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Transthoracic Cardiac Ultrasonic Stimulation Induces a Negative Chronotropic Effect

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Abstract

The objective of this study is to investigate cardiac bioeffects resulting from ultrasonic stimulation using a specific set of acoustical parameters. Ten Sprague–Dawley rats were anesthetized and exposed to 1-MHz ultrasound pulses of 3-MPa peak rarefactional pressure and approximately 1% duty factor. The pulse repetition frequency started slightly above the heart rate and was decreased by 1 Hz every 10 s, for a total exposure duration of 30 s. The control group was composed of five rats. Two-way analysis of variance for repeated measures and Bonferroni *post hoc* tests were used to compare heart rate and ejection fraction, which was used as an index of myocardial contractility. It was demonstrated for the first time that transthoracic ultrasound has the potential to decrease the heart rate by ~20%. The negative chronotropic effect lasted for at least 15 min after ultrasound exposure and there was no apparent gross damage to the cardiac tissue.

I. Introduction

Although diagnostic ultrasound is well established in cardiology, there is an unexplored potential for therapeutic applications. The available literature indicates the possibility of using ultrasound for nonpharmacological treatment of heart failure [1], defibrillation [2], pacing [3], cardiac gene therapy [4], and ablation for treating arrhythmias [5].

Ultrasound is a mechanical wave and it is known that the heart can be affected by mechanical disturbances. Such disturbances are able to influence the origin and the spread of cardiac electrical excitation by intra- or extracardiac mechanisms. This process is called

mechanoelectric feedback and is involved in a variety of clinical manifestations. It affects the physiological heart rate modulation, and underlies the mechanical induction (e.g., commotio cordis) and termination (e.g., precordial thump) of heart rhythm disturbances. This feedback involves mechanosensitive ion channels and mechanisms that affect modulation of cellular Ca^{2+} handling [6], [7].

In a preliminary study [8], we tested how different operation modes of ultrasound application [i.e., continuous wave, pulsed wave at a single pulse repetition frequency (PRF), and pulsed wave at variable PRFs] could produce cardiac bioeffects. At that point, the variable PRF seemed to be quite interesting for further investigation. Thus, the objective of this study is to investigate cardiac chronotropic effects resulting from ultrasonic stimulation using a variable PRF. The experimental group was compared with a control group that went through the same experimental procedures, except for the application of ultrasound.

II. Methodology

A. Ultrasound Transducer

The 1-MHz ultrasound transducer was constructed using a 25-mm-diameter PZT-4 piezoceramic (Morgan Technical Ceramics, Windsor, UK). The transducer calibration was conducted in a tank containing distilled, degassed water at 22°C. A calibrated polyvinylidene fluoride (PVDF) membrane hydrophone (Y-34-3598 EW295, GEC Marconi, Chelmsford, UK), with a 0.5-mm-diameter active element was used. The custom-made transducer was held in a fixed position while the hydrophone was moved by a micropositioning system. This system allows movement along the three translational axes (2- μm accuracy), and around two angular axes (0.02° accuracy). A signal generator (33250A, Agilent Technologies Inc., Santa Clara, CA) and an RF power amplifier (A150, Electronic Navigation Industries, Rochester, NY; 0.3 to 35 MHz; 55 dB) were used to drive the transducer with 50-cycle bursts and voltages ranging from 25 to 275 V_{pp} . In response to the applied voltage, the transducer output pressure behaves quite linearly, ranging from approximately 0.3 to 3 MPa peak rarefactional pressure, as shown in Fig. 1.

B. Animal Studies

The experimental protocols were approved by the University of Illinois Institutional Animal Care and Use Committee (protocol #10104). Ten Sprague–Dawley rats (Harlan Laboratories Inc., Indianapolis, IN) were exposed to the experimental sequence. Animals were anesthetized with 5% isoflurane for induction of anesthesia and then 1.5% to 2% isoflurane for maintenance of anesthesia via face mask. Although isoflurane does not impact cardiac contractility [9], it is a respiratory depressant and it affects the cardiac function, reducing the heart rhythm [10]. Therefore, five additional rats were used as a control group, exposed to the same study protocol, except for the application of ultrasound. Fig. 2 presents the block diagram of the experimental setup.

