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RESEARCH ARTICLE

Effects of Maternal Undernutrition on Coronary Vasculature of Fetuses and Neonates in Rabbits (*Oryctolagus cuniculus)*

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A B S T R A C T

This experiment was designed to determine the maternal under-nutritional effects on coronary vessels in rabbit fetuses. Forty healthy pregnant female, adult rabbits (does) were divided into two groups, well-nourished (WN) and under-nourished (UN). Five pregnant does from each group were euthanized after specific time intervals (two weeks, three weeks, four weeks and after parturition). Fetuses were recovered and fetal hearts fixed in 10 percent neutral buffered formalin solution, then subjected to paraffin tissue preparation technique. The cross-section images of the coronary vessels were analyzed with image analysis software ImageJ®. The lowest mean (9.96±1.34um) coronary intima media thickness (IMT) was found in WN fetuses (2nd week) and highest in UN neonates (46.14±1.50um). The lowest mean coronary vessel luminal diameter was found in UN fetuses at 2nd week of development (25.52±6.48 um) and highest in WN neonates (120.31±22.31 um). The lowest mean coronary vessel luminal diameter was found in WN fetuses at 2nd week of development (24.88±6.45um) and highest in UN neonates (120.31±22.31um). The lowest mean coronary vascular diameter was observed in WN fetuses at 2nd week (39.06±12.84um), highest in WN neonates (146.27±14.84um). The lowest mean coronary vascular perimeter was found in UN fetuses at 2nd week of development (122.7±46.60um) and highest in UN neonates (458.1±46.63um). Thus, maternal undernutrition and developmental age increased coronary IMT, however, other parameters decreased with maternal undernutrition and increased with age of fetuses.

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INTRODUCTION

Coronary artery disease CAD (the most prevalent kind of CVDs) resulted in about 17.9 million deaths and affect hundreds of millions of people, annually. This makes it the most common cause of human deaths globally, with 32% of all deaths. It involves reduced blood flow to the cardiac muscles due to plaque (a waxy substance) build-up in the arteries and arterioles of the heart. Due to decreased vascular lumen, coronary arteries cannot deliver enough oxygenated blood to the heart, which may lead to death (WHO, 2022).

Prediction of CVD risk is one of the best available tools for CVD control (Yang *et al.*, 2020). Studies have shown that carotid and aortic intima and media thicknesses (IMT) can help predicting the risk of CVDs in human subjects (Kume *et al.*, 2005; Kokubo *et al.*, 2018).

An increased peripheral resistance characterized by elevated blood pressure involves small blood vessels, especially small arteries of 150-300µm luminal diameter and larger arterioles of 50-150µm luminal diameter. These are the most important locations in the arterial bed that undergoes changes leading to increased peripheral resistance characterized by elevated blood pressure (Schiffrin, 1992; Woodman, 2009; Delong and Sharma, 2022).

There have been concrete evidences about links between compromised maternal nutrition and impaired fetal growth to the high incidence of CVDs in adulthood and later stages of life (Painter *et al.*, 2007; Pullar *et al.*, 2019), supporting the developmental origins of health and disease (DoHAD) concept regarding the fetal origin of various diseases. According to this concept, an individual's short and long-term health is influenced by exposure to certain environmental factors during certain periods of development and growth (Mandy and Nyirenda, 2018).

The gravity of maternal and children undernutrition issues in Pakistan can be understood by the fact that about 10 million Pakistani children face stunting, one of the major causes being maternal undernutrition (UNICEF, 2022). The World Bank committed the US $47.95 million in 2014 to help Pakistan improve the nutrition status of pregnant and lactating women. This situation can be generalized to women and children living in different developing countries (World Bank, 2014).

However, there is non-availability of literature about the influence of maternal undernutrition upon the IMT. Furthermore, IMT of coronary vessels has not been studied for relationship with CVDs risk, despite that coronary arteries are very much involved in the myocardial infarctions themselves. So, this study was designed to elucidate the effects of maternal undernutrition on the IMT of coronary vessels during different developmental stages in rabbit fetuses.

MATERIALS AND METHODS

Permission was obtained from Institutional Biosafety and Bioethics Committee (IBC), University of Agriculture, Faisalabad, vide letter D. No. 477/ ORIC, dated 01-02-2022, about ethical use of animals in research trial as per guidelines.

