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Alteration of antioxidant defense status precedes humoral immune response abnormalities in macrosomia

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: This study aimed to investigate whether the anomalies affecting the antioxidant and humoral immune defenses could start at birth and to check whether the decrease in antioxidant defenses may precede the immune abnormalities in macrosomic newborns.

Material/Methods: Thirty macrosomic and 30 sex-matched control newborns were recruited for a retrospective case-control study at the Maghnia Maternity Hospital of Tlemcen Department (Algeria).

Results: The serum IgG levels were similar in both groups. However, plasma ORAC, albumin, vitamin E, SOD, CAT and GSH-Px levels were significantly decreased in macrosomic as compared to control newborns, yet no difference was observed after adjustment for weight. Additionally, serum concentrations of complement C3, MDA and XO were significantly higher in macrosomic as compared to controls before adjustment for weight. Moreover, macrosomia was significantly associated with high levels of complement C3 (OR=8, p=0.002); whereas no association with those of IgG was observed (OR<1, p>0.05). Furthermore, macrosomia was significantly associated with low levels of ORAC (OR=4.96, p=0.027), vitamin E (OR=4.5, p=0.018), SOD (OR=6.88, p=0.020) and CAT (OR=5.67, p=0.017), and with high levels of MDA (OR=10.29, p=0.005).

Conclusions: Abnormalities of the humoral defense system in excessive weight could be preceded by alterations of the anti-oxidative defense and by inflammatory response and activation of innate immunity at birth. Additionally, excessive weight could be a potential factor contributing to decreased anti-oxidative capacity and increased oxidative stress.

key words: **antioxidant defense • humoral response • macrosomic newborns • oxidative stress**

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Background

The term macrosomia is used to describe a full-term newborn with excessive birth weight due to somatic growth. It has been defined in several different ways, including birth weight of 4000–4500 g, or above the 90th percentile based on intrauterine growth curves.

Newborns with excessive weight have an increased risk of developing breast cancer, acute lymphoblastic leukemia, Sjogren's syndrome, lupus erythematosus disseminatus and rheumatoid arthritis in adulthood [1]. These pathologies could be a consequence of increased oxidative stress and chronic inflammation associated with overweight and obesity**.** The lipid substrates accumulated in the adipose tissue are the main targets of free radicals responsible for the generation of oxidative stress [2–4]. They are also responsible for starting inflammation in and outside the adipose tissue. The trigger for this inflammation is unknown but may involve hypoxia [5] and hypoxia-induced fibrosis [6], adipose tissue cell death [7], adipocyte stress [8], and adipocyte production of chemokines [9].

In many cases, obesity in adults has been linked to childhood or adolescence obesity and with excessive weight at birth. In addition to inflammation and oxidative stress, obesity has been associated with impaired immunity [10,11]. However, to our knowledge, there are no studies addressing the alteration of the humoral immunity and antioxidant defenses in newborns with overweight.

Reactive oxygen species are physiologically produced during metabolic processes and are essential for several biochemical processes, such as signal transduction [12,13] and gene expression [14]. However, the excessive production of reactive species, collectively called oxidative stress, has been involved in many pathological states and immune system dysfunction [15]. Oxidative stress occurs when the balance between oxidation and antioxidation is tilted in favor of the former [16,17]. Common oxidative markers include the lipid peroxidation product malondialdehyde (MDA) and protein carbonyls (PC), which are formed as a result of protein modification by various oxidants or covalent linkage of aldehyde products of lipid peroxidation [18]. Antioxidants help defend the body against free radical attack and many diseases have been associated with low antioxidant levels. There are 3 levels of defense against free radical attack – preventative antioxidants to prevent the formation of free radicals, scavenging antioxidants to remove reactive species once formed, and repair enzymes to repair damaged biomolecules.

In this study we attempted to evaluate the immune and antioxidative defenses status in macrosomic newborns to investigate whether the anomalies affecting the antioxidant defenses and humoral immunity could start at birth and to check whether the decrease in antioxidant defenses may precede the immune abnormalities in macrosomic newborns. To this end, we conducted a retrospective case-control study at the Maghnia Maternity Hospital of Tlemcen Department (Algeria).

