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Perinatal Cardiovascular Evaluation in Dorper Sheep Echocardiographic Description and Behavior of the Heart Rate Variability on the Field

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Abstract: The heart is innervated by the autonomic nervous system, with a predominance of sympathetic and parasympathetic activity associated, respectively, with increases and decreases in the heart rate, as illustrated by the Heart Rate Variability (HRV). Pregnancy induces significant changes in the maternal cardiovascular and hemodynamic systems. This study aimed at evaluating changes in the clinical and echocardiographic parameters of pregnant sheep, as well as the HRV indexes due to the physiological alterations that happen at this stage implicating in high maternal metabolic demands. For this purpose, the study evaluated 10 Dorper sheep through their pregnancy, starting from the second month until the day after delivery (24 h after birth), being evaluated clinical parameters, some of the echocardiographic measurements and electrocardiographic examinations focused on the HRV. The HRV indexes were obtained through the Televet 100® system. There were increases in the thickness of the interventricular septum during diastole starting from the third month and in the internal diameter of the left ventricle during systole and diastole at the second and third months, that diverged across the moments studied, while the ejection fraction increased as the pregnancy progressed. There were increases in the LVFWs values in the 2nd and 5th months. The size of the left atrium increased starting at the second month. The SDNN, RMSSD and PNN 50 HRV indexes were higher at the fifth month of pregnancy and after delivery. There were no significant differences in the frequency-domain HRV indexes during pregnancy. As pregnancy progresses, there is an increase in some echocardiographic parameters as well as a predominance of parasympathetic autonomic nervous system activity. The gestation leads to alterations in the clinical parameters and the activity of the autonomic nervous system.

Keywords: Sheep, Gestation, Heart Rate Variability, Echocardiogram

Introduction

Pregnancy induces significant alterations in the maternal cardiovascular and hemodynamic systems, including increases in the Cardiac Output (CO) and reductions in Arterial Blood Pressure (ABP) and vascular resistance, as well as a systemic vascular dilation (Orabona *et al.*, 2019). The Renin-Angiotensin-Aldosterone System (RAAS) is activated to increase the circulatory volume (Ferrazzi *et al.*, 2018), since the volume of blood increases during pregnancy due to increases in the volume of plasma and, to a lesser degree, in the volume of red blood cells (De Hass *et al.*, 2017).

The maternal and fetal cardiovascular functions may be evaluated based on both heart rate and Heart Rate Variability (HRV) records, that is to say, short-term and long-term fluctuations in the heart rate HRV reflects the oscillatory influence of the sympathetic and parasympathetic branches of the ANS on the sinus node and recording the HRV allow an analysis of ANS response to stress. Reductions in the HRV reveal sympathetic predominance, while increases in HRV reveal increases in the parasympathetic tone (Giese *et al*., 2018).

A constant worry for pregnant women is the development of pregnancy heart diseases, which may put the maternal health at risk (Fragkou *et al*., 2010). The echocardiographic evaluation allows an early diagnosis of these heart diseases and further studies in sheep using this method may contribute towards reducing the maternal morbidity and mortality rates (Nagel *et al*., 2010; Hallowell *et al*., 2012). Also, sheep have been widely employed in several experimental protocols in human medicine, as a model for remodeling in cases of heart failure (Navarro *et al*., 2010), in fetal electrocardiography in obstetrics and in pharmacological protocols in anesthesiology due to the similarities with human cardiovascular anatomy and physiology, as well as availability, size, low maintenance costs and short pregnancy.

Production animals may generate highly valued individuals due to advancements in reproduction techniques and neonatal mortality happens due to several factors, such as unfavorable environment, infectious and parasitic diseases, congenital anomalies and maternal frailty. In this scenario, the perinatal evaluation (for sheep, between the 60th day after conception and the 28th day after birth) in production animals may contribute significantly towards reducing losses during the pregnancy and the neonatal period (Grazul-Bilska *et al*., 2011).

Since pregnancy causes physiological changes in the maternal organism, in the present study we aim to illustrate echocardiographic measurements in sheep during pregnancy, considering the high maternal metabolic demands during the pregnancy, as the exam is influenced by hemodynamic changes. We also aim to illustrate the activity of the ANS in this period through HRV parameters aiming to employ such methods to detect possible changes that may incur risk for pregnant sheep reducing the risk of compromising the health of the mother and the viability of the fetus, as well as to compare with changes in women to assist in future studies using translational research contributing in human medicine related to pathologies that occur during pregnancy in women.

Our hypotheses are whether the gestational period leads to changes in the ANS activity in sheep, which can be illustrated by the HRV indices and whether clinical parameters, echocardiographic examination and HRV can illustrate the hemodynamic changes that occur during pregnancy also in the sheep species.

Materials and Methods

Study Location

This study was conducted according to the animal well-being guidelines and approved by the Ethics Commission on Animal Use (CEUA, Comissão de Ética no Uso de Animais) of the School of Veterinary Medicine and Animal Science at Universidade Estadual Paulista "Júlio de Mesquita Filho", Botucatu Campus, under protocol CEUA0174/2016. The owners of the animals consented to the experimental plan and to the procedures performed. The study was conducted at the city of Botucatu, State of São Paulo, Brazil, at the Rubião Júnior District, latitude S-22.902107 and longitude W48.516534, from July 2017 to December 2017. The present study was conducted without any type of anesthetic protocol.

Animals

Ten Dorper Sheep (Ovis aries) were evaluated during pregnancy (152 days, or approximately 5 months - mean of days 150.40±1.57). The evaluations started in the second month of pregnancy (aiming to avoid early losses due to handling stress) and were carried out up to 24 h after delivery.

Maternal parameters for weight, age, number of births and number of descendants were registered. The selected sheep underwent a clinical examination and animals presenting abnormalities were excluded from the study.

The clinical parameter compiled were: Pulse rate (beats per minute - bpm), respiratory rate (movements per minute - mpm), rectal temperature (°C), Capillary Refill Time (CRF), color of the mucosae, Systolic Arterial Pressure (SAP), Mean Arterial Pressure (MAP) and Diastolic Arterial Pressure (DAP).

Table 1 illustrates the respective moments when the exam parameters were performed.

Measurement of the Systemic Arterial Pressure

The measurement of the arterial blood pressure was performed through the Pet Map® (Blood Pressure Measurement Device. Ramsey Medical, Inc. Patent No. D531, 313 S) oscillometric method, validated for the species (Ulian *et al*., 2016).

