



# Leptin in breast milk prevents hyperlipidemia in adult female rats born small for gestational age

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**Background:** To investigate the effect of breast milk leptin on prevention of hyperlipidemia in adult rats born small for gestational age (SGA).

**Methods:** SGA rat pups were cultivated through restricted feeding of dams and were randomly divided into four groups, named SB, SF, SFL, and SBLA, which were fed with breast milk, formula milk, leptin-added-formula milk, and breast milk with a leptin antagonist, respectively, within 20 days after birth. Appropriate-for-gestational-age (AGA) pups were fed with breast milk and designated as the AB group. Body biometrics and serum leptin, total cholesterol (TC), and triglyceride (TG) levels were quantified on days 18, 30, 90, and 120 of life.

**Results:** Compared with the breast-fed AGA rats, the breast-fed SGA rats had lower weight and body mass index (BMI) at all age groups and higher TC on days 30 and 90 ( $P < 0.05$  for all comparisons). Formula-fed SGA rats had higher TC and TG level compared with breastfed and leptin-added-formula-fed SGA rats. The leptin-antagonist-added breast-fed SGA rats had higher BMI, TC, and TG levels compared with pure breast-fed SGA rats on days 90 and 120 ( $P < 0.05$  for all comparisons).

**Conclusions:** Leptin in breast milk might prevent adulthood hyperlipidemia in SGA infants.

**Keywords:** Breast milk; leptin; small for gestational age (SGA); hyperlipidemia

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## Introduction

Small-for-gestational-age (SGA) infants, namely those born at a weight below the tenth percentile for gestational age, experience rapid weight gain during early childhood and have a high risk of developing obesity, cardiovascular diseases, and type 2 diabetes during adulthood (1-4). Such conditions may be caused by nutritional programming (5,6); however, the underlying mechanisms remain unclear. Obesity has been associated with premature birth and low birth weight (7-9). Breastfeeding during early life might

prevent obesity in children and adults (10-12). Human milk contains hormones, growth factors, immunoglobulins, cytokines, and enzymes that support the growth and passive defenses of infants (13-15), in which leptin plays an essential role (16).

Leptin regulates body fat by inhibiting food intake and stimulating catabolic, autonomic, and neuroendocrine responses that direct nutrient stores away from fat compartments. Circulating leptin concentrations are closely correlated with body mass index (BMI) (17-19). Leptin in milk during the early stages of lactation may provide a

mechanism for thermoregulation, satiation, and homeostatic endocrine and metabolic control in the neonate (20,21). Marked decreases in leptin and insulin levels in the mature milk of mothers of SGA newborns contributed to rapid weight gain in these newborns (22); however, the exact mechanisms are unclear. To explore whether breast milk leptin may prevent lipid metabolism disorder in adulthood in SGA infants, we assessed the role of leptin through subcutaneous injections of a leptin antagonist in breast-fed SGA pups and oral supplementation of leptin in formula-fed SGA pups. We subsequently evaluated the pups' body weight (BW), BMI, and serum fat profiles at various time points after birth.

## Methods

### *Animal model*

The experimental protocol was approved by the Medical Animal Care & Welfare Committee of Shantou University Medical College (No. SUMC 2016-145). Three-month-old Sprague-Dawley rats were examined and quarantined for 1 week. Mating was confirmed by verifying the presence of spermatozoa in vaginal smears. The animals were housed in a regulated environment with a constant temperature of 25 °C, 50% humidity, and a 12:12-h light-dark cycle. Pregnant rats were divided into two groups (n=10 per group), *ad-libitum*-fed group and restrictedly-fed group, who were fed *ad libitum* or restrictedly to 30% of regular food intake throughout pregnancy respectively. After birth, the BW, nose-to-anus length, and abdominal circumference (AC) of all pups were recorded. BMI was calculated by dividing BW (g) by nose-to-anus length (cm<sup>2</sup>). Pups with a birth weight within the 95% confidence interval limits for litter and sex in the *ad-libitum*-fed group were designated as appropriate for gestational age (AGA) rats. Pups in restrictedly-fed group weighed less than two standard deviations (SDs) below the mean AGA weight were designated as SGA rats. Because male and female rats differ in terms of weight, BMI, and leptin level, we selected female rats as the experimental subjects.