Material and Methods

Subjects

Thirty macrosomic and 30 sex-matched full-term controls were recruited for a retrospective case-control study at the Maternity Hospital of Maghnia (Tlemcen, Algeria). The mean weight (± standard deviation) of macrosomic subjects (17 males, 13 females) at birth was 4205.6±42.83 g, and that of the controls (19 males, 11 females) was 3375.8±44.42 g (p=0.000). Infant were categorized according to birth weight for gestational age status based on the intrauterine reference percentiles growth charts of Lubchenco [19]. Infants born over the 90th percentile were classified as LGA (large for gestational age, or macrosomic) and those born between the 10th and 90th percentiles as AGA (appropriate for the gestational age). The main criteria for inclusion were macrosomia and birth without any disease and without complications. The major exclusion criteria were birth from mothers who were unhealthy, obese, hypertensive, or with some behaviors that are related to infantile overweight or obesity. Informed written consent was obtained from all parents of newborns. The study protocol was reviewed and approved by the Ethics Committee of Maghnia Hospital of Tlemcen Department (Algeria).

Samples

Blood samples were collected aseptically from umbilical cords immediately after delivery. Plasma was obtained by centrifugation of samples collected into EDTA tubes, used for total anti-oxidant capacity, antioxidant enzymes and lipid peroxidation assays, or into tubes containing heparin, used for vitamins, xanthine oxidase and protein carbonyl assays. Serum samples were obtained following centrifugation of blood collected into tubes without anticoagulant, and were used for IgG, albumin and complement C3 assays.

Biochemical and immunological analyses

IgG immunoassay

Serum IgG levels were determined by immunoturbidimetric assay based on the measurement of antigen-antibody reaction by the end-point method. A standard sample was used for preparing the reference curve and a protein sample was used for accuracy control. Measurements were carried out using kits from Cypress Diagnostics (IgG turbidimetric kit, 33232, Protein Standard Turbidimetry; 33222S, Protein Control Turbidimetry; 33222C, Cypress Diagnostics, Langdorpsesteenweg 160, 3201 Langdorp, Belgium).

Complement C3 assay

Serum complement C3 levels were measured by electrophoresis method on agarose gel with high sensitivity, using Sebia Hydragel β1-β2 kit, according to the manufacturer's instructions (Sebia Hydragel β1-β2, Hydragel Protein(e) K20 [amidoschwarz], 3000, France).

Scavenging capacity of plasma determination

Plasma total antioxidant capacity (TAC/ORAC, oxygen radical absorbance capacity) was measured as an indicator of overall anti-oxidative status [20]. Plasma ORAC was measured

according to Cao et al. [21] as previously described [22]. Allophycocyanin was used as a fluorescent protein and Trolox as a reference antioxidant for calculating the ORAC values. Performance monitoring was conducted by the KRL (Spiral/KIRIAL, Dijon, France) biological test [23,24] based on the hemolysis induced by radical attack [25,26].

Antioxidant enzyme assays

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using a commercial kit obtained from Randox Laboratories Ltd (Crumlin, UK), employing xanthine and xanthine oxidase to generate superoxide radicals (O_2^-), which react with p-iodonitrotetrazolium salts (2-(4-iodophenyl)-3-(4-notrophenol)-5-phenyltetrazolium chloride) (INT) to produce a red formazan dye. SOD present in the sample competes with the INT for superoxide radicals and so inhibits the production of the formazan dye by converting the superoxide radical to oxygen. The SOD activity was then determined spectrophotometrically at 505 nm by the degree of inhibition of this reaction. Catalase (CAT, EC 1.11.1.6) activity was determined spectrophotometrically at 240 nm by the measure of amount of hydrogen peroxide decomposition according to the method previously described [27]. Activity of glutathione peroxidase (GSH-Px, EC 1.11.9) was assayed spectrophotometrically at 340 nm according to the method of Paglia and Valentine, based on the reaction of the oxidation of glutathione by cumene hydroperoxide [28]. Enzyme activities were reported in U/mL for SOD and IU/mL for CAT and GSH-Px.