Rats were depilated and placed on a platform in dorsal recumbency for ultrasonic cardiac exposure. Four electrocardiography (ECG) electrodes on the platform were coated with gel to contact the paws. Temperature, respiratory rate, heart rate, and ECG were monitored. Ultrasound was applied to the thorax, and thus to the whole heart, including the sino-atrial node and the lower myocardium. Only one animal was euthanized (CO_2 for 5 min) for histological evaluation, which revealed no evidence of damage as determined by a board-certified pathologist. Because there were no signs of major problems via gross observations, all the other animals were allowed to recover.

Each ultrasound application lasted 30 s and consisted of 1-MHz bursts of 3-MPa peak rarefactional pressure, with a PRF starting slightly greater than the heart rate and decreasing

by 1 Hz every 10 s. The duty factor was approximately 1%, which means that for a PRF sequence of 6, 5, and 4 Hz (i.e., 167, 200, and 250-ms pulse repetition periods, correspondingly), a 2-ms pulse duration was used.

A VisualSonics Vevo 2100 (VisualSonics Inc., Toronto, ON, Canada) high-frequency ultrasound imaging system was used to dynamically monitor the heart through B-mode and M-mode real-time ultrasound images [acquired by a registered diagnostic medical sonographer (RDMS)]. Ejection fraction and other cardiac parameters were calculated by the ventricular trace tool from the Vevo 2100 workstation. This tool is used to trace the position of the inner and outer ventricular walls over one or more heart cycles on a long-axis M-mode tracing of the left ventricle. The left ventricular internal diameter during systole and diastole are used to calculate the end systolic and the end diastolic volumes by the Teichholz method. The stroke volume is then obtained by subtracting the end systolic volume from the end diastolic volume, and the ratio of the stroke volume and the end diastolic volume represents the ejection fraction [11].

M-mode images were acquired three times: approximately 6 min before, 3 min after, and 15 min after the 30-s duration ultrasonic stimulation. The values obtained 3 min and 15 min after were normalized to the values obtained 6 min before ultrasonic stimulation. The statistical analysis was performed in Matlab 7.0.1 (The MathWorks Inc., Natick, MA), using two-way analysis of variance (ANOVA) for repeated measures and Bonferroni *post hoc* tests. The significance level was set at 0.05.

III. Results

Heart rate decrease occurred for all the animals of the experimental group. Fig. 3 shows an example of the heart rate decrease during the 30-s duration ultrasonic stimulation. In some other cases, the negative chronotropic response was only observed right after ultrasound application.

The physiological parameters initial absolute values and normalized values after ultrasound stimulation are presented in Tables I and II, respectively. All values in both tables are expressed as mean and standard error of the mean.

Two-way ANOVA for repeated measures (Table III) was performed to verify whether physiological parameters (heart rate, cardiac output, stroke volume, ejection fraction, and respiratory rate) were modified as a response to ultrasound, and whether the effects lasted. Bonferroni *post hoc* tests were applied to compare the results.

For heart rate, there was a significant difference between groups ($p < 0.0001$) and between times ($p = 0.046$). However, time did not influence each group differently, because the interaction is not significant ($p = 0.81$). Heart rate decreased with time for both the experimental group and the control group (see Table II). The Bonferroni test (Fig. 4) showed significant differences between the experimental and control values, for both points in time, depicted by the absence of superposition of horizontal bars that correspond to a 95% confidence interval.