Blood pressure was measured repeatedly four times, discarding the first measurement. The measurements was performed on the left thoracic limb respecting the dotted lines indicated in the cuff, which was chosen according to the size of the animal (40% of the diameter of the limb) in order to ascertain the precision of the reading. This method detects the arterial pulse through oscillations and also registers the HR values.

The maternal arterial blood pressure measurement was performed during the gestational months, once a month, starting from the second month and it was also performed 2 h after delivery and 24 h after delivery.

Echocardiogram

Echocardiographic assessments started in the second month of pregnancy and were performed every month (once a month) and 24 h after delivery. 3

The echocardiographic examination was performed using an ultrasound device (Mturbo Sonosite) with

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doppler function and a 2 - 8 MHz multi frequency sectorial transducer. The following measurements were taken during diastole through the right parasternal window, in a transversal section, on M mode, with the sheep were standing, at the papilar plane: Thickness of the Interventricular Septum (IVSd); Internal diameter of the Left Ventricle (LVIDd); and thickness of the Left Ventricle Free Wall (LVFWd). During systole, the following measurements were taken: Thickness of the interventricular septum (IVSs), Internal Diameter of the Left Ventricle (LVIDs and thickness of the left ventricle free wall (LVFWs). The means were calculated based on three measurements, All Taken by the Same Operator (ASCA). The Left Ventricle Shortening Fraction (LVSF) and the Ejection Fraction (EF) were obtained. The following formula was used to calculate the LVSF: (LVIDd - LVIDs/LVIDd) x 100.

The diameter of the left atrium (LA) was obtained during ventricular systole, the diameter of the Aorta (Ao) during ventricular diastole, the Left Atrium/Aorta Ratio (LA/Ao) were obtained by a transversal section, throught the right parasternal window, at the aortic plane. The Pulmonary Flow Velocity (PFV) and the pressure gradient between the right ventricle and the pulmonary artery (pulmonary pressure gradient) were also recorded and obtained by a transversal section at the pulmonary plane.

Measurement and Analysis of Maternal HRV

The Maternal Electrocardiogram (ECG) was performed using a Televet 100 system (Kruuse®, version 4.1.3, Marslev, Denmark) as per the technique described by Nagel *et al*. (2011), which detects and registers the ECG via Bluetooth technology. This device employs an ECG filter that enables the visualization and analysis of the maternal and fetal cardiac activity, both at the same time and separately (Nagel *et al*., 2012). The filter extracts and amplifies the fetal ECG signal from the abdominal ECG signal of the mother. The maternal signal is recorded as a modified version of a conventional ECG register, while the fetal signal depends on the position of the fetus inside the uterus and, therefore, is not a standardized record due to the vector modifications, although the standard of electrocardiographic waves is maintained (Quevedo *et al*., 2019).

The electrodes used were adhesive hydrogel electrodes and were placed on the sheep as follows: The green electrode was placed three centimeters from the sternum, the yellow and black electrodes were placed respectively 20 and 30 cm below the withers at the left side of the thorax (Fig. 1) and the red electrode was placed similarly to the yellow one at the right side of the thorax (Ille *et al*., 2014) (Fig. 2). Data was recorded for eight minutes and transferred to a computer via Bluetooth technology.

The analysis of the HRV employed software Kubios (Biomedical Signal Analysis Group, Applied Physics Department of the University of Kuopio, Finland). Before being input in the software, the data was corrected manually in an Excel spreadsheet. The manual correction was performed due to artefacts that could possibly compromise the analysis, as described by Jonckheer-Sheehy *et al*. (2012).

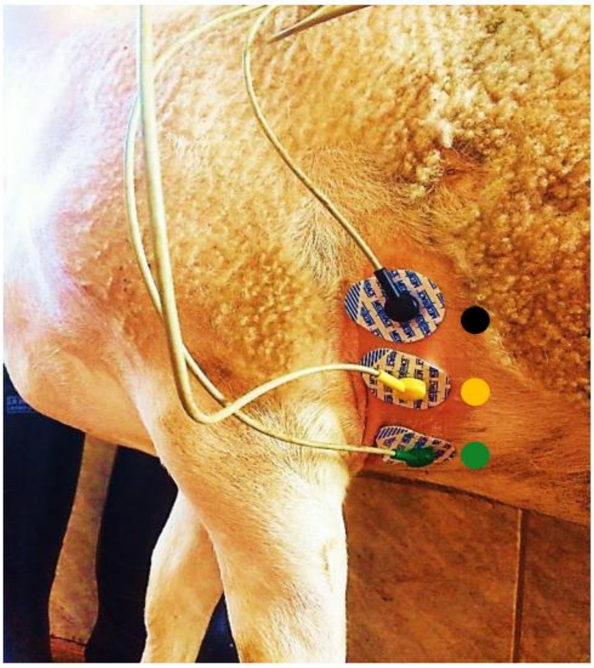

Fig. 1: Position of the electrodes (left side of the thorax) for acquisition of HRV data in Dorper sheep

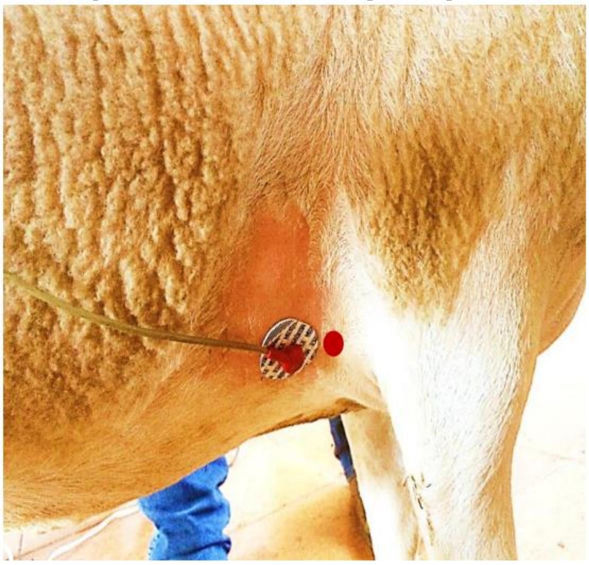

Fig. 2: Position of the electrodes (right side of the thorax) for acquisition of HRV data in Dorper sheep

Table 1: Illustrates the respective moments when the exam parameters were performed

Exams	2 months	3 months	4 months	5 months	2 h after delivery	24 h after delivery
PR	●	●	●	●		
RR	●	●	●	●		
T°C	●	●	●	●		
Blood pressure	●	●	●	●	●	●
Echocardiogram	●	●	●	●		●
HRV	●	●	●	●		●

The minimum, mean and maximum values for PR and the HRV parameters were obtained from all records taken of an individual sheep. Based on the registered RR intervals, the HRV indexes SDNN (Standard Deviation of RR Intervals) and RMSSD (square root of the mean of successive differences between adjacent RR intervals) were calculated (Tarvainen *et al*., 2014). The time-domain index PNN50% (proportion of differences between successive RR intervals exceeding 50 milliseconds) was also recorded.