Non-enzymic antioxidant assays

Plasma vitamin A (retinol) and vitamin E (α-tocopherol) concentrations were determined by reversed phase high-pressure liquid chromatography (RP-HPLC) method with spectrophotometric detection. Retinol was detected at 340 nm and α-tocopherol was detected at 292 nm. Serum albumin levels were performed using Sebia Hydragel β1-β2 electrophoresis assay.

Enzymic oxidant assay

Xanthine oxidase (XO, EC 1.17.3.2) activity was measured as a biomarker for enzymatic oxidation. It was determined by measuring uric acid formation from xanthine at 293 nm according to the Hashimoto method [29]. The XO concentration was expressed in mIU/mL.

Lipid peroxidation analysis

The determination of plasma lipid peroxidation as malondialdehyde (MDA) was measured spectrophotometrically at 535 nm using the thiobarbituric assay as previously described [30].

Protein oxidation analysis

Protein oxidation was determined by measurement of protein carbonyls (PC) concentrations using enzyme-linked immunosorbent assay (ELISA), based on the detection of 2,4-dinitrophenylhydrazine (DNP) by specific antibody (Biocell Protein Carbonyl ELISA kit, ALX-850-312-KI01, AXXORA DEUTSCHLAND GmbH, Germany).

Table 1. Characteristics of macrosomic and control newborns.

Variable	Controls (n=30)	Macrosomes (n=30)	*p*
Gestational age (week)	39.1±0.12	39.07±0.11	0.837
Gender (M/F)	19/11	17/13	0.601
Birthweight (g)	3375.8±8.11	4205.6±7.82	0.000
Mother's age (year)	29.71±0.57	27.88±0.43	0.380
Mother's BMI (kg/m^2)	23.21±0.36	23.64±0.29	0.356
Mode of delivery (vaginal birth/cesarean section)	27/3	23/7	0.299

$p<0.05$ was considered statistically significant. The variables are presented as mean ± standard error. BMI – body mass index; M – male; F – female.

Statistical analyses

Two-tailed Student's *t* test and a one-way analysis of covariance (ANCOVA) with adjustment for weight were performed to compare mean values. The chi-square analysis was used to compare the frequency of male and female sex. Odds ratio (OR) were calculated using the 90th percentile of the circulating levels of IgG, complement C3, XO, MDA and PC and the 10th percentile of ORAC, SOD, CAT, GSH-Px, vitamins and albumin levels in the control groups as cut-off levels. Statistical analyses were performed using SPSS (version 16.0, SPSS Inc., Chicago, IL, USA), and STATISTICA (STATISTICA Version 5.0, '97, StatSoft, Paris, France) software. *P* values <0.05 were considered significant.

Results

The circulating levels of IgG and complement C3 and the oxidant/antioxidant balance were evaluated in 30 macrosomic and 30 control newborns. XO, MDA and PC were determined as markers of oxidative stress, while SOD, CAT, GSH-Px, vitamin A, vitamin E and albumin were used as markers to evaluate the antioxidant status in each group. The overall anti-oxidative status was determined by measuring the plasma ORAC. The range between the 10th and 90th percentile was defined as the reference range. Values outside this range were statistically considered positive and were used to analyze the association between immune or oxidative stress biomarkers and macrosomia.

Characteristics of macrosomic and control newborns are presented in Table 1.

Except for birthweight, gestational age, sex, mother's age, mother's BMI, and mode of delivery were not different between the 2 groups (for all comparison, $p>0.05$).

The plasma levels of ORAC and serum levels of IgG and complement C3 are shown in Figures 1 and 2, respectively.

As indicated in Figure 1, the plasma levels of ORAC are significantly decreased in macrosomic as compared to control newborns ($p=0.000$). However, Figure 2 shows that serum

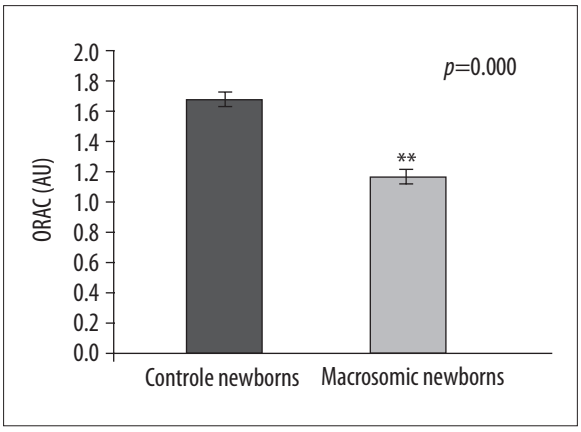

Figure 1. Total antioxidative defense status in macrosomic and control newborns. ORAC – total antioxidant capacity/oxygen radical absorbance capacity. ** $p<0.01$. The variables are presented as mean ± standard error. $p>0.05$ after adjustment for birthweight.

