used to compare the mean values of females with those of males of the same group at the same age. The Kolmogorov–Smirnov test was used to study the normal distribution of the studied parameters. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS statistical software version 18 (SPSS, Chicago, IL).

Results

Body weight

The follow-up of F1 offspring of the different pregnancies indicated that the male offspring of obese and malnourished mothers were significantly heavier than offspring of control mothers from the age of 15 week, irrespective of the type of postnatal diets with the heaviest offspring being those of obese mother maintained under HCD (Fig. 1a). Female offspring showed similar but milder pattern of changes as male with the exception that the offspring of malnourished...
Table 1 Maternal weights and metabolic parameters before gestation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Obese</th>
<th>Malnourished</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>188 ± 14.0</td>
<td>254 ± 24.5*</td>
<td>145 ± 13.4**</td>
</tr>
<tr>
<td>Fasting blood sugar</td>
<td>85 ± 15</td>
<td>108 ± 18*</td>
<td>78 ± 11**</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td></td>
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<tr>
<td>NEFA (mmol/L)</td>
<td>1.4 ± 0.11</td>
<td>1.6 ± 0.2*</td>
<td>1.2 ± 0.15**</td>
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<td>TG (mg/dl)</td>
<td>104 ± 13.4</td>
<td>184 ± 11.1*</td>
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</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>157 ± 13.1</td>
<td>198 ± 15.4*</td>
<td>144 ± 14.2**</td>
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<tr>
<td>LDL-cholesterol (mg/ dl)</td>
<td>85.5 ± 11.4</td>
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<td>80.5 ± 13.4^a</td>
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<td>HDL-cholesterol (mg/dl)</td>
<td>51 ± 4.2</td>
<td>41 ± 5.1*</td>
<td>47 ± 4.5^*</td>
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</tbody>
</table>

Data presented as Mean ± SD
* Significantly different compared to control mothers using ANOVA (p < 0.05)
# Significantly different compared to obese mothers using ANOVA (p < 0.05)

mothers under control diet (MF1/CD) showed no significant increase in the weight compared to control (Fig. 1b) as reported previously [9]. Obese and malnourished displayed expected disruptions of blood glucose levels as well as lipid profile as reported in Table 1.

Serum lipid profile

The male F1 offspring of control pregnancies under CD (CF1/CD) and of malnourished pregnancies under HCD (MF1/HCD) showed lower HDL-C than female offspring (Table 2) at most ages. In contrast, the HDL-C level showed significant decline in the offspring of control mothers as a result of HFD as early as 10th week of age in females and 30th week of age in males (Table 2). The HDL-C level showed significantly lower level in the male and female MF1/HCD offspring and female OF1/HCD offspring from the 10th week of age and in male OF1/HCD offspring at 30th week of age compared to control offspring (CF1/CD). The lowest HDL-C level was observed in the male offspring of malnourished mother under HCD (Table 2).

HCD postnatal feeding of F1 offspring of control pregnancies resulted in significant elevation of triglycerides and total cholesterol only at the 30th week of age in males and females while the LDL-C showed no significant change during the follow-up period. On the other hand, the postnatal feeding with HCD resulted in significant elevation in the triglyceride level as early as 10th week of age in the male offspring of OF1/HCD and by 20th week of age in MF1/HCD male offspring compared to control offspring even under HCD.

The F1 offspring of obese or malnourished mothers under CD showed mild nonsignificant changes in the lipid profile parameters with the exception of triglycerides level which showed significant elevation in the male offspring (OF1/CD and MF1/CD) only at the 20th week of age (Table 2).

From Fig. 2a, it was clear that the serum level of NEFA showed significant higher level in all male offspring compared to control offspring under CD as early as 10th week of age with the exception of the male offspring of malnourished mother under CD (MF1/CD) which showed near-normal or even lower value (Fig. 2a). In female, only the offspring of control and obese mother under HCD (CF1/HCD and OF1/HCD) showed significant higher NEFA level than CF1/CD as early as 10th week of age, and the MF1/HCD female offspring showed significant higher level only at the 30th week age (Fig. 2b).