### *Groups and treatments*

After birth, female SGA rats were randomly assigned to the following four groups: (I) SB: breast-fed SGA rats; (II) SF: formula-fed SGA rats; (III) SBLA: breast-fed SGA rats that received leptin antagonist injections (Triple Rat

Recombinant; ProSpec, Israel); and (IV) SFL: formula-fed SGA rats that received orally fed leptin (PeproTech, USA). The leptin antagonist was dissolved in 0.4% NaHCO<sub>3</sub> at a concentration of 1 mg/mL, and a dose of 2.5 µg/g was subcutaneously injected between 16:00 and 17:00 once daily from day 1 to day 20. The dosage used in the experiment was based on our pilot data in the previous study (23). Recombinant murine leptin (1 ng/µL) was dissolved in water. From day 1 to day 20 of life, during the first 2 h of the light cycle, 20 µL of the recombinant murine leptin solution was administered orally to the pups by using a pipette and the amount was as follows: 1.0, 2.0, 3.0, 4.0, 5.0, 6.3, 7.5, 8.8, 10.0, 11.3, 15.6, 17.2, 18.8, 20.3, 21.9, 23.5, 25.0, 26.6, 39.4, and 43.8 ng, respectively (24). This leptin dosage was five times the amount of daily leptin ingested from maternal milk (25).