Forty clinically healthy, adult female rabbits (does) of mixed local breeds, with minimum body weight difference, were procured from local market and housed at experimental animal house, Faculty of Veterinary Science, University of Agriculture Faisalabad, in individual cages (measuring 75x75x30cm) for this trial. Animals were kept at temperature 10-25°C, during December to March. The animals were exposed to 14 hours' light and 10 hours' darkness), with light intensity of 60 lux. The does were treated with GnRH in a dose of 0.25 ml/animal to induce ovulation (0.8µg/0.5 ml, Receptal inj., ICI, Pakistan) (Hassanein *et al.*, 2021). Then, the does were put to male's cage one by one for mating. Ten healthy males were selected for breeding purpose. Pregnancy was confirmed by ultrasonography (Idris *et al.*, 2016). The pregnant females were divided into two main groups namely WN and UN.

Maintenance energy for pregnant does was calculated as per following formula:

ME=1.35(body weight$^{0.75}$ × 100(Kcal)(Blas and Wiseman, 2020)

Ration for the does was formulated by consulting Institute of Animal Nutrition, University of Agriculture, Faisalabad (Table 2) (Symeon *et al.*, 2015). UN female rabbits were supplied with 50% of their maintenance requirements.

For collection of fetuses, five pregnant females from each group were euthanized at second, third and fourth weeks of pregnancy, while new borns were collected from those who parturated at 30±2 days. Weights of pregnant does were determined at the onset of pregnancy and at the time of euthanasia. Difference of weights was calculated as maternal weight gain. Maternal litter size (the number of fetuses/ neonates recovered/ born from each doe) and the litter weight (weight of all the fetuses/ neonates recovered/ born from each female rabbit) were also determined.

Heart of each fetus/ neonate was collected, weighed, measured, and a vasodilator, nitroglycerine, was injected into the coronary vessels to keep them in a maximal state of dilation. Hearts were put in 10 percent buffered formalin solution, at least for 72 hours, for fixation purpose. Hearts were, then subjected to paraffin tissue preparation technique for histological studies and stained with hematoxylin and eosin stains (Kim *et al.,* 2019).

For histological analysis round cross- sectional profiles of arterial vessels were randomly chosen. The images of the coronary vessels and arterioles sections were captured with a digital camera at X40, X100, and X200 magnifications. Captured images were analyzed with an image analysis software Image J® (Rueden *et al.*, 2021). Calibration of the software was done by capturing images of a stage micrometer at the 40X, 100X and 200X magnifications. This image was opened in image J®. Calibration was done by drawing a straight line between two points of known distance and then putting this distance in the "set scale" option of software (Analyze>set scale).

IMT, luminal diameter, vascular diameter and vascular perimeter of the coronary vessels, were compared in the UN and control fetuses to determine if maternal undernutrition has a link with IMT and does affect the IMT.

Data was computed with Microsoft Excel® and analyzed using analysis of variance (ANOVA) with Completely Randomized Design under Factorial experiment. Statistix 8.1 program was used for data analysis. Regression analysis was done to elucidate correlation between different study parameters. Tukey's honestly significant difference test (HSD) was done to compare the means of parameters between groups at α = 5%.

RESULTS

Maternal undernutrition severely affected body weight and other morphologic parameters, not only in the developing fetuses, but also of dames. The results have been presented below:

Maternal parameters

Maternal weight gain: During pregnancy differed significantly ($P<0.05$) among all groups, except at third week (WN) and fourth week (UN), with respect to the nutritional status of does as well as developmental stage (Table 2). The maternal weight reduced in UN fetuses at 2nd week of development and highest value in WN neonates.

Maternal litter size: Didn't differ significantly ($P< 0.05$) among most groups, with respect to the nutritional status of does as well as developmental stage (Table 2). However, the lowest litter size was found in UN fetuses at 2nd week of development and highest in WN fetuses at fourth week of development.

The litter weight: Differed significantly ($P< 0.05$) among most groups, with respect to the nutritional status of does as well as developmental stage, except WN groups at 4th

Table 1: Nutritional composition as calculated by chemical composition (percent share) of the ingredients used in this study, per Kg live weight of pregnant does.