The cardiac output decreased considering the baseline values in the experimental group (both points in time) and in the control group after 15 min (Table II). In spite of that, there was neither significant difference between experimental and control groups ($p = 0.28$) nor between points in time ($p = 0.1$), as presented in Table III. The Bonferroni test also showed no difference between groups (superposition of the 95% confidence interval horizontal bars, Fig. 5).

Relative to the effect of ultrasound on stroke volume, there was a significant difference ($p = 0.005$) between groups. The stroke volume increased in the experimental group and decreased in the control group, particularly after 15 min, as shown in Fig. 6.

The ejection fraction, defined as the ratio of the stroke volume and the end diastolic volume, was used to assess possible cardiac contractility alterations. The ANOVA showed a significant difference between experimental and control groups ($p = 0.026$) when looking at the set of points in time. Nevertheless, the Bonferroni test showed no difference between groups, regardless of the point in time (superposition of the 95% confidence interval horizontal bars, Fig. 7), suggesting that major cardiac damage was unlikely.

The respiratory rate difference was only significant for time ($p = 0.03$), but group $\times$ time interaction was not significant ($p = 0.94$), meaning that this change with time was not different between groups. Respiratory rate decreased in both groups (see Table II) and it was probably a result of isoflurane anesthesia.

IV. Discussion

Ultrasonic waves are known to interfere in the cardiac activity of turtle [12], dog [2], frog [13], mouse [14], pig [3], and guinea pig [1]. However, negative chronotropic effect has never been reported previously. In this study, trans-thoracic therapeutic ultrasound, delivered at a decreasingly variable PRF, has shown the capability to decrease the heart rate without apparent damage to the cardiac tissue. After removal of ultrasonic stimulation, the negative chronotropic effect lasted until the end of each experiment (at least 15 min). The delayed effect suggests that there might be a more subtle effect that yet needs to be better understood, and that will need to be evaluated in another more comprehensive ultrasound-induced bioeffect study.

For 3-MPa peak pressure and 1% duty factor, the spatial-peak temporal-average intensity (I_{SPTA}) applied was around 3 W/cm^2 . The insonification scheme used in this study excludes bulk (temporal average) temperature effects (maximum temperature rise of $0.6^\circ\text{C} \pm 0.05^\circ\text{C}$), as previously reported [8]. One may also consider a maximum temperature increase from a single pulse, $\Delta T_{\max} = (\dot{Q}\Delta t)/C_v$, where Δt is the pulse duration (2 ms for a single pulse), C_v is the medium's heat capacity per unit volume ($4.18 \text{ J/cm}^3\cdot^\circ\text{C}$ for biological tissue), and $\dot{Q} = 2\alpha I_{TA}$ (the absorption coefficient $\alpha \approx 0.05/\text{cm}$ at 1 MHz; $I_{TA} \approx 300 \text{ W/cm}^2$) is the rate of heat generation per unit volume [15]–[17]. Therefore, $\Delta T_{\max} \approx 0.014^\circ\text{C}$, and assumes no heat removal. Therefore, the observed effect results from a nonthermal mechanism, possibly a combination of tissue vibration, promoted by the propagating ultrasonic wave, and radiation force mechanism.

The radiation force has been associated with cardiac changes in frogs [13] and pigs [3]. It is a second-order effect of the propagating wave which is able to transiently push matter away from the source of ultrasound. In biological tissues, the radiation force is estimated to range from 0.1% to 1% of the instantaneous pressure. High-intensity ultrasound pulses produce greater radiation force effects [18]. Considering the radiation pressure to be 1% of a 3-MPa wave, a transient pressure of 30 kPa (or 0.3 atm or 225 mmHg) would be created on the heart. This pressure rise is close to that described to occur during a precordial thump, which is a single blow that has potential to promote defibrillation. In this case, cell membranes are deformed, thus activating stretch-sensitive ion channels and increasing transmembrane current flow by means of mechano-electrical coupling [19].

