Efeitos da fluoxetina sobre a ultraestrutura mitocondrial no ventrículo direito de ratos expostos ao estresse pelo frio

Fluoxetine effects on mitochondrial ultrastructure of right ventricle in rats exposed to cold stress

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Resumo

Objetivo: **Analisar os efeitos da fluoxetina sobre a estrutura mitocondrial do ventrículo direito de ratos expostos ao estresse pelo frio.**

Métodos: **Os procedimentos do estudo foram realizados em ratos Wistar-EPM (250-300g) machos. Os ratos (n=40) foram divididos em quatro grupos: 1) Controle (CON); 2) Fluoxetina (FLU); 3) Induzidos à hipotermia (IH) e; 4) Induzidos à hipotermia tratados com fluoxetina (IHF). O grupo FLU foi tratado com gavagem contendo 0,75 mg/kg/dia de fluoxetina durante 40 dias. O estresse induzido pelo frio foi realizado mantendo os grupos 3 e 4 em um freezer (-8ºC) por quatro horas. Os animais foram sacrificados e fragmentos do ventrículo direito (VD) foram removidos e processados antes de serem conduzidos para a microscopia eletrônica.**

Resultados: **As alterações ultraestruturais dos cardiomiócitos foram quantificadas pelo número padrão de cristas mitocondriais (cristólises). Os grupos CON (3,85%), FLU (4,47%) e IHF (8,4%) mostraram aspecto normal de suas estruturas celulares com a citoarquitetura dos cardiomiócitos preservada com integridade sarcoplasmática contínua. Por outro lado, o grupo IH (34,4%) apresentou edema mitocondrial e lise nas cristas.**

Conclusão: **A análise ultraestrutural revelou que a fluoxetina previne fortemente cristólises mitocondriais em miocárdio de ratos, sugerindo possível efeito protetor na condição de estresse induzido pelo frio.**

Descritores: **Fluoxetina. Cirurgia. Miócitos cardíacos. Isquemia fria.**

Abstract

Objective: **To assess fluoxetine effects on mitochondrial structure of the right ventricle in rats exposed to cold stress.**

Methods: **The experimental study procedures were performed in 250-300g male EPM-Wistar rats. Rats (n=40) were divided into four groups: 1) Control group (CON); 2) Fluoxetine (FLU); 3) Induced hypothermia (IH) and; 4) Induced hypothermia treated with fluoxetine (IHF). Animals of FLU group were treated by the administration of gavages containing 0.75 mg/kg/day fluoxetine during 40 days. The induced hypothermia was obtained by maintaining the groups 3 and 4 in a freezer at -8°C for 4 hours. The animals were sacrificed and fragments of the right ventricle (RV) were removed and processed prior to performing electron microscopic analysis.**

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Results: **The ultrastructural changes in cardiomyocytes were quantified through the number of mitochondrial cristae pattern (cristolysis). The CON (3.85%), FLU (4.47%) and IHF (8.4%) groups showed a normal cellular structure aspect with preserved cardiomyocytes cytoarchitecture and continuous sarcoplasmic membrane integrity. On the other hand, the IH (34.4%) group showed mitochondrial edema and lysis in cristae.**

Conclusion: **The ultrastructural analysis revealed that fluoxetine strongly prevents mitochondrial cristolysis in rat heart, suggesting a protector effect under cold stress condition.**

Descriptors: **Fluoxetine. Surgery. Myocytes, cardiac. Cold ischemia.**

INTRODUCTION

Induced cold state by low temperature exposure may be considered as a significant stressing agent [1]. Neurogenic lesion relayed on hypertrophic cardiomyopathy or change on myocardial tissue metabolism can be also caused by the cardiovascular reactivity to cold stress that is hypothesized to be a marker for subsequent cardiovascular disease as well as a predictor of hypertension [2].

Experimental evidences suggest that cellular organelles profile such as mitochondria integrity reflects the injury intensity in the myocardium tissue so that a mitochondrial dysfunction process might thus contribute to the heart disease pathogenesis. Mitochondrial lesions were defined as a partial or complete cristae lysis and their substitution by lacunar areas [3]. The ultrastructural changes in the cardiomyocytes of rats subjected to cold stress are characterized by myofilament dearrangement, increasing the gap between mitochondrial cristae sometimes causes erasings, and partial or total rupture of cristae originating in lacular areas [4-7].