Ingredients	Quantity (g)	DM	CP	Digestible energy (kcal/kg)	Lysine	Meth	Fat (%)	Ash	CF	Ca	P
Lucerne fodder	38.5	34	7.7	799.645	2.12	0.616	0	4.428	10.395	6.57	0.85
Maize	13	12	1.17	453.57	0.03	0.0234	0.52	0.702	0.325	0.056	0.35
Wheat bran	16	14	2.4	454.72	0.088	0.0368	0.688	0.896	2.048	0.195	1.54
Molasses	4	2.9	0.12	128.96	0	0	0.004	0.584	0	0.27	0.20
Soybean meal	9	7.9	4.14	335.25	0.261	0.0585	0.09	0.64	0.76	0.31	0.55
Wheat middlings	15.4	14	1.85	485.41	0.054	0.032	0.54	0.66	0.39	0.176	1.20
Sunflower meal	2	1.8	0.64	54.92	0.019	0.011	0.26	0.142	0.29	0.078	0.206
DCP	2	2	0.72	0.67	0.094	0.014	0	1.7	0.03	0.6	0.28
Coccidiostat	0.1	0.1	0	0	0	0	0	0	0	0	0
Total	100	88.7	18.2	2712.475	2.57	0.777	2.1	8.052	14.208	7.655	4.896

DM: Dry matter; CP: Crude protein; Meth: Methionine; CF: Crude fibre; Ca: Calcium; P: Phosophorous

Table 2: Means ±SEM values of maternal parameters from among the UN and WN rabbit does.

Week of Pregnancy	Nutritional Status	N	Maternal weight gain (Mean±SEM) (g)	Maternal litter size (Mean±SEM)	Maternal litter weight (Mean ± SEM) (g)
2nd	UN	4	-109.25±8.19^{d}	3.25±0.39^{b}	25.63±8.93^{f}
	WN	5	-57.80±6.27^{d}	4.60±0.35^{ab}	46.86±7.98^{e}
3rd	UN	4	36.50±5.19^{c}	5.15±0.38^{a}	66.91±8.93^{de}
	WN	4	72.50±4.39^{b}	3.50±0.39^{ab}	93.72±8.95^{cd}
4th	UN	4	45.75±4.17^{b}	4.5±0.39^{ab}	126.75±7.93^{bc}
	WN	4	82.50±5.19^{a}	5.25±0.39^{a}	207.85±10.93^{a}
Born	UN	5	52.40±2.58^{bc}	3.8±0.34^{ab}	144.40±7.99^{b}
	WN	5	102.20±8.27^{a}	4.8±0.33^{ab}	215.8±8.99^{a}

abcdef: Values with different alphabets differ significantly from one another in a row statistically different at P<0.05

Table 3: Means ±SEM values of fetal microscopic parameters from week 2 till birth among UN and WN rabbit fetuses.

Fetal microscopic parameters (Mean±SEM)	Week 2		Week 3		Week 4		At birth	
	UN	WN	UN	WN	UN	WN	UN	WN
Fetal heart weight (g)	0.05±0.007^{e}	0.064±0.008^{de}	0.051±0.007^{e}	0.082±0.007^{d}	0.31±0.007^{c}	0.33±0.007^{c}	0.38±0.007^{b}	0.42±0.006^{a}
Fetal heart length (µm)	1719.3±181.39^{g}	2161.7±133.11^{g}	3307.8±102.31^{f}	4242.9±105.31^{e}	4802.7±107.81^{d}	5889.5±101.31^{c}	6842.8±110.91^{b}	8241.4±115.31^{a}
Fetal heart width (µm)	1014.4±110.97^{f}	864.6±151.23^{f}	2751.9±89.47^{e}	2447.8±89.47^{e}	4473.8±89.47^{c}	3938.2±83.43^{d}	6510.7±83.47^{a}	5816.4±86.47^{b}
Fetal coronary vessel lumen diameter (µm)	24.88±6.45^{c}	60.76±14.86^{bc}	28.07±7.34^{c}	49.49±11.45^{bc}	56.04±12.88^{bc}	95.32±11.45^{b}	81.12±14.40^{bc}	120.31±22.31^{a}
Fetal coronary vessel IMT (µm)	14.22±1.378^{de}	9.96±1.34^{e}	15.18±1.20^{d}	14.23±1.55^{de}	21.08±1.25^{bc}	18.01±1.50^{cd}	27.14±1.50^{a}	23.19±1.2^{b}
Fetal coronary vessel diameter (µm)	39.06±12.84^{c}	70.63±13.84^{bc}	43.28±14.84^{c}	61.99±18.87^{bc}	78.15±13.84^{bc}	109.09±14.84^{b}	123.13±14.84^{b}	146.27±14.84^{a}
Fetal coronary vascular perimeter (µm)	122.7±46.60^{c}	221.8±26.68^{bc}	135.9±36.60^{c}	194.6±16.604^{bc}	245.4± 23.46^{bc}	342.6±46.67^{b}	386.6±48.60^{b}	458.1±46.63^{a}

