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PREGNANCY-DRIVEN CARDIOVASCULAR MATERNAL MIR-29 PLASTICITY IN OBESITY

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Abstract

Background—Obesity in pregnancy (MO) is a risk factor for maternal and/or fetal cardiovascular system disorders. This study evaluated maternal CVS expression of Micro-RNA-29 family and its target molecules in MO to test the hypotheses: CVS miR-29 concentrations are increased in pregnancy and decreased in MO.

Methods—Non-pregnant (n=4), pregnant obese (POb, n=4), and pregnant non-obese (PnOb, n=4) baboons (*Papio spp.)* were studied. Maternal left ventricle (LV), left atrium (LA), and aortic arch (AA) were collected at the end of gestation. Expression of MiR-29 and elastin (*ELN*) mRNA were quantified.

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The authors report no conflict of interest.

This work was performed at the Southwest National Primate Research Center and University of Tennessee Health Sciences Center

Results—LA miR-29 (a, c) expression was highest in PnOb. In the LV, miR-29b expression trended lower (p=0.059) for PnOb animals. *ELN* mRNA expression correlated positively with miR-29b expression in AA (r=0.76, p=0.03).

Conclusion—Maternal obesity diminishes miR-29 adaptation to pregnancy. Pharmacologic, tissue-specific targeting of miRNA-29 may represent a strategy for prevention and treatment of MO complications.

Keywords

Maternal obesity; non-human primate; vascular remodeling

INTRODUCTION

Recognized as a major health problem [8], maternal obesity is associated with an increased risk of cardiovascular system (CVS) complications in mothers and their offspring [5, 50, 56] through poorly understood mechanisms. Maternal CVS adaptations are critical for a successful pregnancy [6, 13, 44] and include local [10, 11] and systemic remodeling [2, 42]. Pathological remodeling is associated with CVS disorders [10] and fetal growth restriction (FGR) [63]. A central process of remodeling is extracellular matrix (ECM) reorganization [38], which includes changes in key molecules, determining CVS compliance-elastin and collagen [60]. Obesity, accompanied by arterial stiffness [24], cardiac fibrosis [36, 65] and consequent diminished diastolic function [31, 32], might interfere with normal pregnancy-related CVS remodeling,

MicroRNAs (miRNA) are small non-coding molecules (19–25 nucleotides long) that regulate biological processes by silencing target mRNAs [29] to repress translation of specific genes. The miR-29 microRNA family regulates at least 16 ECM genes and proteins involved in cardiovascular remodeling [4, 33, 57, 62], especially collagen (colA1, col 3A1), fibrillin, and elastin [39]. The regulatory elements for miR-29 are overrepresented in elastin and type 1 collagen genes [46]. miR-29 has been proposed as a marker for cardiac hypertrophy, fibrosis [15, 52] and atrial fibrillation [15]. MiR-29 is also involved in pregnancy regulation [30, 40]. Down-regulation of miR-29 in the ruminant model of FGR is associated with increased arterial stiffness [30] and up-regulation of this molecule is linked to the deceleration of postnatal body growth [26]. The central position of the miR-29 family in both CVS structural changes and in adaptations to pregnancy makes this microRNA family an ideal target and marker for adequate pregnancy-driven remodeling. The goal of this study was to evaluate maternal cardiovascular miR-29 family expression in obese and non-obese, pregnant and non-pregnant baboons. Based on data showing that pregnancy is associated with the left ventricular hypertrophy, that under physiologic conditions cardiac hypertrophy parallels an increase in miR-29 expression [57] and overfeeding-induced obesity decreases the concentrations of this molecule in the animal model [27], we hypothesized that cardiac miR-29 expression would be increased in pregnant animals and that maternal obesity would reduce these changes.

MATERIAL AND METHODS

Humane Care Guidelines

Colony characteristics and procedures have been described in detail elsewhere [55]. Briefly, pregnant baboons were housed in harem cages, typically housing one male and 10-15 females, in AALAC approved facilities at the Southwest National Primate Research Center at the Texas Biomedical Research Institute as described previously [19]. The harem cages were part of the complex with a covered area of 4988 sq. ft., of which 3200 sq. ft. is divided into eight 400-sq. ft. cages [20]. The Animals were fed LEO5 monkey diet with the composition identical to Purina 5038 (LEO 5, Purina, St. Louis, MO, USA) and given water *ad libitum* [43], environmental enrichment was provided in the form of toys, mirrors, and pools [53]. Subjects were divided into two groups, obese (POb, n=4) and non-obese (PnOb, n=4), based on the Rh index, which is an obesity index defined as body weight divided by the square of the crown-rump length [19]. Tissues from four non-pregnant, reproductive age animals were collected during routine pathological examinations at the department of pathology at the Southwest National Primate Center (Table 1). All procedures were approved by the Animal Care and Use Committee of the Texas Biomedical Research Institute.