The heart rate reduction probably involves the activation of mechanosensitive channels. These channels are able to change their open probability in response to a mechanical stimulus, and have been roughly divided into stretch-activated channels (SAC) and volume-

activated channels (VAC). They can be further subdivided by their ion selectivity, such as cation-nonselective, potassium-selective, and chloride-selective channels (e.g., SAC_{NS} , SAC_K , VAC_{Cl}) [20]. In the past, cell swelling was assumed to accelerate spontaneous pacemaking rate via activation of VAC_{Cl} . Nevertheless, experiments demonstrated that the opposite occurs: spontaneously active sino-atrial node cells reduce their pacemaking rate by approximately 24% during swelling [21]. In spite of its name, VAC_{Cl} is not only stimulated by osmotic and hydrostatic increases in cell volume, but also by direct mechanical stretch. VAC_{Cl} is largely distributed throughout the heart and plays a role in arrhythmogenesis, myocardial injury, preconditioning, and apoptosis of myocytes [22].

Because decreasing the heart rate through parasympathetic stimulation of the heart has been shown to protect against the development of some life-threatening arrhythmias [23], the ultrasound sequence proposed here might be protective as well. The cardiac chronotropic response to ultrasound may also include a reflex component, which has not been examined in the present study, and is under current investigation.

The cardiac output is the product of heart rate and stroke volume. The decrease in heart rate would directly lead to a decrease in cardiac output. However, this did not happen after ultrasound application, apparently because of the significant increase in stroke volume. The latter is likely to have occurred via Frank–Starling mechanism, as a result of enhanced preload, resulting from greater ventricular filling during the long diastolic interval. Thus, the negative chronotropic effect of ultrasound seems to have been fully compensated for, and did not result in impairment of cardiac blood delivery to circulation.

At this point, the study protocol excluded measurements of blood pressure and peripheral vascular resistance, which are important data needed to fully understand the hemodynamic effects of cardiac ultrasound application. To overcome these limitations, additional experiments are being conducted, using the specific set of acoustic parameters proposed here.

V. Conclusion

This is the first study to demonstrate that a specific sequence of pulsed ultrasound delivered transthoracically has potential to induce a negative chronotropic effect without major impairment of myocardial contractile activity. Ultrasound was able to decrease the heart rate by 18.7% immediately after stimulation was discontinued. After removal of ultrasonic stimulation, the negative chronotropic effect lasted until the end of each experiment (15 to 45 min). The acoustic parameters used herein were frequency of 1 MHz, peak rarefactional pressure of 3 MPa, approximately 1% duty cycle (2- to 2.5-ms pulses), and variable PRF that ranged from slightly above the heart rate to 2 Hz less than the heart rate. The stimulation lasted 30 s, changing the PRF every 10 s.

Production of this effect requires, in addition to an ideal range of ultrasound parameters (frequency, rarefactional pressure, and duty cycle), a particular protocol of ultrasound application, with PRF close to the heart rate. Variation of PRF was a clear requisite for production of the negative chronotropic effect, as the latter was not observed in preliminary experiments with constant PRF.

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Biographies

Elaine B. Buiochi received a degree in dental surgery from the Federal University of Rio de Janeiro, Brazil, in 2001, an M.Sc. degree in biomedical engineering from the Alberto Luiz Coimbra Institute, Graduate School and Research in Engineering, Federal University of Rio de Janeiro, Brazil, in 2004, and a doctoral degree in electrical engineering (biomedical engineering) from the University of Campinas, Brazil, in 2011. She developed part of her doctoral research at the Acoustics Institute, Spanish National Research Council (CSIC), Madrid, Spain, and at the Bioacoustics Research Laboratory, University of Illinois at Urbana–Champaign, Urbana, IL. Her research interests include the development of ultrasonic transducers and medical applications of ultrasound.