abcdef: Values with different alphabets differ significantly from one another in a row statistically different at P<0.05.

week and at birth (Table 2). The lowest litter weight was noted in UN does at second of development and highest in WN born neonates.

Fetal parameters

Fetal heart weight and width: Differed significantly (P<0.05) among all groups, except at 4th week of pregnancy, with respect to the nutritional status of does as well as developmental stage (Table 3). The lowest fetal heart weight was found in UN at 2nd week of development and highest in WN neonates. The lowest fetal heart width was found in WN fetuses at 2nd week of development, highest in UN neonates. Interestingly, fetal heart width was increased due to maternal undernutrition.

Fetal heart length: Differed significantly (P<0.05) among all groups, except 2nd week (Fig. 2). The lowest value of fetal heart length was found in UN fetuses at 2nd week of development and highest in WN neonates.

Fetal coronary vessel parameters: No coronary arteries (with tunica media having more than 3 smooth muscle layers) could be identified in these developmental stages. Coronary arterial vessels only included arterioles (with 1-3 layers of smooth muscles in the tunica media). Fetal coronary vessel luminal diameter differed significantly (P<0.05) among most groups. The smallest and the largest values were found in UN- 2nd week and WN neonates at birth, respectively (Table 3).

Fetal coronary IMT generally increased with developmental age, higher in UN and low in WN groups. The lowest value of coronary IMT was found in the coronary arterioles of WN fetuses at 2nd week and highest in UN neonates (Table 3).

Fetal coronary vessel diameter and perimeter generally increased with developmental age, higher in WN and low in UN groups. The lowest coronary vascular diameter and perimeter were found in WN fetuses at 2nd week and highest in WN neonates (Table 3, Fig. 1).

DISCUSSION

Maternal undernutrition during pregnancy effects maternal as well as fetal morphological and histological parameters, as evident from results of the present study. The maternal weight reduced during initial two weeks and the highest gain was observed in WN does at birth. These are in line with findings in sheep (Welsh Mountain ewes) with 50% decreased feed intake, where a huge reduction in maternal weight gain was observed in pregnant ewes as compared to control group during early 28 days of gestation (Cleal *et al.*, 2007). Another feed restriction study in nulliparous rabbit does (mixed breeds) has also shown a significantly reduced weight gain in UN does