Myocardial triglycerides (TG) content

The myocardial TG content was higher in the offspring of obese and malnourished mothers maintained postnatal on HFD (OF1/HCD and MF1/HCD, respectively) from as early as 15th in males (Fig. 3a) and 20th week of age in females (Fig. 3b) and thereafter. Also, the male offspring of obese mothers under control diet and the control offspring under HCD showed mild but significant elevation in the TG content compared to control, while the male offspring of malnourished mother under CD showed significantly higher level only at the 30th week of age. At the age of 30 week, the female offspring of obese under CD and of control offspring under HCD showed significantly higher TG content than CF1/CD (Fig. 3).

Plasma level of homocysteine (Hcy)

Figure 4 summarizes the results of plasma homocysteine level in the male (A) and female (B) offspring. It is clear that, the offspring of malnourished mothers (MF1/CD and MF1/HCD) have significantly higher plasma level of Hcy as early as fifth week of age compared to CF1/CD. Those offspring under HCD showed higher levels compared to those under CD, and the male offspring showed higher level than female ones (Fig. 4a, b). The offspring of obsess mother showed similar pattern of increased Hcy but milder than that observed in the offspring of malnourished mothers (Fig. 4a, b).

Plasma level of nitric oxide end products (NOx)

As indicated in Fig. 5a, b, the plasma level of NOx showed higher values in the offspring of obese and malnourished mother under HCD (OF1/HCD and MF1/HCD) compared to control offspring under CD as early as 10th week in males (Fig. 5a) and 15th week of age in females (Fig 5b). Only male offspring of obese and malnourished mother under
CD (OF1/CD and MF1/CD) showed significant higher NOx level than control offspring at age of 30 week (Fig. 5a).

**Discussion**

In our study, we represented two different models of maternal complications that have devastating effects directly on fetal health and even later in life on the susceptibility to chronic diseases, namely these models are maternal malnutrition and maternal obesity. From this study, these two maternal challenges appear to increase the risk of cardiovascular disease through different mechanisms.

It has been evidently documented in many studies that maternal malnutrition has a direct effect on the fetal growth as it causes intrauterine growth retardation (IUGR) [13, 14]. There is a concrete relation between fetal growth restriction and the prevalence of cardiovascular risk factors and the development of insulin resistance and type 2 diabetes [15]. Moreover, impaired endothelial function has been proven to be firmly related to IUGR [16]. So these studies and many others interpreted that IUGR due to maternal restriction and the prevalence of cardiovascular risk factors

<table>
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<th>Age (week)</th>
<th>CF1/CD</th>
<th>CF1/HCD</th>
<th>OF1/CD</th>
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<th>MF1/CD</th>
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<td>100 ± 18</td>
<td>103 ± 21</td>
<td>113 ± 15</td>
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<td>111 ± 18</td>
<td>124 ± 18</td>
<td>134abd ± 29</td>
<td>156abc ± 16</td>
<td>122d ± 13</td>
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<td>124 ± 22</td>
<td>131 ± 18</td>
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<td>40 ± 4</td>
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</table>

Data presented as Mean ± SD and n = 10 rats for all groups (MF1/HCD)

F1 offspring of control mothers under control diet (CF1/CD), F1 offspring of control mothers under HCD (CF1/HCD), F1 offspring of obese mothers under control diet (OF1/CD) and F1 offspring of obese mothers under HCD (OF1/HCD), F1 offspring of malnourished mothers under control diet (MF1/CD) and F1 offspring of malnourished mothers under HCD

* Significant difference compared to females of the same group at the same age by t test (p < 0.05)

a Significantly different compared to CF1/CD, b significantly different compared to CF1/HCD, c significantly different compared to OF1/CD, d significantly different compared to OF1/HCD and e significantly different compared to MF1/CD by ANOVA (p < 0.05)
malnutrition is undoubtedly a major predictor of cardiovascular disease in the adult offspring [17, 18].