Fluoxetine is a commonly used antidepressant compound having selective serotonin reuptake inhibitor (SSRI) properties [7]. Other neural psychological disorders like panic syndrome, obsessive-compulsive disorder, atypical depression, premenstrual tension, eating disorders, dementia, personality disorders with aggressive or impulsive features and chronic pain have being treated with fluoxetine administration and its clinical efficacy evaluated [8]. Although atrial fibrillation and tachycardia episodes at overdose situations occurred, it is not proved if the long-term of fluoxetine treatment affect the heart in general [9]. We speculate that through its sympathetic activity decrease effect, fluoxetine may reduce the mithocondrial heart damage caused by the cold stress exposure.

In view of the above consideration, this investigation was undertaken to evaluate the effects of fluoxetine on mitochondrial ultrastructural of myocardial tissue of rats exposed to cold stress.

METHODS

Animals

Experiments were performed on 40 adult male rats (*Rattus novergicus albinus*, Rodentia Mammalia), EPM-Wistar, weighing 250-350 grams. Rats were obtained from the Central Biotery of our University. Temperature was monitored as 22ºC, air humidity nearly 60% and the clear-dark cycle was controlled and established as twelve hours each one. Animals had free access to food and water. After an adaptation period of nearly one week animals were randomly selected and separated into four groups: Control Group (CON, n=10): rats treated by the administration of gavages containing 1 mL of water at 10:00 a.m. during 40 consecutive days; Fluoxetin Group (FLU, n=10): rats treated by the administration of gavages (1 mL) containing 0.75 mg/kg fluoxetine at 10:00 a.m. during 40 consecutive days; Stress Group (S, n=10): rats treated by the administration of gavages containing 1 mL of water at 10:00 a.m. and exposed to -8ºC during four hours on the last day (40th) and; Fluoxetin + Stress Group (FLU+S, n=10): rats treated by the administration of gavages (1 mL) containing 0.75 mg/kg fluoxetine at 10:00 a.m. during 40 consecutive days and exposed to -8ºC during four hours on the last day (40th). All procedures were performed in accordance with ethical guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethical Committee in research of our University (number 0771/01).

Cold stress procedure

Rats were exposed to cold stress by maintaining them at -8ºC for 4 hours in a open refrigerated compartment in wire mesh cages. Cold stress was performed only once and rats behavior was examined during all the procedures [3]. Rats body temperature were examined and maintained near 37°C. No rats died during cold stress exposure procedure.

Ultrastructural study

Immediately after light halothane anesthesia, we verified tail tonus and response to external stimuli before and during surgical procedure through evaluation of vibrissa movements, all animals were submitted to a thoracotomy. The thorax of each rat was opened and the right ventricle exposed and removed. Cold 2% glutaraldehyde solution was dropped on the still beating heart in order to prevent other ultra structural modifications. Right ventricle

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myocardium fragments were fixed in formaldehyde with glutaraldehyde in a 2% phosphate buffer solution at 4ºC (0.1M; pH=7.4). Fragments were cut into small 1 mm³ pieces and post-fixed in a 1% OsO_4 solution for 2 hours, dehydrated and embedded in araldite. Silver or gray thin sections (60-90 nm) were selected on a Porter-Blum MT-B ultramicrotome. The ultra-slices were mounted on copper silver grids with 200 patches and stained with uranyl acetate and lead citrate. The preparations were examined under the electronic microscope (Model EM 90, Carl Zeiss, using a tension of 80 kV). All electron micrographs were taken under the same magnification parameters and their dimensions at the end of the process were 18x24 cm² (final amplification x 13,200) [3].

Data analysis

The mitochondrial morphology was analyzed under electron microscopy, with emphasis on the cristae integrity. Mitochondrial lesions were defined as a partial or complete cristalysis and their substitution by lacunar areas. Electron micrographs of the right ventricle myocardium were obtained to each examined group. The samples were examined by three independent investigators with the same and standardized criteria [2]. Counting was done in order to determine how many injured mitochondria were presented in each sample analysis, as well as the means of injured mitochondria. The number of injured mitochondria compared to the amount was entitled "crystolysis index": injuried mithocondria / total mithocondria x 100 [3].

Statistical analysis

In order to evaluate the data associated to crystolysis index, comparison among independent groups, Kruskall-Wallis and Tukey post-hoc tests were applied (P < 0.05, level of significance). Concordance of measurements performed by the three investigators was evaluated and analyzed by Bartko's intra-class correlation coefficient according to Fleiss guidelines [9].