Tissue collection and processing

Cesarean sections were performed near term (92% of gestational length) with tissue collections as detailed by Farley *et al.* [19]. Animals were premedicated with ketamine hydrochloride (10 mg/kg) as described in (34). After intubation, isoflurane (2%, 2 l/min) was administered to maintain a surgical plane of anesthesia throughout surgery and fetal sampling [20, 54]. The euthanasia was performed according to the guidelines of the American Veterinary Medical Association (AVMA) Panel on Euthanasia [1] with exsanguination under general anesthesia. Samples of the maternal heart left ventricle (LV), left atrium (LA), and aortic arch (AA) were collected and divided into two parts in POb and PnOb animals. One part was flash-frozen in liquid nitrogen and stored at −80°C until evaluation. The second part was fixed in 10% neutral buffered formalin, embedded in paraffin, cut at 5 μm and processed for immunohistochemistry and morphometry as described below. NP baboon tissues were frozen and stored at −80°C until evaluation.

RT-PCR

Total RNA was extracted from 200 mg tissue from LA, LV, and AA using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions [9]. cDNA synthesis for elastin quantification was performed using a first-strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) from 1-5 μg of total RNA; amount of total RNA was the same within the pair transcript-gene of interest. MiRNA quantification was performed from 2.5 μg of total RNA using the NCode™ miRNA First-Strand cDNA Synthesis Kit (Life technologies, cat #45-6612) with GoTaq® qPCR Master Mix (Promega A6002, Madison, USA) [18, 45]. qPCR with 10 ng/reaction was performed on the CFX96™ Real-Time PCR Detection System using SYBR Green supermix (Kapa Bio systems Inc. Woburn, MA, USA). Quantitative PCR was performed in duplicates with sequence-specific oligonucleotides, that were custom-synthesized [59] (Thermo Fisher

Scientific Inc, Waltham, MA, USA and Invitrogen, Carlsbad, CA, USA) and also commercially available (Roche, Applied sciences, Indianapolis, IN, USA) (Table 2) [16]. Data were collected on a LightCycler® 480 (Roche, Applied Sciences, Indianapolis, IN, USA) for *ELN* expression. Gene expressions were compared to suitable reference genes (i.e., β-actin, s-19, and UC6) and normalized using the $2^{-\Delta\Delta C}{}_{T}$ method.

Histomorphometry

Elastin fibers were demonstrated using the Verhoeff-Van Gieson Elastic Stain Kit (Sigma Aldrich, LLC, USA) according to the manufacturer's instructions. Slides where deparaffinized and hydrated with distilled water followed by the addition of Verhoeff working solution. After rinsing in distillated and tap water, each slide was differentiated individually in 2% ferric chloride with agitation, washed in tap water, then placed in Sodium Thiosulfate 5% Aqueous and counterstained with Van Gieson stain solution. Finally, the slides were dehydrated in 95% alcohol. This process resulted in elastic fibers and nuclei being blue/black to black and collagen being a red color (Fig.1).

Slides were scanned using an Aperio ScanScope® instrument at 40× magnificatoin. Image analysis with ImageScope™ v11.1.2.752 by Aperio® available positive pixel count algorithm quantified elastin content in LV and LA, using the histoscore calculation method as described in detail elsewhere (Fig. 1A) [9]. Minimal thickness of the aortic ring (Fig. 1C), number of elastic fibers (Fig. 1D) [58] and percentage of collagen fibers in LA and LV were evaluated as described previously [22].

Statistical methods

Kruskal-Wallis, non-parametric, one-way analysis compared three groups: obese, and non-obese pregnant groups, and the non-pregnant group. The Wilcoxon test was used for the analysis of obese and non-obese baboon groups and Wilcoxon Signed-rank test for differences between LA and LV for their collagen and elastin contents, aortic thickness and number of fibers. The association between two continuous variables was assessed by Spearman's rank correlation. Data are presented as mean ± SE, unless indicated otherwise. Significance was set at 5%. Reported p values were not adjusted for multiplicity.