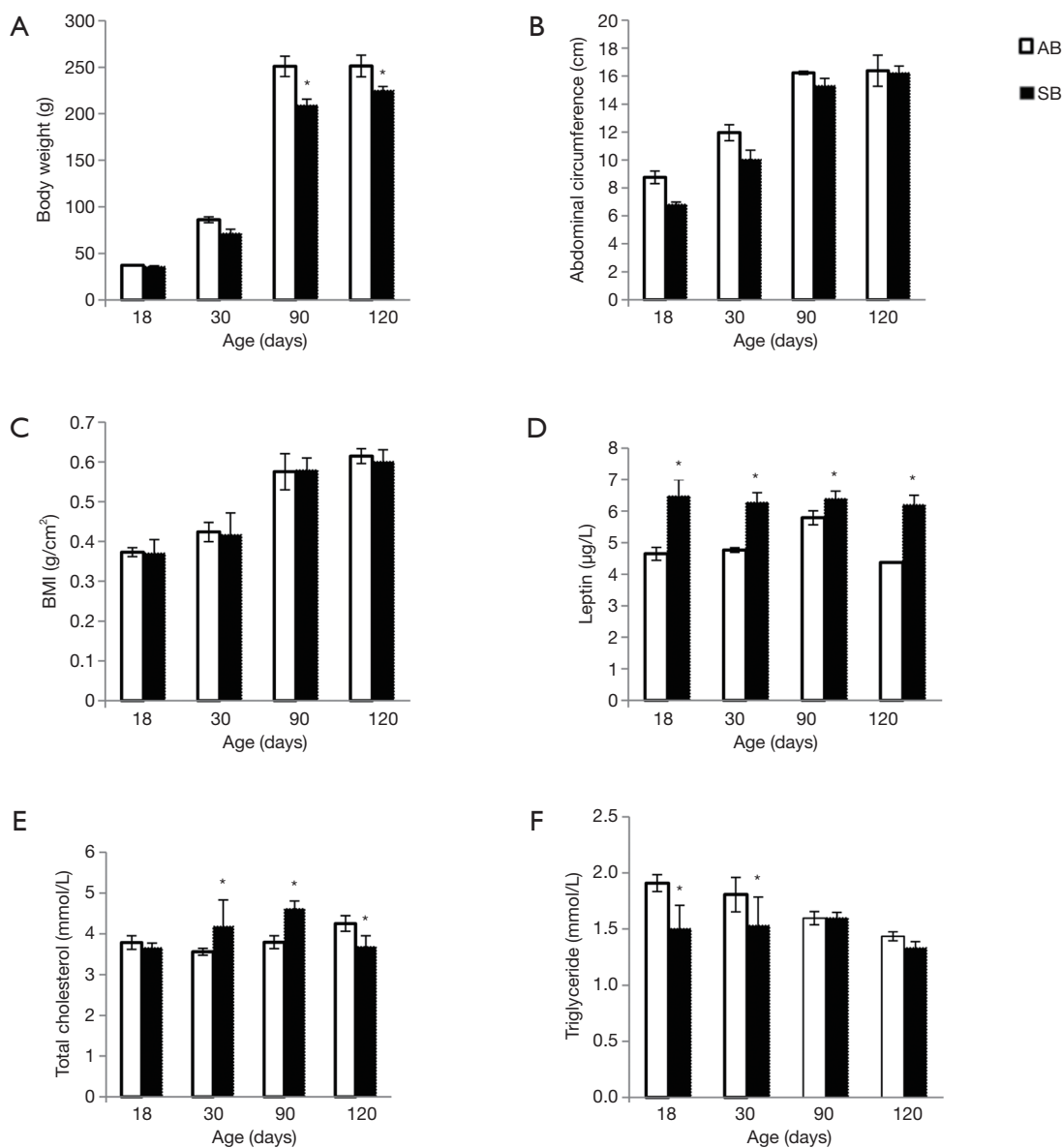
Breast-fed AGA pups were designated as AB group. The rats in breast-fed groups, namely AB, SB, and SBLA, were fed by the mother rats. The rats in formula-fed groups, namely SF and SFL, were separated from the mother rats and artificially fed through a thin tube. After day 20, all rats were fed rat chow. The composition of formula milk was close to that of breast milk. Standard laboratory chow comprising 6% fat, 21% protein, and 55% carbohydrates with an energy content of 3.66 kcal/g (Laboratory Animal Center of Shantou University Medical College) was provided. BW determined food consumption from day 21 to day 120 of life. No significant differences were observed among the study groups at any age (P>0.05 for all comparisons; data not shown).

### *Enzyme-linked immunosorbent assay (ELISA) analysis*

On days 18, 30, 90, and 120 of life, the blood was drawn by puncturing the retro-orbital plexus under light ether anesthesia after overnight fasting (10 h) to assess serum leptin, total cholesterol (TC), and triglyceride (TG) levels. Blood samples were centrifuged at 2,500 ×g for 15 min at 4 °C. The entire serum was immediately frozen at -80 °C until subsequent analysis. Leptin levels were measured using a rat/mouse leptin ELISA kit (Santa Cruz, USA). TC and TG levels were measured using a rat/mouse cholesterol/TG ELISA kit (Santa Cruz, USA).

### *Statistical analysis*

Results were analyzed by conducting analyses of variance (ANOVAs). For all analyses, the level of significance was set