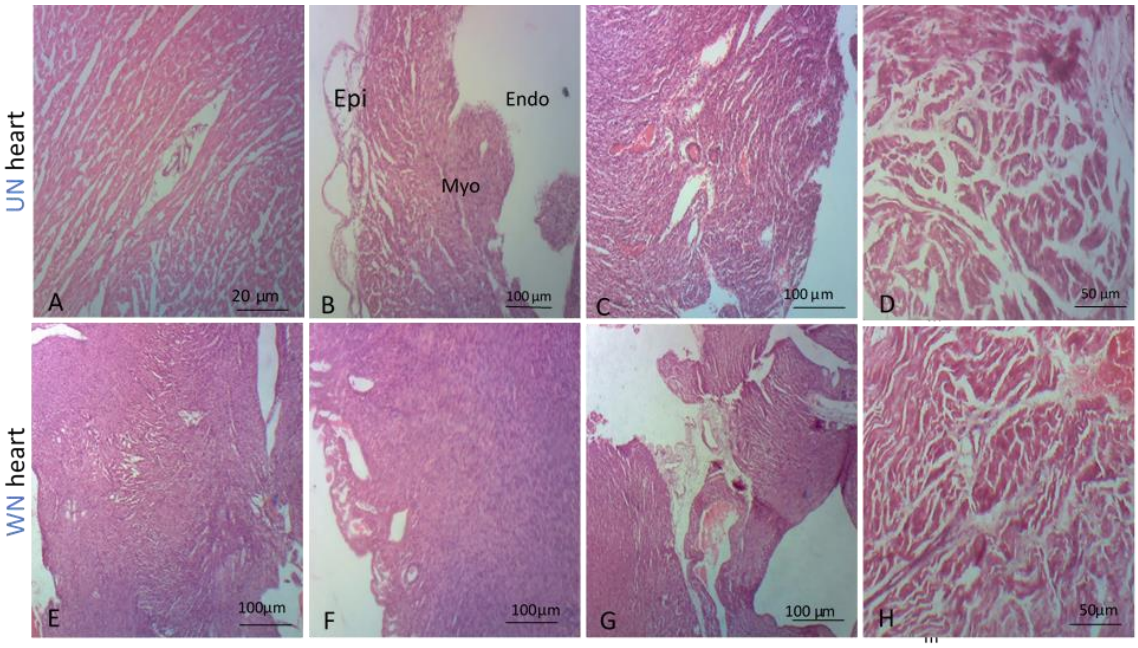

Fig. 1: Light micrographs showing coronary vessels in the cardiac wall of UN and WN fetuses at 2nd week (A &E), 4th week (B& F), 6th week (C &G) and at birth (D &H). Endo: endocardium, Myo: myocardium, Epi: epicardium. Stain H& E. Image A captured at 200X, B,C, E, F and G at 40X and D, H at 100X.

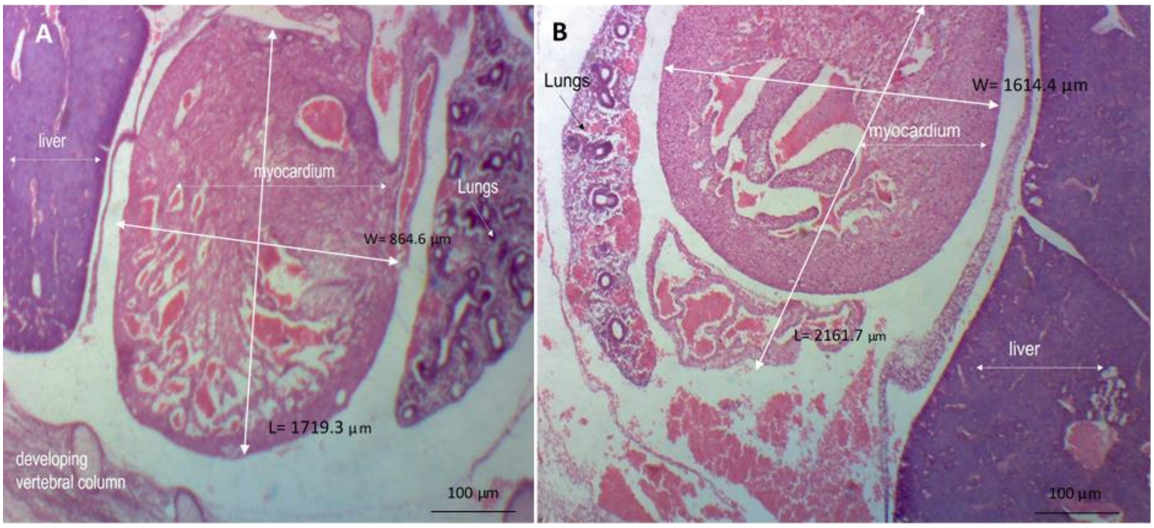

Fig. 2: Light micrograph showing initial stages of cardiac development and surrounding organs in UN (A) and WN (B) fetus. L: mean fetal heart length; W: mean fetal heart width. Stain H& E, X40.

