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ORIGINAL ARTICLE

Sex differences in the coagulation process and microvascular perfusion induced by brain death in rats

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SUMMARY

Brain death (BD) leads to a systemic inflammation associated with the activation of coagulation, which could be related to decreased microcirculatory perfusion. Evidence shows that females exhibit higher platelet aggregability than males. Thus, we investigated sex differences in platelets, coagulation and microcirculatory compromise after BD. BD was induced in male and female (proestrus) Wistar rats. After 3 h, we evaluated: (i) intravital microscopy to evaluate mesenteric perfusion and leucocyte infiltration; (ii) platelet aggregation assay; (iii) rotational thromboelastometry; and (iv) Serum NO_x^-. Female rats maintained the mesenteric perfusion, whereas male reduced percentage of perfused vessels. Male BD presented higher platelet aggregation than the controls. In contrast, female BD had lower platelet aggregation than the control. Thromboelastometry indicated a reduction in clot firmness with increased clotting time in the female group compared with the male group. Serum NO_x^- level in female BD was higher than that in the male BD and female control. There is sex dimorphism in platelet function and clotting process, which are altered in different ways by BD. Thus, it is possible to connect the reduction in microcirculatory perfusion in males to intravascular microthrombi formation and the maintenance of perfusion in females to a higher inflammatory response and NO synthesis.

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Key words
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Introduction

Organ donation after brain death (BD) is an important source of transplantable organs. Therefore, understanding BD-generated changes contributes to the optimization of clinical management and the acquisition of adequate organs for transplantation. Sexual dimorphism can affect transplantation in multiple aspects. Clinical studies have shown differences, attributable to donor sex, in the short- and long-term prognosis of transplanted organs [1–4]. Hormonal differences, chromosomal differences and antigen development are some of

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the challenges that have to be considered when donor/recipient sex does not match [1]. Also, the gender-based differences on transplant outcomes should be influenced by several mechanisms which include hormonal factors [2].

Previous studies have pointed to microcirculatory changes in male rats subjected to BD, suggesting that hypoperfusion could reduce the viability of various organs [5–11]. In female rats, the initially high estradiol concentration before BD and elevated expression of eNOS seemed to favour the maintenance of microvascular perfusion/flow [12]. In this context, despite better perfusion, female rats present higher inflammatory infiltrates in the organs and higher concentrations of inflammatory markers associated with female sex hormone reduction [13].

It is well established that inflammatory response is accompanied by the activation of the coagulation system. The activation of this system is characterized by small variations in coagulation factors and may culminate in the induction of a disseminated intravascular coagulation process, as observed in patients with severe sepsis [14]. The brain-dead donor can possibly be in a procoagulant state, which is linked to the inflammatory process triggered by BD [15]. The activation of the coagulation system induced by BD seems to be associated with fibrin and thrombin generation in patients [16]. Furthermore, an experimental study on the different pathways of coagulation activation indicates that BD leads to hypercoagulation [17]. However, these studies were performed only in males, not taking into account the possible influence of sex on the responses. Therefore, the evaluation of effector pathways of platelet aggregation and coagulation in parallel with microvascular perfusion analysis may contribute to differentiating the clinical characteristics in relation to the donors' sex. Here, we aimed to investigate the sex-influenced differences in platelet behaviour, coagulation processes and microcirculatory compromise after BD in rats.

Materials and methods

Animals

A total of 48 male and female Wistar rats weighing 250–300 g (6–8 weeks old) from our institutional animal facilities were used in this study. They were housed in groups of three rats per cage (12:12-h light-dark cycle, 21 ± 2 °C) with free access to food and water. All animals received humane care under the ethical principles for animal research adopted by the Brazilian College of Animal Experimentation and the Principles of Laboratory Animal Care (National Society of Medical Research). The Animal Subject Committee of Sao Paulo University Medical School approved the experimental protocol under the number SDC 4877/19/096.

Study groups

Rats subjected to BD were assigned to two groups ($n = 12$): (i) female rats in the proestrus phase of the oestral cycle and (ii) male rats. Oestrous cycle phases were determined based on the morphological features of cells in vaginal smears. Female and male non-manipulated animals (control, $n = 24$) were used as controls.

Anaesthesia and brain death model

The animals were anesthetized with 5% isoflurane in a closed glass chamber, intubated, mechanically ventilated with a volume control ventilator (Harvard Apparatus, Inc., Holliston, MA, USA) at a tidal volume of 10 ml/kg, frequency of 70 breaths/min and oxygen fraction of 100%, and then maintained with 2% isoflurane. All animals were monitored for 3 h through a catheter in the carotid artery to monitor the rats' blood pressure, and the jugular vein was catheterized for fluid administration (saline solution, 2 ml/h).

A 1-mm hole was drilled through the skull, and a Fogarty-4F catheter (Fogarty Arterial Embolectomy Catheter: 4 French; Edwards Lifesciences, Irvine, CA, USA) was inserted intracranially. To increase intracranial pressure and induce BD, the balloon of the catheter was rapidly inflated with 400–500 μl saline solution. BD was confirmed by apnoea, drop in mean arterial pressure (MAP), maximally dilated and fixed pupils, and absence of reflexes. After BD, anaesthesia was interrupted.