In the frequency domain, spectral analysis was conducted through the Fast Fourier Transform (FFT) algorithm and the following indexes were compiled and expressed in normalized units (n.u.): The High Frequency Component (HF) varying between 0.15 and 0.4 Hz; and the Low Frequency Component (LF), varying between 0.04 and 0.15 Hz.

Statistical Analysis

The results are shown as the mean, standard deviation and minimum/maximum values. The test employed to verify the normality of the data was the Kolmogorov Smirnov test, while the test employed to compare the proposed moments was Friedmann's test. The significance level adopted for the analysis was $p<0.05$. Friedmann's test was also used to verify if there were differences in the parameters during the pregnancy.

Results

The mean age of the sheep was four years (4.40±1.30); the minimum age was two years and the maximum age seven years) and the mean weight was 70.83±14.01 kilograms (kg). The mean number of births of the females used in the study was three births (3.15±1.72) per animal, while the mean number of the descendants was four (4.30±2.99) per female.

Clinical Parameters and Blood Pressure Measurement

Table 2 shows the maternal clinical parameters during pregnancy and 24 h after birth. An increase in Pulse Rate (PR) and respiratory rate was observed during the last month of pregnancy and the difference was statistically significant when comparing with other periods in the pregnancy.

There were also significant differences in the PR values obtained through the oscillometric method at distinct moments, with higher values occurring at the fourth month of pregnancy and peaking 24 h after birth. The diastolic arterial blood pressure obtained through the same method also presented differences between the moments and peaked 24 h after birth. On the other hand, the systolic blood pressure presented a marked increase during the second month of pregnancy, subsequently decreasing over the remainder of the pregnancy before increasing again 24 h after birth, as shown in Table 2.

Maternal Echocardiographic Measurements

Table 3 shows the maternal echocardiographic measurements obtained during pregnancy. There were statistically significant increases in the IVSd values starting from the third month of pregnancy and in the LVFWs values in the 2nd and 5th months. LVIDd and LVIDs diverged significantly across the moments studied, increasing during the second and third months of pregnancy. On the other hand, LVFWd did not diverge significantly across the moments. The EF increased significantly, as the pregnancy progressed, before decreasing after birth. There were no statistically significant differences in the LVSF values. The differences between the echocardiographic measurements and the moments evaluated are represented in Table 2 as superscript letters.

The Left Atrium (LA) presented a statistically significant increase in size from the 2nd to the 4th month of pregnancy before decreasing in the 5th month in comparison with the previous values and after birth in comparison with pregnancy as a whole. There was an increase in the LA/Ao ratio during the pregnancy, but there were no statistically significant differences between the moments analyzed.

Maternal HRV Indexes

Table 4 shows the maternal HRV indexes during pregnancy and after birth. According to the results, there was a statistically significant difference in the duration of the RR interval across the moments studied, which was longer during the 5th month of pregnancy in comparison with the previous months, before decreasing after birth. The shortest durations were recorded on the 2nd and 4th months of pregnancy.

Table 2: Maternal clinical parameters (mean, standard deviation, minimum, maximum) obtained during the pregnancy and after the birth of the lambs

Clinical Parameters	2 months of pregnancy	3 months of pregnancy	4 months of pregnancy	5 months of pregnancy	Hours after birth		P
PR (bpm)	80.00±12.75^{abc}	90.00±23.05^{ab}	84.00±20.48^{abc}	106.00±27.47^{c}	-		*0.007
	(64.00;112.00)	(44.00;140.00)	(58.00;124.00)	(48.00;152.00)			
RR (mpm)	47.00±19.82^{a}	50.00±22.56^{a}	42.00±12.72^{a}	83.00±34.23^{b}			
	(28.00;112.00)	(24.00;100.00)	(20.00;60.00)	(44.00;140,00)	-		*0.000
T (°C)	38.80±0.25^{ac}	38.50±0.42^{b}	38.90±0.45^{abc}	39.30±0.26^{abc}			
	(38.50; 39.30)	(38.30;38.80)	(38.30;39.80)	(39.10;39.90)	-		*0.000
Mucosae	1	1	1	1	-		
CRT	2	2	2	2	-		
ABP (mmHg)					2 h	24 h	
Diastolic mean	112.00±43.20^{abc}	108.00±31.87^{ab}	120.00±37.06^{abc}	119.00±27.30^{abc}	127.00±27.14	146.00±35.34	
	(60.00;225.00)	(60.00;170.00)	(60.00;185.00)	(65.00;170.00)	(75.00;165.00)^{c}	(100.00;225.00)^{abc}	0.001
	143.00±35.64^{a}	130.00±28.80^{cd}	138.00±34.99^{abd}	142.00±24.75^{abcd}	144.00±27.72	154.00±31.79	*0.004
	(95.00;230.00)	(80.00;190.00)	(75.00;205.00)	(85.00;180.00)	(85.00;175.00)^{abcd}	(90.00;230.00)^{d}	
Systolic	195.00±38.26^{ab}	173.00±31.66+	165.00±44.20^{c}	171.00±25.89^{abc}	165.00±32.43	184.00±35.28	*0.003
	(130.00;270.00)	(130.00;230.00)	(90.00;250.00)	(125.00;220.00)	(100.00;205.00)^{abc}	(105.00;240.00)^{a}	
HR Pet Map	98.00±23.64^{a}	88.00±24.04a	114.00±27.64^{b}	126.00±24.22c	128.00±24.78	131.00±28.45	*0.000
	(70.00;155.00)	(40.00;135.00)	(60.00;170.00)	(75.00;170.00)	(75.00; 175.00)^{d}	(80.00;205.00)^{e}	