Rita J. Miller received her D.V.M. degree at the University of Wisconsin–Madison in 1992. Dr. Miller completed a small animal medical/ surgical internship at the University of Illinois at Urbana–Champaign in 1993. She then worked as a Veterinary Poison Information Specialist at the National Animal Poison Control Center, also at the University of Illinois at Urbana–Champaign. She has been a member of the Bioacoustics Research Laboratory since 1998. While at the University of Illinois, she has been involved with a variety of research projects: Evaluation of the efficacy of a new treatment for ehrlichiosis in dogs, Rehabilitation with electrical muscle stimulation for dogs with surgically treated cranial cruciate ligament deficient stifles, Role of IGF-1 (insulin-like growth factor 1) and the IGF-1R (type one insulin-like growth factor one receptor) in the uterotrophic effect induced by the administration of tamoxifen, Identification and cloning of canine MMP-2 (matrix metalloproteinase-2), RT-PCR (reverse-transcription polymerase chain reaction) profiling of canine spontaneous tumors for the presence of MMP-2, and The assessment of ultrasound-induced lung damage and attenuation coefficient determination of intercostal tissues. Her current research involves the assessment of the biological effects of ultrasound on tissue, including the heart, capillary beds, and large arteries, and the interaction of contrast agents with ultrasound.

Emily L. Hartman completed a B.S. degree in radiologic sciences with a specialization in ultrasound from Southern Illinois University Carbondale in 2001. She became ARDMS registered in abdomen/small parts and OB/gynecology in 2002, and is certified by the NTQR for prenatal nuchal translucency and nasal bone screening. She began working for Carle Clinic Association/Carle Hospital in 2001 in the radiology and OB departments before training to perform targeted ultrasounds and fetal echocardiography on high-risk patients. While at Carle, she held the positions of lead sonographer and coordinator of radiology and OB ultrasound. Since coming to the University of Illinois at Urbana–Champaign in 2010, she assisted in the establishment of the Ultrasound Imaging Laboratory in the Beckman Institute Biomedical Imaging Center and works with a variety of investigators on a wide array of projects. She performs the imaging for studies including evaluation of ultrasound–tissue interaction, monitoring of tumor growth, and cardiac function on species including rats, mice, and frogs. She is a member of the Society of Diagnostic Medical Sonographers.

Flávio Buiochi received the B.S., M.Sc., and doctoral degrees in mechanical engineering from the School of Engineering, University of São Paulo, Brazil, in 1990, 1994, and 2000, respectively. He started his teaching career in the Department of Mechatronics Engineering, University of São Paulo, in 1992, where he is currently an Associate Professor. From September 2001 to March 2003, he was a postdoctoral Fellow with the Acoustics Institute,

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Rosana A. Bassani graduated in biological sciences and received the Sc.D. degree in physiology and biophysics from the University of São Paulo, São Paulo, Brazil. She was a Postdoctoral Fellow at the University of California, Riverside, and at Loyola University of Chicago, School of Medicine, Chicago, IL. Since 1996, she has been a Senior Researcher at the Center for Biomedical Engineering, University de Campinas, São Paulo, Brazil. Her current research interests include calcium homeostasis and its regulation in the myocardium. She also teaches physiology to biomedical engineering graduate students and has been an editor with *Frontiers in Bioscience* (USA) and the *Brazilian Journal of Biomedical Engineering* (Brazil). Dr. Bassani is a member of the American and Brazilian Biophysical Societies and of the International Society for Heart Research.

Eduardo T. Costa received his B.S. degree in electrical engineering in 1978 from São Carlos School of Engineering, São Paulo University (EESC/USP), São Carlos, SP, Brazil; the M.Sc. degree in electrical engineering in 1985 from the University of Campinas (UNICAMP), Campinas, SP, Brazil; and the Ph.D. degree in medical engineering and physics in 1989 from the King's College School of Medicine and Dentistry, London, UK. Since 1981, he has been with the Department of Biomedical Engineering of the School of Electrical and Computer Engineering (FEEC) of UNICAMP, as a full professor since 2004. Professor Costa was Director of the Biomedical Engineering Center (CEB) of UNICAMP for the term 2005–2011. His research interests include biomedical instrumentation, ultrasound transducers and instrumentation, image processing, and tissue characterization. Professor Costa is a full member of the Brazilian Biomedical Engineering Society (SBEB), where he was President of the Administrative Council. He was President of SBEB in the term 2002–2004, and has been elected for another term (2011–2012).