RESULTS

Maternal morphometry

The weight of maternal heart tended to be higher (p=0.08) in PnOB animals (61.68 ± 2.1g) compared to NP (52.48 ± 4.1g). The weight of maternal heart in POB animals (57.1± 7.5g) was also higher than in NP, but less than PnOB animals. The weight of the pericardial adipose tissue was higher in POB than PnOB baboons, but did not reach significance (Table 1).

miR-29 expression

In the LA, combined expression of miR-29 (a, b, and c) was highest in PnOb baboons, while only miR-29a and miR-29c expression reached significance. (Table 3B). In the LV,

combined expression of miR-29 (a, b, and c) was lowest in PnOb baboons compared to NP and POb non-pregnant baboons (p=0.056) (Table 3).

Elastin and collagen expression

ELN mRNA expression correlated positively with miR-29b expression in the AA (r=0.76, *P* =0 .03), but not in the LV and LA. There were no differences in elastin protein expression, collagen content, aortic thickness, or number of elastic fibers between the obese and non-obese animals (Table 4). In both groups of pregnant (PnOB and POB) animals elastin protein expression and collagen content differed between the left atrium and left ventricle (p=0.13), but did not reach significance (Table 4).

DISCUSSION

Structural and functional cardiac remodeling in obesity [3] and pregnancy [42] is well documented. Both conditions are associated with left ventricular hypertrophy and left atrial enlargement as a result of adaptation to endocrine and metabolic changes, and expansion of blood volume and inflammation that occur in pregnancy. In our study, only pregnancy, but not obesity in pregnancy, was paralleled by an increase in cardiac weight. This finding can possibly be explained by the diminished pregnancy driven heart remodeling in obesity. The mechanism could involve paracrine regulation of the myocardial growth by the cardiac fat [9, 12, 21, 28]. Epicardial, perivascular and intracardial fat are not separated from the underlying tissues by fascia, therefore the adipocytokines from adipose tissue have direct access to the cardiomyocytes [35, 47] (Fig 1B).

Atrial and ventricular changes

Obesity, in general, is a profibrotic state [31] with such common complications as atrial fibrillation (AF) [64]. AF is associated with pathological atrial remodeling including perivascular fibrosis [14]. Interestingly miR-29 regulates cardiac fibrosis [4, 62] and expression of this micro-RNA is decreased in atrial fibrillation [15]. Conceivably, obesity in pregnancy may stimulate cardiac fibrosis through an miR-29 related pathway. In our study, atrial miR-29a and miR-29c (but not miR-29b) expressions were decreased in obese pregnant animals, however miR-29 expression did not correlate with atrial collagen content. This discrepancy could be explained by additional mechanisms that regulate collagen expression, e.g., mechanical changes in the blood flow parameters [7] and pregnancy-specific changes [41]. Interestingly, miR-29 regulates the gene cluster associated with body growth, down regulating growth-promoting genes, including *Igf1* [26]. Thus the observed decrease in miR-29 might represent obesity-specific cardiac growth promoting adaptations.

MiR 29b regulated LV specific collagen content in mouse models infected with a miR-29b sponge carried by a cardiotropic adeno-associated virus [15]. Additionally recently, antagomir 29b had been shown to inhibit endometrial fibrosis [37]. MiR-29c is decreased in cardiac hypertrophy [61]. In agreement with this observation, our model displayed decreased miR-29b expression paralleled by the increased cardiac weight in pregnant, compared to the non-pregnant animals.

The topographic differences in miR-29 expression (in the LA, but not in the LV) between non-obese and obese animals are in agreement with the fact that miR-29 pathway [51, 66] is under the control of the major mediators of differential molecular signaling between LA and LV [17, 23, 37, 48, 49].

Changes in the aortic arch

The Framingham offspring cohort study demonstrated that arterial stiffness precedes increases in systolic blood pressure in obesity [25]. Pregnancy decreases total vascular resistance by at least 25%. The obese animals in this study did not manifest structural changes in the AA, but their *ELN* expression correlated positively with miR-29 expression. This relationship supports the reported role of miR-29 in profibrotic changes of other tissues, namely urinary, hepatic and pulmonary fibroses [48, 51].

CONCLUSION

This study's data describes the maternal cardiac changes in miR-29 and its down-stream molecules in pregnancy and obesity.