Mucosae: 1= normal color, 2= pale, 3= congested; CRT: 1= > 2 seconds, 2= < 2 seconds. Comparison between moments: Friedmann's test *significance
$p<0.05$; ^{ab} different superscripted letters in the same line indicate significant differences between the periods evaluated

Table 3: Maternal echocardiographic parameters (mean, standard deviation, minumum, maximum) in Dorper sheep obtained during pregnancy and 24 h after birth

Echocardiographic Parameters	2 months of pregnancy	3 months of pregnancy	4 months of pregnancy	5 months of pregnancy	24 h after birth	p
IVSd (cm)	0.91±0.21	1.04±0.17	1.00±0.17	1.01±0.17	1.08±0.24	
	(0.57;1.51)^{a}	(0.76;1.39)^{abc}	(0.78;1.39)^{bc}	(0.69;1.33)^{bc}	(0.76;1.63)^{bc}	*0.045
LVIDd (cm)	4.86±0.52	4.53±0.67	4.24±0.55	4.28±0.69	4.26±0.68	
	(3.90;5.89)^{a}	(3.54;6.05)^{b}	(3.02;5.06)^{abc}	(2.65;5.55)^{abc}	(3.11;5.20)^{ac}	*0.009
LVFWd (cm)	1.10±0.31	1.04±0.24	1.05±0.15	1.21±0.26	1.14±0.21	
	(0.76;2.02)	(0.61;1.58)	(0.63;1.26)	(0.88;1.89)	(0.79; 1.51)	0.178
IVSs (cm)	1.46±0.21	1.53±0.24	1.51±0.18	1.55±0.24	1.57±0.19	
	(1.17;1.95)^{a}	(1.19; 2.08)^{ab}	(1.29;1.83)^{a}	(1.20; 2.21)^{ac}	(1.20;1.88)^{ad}	0.048
LVIDs (cm)	3.15±0.42	2.89±0.5	2.65±0.47	2.56±0.49	2.64±0.55	
	(2.39;3.91)^{a}	(1.86;3.97)^{b}	(1.95;3.59)^{bc}	(1.58;3.41)^{c}	(1.67;3.34)^{b}	*0.004
LVFWs (cm)	1.76±0.27	1.54±0.29	1.46±0.23	1.62±0.20	1.56±0.27	
	(1.23;2.15)^{ab}	(1.05;2.39)^{a}	(0.88;1.75)^{ab}	(1.32;2)^{b}	(1.20;2.08)^{ab}	*0.006
EF (%)	64.00±5.70	66.00±6.60	68.00±8.50	70.00±7.33	68.00±6.60	*0.040
	(55.00;76.00)^{a}	(53.00;81.00)^{b}	(53.00;83.00)^{bc}	(59.00;83.00)^{ac}	(61.00;82.00)^{bc}	
LFSF (%)	35.00±4.33	36.00±5.18	38.00±6.85	40.00±6.07	38.00±5.40	0.062
	(29.00;45.00)	(27.00;49.00)	(28.00;51)	(31.00;52.00)	(33.00;50.00)	
LA (cm)	4.08±0.42	4.15±0.25	4.20±0.36	3.83±0.46	3.90±0.43	*0.008
	(3.56;4,96)^{a}	(3.58;4.50)^{bc}	(3.33;4.84)^{bc}	(3.04;4.73)^{abc}	(3.32;4.89)^{abc}	
Ao (cm)	2.36±0.34	2.25±0.29	2.31±0.37	2.26±0.28	2.33±0.31	0.415
	(1.87;2.96)	(1.76;2.78)	(1.44;2.98)	(1.84;2.94)	(1.84;2.92)	
LA/Ao	1.74±0.19	1.86±0.21	1.85±0.28	1.73±0.24	1.67±0.14	0.065
	(1.45;2.19)	(1.51; 2.24)	(1.42;2.51)	(1.38;2.40)	(1.50;2.04)	
Pulmonary Velocity (cm/s)	79.99±11.40	79.28±11.64	79.41±12.51	79.93±11.05	79.69±11.70	0.973
	(65.70;106.20)	(61.00;111.30)	(64.60;105.00)	(61.00;103.60)	(61.00;104.10)	
Pressure Gradient (mmHg)	2.64±0.77 (1.73;4.51)	2.58±0.78 (1.49;4.96)	2.62± 0.85 (1.67;4.41)	2.64±0.75 (1.49;4.31)	2.66±0.73 (149.00;4.33.00)	0.942

Comparison between moments: Friedmann's test; diastole (d), systole (s), Interventricular Septum (IVS), Left Ventricle Internal diameter (LVId), Left Ventricle Free Wall (LVFW), Ejection Fraction (EF), left ventricle shortening fraction (LVSF), Left Atrium (LA), Aorta (AO), velocity of the blood flow in the pulmonary valve (pulmonary velocity), pressure gradient between the right ventricle and the pulmonary artery (pressure gradient). *Significance: $p<0.05$; ^{ab} different superscripted letters in the same line indicate significant differences between the periods evaluated

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Table 4: Maternal HRV indices (mean, standard deviation, minimum and maximum) during the gestational period and 24 h after parturition in dorper ewes