Another aspect of the damaging effect of fetal growth restriction due to maternal malnutrition is that growth restriction is usually compensated by fast catch-up growth or in other words accelerated postnatal growth [19]. In this study, the offspring of maternal obesity and malnutrition show obesogenic behaviors as indicated by higher weights of the offspring, especially those fed postnatal with HCD. The relation between the catch-up growth in infancy and the vulnerability to develop cardiovascular disease is proven to be an indubitable fact. Hence, adherence to postnatal low-fat diet in order to restrict postnatal accelerated growth may prevent the reinforcement of cardiovascular risk factors [20].

Drake et al. [21] documented that the offspring of obese mothers suffer from impaired endothelial function, hypertension and disturbed vascular fatty acid content concordant with our study which indicated elevated NEFA in plasma of the offspring of obese mother irrespective of the type of diets and in the offspring of malnourished mothers only under HCD. This excessive production of NEFA was shown to be associated with accumulation of triglycerides in the myocardial tissues, especially in the male offspring under HCD (Fig. 3). The firm relationship between maternal obesity and increased susceptibility to cardiovascular disease in the offspring is an established fact [21, 22]. In addition, maternal obesity is a radical cause in developing insulin resistance and disturbed glucose homeostasis in the adult offspring [9, 23].

We clarified that prenatal nutrition and postnatal diet play a substantial role in the negative or positive reinforcement of metabolic and cardiovascular diseases. Another variable that is involved in shifting that reinforcement is sex. We observed that male offspring are more vulnerable toward developing cardiovascular disease. So, logically the male offspring of malnourished mothers under HCD presented the most susceptible subjects. Male offspring of both malnourished and obese mothers showed significantly higher levels of triglycerides along with lower value of HDL-C at old ages which all are identified as prevalent cardiovascular risk factors [24].
On the other hand, female offspring only showed mild change in these parameters in comparison with their change in male offspring. We think that the milder changes in these parameters and cardiovascular risk factor may be thanks to ovarian steroid hormones in female. As our previous study showed that the decline in ovarian steroid hormones, especially estrogen, causes disturbances in lipid profile, through increasing cholesterol, triglycerides, LDL-cholesterol and NEFA significantly, as well as a significant decrease in HDL-cholesterol [25].

Further evidence enlightening the relation between impaired endothelial function and production of NO is provided. In a study of Beckman et al., it is demonstrated that NO combines with \((\text{O}_2)^-\) to form peroxynitrite \((\text{ONOO})^-\). Peroxynitrite is an oxidizing agent that oxidizes tetrahydrobiopterin \((\text{BH}_4)\) which is the essential cofactor in production of NO by eNOS and the lack of \(\text{BH}_4\) compels the NOS to produce \((\text{O}_2)^-\) instead of NO in a process known as NOS uncoupling [26]. In addition, peroxynitrite \((\text{ONOO})^-\) production is responsible of generating oxidative/nitrative stress resulting in endothelial dysfunction [27].

As observed in this study, male offspring of both malnourished and obese mothers, particularly the ones under HCD, showed a significant age-dependant elevation (at 15th week of age) in levels of NOx (indicators of nitric oxide). Meanwhile, female offspring showed mild changes in NOx levels at older age. These observations reinforce the evidence that male offspring of maternal malnutrition and maternal obesity are more vulnerable to develop cardiovascular disease than female offspring.