William D. O'Brien, Jr. (S'64–M'70–SM'79–F'89–LF'08) received the B.S., M.S., and Ph.D. degrees from the University of Illinois at Urbana–Champaign. From 1971 to 1975, he worked with the Bureau of Radiological Health (currently the Center for Devices and Radiological Health) of the U.S. Food and Drug Administration. Since 1975, he has been at the University of Illinois, where he is the Donald Biggar Willet Professor of Engineering. He also is Professor of Electrical and Computer Engineering and of Bioengineering, College of Engineering; Professor of Bioengineering, College of Medicine; Professor of Nutritional Sciences, College of Agricultural, Consumer, and Environmental Sciences; Professor in the Beckman Institute for Advanced Science and Technology; and Professor in the Coordinated Science Laboratory. He is the Director of the Bioacoustics Research Laboratory. His research interests involve the many areas of ultrasound–tissue interaction, including biological effects and quantitative ultrasound imaging, for which he has published 371 papers. Dr. O'Brien is a Life Fellow of the IEEE, a Fellow of the Acoustical Society of America, a Fellow of the American Institute of Ultrasound in Medicine, and a Founding Fellow of the American Institute of Medical and Biological Engineering. He was recipient of the IEEE Centennial Medal (1984), the AIUM Presidential Recognition Awards (1985 and 1992), the AIUM/WFUMB Pioneer Award (1988), the IEEE Outstanding Student Branch Counselor Award for Region 4 (1989), the AIUM Joseph H. Holmes Basic Science Pioneer Award (1993), the IEEE Ultrasonics, Ferroelectrics, and Frequency Control Society Distinguished Lecturer (1997–1998), the IEEE Ultrasonics, Ferroelectrics, and Frequency

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Fig. 1.
Transducer pressure responses to 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, and 275 V_{pp} .

Fig. 2. Block diagram of the experimental setup: anesthesia, ultrasonic stimulation, and physiological monitoring.

Fig. 3. Heart rate response during 30-s duration ultrasonic stimulation: (a) ECG record of the first and last seconds; (b) heart rate as a function of time for the 30-s duration ultrasonic stimulation.

Fig. 4. Results of Bonferroni *post hoc* test for heart rate: Values expressed as mean (circles) and 95% confidence interval (horizontal bars) for both groups and for both times.

Fig. 5. Results of Bonferroni *post hoc* test for cardiac output: Values expressed as mean (circles) and 95% confidence interval (horizontal bars) for both groups and for both times.

Fig. 6. Results of Bonferroni *post hoc* test for stroke volume: Values expressed as mean (circles) and 95% confidence interval (horizontal bars) for both groups and for both times.

Fig. 7. Results of Bonferroni *post hoc* test for ejection fraction: Values expressed as mean (circles) and 95% confidence interval (horizontal bars) for both groups and for both times.

Initial Absolute Values of Physiological Parameters Expressed as Mean and Standard Error of the Mean (SEM) for the Experimental and Control Groups.