Maternal HRV	2 months of pregnancy	3 months of pregnancy	4 months of pregnancy	5 months of pregnancy	24 h after birth	p
Min PR	86.00±23.00[ab] (43.00;122.00)	41.00±3.37[a] (39.00;52.00)	75.00±9.27[ab] (63.00;99.00)	44.00±4.45[b] (39.00;53.00)	46.00±5.22[ab] (42.00;61.00)	*0.000
Mean PR	114.00±23.27[a] (66.00;148.00)	81.00±9.48ab (65.00;103.00)	114.00±12.10[ab] (93.00;134.00)	81.00±15.00[ab] 94.00±15.00[b]	(65.00;132.00) (72.00;117.00)	*0.000
Max PR	158.00±21.78[ab] (96.00;186.00)	141.00±17.20[a] (99.00;150.00)	183.00±8.58ab (165.00;197.00)	136.00±12.48[b] (101.00;149.00)	138.00±16.44[ab] (98.00;150.00)	*0.000
RR (ms)	554.00±136.68[a] (406.00;914.00)	749.00±90.67[ab] (580.00;925.00)	529.00±57.20[ab] (446.00;643.00)	757.00±113.46[ab] (455.00;926.00)	653.00±107.00[b] (515.00;835.00)	*0.000
SDNN (ms)	9.95±4.60[a] (4.70;18.40)	12.51±7.45[ab] (6.70;32.60)	(4.20;55.10) 17.20±14.84[b]	(9.60;96.70) 36.83±24.32[ab]	(11.50;93.20) 45.01±22.37[ab]	*0.000
RMSSD (ms)	6.42±3.59[a] (2.80;15.20)	10.49±5.40[b] (5.70; 24.40)	11.58±9.84[bc] (2.70;33.60)	29.23±23.55[abc] (7.60;91.60)	36.74±28.47[abc] (12.30; 127.40)	*0.000
PNN50 %	0.29±0.77[ab] (0.00;2.41)	1.04±1.50[a] (0.00;5.36)	2.46±5.27[ab] (0,00;18.75)	7.84±13.40[b] (0.00;50.00)	6.24±4.83[ab] (0.80;16.57)	*0.000
LF (n.u.)	75.91±14.98 (46.18;94.79)	75.25±8.56 (58.03;86.80)	73.69±9.19 (60.33;91.98)	74.58±17.89 (25.34;95.46)	76.07±14.21 (48.16;93.71)	0.653
HF (n.u.)	24.05±14.98 (5.14;53.81)	24.96±9.15 (13.17;45.85)	25.33±8.90 (7.98;39.56)	25.28±17.86 (4.53;74.61)	23.88±14.18 (6.25;51.83)	0.653
LF/HF	5.58±5.22 (0.86;18.43)	3.51±1.59 (1.18;6.59)	3.58±2.63 (1.53;11.53)	5.17±5.03 (0.34;21.07)	5.70±4.14 (1.39;14.99)	0.421

Comparison between moments: Friedmann's test *significance: $p<0.05$; n. u. = normalized units. HR: Heart rate; Min: Minimum, Max: maximum; RR: RR intervals; SDNN: Standard deviation of all RR intervals, RMSSD: Square root of the mean of successive differences between adjacent RR intervals; PNN50: Proportion of differences between successive RR intervals exceeding 50 milliseconds; HF: High Frequency, LF: Low Frequency, [ab] different superscripted letters in the same line indicate significant differences between the periods evaluated

The mean PR obtained through the HRV analysis diverged significantly between the moments, with marked reductions on the 3rd and 5th months of pregnancy. The values increased again after birth, although without surpassing the ones recorded during the 2nd and 4th months of pregnancy. Minimum PR diverged significantly across the moments evaluated, with the highest values recorded on the 2nd and 4th months of pregnancy before decreasing during the 5th month and increasing slightly after birth. Maximum PR also diverged significantly, decreasing markedly on the 5th month of pregnancy and after birth.

The time-domain HRV index SDNN also presented statistically significant differences between the moments, increasing on the 5th month of pregnancy and after birth. Similar phenomena were observed for the RMSSD and PNN50 indexes, both of which represent parasympathetic activity. The Normalized (n. u.) LF and HF indexes and the LF/HF ratio did not diverge significantly across the moments studied.

The analysis of each specific moment during pregnancy revealed statistically significant differences for minimum and maximum PR on the 3rd and 5th months of pregnancy; mean PR and RR interval on the 2nd month and after birth; SDNN between the 2nd and 4th months; RMSSD between the 2nd and 3rd months and between the 2nd and 4th months; and PNN50 between the 3rd and 5th months.

Discussion

Clinical Parameters

In this study, PR was obtained by two methods (maternal clinical examination and oscillometric), but in both PR was high during the last month of pregnancy. The concentrations of progesterone remain high in sheep until the last week of pregnancy, with the placenta producing five times more progesterone than the ovaries towards the end of the gestation. In the final third part of the pregnancy, estrogen causes a dilation in the cotyledonary vessels, effectively doubling the amount of blood that reaches the placenta (Prestes and Landim-Alvarenga, 2006).

Therefore, high PR values in sheep may be explained by the high levels of progesterone, which promotes vasodilation, reacting with receptors in vascular smooth muscle promoting vascular relaxation (Costantine *et al*., 2014) and by the increased blood flow to the placenta. Fetal growth is also accelerated during the last months of pregnancy, which leads to more compressed abdominal organs and, therefore, higher sympathetic activity.

These increases in PR may also be explained by the increased venous return during pregnancy (Almeida *et al*., 2017). Despite the clinical PR increase, the mean for maternal PR remained within the reference values for the species (90-115 bpm).

There was an increase in the values of MAP and SAP in the last month of pregnancy in comparison with the previous month. The action of progesterone promotes natriuresis (Ganaie *et al*., 2009) and reduction of the peripheral vascular resistance (Juengel *et al*., 2004) during pregnancy, while estrogen cause an increase in levels of plasma renin substrate, plasma renin activity and angiotensin leading to increased aldosterone concentration and consequently volume increase of volume through sodium retention. Thus, the increased blood volume during pregnancy is also caused by the activation of the RAAS (Gorrill *et al*., 1986).

We further hypothesized that the high PR and ABP values observed during the last few months of pregnancy could also be related to by an increase in the energetic metabolism of the mother due to the increase size of the fetus and the high concentrations of thyroid hormones during this period.

Pregnant sheep presented increase concentrations of thyroid hormones and this increase is related to the energy metabolism. In non-pregnant animals, these hormones are considered indicators of the metabolic and nutritional state of the herd. In pregnant sheep, the increased activity of the thyroid in comparison with other animals happens due to the increases in the concentrations of ligating proteins, in the secretion of thyrotrophic factors by the placenta and in the response of the hypophysis to the Thyroid-Stimulating Hormone (TSH) related to the secretion of the Thyrotropin-Releasing Hormone (TRH) by the hypothalamus (Araujo *et al*., 2014).

Further studies are needed to verify whether the alterations in ABP in sheep are equivalent to those observed in women with pre-eclampsia to further underly whether sheep are a reliable model for human diseases.

Pre-eclampsia is a syndrome that affects 3% to 5% women and is diagnosed when the pregnant woman presents high blood pressure caused by the pregnancy during the last months of pregnancy. The syndrome is characterized by excessive retention of sodium and water in the kidneys of the mother (Mol *et al*., 2016). In these cases, there are often injuries in the vascular endothelium, leading to arterial spasms mainly in the kidneys, brain and liver. The causes of pre-eclampsia are still not fully understood. Some women are genetically predisposed to developing the affliction and associations have been identified between pre-eclampsia and genetic variants involved in inflammation, oxidative stress and the RAAS (Rana *et al*., 2014).