Also, we can link between milder changes in NOx in female offspring compared to male and ovarian sex hormones production. As we found from our previous work that there is metabolic interaction between insulin and female sex hormones; for example, the low levels of female sex steroids are related to insulin resistance and a decline in whole-body insulin-mediated glucose uptake [25].
Furthermore, another variable involved in disturbing NOx levels is circulating Hcy concentration. Numerous studies have provided evidence on the association between high levels of NOx and hyperhomocysteinemia [28, 29] as the high levels of Hcy are considered a trigger to stimulate the endothelial NO production through the stimulation of iNOS resulting in impaired endothelial function [7, 28, 30]. High levels of insulin that are distinctly observed with insulin resistance and impaired insulin sensitivity are accompanied by high levels of circulating Hcy. In the same manner, Hcy and lipid metabolism are strongly related. Obeid et al. accentuated that hyperhomocysteinemia and dyslipidemia are two collateral conditions. They documented that hyperhomocysteinemia through hypomethylation is associated with disturbed plasma lipids or fatty liver. Moreover, they clearly documented the different mechanisms of hyperhomocysteinemia and its prevalent effect on deregulated lipid metabolism including high levels of blood cholesterol and disturbed LDL-C and HDL-C levels along with imbalanced triglycerides and phospholipids synthesis [31]. Subsequently, H. Wang et al. demonstrated that the administration of high-caloric diet did not only increase total cholesterol levels but also doubled Hcy levels in animals. These results indicate that high intake of diet rich in fat and cholesterol contribute to elevation of circulating Hcy levels that result in elevation in plasma lipids and simultaneously increase the risk factor for cardiovascular disease [32]. The stated concept is clinically important in managing cardiovascular risk factors by the adherence to low-fat diet and caloric restriction in order to retain normal values of Hcy concentration [33].

The mentioned evidence is clearly illustrated by our observation in this study, as we noted that male offspring of maternal malnutrition and maternal obesity “particularly the ones under HCD” showed a significant age-dependent elevation in Hcy levels compared to a significant mild elevation observed in female offspring (Fig. 4).

From all these stated evidence and noted observations, we can fairly conclude that the vicious cycle that starts with maternal complications can manifest directly on the offspring during early childhood or even persist more fiercely to adulthood. Maternal malnutrition and obesity increase the risk of the development of cardiovascular diseases in the offspring. The prenatal obesity induces insulin
resistance, impaired glucose homoeostasis, disturbed levels of blood lipid and NEFA and accumulation of TG in myocardial tissues. However, prenatal malnutrition has additive risk on the development of cardiovascular diseases through enhanced production of NO and elevated plasma level of Hcy. Also, the high intake of dietary fat postnatal is evidently a burden added to the susceptibility for cardiovascular disease which implies that controlled diet and caloric restriction are inevitable. A final conclusion is that male offspring of maternal obesity and malnutrition; especially under postnatal HCD, are the more vulnerable to develop cardiovascular disease than the female offspring.

Compliance with ethical standards

Conflict of interest No declared.

Ethical approval All institutional and national guidelines for the care and use of laboratory animals were followed. The animal protocol was approved by the Institutional Animal Care and Use Committee at the Medical Research Institute, Alexandria University, Alexandria, Egypt.

This article does not contain any studies involved human subjects performed by any of the authors.

Informed consent No informed consent.

References


Fig. 5 Age-dependent change in plasma nitric oxide end products level in male (a) and female (b) F1 offspring under control and HCD. Each point represents mean of ten measurements ± SD (plus, number sign, commercial at and dollar sign represent a significant difference between OF1/HCD, OF1/CD, MF1/CD and MF1/HCD rats compared to CF1/CD rats, respectively, Using ANOVA, p < 0.05). Group abbreviation: F1 offspring of control mothers under control diet (CF1/CD), F1 offspring of control mothers under HCD (CF1/HCD), F1 offspring of obese mothers under control diet (OF1/CD) and F1 offspring of obese mothers under HCD (OF1/HCD), F1 offspring of malnourished mothers under control diet (MF1/CD) and F1 offspring of malnourished mothers under HCD (MF1/HCD)