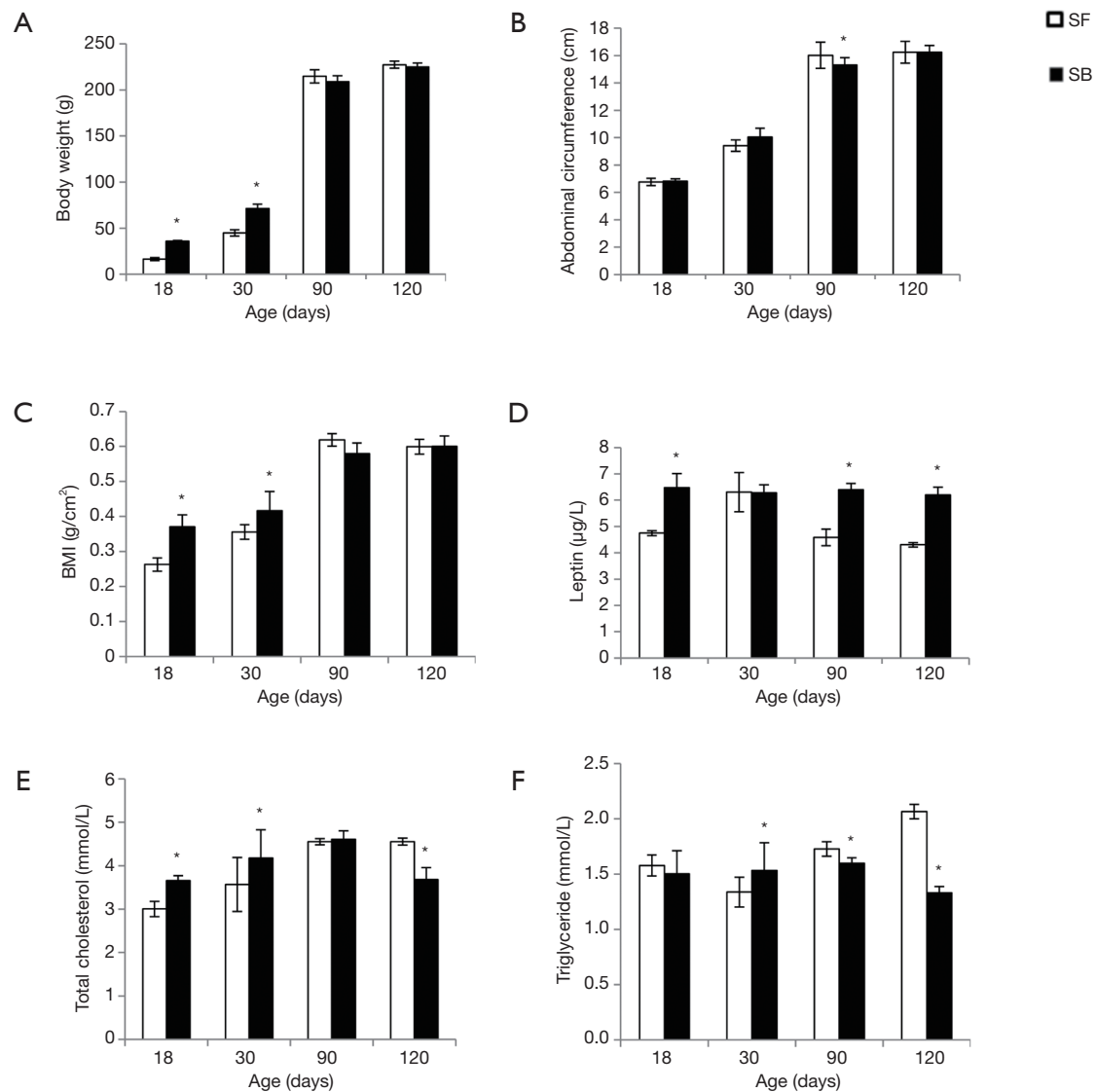
**Figure 1** Comparisons of BW (A), AC (B), BMI (C), serum leptin (D), TC (E), and TG (F) levels between breastfed female AGA and SGA rats. Results are expressed as mean ± SD (n=8 per group). \*, P<0.05 for comparisons between the AB and SB groups, as determined using ANOVA. BW, body weight; AC, abdominal circumference; BMI, body mass index; TC, total cholesterol; TG, triglyceride; AGA, appropriate-for-gestational-age; SGA, small for gestational age; AB, breast-fed AGA pups; SB, breast-fed SGA rats; SD, standard deviation.

at P<0.05. All values are expressed herein as mean ± SD.