Intravital microscopy

An incision of the abdomen was made, and the distal ileum and its accompanying mesentery were exposed for *in vivo* observation of the microcirculation. Krebs–Henseleit solution (37 °C) saturated with a mixture of gases (95% N_2 and 5% CO_2) was used to maintain the mesentery warm and moist. A camera (AxioCam HSc, CarlZeiss Munchen, Hallbergmos, Germany) was connected to a triocular microscope (Axioplan 2; Carl Zeiss), and analyses were performed using image-computer software (Axiovision 4.8; Carl Zeiss). The number of leucocytes, which accumulated in the connective tissue that was

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adjacent to a selected postcapillary venule, was determined in a standard area of 5000 mm^2 using a ×40 light microscopic objective. Three fields were examined for each microvessel. To analyse the density of the perfused small 3rd order vessels (<30 mm in diameter), the animals received 120 μg/kg of FITC-conjugated platelet antibody (Rat anti-CD49b FITC MAB 0.5MG HA1/29; Becton Dickinson, Franklin Lakes, NJ, USA). In five selected fields for each animal, the flow in each vessel was analysed and classified as continuous or absent using a fluorescent microscope with ×10 objective and a 1 mm^2 area on the computer screen.

Leucocytes and platelet blood counts

Blood samples (20 μl) were collected through a small section of the caudal apex of the at baseline (0 min, before the surgical procedure) and at the end of the experiment (3 h after BD induction). Leucocytes and platelets were counted using an automatic haematology analyser (Mindray BC 2800 Vet, Shenzhen, China).

Platelet aggregation analysis

For the platelet aggregation analysis, all animals were sacrificed through abdominal aorta exsanguination, and 3.5 ml blood samples were placed in a tube containing 3.2% sodium citrate and centrifuged for 4–6 min at 200-450 *g* to obtain platelet-rich plasma (PRP). AggRAM equipment (Helena Laboratories, Gateshead, UK) was used to detect light transmission through the platelet-rich citrate plasma in suspension. As platelets aggregated by the addition of an aggregating agent (adenosine 5′diphosphate and adrenaline), turbidity decreased and a proportional increase in light transmission was detected by a photoelectric cell and recorded under a platelet aggregation curve by a recorder coupled to the equipment. The results are expressed as the maximum aggregation amplitude. The test was performed for up to 4 h after sample collection.

Determination of serum total nitric oxide

Total nitrite (NO_x^-) was determined by measuring the formation of the stable oxidation products of nitric oxide (NO), namely, nitrite (NO_2^-) and nitrate (NO_3^-). NO in oxygen-containing solutions is chemically unstable and undergoes rapid oxidation to NO_2^-. The presence of various biological tissue components catalyses this oxidation and promotes further oxidation of NO_2^- to NO_3^-. Therefore, the measurement included both NO_2^- and NO_3^- to accurately determine the level of total NO. NO_3^- in serum was first reduced to NO_2^- by incubating the samples with nitrate reductase (0.15 U/ml), and the concentrations of NO_2^- were determined by the Griess reagent reaction. The optical density (at 540 nm) was recorded using a microplate reader (Spectramax plus 384; Molecular Devices, San Jose, CA, USA), and the nitrite levels were obtained from a standard curve of $NaNO_2$ (1.5–100 mM).

Whole blood coagulation analysis (thromboelastometry)

The whole blood coagulation profile analysis was performed by thromboelastometry (ROTEM®; Pentapharm, Munich, Germany). All animals were euthanized through abdominal aorta exsanguination, and 3.5 ml blood samples were placed in a tube containing 3.2% sodium citrate. Prewarmed (37 °C) plastic cups and kits (ROTEM®) were used to determine coagulation by carrying out extrinsic screening test (EXTEM), intrinsic screening test (INTEM) and fibrinogen screening test (FIBTEM), following the instructions of the manufacturer during a period of 30 min.

Statistical analysis

Data are presented as means ± SEMs. Comparisons between groups were conducted through two-way analysis of variance followed by pairwise Tukey's multiple comparisons test with *P* values adjusted to account for multiple comparisons. All statistical analyses were performed using GRAPHPAD PRISM software, version 8.3.1 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Mesenteric perfused small vessels and migrated leucocytes

As illustrated in Fig. 1a, after BD, the female rats did not present a reduction in the proportion of perfused small vessels (<30 mm in diameter) when compared with the female control rats (control = 71% vs. BD = 61%). Nevertheless, male rats subjected to BD showed a significant decrease in the proportion of perfused small vessels compared with the male control rats (control = 78% vs. BD = 36%). The reduction found in BD males was significant when compared with BD females (BD female = 61% vs. BD male = 36%). Figure 1b shows the number of migrated leucocytes in the postcapillary venules. In BD