Table 2. Levels of oxidative stress biomarkers in macrosomic and control newborns.

Variable	Controls (n=30)	Macrosomes (n=30)	*p*
Albumin (g/L)	30.77±1.13	26.35±1.17	0.009
Vitamin A (µmol/L)	0.98±0.09	0.85±0.07	0.256
Vitamin E (µmol/L)	9.16±0.22	7.82±0.27	0.000
MDA (µmol/L)	2.1±0.09	2.85±0.06	0.000
PC (nmol/mg protein)	0.13±0.02	0.14±0.02	0.726

$p<0.05$ was considered statistically significant. The variables are presented as mean ± standard error. For all comparison, $p>0.05$ after adjustment for birthweight. MDA – malondialdehyde; PC – protein carbonyls.

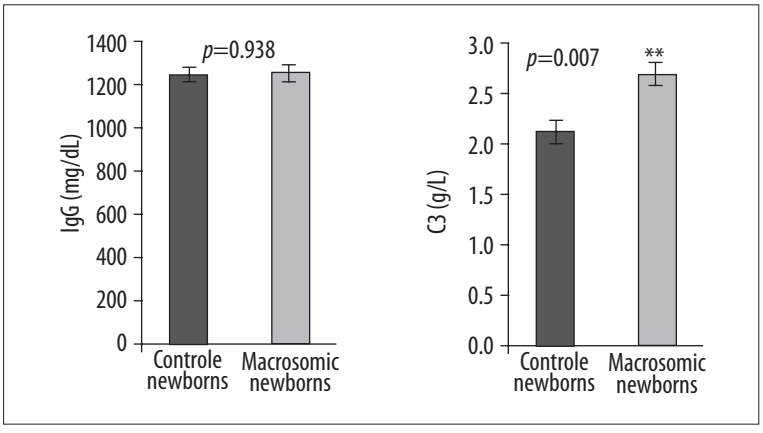

Figure 2. IgG humoral response and complement C3 levels in macrosomic and control newborns. C3 – complement component 3, IgG – Immunoglobulin G. ** $p<0.01$. The variables are presented as mean ± standard error. $p>0.05$ after adjustment for birthweight

IgG levels were similar in both groups (p=0.938). On the contrary, serum concentrations of complement C3 were significantly higher in macrosomic than in control newborns (p=0.007).

The biomarkers of oxidant/antioxidant balance are shown in Tables 2 and 3.

Circulating levels of albumin, vitamin E, SOD, CAT and GSH-Px levels were significantly decreased in macrosomic as compared to control newborns (respectively, p=0.009, p=0.000, p=0.000, p=0.001, p=0.008). However, those of vitamin A are slightly diminished in macrosomic newborns as compared to controls, but the difference was not statistically significant (p=0.256). Additionally, plasma concentrations of MDA and XO were significantly higher in macrosomic as compared to controls (for the 2 comparisons, $p<0.01$). Plasma levels of PC were similar in both groups ($p>0.05$). On the other hand, there are no significant differences for all comparison between the 2 groups after adjustment for weight (p values not shown).

The association analysis between immune or oxidative stress biomarkers and macrosomia is presented in Table 4.

Table 3. Oxidant and antioxidant enzyme activities in macrosomic and control newborns.

Variable	Controls (n=30)	Macrosomes (n=30)	*p*
XO (mIU/mL)	0.29±0.02	0.37±0.02	0.007
SOD (U/mL)	81.77±1.05	75.58±1.17	0.000
CAT (IU/mL)	22582.5±479.62	20271.85±509.93	0.001
GSH-Px (IU/mL)	1.94±0.05	1.69±0.07	0.008

$p<0.05$ was considered statistically significant. The variables are presented as mean ± standard error. For all comparison, $p>0.05$ after adjustment for birthweight. CAT – catalase; GSH-Px – glutathione peroxidase; SOD – superoxide dismutase; XO – xanthine oxidase.