Several strengths of this study include the use of the experimental animal model closest to humans thus allowing translation to the events in pregnant women; the uniform, controlled environment of animal housing (i.e., temperature, humidity, physical activity, dietary composition, etc.) would be impossible to achieve in human population studies. A unique strength of this study was the ability to obtain myocardial tissues from precise topographic locations at precise times for pregnant and non-pregnant animals of reproductive age. Weaknesses of this study include its retrospective design, small sample sizes, and heterogeneity among the non-pregnant cohort. Pharmacologic, tissue-specific targeting of miR-29 may represent a novel strategy to prevent and treat the early consequences of maternal obesity, as it has been shown to suppress lipogenic programs in liver [34].

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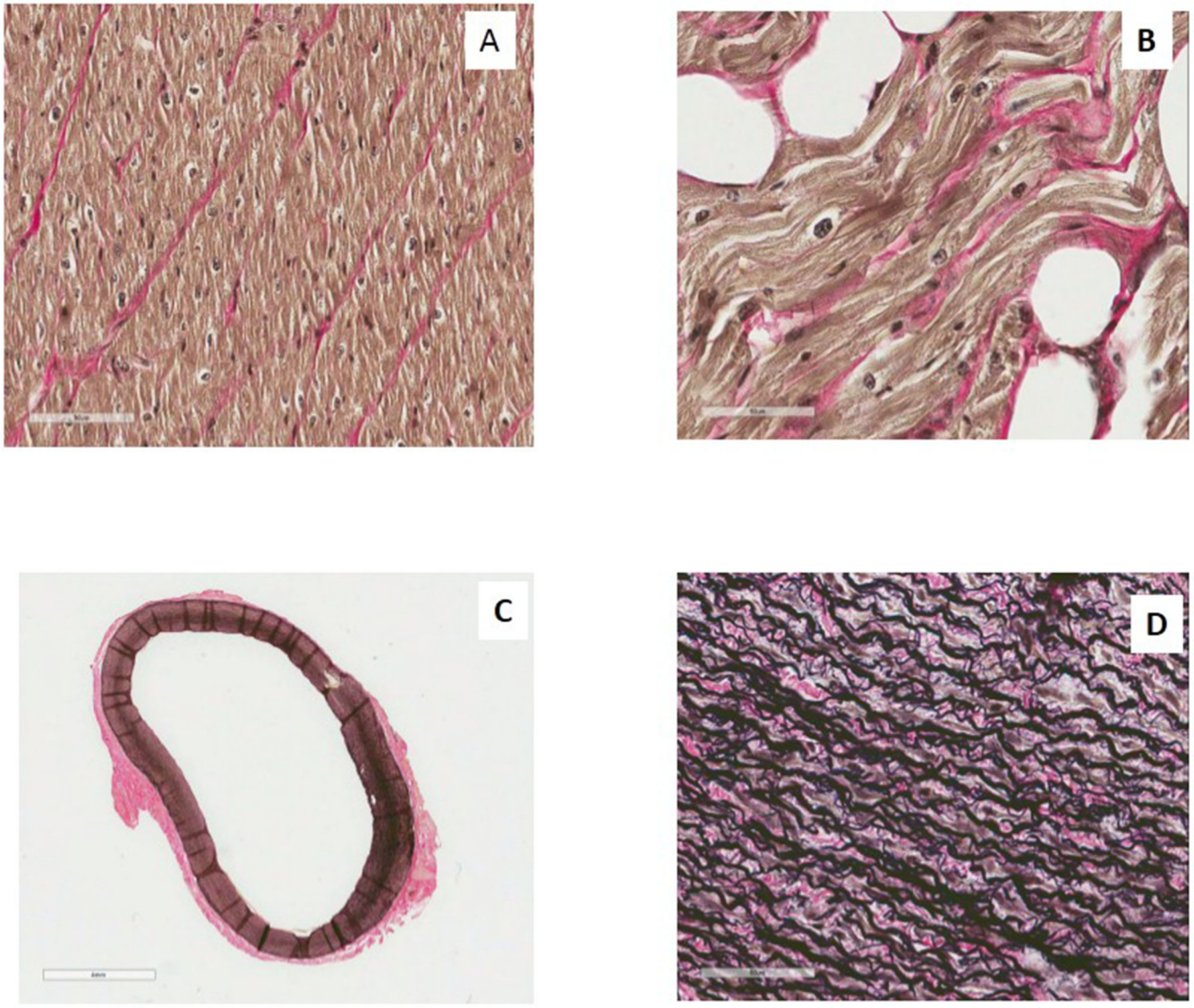

Fig. 1.