The maternal blood pressure values and the PR obtained through the oscillometer after birth were both high in this study. Near to the delivery, the cardiac output increases due to the increase venous return from each uterine contraction, increasing further after birth due to the decompression of the abdominal organs (Neal Schrick *et al*., 1993). In addition, there is an increase in the systolic volume immediately after birth, possibly due to the release in the occlusion of the vena cava, with the CO and the PR returning to the normal values two weeks after birth in women (Hunter and Robson, 1992).

Metcalfe and Parer (1966), in a study with pregnant sheep that aimed to evaluate the hemodynamic alterations and compare them to the human species, noted that pregnancy in sheep leads to increases in CO, PR and blood volume and reductions in peripheral vascular resistance, which is similar to the alterations observed in women towards the end of pregnancy. The authors observed a PR of 108 bpm in the last month of pregnancy, which is similar to the values observed in this study, which presented a mean PR of 106 bpm on the last month of pregnancy. They also observed that the SAP did not diverge between the studied moments during pregnancy (although the animals were evaluated under the effects of general anesthesia), but presented an increase 24 h after birth.

This is in line with the results of this study, which observed peak blood pressure values 24 h after birth.
The main limitation with our pressure measurement is that the method used to measure the arterial blood pressure and the stress caused by handling may have contributed to the high blood pressure values observed in the species. The animals proved to resist being handled and the blood pressure was conducted with the sheep in station, which also reflects the difficulties faced when working in the field.

Even though the oscillometric method has been validated for the species, it overestimates the pressure values. The measurement of the arterial blood pressure is fraught with errors. Mercury sphygmomanometry remains the gold standard, but the method has been replaced by a plethora of devices, including aneroid and automatic machines. However, these devices present inherent limitations, with aneroid manometers presenting a deterioration in precision over time and requiring recalibration each 6 to 12 months (Pettit and Brown, 2012).

We observed that the respiratory rate was higher during the last month of pregnancy than in the previous months. There is a significant increase in the demand for oxygen and a 40-50% increase in minute ventilation during a normal pregnancy. This happens due to a 15% increase in the metabolic rate and a 20% increase in oxygen consumption (Soma-Pillay *et al*., 2016). However, it is believed that the high levels of progesterone during pregnancy help increase the minute ventilation even further, considering that progesterone increases the sensitivity of the respiratory core to carbon dioxide, which promotes an increase in minute ventilation. Simultaneously, the growing uterus compresses the contents of the abdomen and applies pressure against the diaphragm, reducing the total distension of the diaphragm and leading to an increase in the respiratory rate to maintain the extra ventilation (Guyton and Hall, 2017).

Echocardiographic Measurements

The echocardiographic evaluation of the structure and function of the heart during pregnancy requires understanding the normal physiological alterations that happen during this period, particularly the increased blood volume (preload), the reduced systemic vascular resistance (after load) and the increased cardiac output. In addition, the cardiac output increases by 30 to 60% in women during the first and second trimesters of pregnancy, first as a result of the increased preload and systolic volume and later as a result of the increased PR. There are divergences in the literature regarding whether the cardiac output increases even further during the third trimester and these divergences may be explained by the individual variation of the mothers (Nagel *et al*., 2016).

In this study, we observed increases in the thickness of the IVS in diastole and increases of the LVFW in systole in comparison with the start of pregnancy in the ewes. In women, the mass of the left ventricle increases 5 to 10% during pregnancy before returning to normal levels six months after birth. Ventricular hypertrophy happens proportionally to the increase of the workload imposed on the heart by pregnancy (Simmons *et al*., 2002).

The maternal echocardiographic parameter LVIDD presented increases in this study, particularly during the second and third months of pregnancy. Due to the hemodynamic modifications that happen during pregnancy, the venous return increases during this period due to the increased blood volume, particularly during the first trimester of pregnancy. This increase in the blood volume and in the cardiac output during pregnancy results in a physiological state of volume overload, which leads to a dilation of the heart and reversible eccentric hypertrophy (Eghbali *et al*., 2005). Serial measurements of the final diastolic dimensions of the left atrium and left ventricle in M-Mode highlights these alterations (Pettit and Brown, 2012). Despite the increased volume, the pressures within the pulmonary vessels and the central venous pressures do not increase during pregnancy, possibly due to the dilation and reduction of vascular resistance in the systemic and pulmonary vascular beds (Soma-Pillay *et al*., 2016).

The systolic and diastolic diameters of the LV were higher in the study presented in this study than in the study conducted by Acorda *et al*. (2016) in both pregnant and non-pregnant sheep. The authors observed LVIDd values of 2.16 cm in pregnant sheep and 2.18 cm in non-pregnant sheep and LVIDs values of 1.19 cm in pregnant sheep and 1.24 cm in non-pregnant sheep. These differences highlight the need for further studies standardizing echocardiographic parameters in sheep according to breed and body weight, since the sheep used in this study presented an average weight of 70 kg, while the ones in Acorda *et al.* (2016) study presented an average weight of 20 kg. The authors cited also observed differences in the echocardiographic parameters according to age and lactation and the internal diameter of the right ventricle also increased during pregnancy, which was not observed in this study.

In the presented study, the EF increased as pregnancy progressed before decreasing 24 h after birth. Studies regarding these indexes in M Mode have shown that the EF and the mean circumferential shortening rate of the fibers increase during pregnancy as a response to the increased systolic volume during this period. This increase happens during first and second trimesters in women before decreasing during the third trimester (Hall *et al*., 2011).

The diameter of the LA increased during the third and fourth months of pregnancy, before decreasing during the last month of pregnancy and increasing again after birth. In a study conducted by Rubler *et al*. (1977) with pregnant women, the diameter of the LA increased as the pregnancy progressed. The same happened in sheep, but there was a decrease in the fifth month. The increased values observed after birth may be explained by the increased venous return after decompression of the abdominal organs.

Locatelli *et al*. (2011) conducted a study to assess if the echocardiographic measurements in sheep are similar to those in humans, concluding that the echocardiographic measurements for the systolic and diastolic functions of the LV in young adult sheep may be extrapolated for human adults, supporting the use of sheep models of human cardiac disease in translational research.