As shown in Table 4, macrosomia is significantly associated with high levels of complement C3 (OR [95%CI]; 8 [2.11–30.34], p=0.002) whereas, no association with those of IgG was observed (OR<1, 95%CI 0.24–2.96, $p>0.05$). Additionally, macrosomia is associated with low levels of ORAC (OR=4.96), albumin (OR=2.25), vitamin E (OR=4.5),

Table 4. Association analysis of immune and oxidative stress biomarkers with macrosomia.

Variable	*p*	OR (95%CI)
IgG (mg/dL)	0.797	0.85 (0.24–2.96)
C3 (g/L)	0.002	8.00 (2.11–30.34)
ORAC (AU)	0.027	4.96 (1.2–20.55)
Albumin (g/L)	0.353	2.25 (0.41–12.48)
Vitamin A (µmol/L)	0.242	2.04 (0.62–6.75)
Vitamin E (µmol/L)	0.018	4.50 (1.29–15.68)
SOD (U/mL)	0.020	6.88 (1.35–35.11)
CAT (IU/mL)	0.017	5.67 (1.37–23.46)
GSH-Px (IU/mL)	0.353	2.23 (0.41–12.48)
MDA (µmol/L)	0.005	10.29 (2.02–52.36)
XO (mIU/mL)	0.240	3.80 (0.41–35.28)
PC (nmol/mg protein)	0.531	0.62 (0.14–2.81)

$p<0.05$ was considered statistically significant. High levels of immune (IgG and complement C3), and oxidant biomarkers (MDA, XO and PC) correspond to the 90th percentile for control subjects. Low levels of antioxidant biomarkers (ORAC, albumin, SOD, CAT, GSH-Px and vitamins) correspond to the 10th percentile for controls. CAT – catalase; CI – confidence interval; C3 – complement component 3; GSH-Px – glutathione peroxidase; MDA – malondialdehyde; OR – odds ratio; ORAC – total antioxidant capacity/oxygen radical absorbance capacity; PC – protein carbonyls; SOD – superoxide dismutase; XO – xanthine oxidase.

vitamin A (OR=2.04), SOD (OR=6.88), CAT (OR=5.67) and GSH-Px (OR=2.23), and with high levels of MDA (OR=10.29) and XO (OR=3.8), but the association did not reach a significant level for albumin, vitamin A, GSH-Px and XO ($p>0.05$). Moreover, no association between macrosomia and high levels of PC (OR [95%CI]; 0.62 [0.14–2.81], $p=0.531$) was found.

Discussion

Macrosomic newborns are usually exposed to risk of perinatal mortality, trauma, congenital malformations, and metabolic disorders, and to the development of obesity and diabetes during adolescence [31–33].

Excess body fat characterizing obesity, mainly represented by the subcutaneous adipose tissue, may be the source of cellular stress and increased lipid peroxidation [34,35]. One of the most frequently used biomarkers providing an indication of lipid peroxidation level is the plasma concentration of malondialdehyde (MDA), one of several by-products of lipid peroxidation processes [36]. Therefore, our results are consistent with those of Yesilbursa et al. [37] and Prazny et al. [38] who observed increased plasma MDA levels in obese compared to non-obese healthy controls. We also found a significant association between macrosomia and MDA in the current study. Additionally, one of the major reactive oxygen species producing enzyme systems is XO systems. The increased levels of XO observed in macrosomic newborns in this work corroborate several studies that have reported the genesis of oxidative stress in macrosomia and obesity [39–41]. Proteins are also targets for oxygen radicals [12,42,43]. Carbonyl groups can be introduced into proteins either *via* non site-specific or site-specific metal catalyzed oxidation of amino acid residues [44]. The PC content can therefore be used as a measure of radical damage to proteins. In our study, the PC levels are in discordance with those reported in adults with excessive weight [40], and, to our knowledge, there are no studies that have evaluated PC in macrosomic newborns.