(30% reduction) as compared to controls (Adeyemo *et al.*, 2018). However, these are in partial disagreement with study in Hyla Nouvelle rabbit does, fed 50% of maintenance energy requirements (during 7-19 days of pregnancy and 20-27 days of pregnancy), maternal weight slightly decreased as compared to the initial weight (Symeon *et al.*, 2015). Since Hyla Nouvelle rabbits are also known as broiler rabbits and are genetically modified for fast growth rate and increased body weight, while ours were indigenous rabbits, whose body weight doesn't cross 2Kg. So, apparently variation from our findings could be due to different breeds or climate. In our case, these indigenous rabbits tend to maintain body weight. It appears from our findings as well as these two references that maternal undernutrition impacts maternal body weight, the most, during early gestational phases. Furthermore, ladies with a gestational weight gain less than 8 kg were around 3 times more likely to give birth to a small for gestational age or low birth weight infants (Young and Ramakrishnan, 2020).

Maternal litter size didn't differ significantly ($P< 0.05$) among most groups, with respect to the nutritional status of does as well as developmental stage. These findings are in line with those reported earlier in nulliparous rabbit does (mixed breeds, 30% feed reduction) (Adeyemo *et al.*, 2018) and Hyla Nouvelle rabbit does fed 50% of maintenance energy requirements (during 7-19 days of pregnancy and 20-27 days of pregnancy) (Symeon *et al.*, 2015).

The litter weight differed significantly ($P< 0.05$) with respect to the nutritional status of does as well as developmental stage. These findings are in line with those reported in nulliparous rabbit does (mixed breeds, 30% feed reduction) (Adeyemo *et al.*, 2018). However, different findings are reported in Hyla Nouvelle rabbit does, fed 50% of maintenance energy requirements (during 7-19 days of pregnancy and 20-27 days of pregnancy), litter weight remained unaffected in undernourished and control groups. However, it was quite high (516- 584g) as compared to our findings (Symeon *et al.*, 2015). This variation from our findings could be due to different breeds, climate and time span of undernutrition during pregnancy.

Fetal heart weight differed significantly (P<0.05) in some groups, increased with fetal age, lower in UN than WN. Literature could not be found to compare the effects of nutrition and gestational age on this parameter in rabbits. However, fetal heart weight in rabbits has previously been reported to range 0.002g at day 18 to 0.34g at days 30 of gestation (Karnak *et al.*, 1999). Furthermore, in the fetuses of normotensive pregnant rabbits, heart rate averaged 246 ± 4 beats per min (Coombs *et al.*, 2020). In sheep fetuses heart weights at 109 and 125 days of gestation were found to be 14-16 and 25- 30g, respectively (Frasch *et al.*, 2007). Another study reported the fetal heart weight to be 32.17g in control group of sheep and 18.77g in placental restriction/ hypoxic group, at 119 days of gestation (Botting *et al.*, 2014). In sheep neonates/ lambs, the UN and WN lamb's hearts weighed 43.6±6.6 and 50.8±5.8g, respectively (Rehan *et al.*, 2014). Variation depicts species and body size and weight attributes of two different species.

The fetal heart length increased with gestational age and nutritional status, lowest in UN fetuses at 2nd week and highest in WN neonates. Literature could not be found to compare the effects of nutrition and gestational age on this parameter in rabbits. However, in New Zealand rabbit fetuses, at 25 days of gestation, cardiac length was reported to be 8.49±0.53mm in control group and 7.08 ± 0.73mm in IUGR group (Garcia-Canadilla *et al.*, 2019), almost in line with our findings. Another study reported ultrasonographic measurements of fetal hearts in New Zealand does, describing values 1.7- 2.8mm at 15th day of gestation, 4-4.9mm at 21st gestational day and 10.3- 13.7mm at 29th day of gestation (Chavatte-Palmer *et al.*, 2008), quite higher from our values at birth. Difference could be the large body size of New Zealand rabbits than our animals.

The fetal heart width increased with gestational age and decreased with nutritional status, lowest in WN fetuses at 2nd week and highest in WN neonates. No literature could be found to compare the effects of nutrition and gestational age on this parameter in rabbits. Width of fetal heart was reported as 1.7-2.6mm, 3.5-5mm and 8.4-10.3mm at 15th, 21st and 29th gestational days in New Zealand white rabbit fetuses (Chavatte-Palmer *et al.*, 2008), higher than our values. Difference could be due to different breeds. Furthermore, In New Zealand rabbit fetuses, at 25 days of gestation, basal diameter was found to be 6.49 ± 0.17mm and 6.59 ± 0.25mm in control and IUGR groups, respectively (Garcia-Canadilla *et al.*, 2019), almost in line with our findings. These findings also suggest that maternal undernutrition leads to increase in heart width during pregnancy, while heart length remains higher in the well- nourished fetuses.