### Results

As showed in *Figure 1*, compared with the breast-fed AGA rats, the breast-fed SGA rats had lower weight on days 90 and 120 (P<0.05 for all the comparisons), but similar BMI on every time point (P>0.05 for all the comparisons). SGA

rats had higher leptin level on every time point (P<0.05 for all the comparisons). Referring to the fat profiles, SGA rats had lower TG level on days 18 and 30 (P<0.05 for all the comparisons), but similar TG level on days 90 and 120 (P>0.05 for all the comparisons) compared with the AGA rats. SGA rats had higher TC level on days 30, 90, but lower TC level on day 120 compared with the AGA rats (P<0.05 for all the comparisons). Although SGA rats were



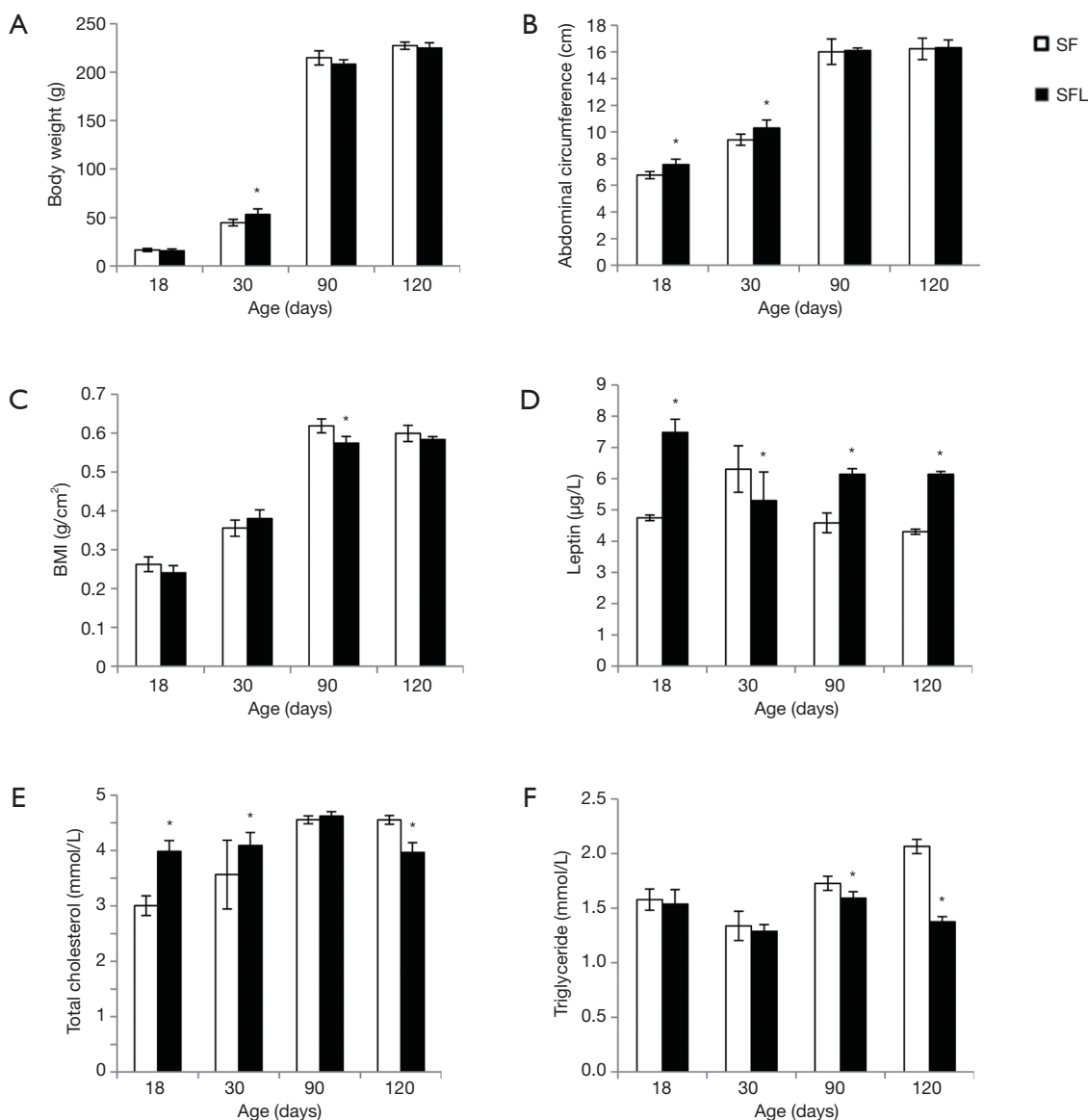
**Figure 2** Comparisons of BW (A), AC (B), BMI (C), serum leptin (D), TC (E), and TG (F) levels between formula-fed and breastfed female SGA rats. Results are expressed as mean  $\pm$  SD (n=8 per group). \*,  $P < 0.05$  for comparisons between the SF and SB groups, as determined using ANOVA. BW, body weight; BMI, body mass index; TC, total cholesterol; TG, triglyceride; SGA, small for gestational age; SF, formula-fed SGA rats; SB, breast-fed SGA rats; SD, standard deviation.