Table 1

Demographic characteristics and cardiac morphometry of study baboons (mean ± SEM)

Animal characteristics	Non-pregnant n=4	Pregnant	
		non-obese n=4	Obese n=4
Age (years)	11.75 ± 1.1	10.5 ± 1.2	11 ± 1.6
Parity	3.5 ± 0.7	4 ± 0.7	3.5 ± 0.9
Weight (kg) *	14.4 ± 1.5	15.2 ± 0.7	16.7 ± 1.1
Rh Index (kg/m^2) *	NA	39.1 ± 3.2 *A*	48.7 ± 1
Heart weight (g) *	52.48 ± 4.1	61.7 ± 2.09	57.1 ± 7.45
Pericardial adipose tissue (g)	NA	14.1 ± 2.4	29 ± 8.4

* Data for pregnant animals are obtained from Ref [9, 19].

A $p<0.05$ compared to obese [19].

Table 2

Primers used in this study

Transcripts	Left (l) and Right (R) Primers	Reference
ELASTIN	L 5'-GGCCATTCCTGGTGGAGTTCC—3' R5'-AACTGGCTTAAGAGGTTTGCCTCCA—3'	[16]
S-19	L 5'GCTTGCTCCCTACGATGAGA-3' R 5'- ACCCCGGAGGTACAGGTG-3'	Roche Applied Biosystems, Indianapolis, IN, USA
B-ACTIN	L 5'-CCAACCGCGAGAAGATGA-3' R 5'-CCAGAGGCGTACAGGGATAG-3'	[9]
MiR-29A	TAGCACCATCTGAAATCGGTTA	Invitrogen, Carlsbad, CA, USA
MiR-29B	TAGCACCATTTGAAATCAGTGTT	Invitrogen, Carlsbad, CA, USA
MiR-29C	TAGCACCATTTGAAATCGGTTA	Invitrogen, Carlsbad, CA, USA
U6	L 5'-CTCGCTTCGGCAGCACA-3' R 5'-ACGCTTCACGAATTTGCGT -3'	Invitrogen, Carlsbad, CA, USA

Table 3

The miR29 expression in the heart of the pregnant obese (POb, n=3-4), pregnant non-obese (nOb, n=3-4) baboons at term and in the non–pregnant (NP, n=3-4) baboons (*Papio spp.*).

		Min	Median	Max	p-value
A. Aortic Arch					
miR-29a	**NP**	0.71	1.25	2.67	0.11
	P-nOB	2.01	4.01	6.54	
	P-Ob	1.70	2.23	10.15	
miR-29b	**NP**	0.003	0.57	2.06	0.22
	P-nOB	0.82	2.17	3.54	
	P-Ob	1.06	1.22	2.93	
miR-29c	**NP**	0.05	1.14	2.34	0.33
	P-nOB	1.15	3.09	4.83	
	P-Ob	1.10	1.43	6.40	
B. **Left Atrium**					
miR-29a	**NP**	0.53	1.31	1.91	0.024
	P-nOB	14.84	27.72	46.65	
	P-Ob	0.70	8.38	10.76	
miR-29b	**NP**	0.37	1.54	3.85	0.080
	P-nOB	6.29	6.84	16.94	
	P-Ob	1.39	1.57	11.53	
miR-29c	**NP**	0.51	1.25	1.45	0.027
	P-nOB	16.66	23.69	44.49	
	P-Ob	0.44	6.95	10.01	
C. **Left Ventricle**					
miR-29a	**NP**	7.36	21.45	207.95	0.25
	P-nOB	0.10	2.25	33.64	
	P-Ob	0.37	6.54	176.70	
miR-29b	**NP**	8.29	10.09	62.06	0.059
	P-nOB	0.51	1.92	6.32	
	P-Ob	0.86	3.81	27.87	
miR-29c	**NP**	7.83	25.87	244.79	0.17
	P-nOB	0.06	1.43	28.54	
	P-Ob	0.21	5.91	146.25	

Table 4

The histoscore of elastin stain and percentage of collagen in the left ventricle and left atrium; morphometry of aortic ring (data presented as mean ±SEM).

	Non-Obese	Obese
Left Atrium		
Elastin	186.7 ± 4.7	181.9 ± 7.5
Collagen (%)	4.5 ± 0.01	8.5 ± 0.05
Left Ventricle		
Elastin	214.5 ± 10.3	220.6 ± 12.7
Collagen (%)	2.1 ± 0.003	3.4 ± 0.01
Aortic arch		
Minimal thickness of aortic ring (μm)	872.9 ± 108.1	809.4 ± 142.3
Number of elastic fibers	60.25 ± 14.0	54.0 ± 11.3