TABLE I

Parameter	Units	Experimental		Control	
		Mean	SEM	Mean	SEM
Heart rate	bpm	344.92	8.25	353.17	8.06
Cardiac output	mL/min	49.55	2.00	56.72	3.24
Stroke volume	μL	143.80	4.51	160.56	8.65
Ejection fraction	%	83.71	2.05	78.10	2.43
End-diastolic volume	μL	172.98	7.55	206.12	11.63
End-systolic volume	μL	29.18	4.73	45.56	6.50
Fractional shortening	%	54.15	2.32	47.96	2.34
End-diastolic diameter	mm	5.9	1.86	6.36	0.16
End-systolic diameter	mm	2.7	0.86	3.31	0.19
Respiratory rate	/min	42.10	2.82	44.00	2.35
Temperature	°C	33.22	0.50	33.82	0.39

Normalized Post-Exposure Values of Physiological Parameters for the Experimental Group and the Control Group Expressed as Mean and Standard Error of the Mean (SEM).

TABLE II

Parameter	Experimental						Control					
	3 min after			15 min after			3 min after			15 min after		
	Mean	SEM		Mean	SEM		Mean	SEM		Mean	SEM	
Heart rate	0.80	0.02		0.75	0.02		0.99	0.01		0.95	0.02	
Cardiac output	0.85	0.04		0.83	0.04		0.97	0.01		0.84	0.03	
Stroke volume	1.06	0.04		1.10	0.05		0.98	0.02		0.88	0.03	
Ejection fraction	0.95	0.02		0.97	0.01		1.00	0.01		1.00	0.01	
End-diastolic volume	1.11	0.05		1.14	0.05		0.98	0.02		0.88	0.03	
End-systolic volume	1.52	0.21		1.45	0.18		0.98	0.04		0.88	0.06	
Fractional shortening	0.93	0.03		0.95	0.03		1.00	0.00		1.00	0.01	
End-diastolic diameter	1.05	0.02		1.06	0.02		0.99	0.01		0.95	0.02	
End-systolic diameter	1.16	0.06		1.14	0.06		0.99	0.00		0.95	0.03	
Respiratory rate	0.85	0.04		0.75	0.04		0.93	0.02		0.82	0.04	
Temperature	0.97	0.01		0.94	0.01		0.97	0.00		0.96	0.01	

Two-Way Analysis of Variance (ANOVA) for Repeated Measures of Heart Rate, Cardiac Output, Stroke Volume, Ejection Fraction, and Respiratory Rate. Factors: Group (Experimental/Control); Time (3 min/15 min).

TABLE III

	Sum of squares (SS)	Degrees of freedom (DOF)	Mean squares (SS/DOF)	F	p
Heart rate					
Group (G)	0.24627	1	0.24627	73.88	0.045 e-7
Time (T)	0.01466	1	0.01466	4.4	0.0458
Interaction (G×T)	0.00019	1	0.00019	0.06	0.8145
Error	0.08667	26	0.00333		
Total	0.35089	29			
Cardiac output					
Group (G)	0.02022	1	0.02022	1.53	0.28
Time (T)	0.03878	1	0.03878	2.94	0.10
Interaction (G×T)	0.01968	1	0.01968	1.49	0.23
Error	0.31663	24	0.01319		
Total	0.38332	27			
Stroke volume					
Group (G)	0.14905	1	0.14905	9.43	0.005
Time (T)	0.00471	1	0.00471	0.3	0.59
Interaction (G×T)	0.03216	1	0.03216	2.03	0.17
Error	0.3794	24	0.01581		
Total	0.56094	27			
Ejection fraction					
Group (G)	0.01082	1	0.01082	5.65	0.0258
Time (T)	0.00097	1	0.00097	0.5	0.4845
Interaction (G×T)	0.00076	1	0.00076	0.4	0.5353
Error	0.04596	24	0.00192		
Total	0.05919	27			
Respiratory rate					
Group (G)	0.04035	1	0.04035	2.79	0.1069
Time (T)	0.07661	1	0.07661	5.3	0.0297
Interaction (G×T)	0.00009	1	0.00009	0.01	0.939

	Sum of squares (SS)	Degrees of freedom (DOF)	Mean squares (SS/DOF)	F	p
Error	0.37617	26	0.01447		
Total	0.50088	29			