Among the microscopic parameters, the coronary vessel luminal diameter increased with nutritional status and gestational age, lowest in UN fetuses at 2nd week and highest in WN neonates. Literature could not be found to compare the effects of nutrition and gestational age in rabbits. In sheep fetuses, luminal diameter of coronary arterioles was found significantly higher in WN fetuses (26.96±1.92µm) than UN fetuses (16.54±1.92 µm) (Rehan *et al.*, 2014), which supports our present findings.

The coronary IMT increased with nutritional status and gestational age, lowest in WN fetuses at 2nd week, highest in UN neonates. No literature could be found regarding the effects of nutrition and gestational age on coronary IMT in rabbits. In sheep fetuses, IMT of coronary arterioles was found significantly higher in UN fetuses (11.51±0.54µm) than WN fetuses (10.21±0.64µm) (Rehan *et al.,* 2014). In rats, however, due to hypoxic pregnancy increase in wall thickness and wall-to-lumen area ratio of the fetal aorta have been reported (Giussani *et al.*, 2012). Results of the present study are contrary to the findings of previous study for carotid and femoral IMT in humans. Maternal under nutrition during pregnancy caused a decrease in carotid and femoral IMT, but an increased prevalence of CAD around 58 years of age to individuals exposed to famine during gestation (during the Second World War) than the unexposed ones (Painter *et al.*, 2007).

The coronary vessel luminal diameter increased with nutritional status and gestational age. No literature was available the effect of maternal under-nutrition upon coronary vessel luminal diameter during pregnancy, in rabbits. However, in sheep, it has already been reported that luminal diameter in coronary arterioles of WN neonates/ lambs was significantly higher than those of UN ones (Rehan *et al.,* 2014). Similar trend has been reported for the hypertensive rats in the mesenteric (178-222µm), femoral (167µm) and cerebral (87µm) in the resistance arteries of hypertensive rats. In the normotensive rats luminal diameters of mesenteric, cerebral and femoral arteries were 194-265, 102 and 199µm, respectively (Ledingham and Ashton, 2005; Souza-Smith *et al.*, 2011). This suggests that decreased luminal diameter in coronary vessels could be due to hypertension.

The fetal coronary vessel diameter increased with nutritional status and gestational age. Literature could not be found about how the maternal under-nutrition affects coronary vascular diameter during pregnancy, in developing fetuses in rabbits. During initial stages of heart development in rabbits, the heart is composed of myocardial walls and the lumen being lined throughout by a layer of endocardial jelly with absent of epicardial layer and coronary blood vessels (Ghazi and Al-jebori, 2014). Similar findings are reported in in human that embryonic heart muscle lacks coronary blood vessels during early stages of heart development (Wilting and Männer, 2013). In sheep fetuses, vascular diameter of coronary arterioles was found significantly lower in UN fetuses (39.56± 2.35µm) than WN fetuses (48.76± 2.35µm) (Rehan *et al.*, 2014). These findings are supported by values of coronary artery diameters in human babies at birth, 1st month and 6 months (Karagol *et al.*, 2021).

Fetal coronary vascular perimeter increased with gestational age and nutritional status. No literature could be found to compare the effects of nutrition and gestational age on fetal coronary vascular perimeter in rabbits. In sheep fetuses, perimeter of coronary arterioles was found significantly lower in UN fetuses (124.22±7.38µm) than WN fetuses (153.10±7.38µm) (Rehan *et al.*, 2014), suggesting that maternal undernutrition had lasting effects on cardiac and coronary structure of the off springs.

Conclusions: Maternal undernutrition decreased maternal, fetal body weights, fetal heart weight and dimensions, decreased coronary luminal and vascular diameters, and increased coronary vascular IMT. Thus, maternal nutritional status is critical for fetal development, in general, and cardiovascular development, in particular.

Authors contribution: This manuscript is from the Ph.D. thesis of SR. SR and ASQ conceived and designed the project. SR executed the experiment and the lab work. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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