Bartko's test formula

R= N(PMS - EMS) / N(PMS) + (K - 1)(RMS) + (N - 1)SEM

R - Bartko's correlation index; **PMS-** Patients Mean Square; **RMS-** Researcher Mean Square; **EMS-** Error Mean Squaren; **N-** Number of events; **K-** Number of investigators.

RESULTS

The exposure of rats to low temperature stress resulted in high number of injured mitochondria when observed by three investigators (O1, O2 and O3) (Tables 1 and 2, Figure

1). We next assessed an organelle protected situation in the presence of previous fluoxetine administration. Electron microscopy analysis revealed that cardiomyocytes of rats exposed to cold stress pretreated with fluoxetine resulted in an ultrastructural level protection (Figure 1).

Table 1. Comparative analysis of mitochondrial crystalysis profile number

Numbers	CON	FLU	IH	FLU+IH
1	2.3	3.0	44.3	8.3
2	3.0	2.0	24.0	9.0
3	3.3	5.0	17.3	7.3
4	3.3	4.5	35.0	7.3
5	4.3	4.0	28.6	8.0
6	2.6	5.5	29.0	7.0
7	3.6	4.0	69.3	10.3
8	4.3	6.3	40.0	11.3
9	8.0	6.0	22.6	7.3
Mean	3.85	4.47	34.4	8.4

R: CON=0.44; FLU=0.88; IH=0.96; IHF= 0.73. The values represented in the columns express the mean result between three independent observers in the control (CON), fluoxetine (FLU), induced hypothermic (IH) and fluoxetine + induced hypothermic (IHF) groups. Variance analysis was performed using the Kruskal-Waalis method. Hc=7.82, Hcalc.=17.61.(*P<0.005). Comparison of means> Zc.=2.6 and Zcalc.=10.77**(**P<0.005). IH > CON; FLU and IHF*

Table 2. Values of mitochondrial profiles with crystalysis for nine photomicrographs analysed by three independent observers (O1, O2, O3) in the Control (CON), Fluoxetin (FLU), Induced Hypotermic (IH), and Fluoxetin + Induced Hypotermic (FLU+IH) Groups

| Photo	CON			FLU			IH			IHF		
	O1	O2	O3	O1	O2	O3	O1	O2	O3	O1	O2	O3
1	2	2	3	3	3	3	38	47	48	7	8	10
2	4	3	2	2	2	2	22	25	25	9	8	10
3	5	3	2	5	4	6	18	16	18	8	7	7
4	4	3	3	6	3	5	31	33	41	9	6	7
5	5	3	5	4	4	4	25	33	28	9	7	8
6	4	2	2	7	5	4	26	26	35	6	8	7
7	4	4	3	3	4	5	64	76	68	11	12	8
8	4	7	2	6	7	6	40	45	35	12	12	10
9	5	9	4	8	6	8	19	34	18	8	6	8
Total	37	36	26	44	38	43	380	335	316	79	74	75

The values represented in the columns express the mean result between three independent observers in the control (CON), fluoxetine (FLU), induced hypothermic (IH) and fluoxetine + induced hypothermic (IHF) groups. Variance analysis was performed using the Kruskal-Waalis method. Hc=7.82, Hcalc.=17.61.(*P<0.005). Comparison of means> Zc.=2.6 and Zcalc.=10.77**(**P<0.005). IH > CON; FLU and IHF*

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Comparison among control (CON), fluoxetine pre-administration (FLU), induced hypothermic (IH) and IH with fluoxetine pre-administration (IHF) were performed. In CON as well as in FLU cytoarchitecture had a normal ultrastructural profile (Figure 2).

Electron microscopy description analysis showed in CON and FLU a regular myocardium with preserved fibers and sarcoplasmic reticulum. In the paranuclear region it was identified an expressive number of mitochondria with preserved layers. Intact sarcomere limited by two clear Z bands and several electron dense granules were observed near the nucleus.
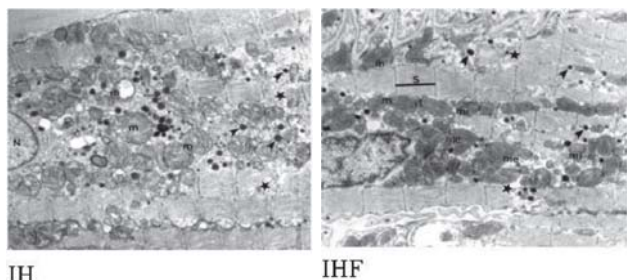
IH   IHF