According to the maternal measurements observed in the study presented in this study, the septum hypertrophy was observed. LVIDs and LVIDD also increased, particularly at the beginning of pregnancy, but these results may have been influenced by the pregnancy hormones, since according to Fthenakis *et al.* (2012), the blood flow of the sheep during the estrous cycle increases by 10 mL/minute due to the high concentrations of estrogen and progesterone. In case of fertilization, this flow decreases before starting to increase gradually starting at the 20th day of pregnancy, which may have contributed to the increased LVIDs and LVIDD values observed in this study during the second and third months of pregnancy.

The changes observed in the present study in relation to echocardiographic measurements are similar to the hemodynamic repercussions that occur in women (Pettit and Brown, 2012; Simmons *et al*., 2002), so the results of the present study can contribute to research in women who may develop pathologies during the gestational period, as well as help studies in sheep during pregnancy contributing to follow-up of pregnancy in sheep.

Maternal HRV

Regarding the maternal HRV indexes and the behavior of the PR, the highest records for minimum, mean and maximum PR happened during the fourth month pregnancy and after birth and they came accompanied by inversely proportional variations in the RR intervals as expected. Carpenter *et al*. (2015) observes that changes happen in the HRV during pregnancy and they seem to reflect a substantial reduction in the parasympathetic tone and an increase in sympathetic activity until the end of the first trimester of pregnancy in women.

In contrast with the findings of Carpenter *et al*. (2015), Alam *et al*. (2018), in a study aiming to evaluate the behavior of HRV in pregnant women, observed more parasympathetic dominance during the first trimester of pregnancy and a gradual reduction in vagal activity during the second and third trimesters, while sympathetic activity was lower during the first trimester before increasing gradually during the second and third trimesters, together with an increase in PR. We believe, according to our results, that, during the evaluation of the PR in production animals in the field, environmental factors that might result in increased PR, such as the stress caused by handling, should also be considered, since this fact is a reality in farm animals. Blood pressure is a parameter that we believe is influenced by manipulation since the sheep in the present study were extremely agitated with the manipulation of the limbs. The animals proved to resist being handled and the blood pressure was conducted with the sheep in station, which also reflects the difficulties faced when working in the field.

We observed increases in the maternal HRV indexes RMSSD and PNN50 as pregnancy progressed, revealing an increase in parasympathetic activity. The same happened for the index SDNN, which illustrates activity of both ANS branches.

The increased blood flow may have contributed to increase the distention of the arterial walls and, therefore, promote the activation of the barore flex, the inhibition of the bulbar vasoconstrictor center and the stimulation of the vagal parasympathetic center (Pettit and Brown, 2012).

Therefore, as pregnancy progresses there seems to be a predominance of parasympathetic activity, which may represent an attempt by the ANS to maintain the balance of the reactions that happen during this period, considering that the uterus increases in size as the pregnancy advances, increasing the compression of the vena cava and abdominal aorta, decreasing the venous return and increasing sympathetic activation (Humphries *et al*., 2018). The parasympathetic predominance may be caused by this increase in the size of the uterus as the pregnancy advances, which promotes an increase of the intrathoracic pressure and leads to increased vagal stimulus. In addition, the distention of the forestomach in sheep due to feeding and the size of the uterus may have exacerbated the parasympathetic stimulus.

We believe that a series of events during pregnancy, such as the size of the uterus, hormonal action and the barore flex, all contribute towards that the changes that happen in the ANS activity during this period, but these mechanisms are complex and still not fully understood, warranting further research in the field.

Individual factors should also be considered during the HRV analysis in production animals and when data is collected on the field. The HRV analysis is a method that may be employed to evaluate the ANS in sheep, but when interpreting the indexes recorded in this study, one should consider that they reflect the HRV of animals under conditions of stress. In addition to the influence of pregnancy, there is also an influence of how the animals are being handled, which means that when obtaining HRV data to use as reference, it is necessary to consider under which conditions these references are obtained, considering factors such stress in the field, nutrition and afflictions.

Trenk *et al*. (2015) assessed PR and HRV in bovines during pregnancy and observed that the HRV indexes SDNN and RMSSD did not present any significant alterations during the last 14 weeks of pregnancy, but SDNN was higher in cows than in heifers, suggesting a higher sympathovagal tone in the cows and that age has a degree of influence on the autonomic activity. Quevedo *et al*. (2019), in a study assessing maternal and fetal HRV in Holstein cows, observed a significant decrease in the maternal index SDNN during the last month of pregnancy. The index RMSSD also decreased during the period, but without statistical significance. The age of the animals used in this study may have contributed towards the results observed for the HRV indexes, which is in line with Trenk *et al*. (2015) who observed differences in the index SDNN between cows and heifers.

There were no statistically significant differences for the frequency-domain HRV indexes, but that may be explained by the relatively small sample size in this study. The HF indexes corresponds to respiratory modulation and is an indicator of the action of the vagal nerve over the heart. The LF indexes is a result of the joint action of the sympathetic and parasympathetic components over the heart, with sympathetic predominance (Von Booth *et al*., 2007; Brown *et al*., 1993; Eghbali *et al*., 2005).

Since the HF component of HRV is centered around the RR, breathing is a factor that should be considered when analyzing the HRV because the RR varies between individuals and change according to physiological conditions, such as exercise (Jensen *et al*., 2008). Therefore, the RR in sheep is a factor that should be considered when interpreting the HF index, since the animals in this study presented increased respiratory rates. In addition, the blood flow to the respiratory system increases during pregnancy (Levy, 1990) and evaporation through breathing is an important heat loss in sheep exposed to high temperatures, which is something that may have interfered with the results observed in this study for RR and the HF index.

Chaswal *et al*. (2018), in a study aiming to assess the behavior of HRV in pregnant women, observed sympathetic hyperactivity and sympathovagal imbalance in patients with pre-eclampsia in comparison with controls comprised of women with normal blood pressure and non-pregnant women. The decrease in parasympathetic activity was obvious due to the significantly lower values for the RMSSD and HF (n. u.) indexes in the group with pre-eclampsia. The increase in the LF (n. u.) index reveals increased sympathetic activity, while the higher LF/HF ratio indicates a sympathovagal imbalance, attributed to the sympathetic dominance and reduction of the vagal tone in women with pre-eclampsia. This shows that the HRV is an index capable of revealing afflictions that could develop as the pregnancy advances and potentially compromise the health of both mother and fetus.