smaller than the AGA rats, they were prone to get lipid metabolism disorder when growing up.

As showed in *Figure 2*, the weight and BMI of the formula-fed SGA rats were lower than those of the breast-fed SGA rats on days 18 and 30 ( $P < 0.05$  for all the comparisons), but were similar on days 90 and 120 ( $P > 0.05$  for all the comparisons). Compared with breastfed SGA rats, formula-fed SGA rats had lower leptin level on days 18, 90, and 120 and higher TC and TG levels on day 120

( $P < 0.05$  for all the comparisons). Hyperlipidemia was present in the adult SGA rats that were fed with formula milk during the neonatal period.

As the leptin level was low in formula-fed SGA rats, which might be related to the hyperlipidemia, we investigated the effect of leptin in breast milk by supplying leptin to formula-fed rats or injection of leptin antagonist to breast-fed rats. As leptin was added to the formula milk, the leptin-added-formula-fed rats, which were named



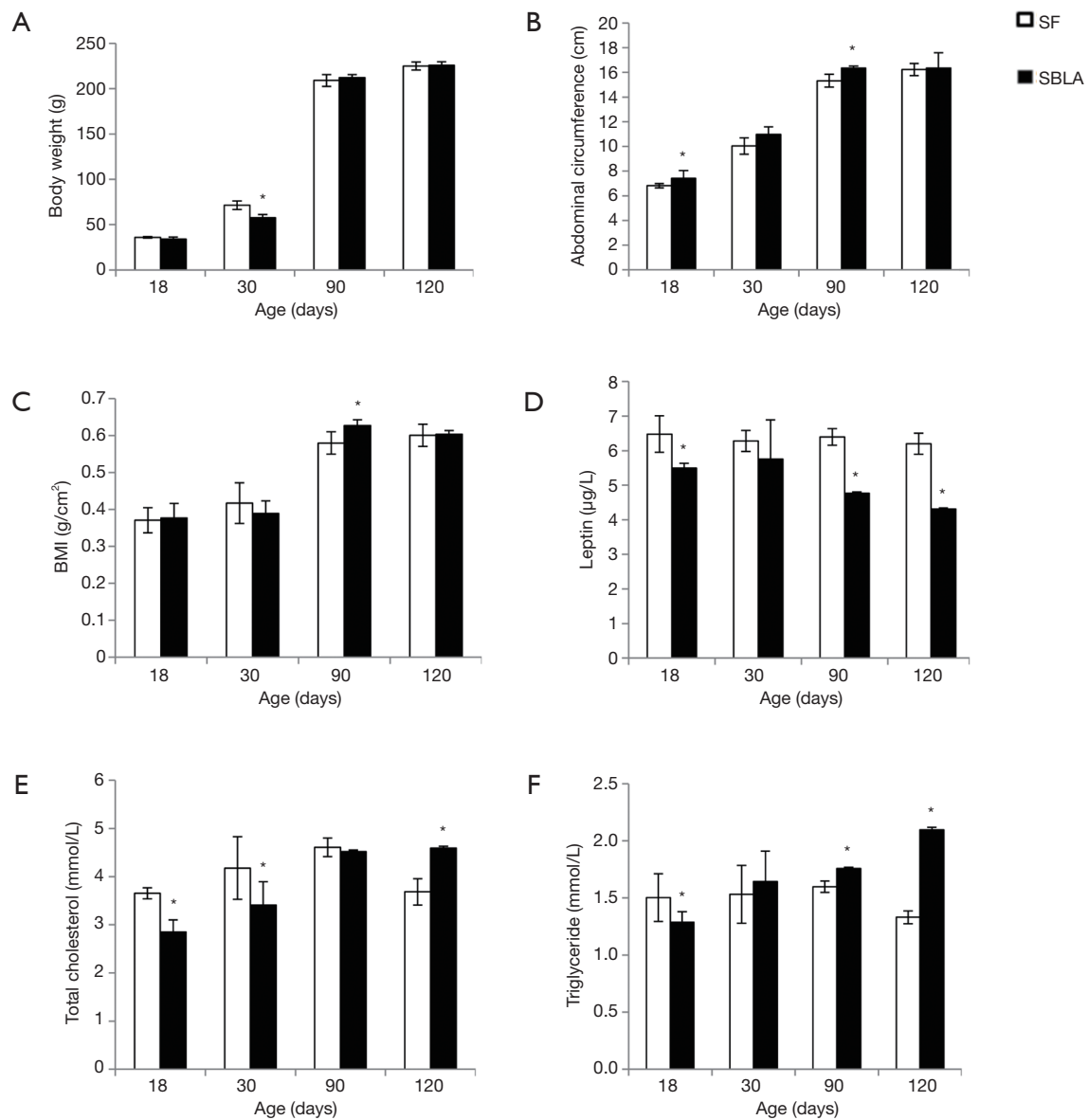
**Figure 3** Comparisons of BW (A), AC (B), BMI (C), serum leptin (D), TC (E), and TG (F) levels between formula-fed SGA and formula-fed SGA + leptin female rats. Results are expressed as mean ± SD (n=8 per group). \*, P<0.05 for comparisons between the SF and SFL groups, as determined using ANOVA. BW, body weight; BMI, body mass index; TC, total cholesterol; TG, triglyceride; SGA, small for gestational age; SF, formula-fed SGA rats; SFL, formula-fed SGA rats that received orally fed leptin; SD, standard deviation.

SFL group, had higher leptin levels and lowered BMI, TC, and TG levels on days 90 and 120 compared with the formula-fed rats (P<0.05 for all comparisons; *Figure 3*). Otherwise, after leptin antagonist was injected, the rats in SBLA group had lower leptin level and higher BMI, TC, and TG levels compared with the breast-fed SGA rats on days 90 and 120 (P<0.05 for all comparisons; *Figure 4*). The observed hyperlipidemia in formula-fed rats was improved

as leptin was added to the milk; otherwise, the prevalence of hyperlipidemia was increased as the effect of leptin in breast milk was inhibited.