Fig 1 - On the IH group all muscle fibers (ê) were irregular with perversion in its bundle architecture, with partial or total loss of integrity. Necrotizing areas were found replacing the fibers, and mitochondria (m) showed variable degrees of injury some with edema, others with layer replacement for vacuoles (mitochondrial cristolysis) or even blanking. The electrodense grains (%) were spread across the sarcoplasm, presenting irregular shape. Nucleus (N). Z Lines (S). Amplification x 13.200. In IHF group, still in a cold stress situation, a significant number of mitochondria with a normal aspect (m) in contrast to few injured mitochondria (mi/me) were perceived. Only some sarcomere areas were disarranged (ê) in comparison with a clear area depicting well organized ones. Nucleus (N). Z Lines (S). Amplification x 13.200
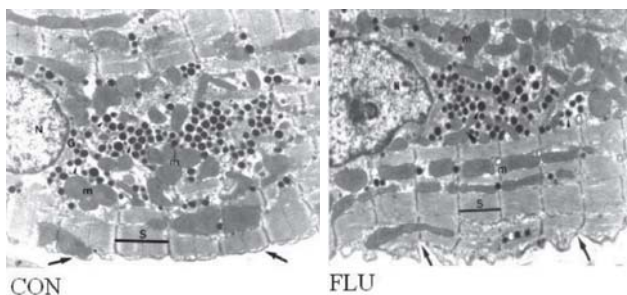
CON   FLU

Fig 2 - Electron micrographs of ventricle cardiomyocytes sliced longitudinally. In CON and FLU groups, clearly sarcomere delimited by the Z lines (S), normal aspects of mitochondria (m), Golgi complex (G) and paranucleate granules with electron dark appearance (%) were identified. Note the sarcoplasmic membrane integrity with a regular aspect (arrows). x13.200

On the other hand, the IH group showed irregular muscle fibers and the ultrastructural analysis revealed dropsy damaged mitochondria with partial or total cristae lysis. Dense-core granules with irregular shape were spread through out the sarcoplasm (Figure 1). The IHF group presented preserved myofibrillae without dystrophy or necrobiotic event neither mitochondrial alteration. Electron dense granules with enhanced membranes extent were observed in the sarcoplasm (Figure 1). Data variance analysis by *Bartko's* correlation index ranged between 0.44-0.96 in all experimental groups depicts the methodology validation. In addition, variation analysis among group's means differences was statistically significant.

DISCUSSION

The establishment of DHCA lead to temporary stop of cardiac contractile function and subsequent loss of pulsatile flow. Under these conditions, flow is diminished and blood pressure within the circulatory system is mainly dominated by hydrostatic factors, rather than cardiac pumping activity. Hence, measured arterial blood pressure is usually very low [10]. On the other hand, a recent study has already evidenced that extreme low temperature exposure cause reduction of cardiomyocytes nucleus size [11]. Our investigation examined the ventricle myocardium tissue of rats exposed to cold stress and evaluated fluoxetine effects on cardiomyocytes of the induced hypothermic group. We showed that the mitochondria-dependent integrity relayed to cardioprotective pathway is one of the mechanisms of cold stress lesion in cardiomyocytes. Besides, it was observed that fluoxetine protected cardiomyocytes against cold stress injury through attenuation of mitochondrial stress. This information is helpful, since important information should be considered in methods used for protecting the myocardium during ischemia in heart surgery.

Despite the fact that the incidence of mitochondrial cristolysis in the Hipothermic Fluoxetine group (8.4) was 130% higher than the control group (3.85), which lead us to suggest that this proposed method is inferior to the recent recourses able to protect heart tissue, in our electron microscopy analysis it was noted that the group exposed to cold stress and treated with fluoxetine presented preserved myofibril without dystrophy or necrobiotic event neither mitochondrial alterations while the opposite was found in the induced hypothermic not treated group.

Therefore, these findings indicate the cardioprotector property of fluoxetine in this investigation. Many drugs were tested to evaluate their effects on myocardium tissue of animals exposed to cold stress [3,12-17]. In the 70 years Elonen [18] indicated that antidepressive drugs have high afinity with myocardial tissue due their high solubility in

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their cellular membrane and it affects cardiomyocytes biological intracelular events.

Moreover, phenothiazine tends to accumulate in the heart tissue [19]. However, the mechanism by which fluoxetine protects cardiomyocytes mitochondrial cristolysis remains undisclosed, although the mechanism could be related to suppression of protein synthesis due to phosphorylation of eEF2 like AMP-activated protein kinase via protection against hypoxic injury [19]. Another hypothesis relies on the Na^+/Ca^{+2} exchanger down-regulation that could increase Ca^{+2} concentration at the sarcoplasmic reticulum and cytoplasm leading to an overload-induced Ca^{+2} loss of mitochondrial membrane potential becoming a target for Akt-mediated protection [20].