Interestingly, obesity has until fairly recently been characterized by a decreased activity of antioxidant defense systems [45]. In our study, the decreased level of ORAC, SOD, CAT, GSH-Px and vitamin E in macrosomic newborns confirms these observations. Additionally, highly significant associations between macrosomia and low levels of ORAC, vitamin E, SOD and CAT were observed in this study. The results showing no alteration in vitamin A levels in macrosomia are in agreement with observations previously reported [41]. Furthermore, the decreased circulating levels of albumin in macrosomic newborns may also reflect the alteration of antioxidant defenses during obesity and excessive weight. Indeed, it has been reported that the albumin is an important antioxidant because it has specific binding sites for copper ions and a free sulfhydryl group, which can scavenge harmful reactive oxygen species [46].

Inflammation has been associated with oxidative stress according to several investigations conducted in the field [47–49]. In fact, it has been observed that the oxidative stress generated by increased production of oxygen free radicals or by antioxidant micronutrients deficiency is implicated in the physiopathology of many acute inflammatory diseases, such as systemic inflammatory response syndrome, septic shock, adult respiratory distress syndrome, extensive burns, polytrauma, and renal failure [50]. This is in agreement with our results showing increased levels of complement C3 and decreased levels of albumin.

Despite the intense interest in the relationship between inflammation and obesity, to our knowledge the role of the complement system has not been explored in macrosomic newborns. Complement C3 is increased in response to inflammation and infection but at a slower rate than for traditional acute phase proteins [51,52]. Both C3 and C4 have shown substantial correlations with obesity [53–56], and high gene expression of these complement components has been reported in omental adipose tissue in obese men [53]. High C3 levels have been reported in subjects with diabetes and insulin resistance [56–59] and with risk of developing a myocardial infarction [60]. Our results corroborate those of Cianflone et al. [61] who reported that complement C3, adiponectin, and cleavage product of C3 (acylation-stimulating protein, ASP) are altered in obesity in very young children. However, ASP deficiency in mice has been associated with resistance to weight gain on a high-fat diet, despite increased food intake [62]. Additionally, high levels of C3 among macrosomic newborns in the current study could be related to its low or reduced consumption resulting from an abnormal regulatory pathway through complement factors H and I, which are usually loaded to avoid a runaway

of the activation pathway of complement. In this case, we may even envisage a gain-of-function mutation that would confer a constitutive enhanced activity either on Factor H or I, or on an unidentified cofactor resulting in a decreased use of C3. On the other hand, we cannot rule out the possibility of a regulatory gain-of-function polymorphism in the promoter or in the enhancer region of the C3 gene [63].

Overweight and obesity can weaken the body's immune system and reduce its ability to fight infections. Though it is unclear exactly what causes obesity to affect the immune response, it was suggested that gaining weight might upset a mechanism in the body that reacts to foreign organisms and would have implications for many bacterial infections. Additionally, obesity can lead to chronic inflammatory responses starting in the adipose tissue [64,65]. The main cells infiltrating the adipose tissue are macrophages [66,67], neutrophils [68], B cells [69], T cells [69–71], and mast cells [72]. B cells have been shown to increase 3 weeks after high-fat diet (HFD), followed by T cells [73]. These are the only cells responsible for antibody production.

The primary antibody of the newborn is the maternal IgG, which confers protection from some specific diseases; however, there are low or nonexistent levels of the other immunoglobulin antibodies in neonates [74]. This immunoglobulin can bind to many kinds of pathogens and is able to activate the classical pathway of the complement system. In our study, serum levels of IgG were similar in both groups of newborns, while the complement C3 levels were higher in macrosomic newborns than in controls and were significantly associated with macrosomia. It is therefore likely that the activation of the complement system in macrosomic newborns is independent of the adaptive immune response, and could be triggered by the alternative or lectin pathway of the innate immunity.

Conclusions

In conclusion, the immunological abnormalities related to adaptive immune response observed in obesity and overweight could be preceded by the alteration of antioxidant defense status and by the innate immunity activation starting from birth. Additionally, overweight could be a potential factor of decreased anti-oxidative capacity and increased oxidative stress. Consequently, treatment strategies and prevention targeting complications related to overweight and obesity should be undertaken at a very early stage of life.

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