In this study and in previous studies from the literature, we highlighted the alterations in the cardiovascular parameters in sheep during pregnancy and emphasized the need to monitor the mothers during this period due to the series of hemodynamic alterations that happen. The changes that occurred in the maternal HRV indices may contribute to studies in sheep and in women to identify early signs of fetal distress, as well as imbalance in the activity of the ANS during the gestational period in both species, which can also be an indicator of maternal pathologies developed during pregnancy.

In addition, extensive studies are conducted on the cardiovascular parameters and HRV of sheep since the species has a heart that is similar to the human heart in several aspects, including the dimensions of the chambers, the coronary anatomy and the magnitude of the hemodynamic parameters such as blood pressure, PR and cardiac output. In addition, the autonomic innervations of the heart are similar in sheep and humans (Von Booth *et al*., 2007; Thayer *et al*., 2012; Partridge *et al*., 2017).

The creation of a physiological extra-uterine environment that can support the continuous growth of the fetus and the development of organs with no connection to the biological placenta has the potential to improve the survival rates and reduce morbidity in premature births (Acharya *et al*., 2004). There are echocardiographic parameters that characterize the clinical state of the fetal cardiovascular system.

Doppler ultrasound is used to assess the cerebrovascular, placental and fetal blood flow, as well as the vascular resistance through the calculation of pulsatility indexes for the umbilical artery and the middle cerebral artery, respectively (Acharya *et al*., 2004). A study conducted by Ozawa *et al*. (2020) aiming to employ an extra-uterine environment (artificial placenta) for neonatal development revealed that is resembled the natural state in the placenta and promoted a stable and sustainable hemodynamic state for a period of three weeks.

Under this light, we want to emphasize the importance of describing the echocardiographic parameters in pregnant sheep and the hemodynamic behavior of the changes that happen during this period considering that studies regarding the development of artificial placentas have gained relevance recently and some of them use echocardiography to monitor fetal development as well as monitor SNA activity through electrocardiographic parameters.

It´s important for translational research monitoring the sheep during pregnancy through a cardiovascular evaluation ensures a healthy pregnancy, avoiding any possible risks to the health of both mother and fetus, which could lead to economic losses, particularly when using Fixed Time Artificial Insemination (FTAI) and the loss of descendants that could potentially contribute towards the profitability of the sector.

As far as the authors know, there is a shortage in the description of diseases in sheep during pregnancy, as is well described in women, for example, eclampsia. The echocardiogram has been an exam of great value in the routine since systemic dysfunction can be diagnosed and/or suspected according to changes that occur in the echocardiographic parameters. We believe that the present study can contribute to refer echocardiographic parameters in ewes during pregnancy as well as future research for pathologies that may affect pregnant ewes.

Study Limitations

This study has some inherent limitations. The high values for the arterial blood pressure may have been caused because the measurements were taken with the animals in station as we were not allowed to position the animals in decubitus due to their cost. The manual restraint of the sheep increased their stress since the animals were not used to handling, which may have influenced the results for ABP.

The small sample size may have contributed towards non-significant results and the choice of breed may have influenced the echocardiographic parameters since the Dorper breed is considered a meat-producing sheep. Control animal parameters were also not performed, which limits analysis to compare normal sheep parameters and during pregnancy.

Conclusion

The echocardiographic evaluation highlighted the hemodynamic alterations that happen in sheep during pregnancy. During this period in sheep, there is a predominance of parasympathetic activity and it is believed that this is an attempt to maintain the sympathovagal balance in the face of the adaptations that happen in the organism of the mother to accommodate the fetus. This is illustrated by the HRV, which has proven to be a feasible technique in production animals that may contribute towards reducing economic losses in the sector, since a healthy pregnancy may generate high performance descendants and, therefore, high zootechnical value, having a positive impact in the agribusiness sector.

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Author's Contributions

Maria Lucia Gomes Lourenço and Simone Biagio Chiacchio: Contributed with conceptualization.

Danilo Otávio Laurenti Ferreira: Contributed with methodology.

Amanda Sarita Cruz Aleixo e Mayra de Castro Ferreira Lima: Contributed with formal analysis.

Lukas Garrido Albertino: Contributed with investigation.

Ana Luísa Holanda de Albuquerque and Raphael Tortorelli Teixeira: Contributed with resources.

Amanda Sarita Cruz Aleixo: Contributed with writing original draft preparation and writin review and editing.

Miriam Harumi Tsnunemi: Contributed with visualization, supervision and statistical analysis.

Maria Lucia Gomes Lourenço and Simone Biagio Chiacchio: Contributed with project administration and funding acquisition.

All authors have read and agreed to the published version of the manuscript.

Ethics

This study was conducted according to the animal well-being guidelines and approved by the Ethics Commission on Animal Use (CEUA, *Comissão de Ética no Uso de Animais*) of the School of Veterinary Medicine and Animal Science at *Universidade Estadual Paulista "Júlio de Mesquita Filho",* Botucatu Campus, under protocol CEUA 0174/2016.

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Abbreviation

ABP: Arterial Blood pressure
ANS: Autonomic Nervous System
AO: Aorta
CO: Cardiac Output
CRT: Capillary Refill Time (CRT)
DAP: Diastolic Arterial Pressure
EF: Ejection Fraction
ECG: Electrocardiogram
FFT: Fast Fourier Transform
FHR: Fetal Heart Rate
FTAI: Fixed Time Artificial Insemination
HF: High Frequency
PR: Pulse Rate
HRV: Heart Rate Variability
IVSD: Thickness of the Interventricular Septum Diastole
IVSs: Thickness of the Interventricular Septum Systole
LA: Left Atrium
LF: Low Frequency Component
LV: Left Ventricle
LVFWD: Thickness of the Left Ventricle Free Wall Diastole
LVFWs: Thickness of the Left Ventricle Free Wall Systole
LVIDD: Internal Diameter of the Left Ventricle Diastole
LVIDs: Internal Diameter of the Left Ventricle systole (LVIDs)
MAP: Mean Arterial Pressure
LVSF: Left Ventricle Shortening Fraction
PNN50%: Proportion of Differences Between Successive RR Intervals Exceeding 50 Milliseconds
PFV: Pulmonary Flow Velocity
RAAS: Renin-Angiotensin-Aldosterone System
SAP: Systolic Arterial Pressure
SDNN: Standard Deviation of RR Intervals
RMSSD: Square Root of the Mean of Successive Differences Between Adjacent RR Intervals
TSH: Thyroid-Stimulating Hormone
TRH: Thyrotropin-Releasing Hormone.