Although we used cold as a stress agent in this study, induced hypothermia is also used during heart surgery procedures, this is the reason for the method used in our investigation. A hypothermic cardiac arrest offers the possibility of survival because of the effects of rapid cooling. Hypothermia causes a decrease in cellular oxygen demand, which is advantageous during periods of ischemia [21]. Cardiac surgeons take advantage of controlled hypothermia to perform surgery under circulatory arrest. The induced hypothermia has positive and negative points; Wypij et al. [22] evaluated the negative effects of hypothermic cardiopulmonary bypass in infant heart surgery, Eggum et al. [23] noted that only minor differences in cytokine levels were detected between those with moderate and those with mild hypothermia during cardiopulmonary bypass.

Moreover, cold agglutinins are predominately immunoglobulin M autoantibodies that react at cold temperatures with surface antigens on the red blood cell. This can lead to hemagglutination at low temperatures, followed by complement fixation and subsequent hemolysis on rewarming. This autoimmune phenomenon is of unique relevance during cardiac operations when hypothermic cardiopulmonary bypass and cardioplegia are instituted [24]. The results of this study indicate the cold stress exposure as a protocol able to induce cardiac cells impairment, which is supported by previous investigations regarding cardiac cells protection [12-17]. Taken together, our results indicate a possible way to prevent or reduce the cardiac cell damage caused by the cold exposure during heart surgery.

This study showed that fluoxetine at the dosage used by us (0.75 mg/kg) is able to protect the heart tissue in a cold stress situation. We should be careful with the dosage that it is used. According to Bachmann and Zbinden [25], higher concentration of tryciclic antidepressive and antipsychotic agents cause oxidative phosphorylation increase, although they did not observe increase of oxygen consummation. Its accumulation on the membrane lipids

became it less flexible to functional disturbs of membrane enzymes and it is also possible that it inhibits the output of ATP through its interaction with membrane lipids [25].

Furthermore, Souza et al. [26] showed that higher concentrations of fluoxetine have toxic effects, they evaluated this drug at much higher dosage than those used in our research (near the highest tolerated by humans) and evidenced increased oxidative phosphorylation in the liver of rats. Considering those and our studies, it can be suggested that the effects of a small dosage of fluoxetine may be occurred through direct action on mitochondrial metabolism. These results might probably open new point of views for more studies and may benefit experimental and clinical investigations.

The protector effect of fluoxetine on the mitochondrial cristolysis of cardiac cells found in our study may put in focus the role of this drug or its components in the circulatory system. Recent studies demonstrated the association between fluoxetine and cardiovascular parameters. Sas et al. [27] evidenced that rabbits treated with this drug presented decreased heart rate and no alterations on blood pressure and corticocerebral flow. Pousti et al. [28] observed decrease in the rate and contractile force of heart in a dose-dependent manner and they suggested that the negative chronotropic and inotropic effect of fluoxetine on isolated guinea-pig atria is probably mediated through an inhibition of the reuptake of adenosine or the A(1) receptor mechanism. Moreover, SSRI were associated with a 50% reduction in the risk of death in a small cohort of pulmonary arterial hypertensive patients [29].

However, Rajamani et al. [30] supported the idea that fluoxetine intoxication may contribute to long QT syndrome, they reported that fluoxetine reduced *human ether-a-go-go-related-gene* potassium current and that it is caused by both direct channel block and indirectly by disruption on channel protein trafficking. Perhaps its actions on the sympathetic system would reduce heart as well as blood pressure, on the other hand, we did not evaluate those parameters. Therefore, we could not confirm its effects on cardiovascular determinants. We consider our data very important, since new protocol models may be developed in future studies endeavoring to prevent cardiac cells ultrastructure in a stress situation during surgery procedures or lesions.

This is the first research to demonstrate that fluoxetine strongly attenuates mitochondrial injury in right ventricle myocardium tissue of rats exposed to cold stress. We believe that these data are important to develop futures therapies aiming to preserve cardiomyocytes ultrastructure in a cold stress situation during surgical repair of complex congenital cardiac abnormalities or lesions of the aortic arch. These findings may possibly open new perspectives

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for more research and may benefit experimental and clinical investigations.

CONCLUSION

In conclusion, our findings suggest that fluoxetine strongly prevents mitochondrial crystolysis of myocardial tissue of rats in a cold stress condition.

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