### Discussion

In this study, we developed a model of undernutrition in pregnant rats by restricting 30% of ad libitum consumption



**Figure 4** Comparisons of BW (A), AC (B), BMI (C), serum leptin (D), TC (E), and TG (F) levels between breastfed SGA and breastfed SGA + leptin antagonist female rats. Results are expressed as mean  $\pm$  SD (n=8 per group). \*, P<0.05 for comparisons between the SB and SBLA groups, as determined using ANOVA. BW, body weight; BMI, body mass index; TC, total cholesterol; TG, triglyceride; SGA, small for gestational age; SB, breast-fed SGA rats; SBLA, breast-fed SGA rats that received leptin antagonist injections; SD, standard deviation.

throughout gestation; this led to the delivery of SGA infants, similar to other studies (26,27). The nutritional status of the mother during pregnancy or that of the infant during the first year of life can exert long-term effects on metabolism in adulthood (28). These effects are referred to as programming. Undernourishment during development can cause an adaptive response that programs offspring to prioritize organ growth and increases the metabolic

efficiency in preparation for an environment with sparse resources; this is the core theory of the thrifty phenotype hypothesis. Programming becomes detrimental when postnatal nutrition is more plentiful than prenatal nutrition; offspring exhibit rapid catch-up growth and subsequent obesity, which is a critical risk factor for noncommunicable diseases in adulthood, including metabolic syndrome and coronary heart disease (29). Epidemiological studies

in humans and controlled animal studies have revealed that nutritional programming in early periods of life is a phenomenon that affects metabolic and physiological functions throughout life and can even affect the next generation (30,31). In our study, the observed BW, AC, and BMI levels in SGA female rats were lower than those in AGA rats during the neonatal period; however, the adult SGA female rats developed a high BMI and hyperlipidemia, which closely resembled clinical and metabolic abnormalities observed in humans born with a low weight.

The regulation of nutritional imprinting on hormonal and epigenetic mechanisms is complimentary. The central part, including the hypothalamic-pituitary-adrenal axis (32) and growth hormone-insulin-like growth factor axis (33), as well as peripheral tissue such as adipose tissue (34), may play a crucial role in regulation induced by nutritional programming. In these complex systems, leptin is critical, particularly in the process of lipid metabolism (35). Leptin is produced primarily in fat cells but is also produced in other organs and tissues such as the placenta and breast milk tissues. In our study, we found that within 18 days of birth, the leptin levels were higher in the breastfed SGA group than in the formula-fed SGA group, but the levels were similar in these two groups after the weaning period, thereby indicating that breast milk is the primary source of leptin during lactation. After day 18 of life, the number and volume of fat cells began to gradually increase, thereby enhancing the ability of fat cells to synthesize and secrete leptin. In this study, the peak leptin level was attained on day 90, and the levels gradually decreased after that. These results are similar to those of another study that showed that the leptin level was closely related to the amount of body fat tissue present and an increase in BW (36).

A comparison between breastfed female SGA and AGA rats revealed that there were higher TC levels, but the TG level was lower on days 30 and 90 in the SB group, indicating that female SGA rats experience hypercholesterolemia at the juvenile stage. Compared with the breastfed SGA group, formula-fed SGA rats had a lower leptin level and higher TC and TG levels. After leptin was added to the formula, the SFL group exhibited lower BMI, TC, and TG levels than the formula-fed SGA group did. Leptin deficiency was hypothesized to disrupt the lipid metabolism-regulating effect, leading to hyperlipidemia in the formula-fed female SGA group. To verify this hypothesis, the SBLA group, in which the rats were fed the leptin antagonist to inhibit the effect of leptin, was analyzed; higher BMI, TC, and TG levels were observed in this

group compared with the SB group. These results indicate that breastfeeding may prevent obesity and hyperlipidemia during adulthood in SGA female rats because of the leptin in breast milk. Another study obtained a similar result and determined that peripheral leptin administration reduced BW under normal food intake (37). Leptin can influence the proliferation and differentiation of infant adipocytes and can help prevent obesity in later life.

In summary, breast milk is the primary source of leptin during lactation. Leptin plays a vital role in preventing obesity and hyperlipidemia during adulthood in SGA rats. However, the exact underlying mechanisms require further investigation.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The experimental protocol was approved by the Medical Animal Care & Welfare Committee of Shantou University Medical College (No. SUMC 2016-145).

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