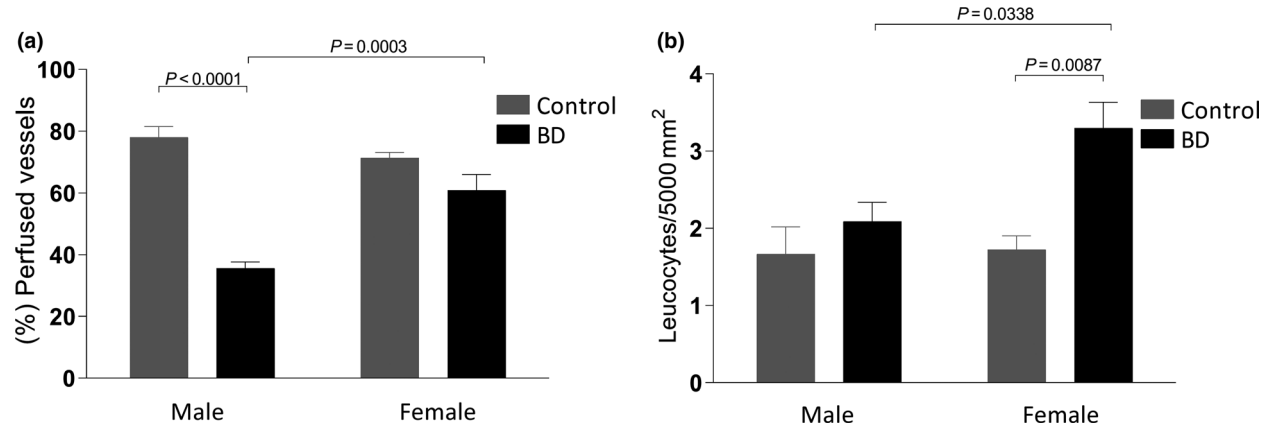


Figure 1 Sex differences in the numbers of perfused small vessels and migrated leucocytes in mesenteric microcirculation after brain death (BD). Percentage of the perfused small vessels (a) and migrated leucocytes (b) in the mesenteric microcirculation of control rats (non-manipulated animals) and BD groups (rats subjected to brain death; $n = 5$ per group). Data are presented as mean ± SEM.

animals, there was a higher number of leucocytes in the female rats than in the male rats.

Platelet counts in whole blood and platelet aggregation

The platelet counts illustrated in Fig. 2 indicate a decrease in the platelet count in the blood of animals subjected to BD, regardless of the sex. The platelet aggregation analysis revealed sex differences at the baseline, with the female rats presenting significantly higher aggregation than the male rats. Conversely, after BD, platelet aggregation increased in male rats and decreased in female rats, featuring a significant inversion in platelet behaviour (Fig. 3). Intravascular thrombus formation was documented in the mesenteric microcirculation by intravital microscopy in the male rats, whereas the same phenomenon was not observed in the female animals after BD (Fig. 3).

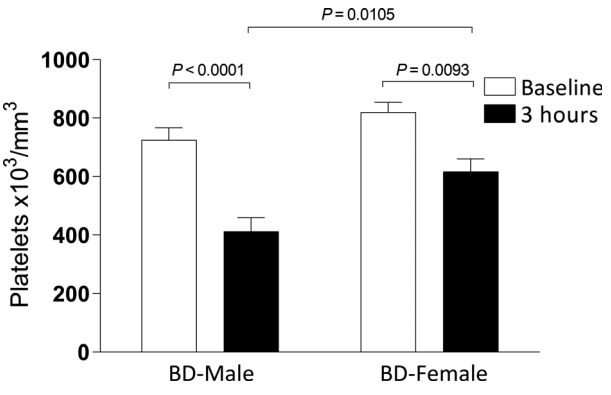


Figure 2 Sex differences in platelet blood counts. Platelet blood counts in male and female animals at baseline and 3 h after brain death ($n = 7$ per group). Data are presented as mean ± SEM.

Effect of BD on the serum levels of NO_x^-

The NO_x^- level in the serum samples of the female BD rats was higher than that in the Male BD and Female Control rats (Fig. 4).

Coagulation activation and clot polymerization parameters

Table 1 presents the coagulation activation and clot polymerization parameters. The coagulation time (CT), which is the time from the start of the test until an amplitude of 2 mm is reached, was increased in BD female group for the FIBTEM pathway, compared to that in the BD male group ($P = 0.021$). The same phenomenon was observed for the other pathways (EXTEM and INTEM), but without statistical difference. In all the pathways analysed, the clot formation time (CFT) analysis, which is the time between 2 mm amplitude and 20 mm amplitude, was significantly higher in the BD female group than in all the other groups. EXTEM: there were statistical differences in relation to control female group ($P = 0.024$) and BD male group ($P = 0.006$); INTEM: significant increase compared with control female group ($P = 0.030$); FIBTEM: there was an increase compared with the control female group ($P = 0.057$) and BD male group ($P = 0.053$). The BD female group had a decreased α-Angle (angle between the baseline and a tangent to the clotting curve through the 2 mm point) in all the pathways. EXTEM: there was a reduction in relation to the control female group ($P = 0.022$) and BD male group ($P = 0.049$); INTEM: the BD female group showed a drop in relation to the other groups, with a significant difference in

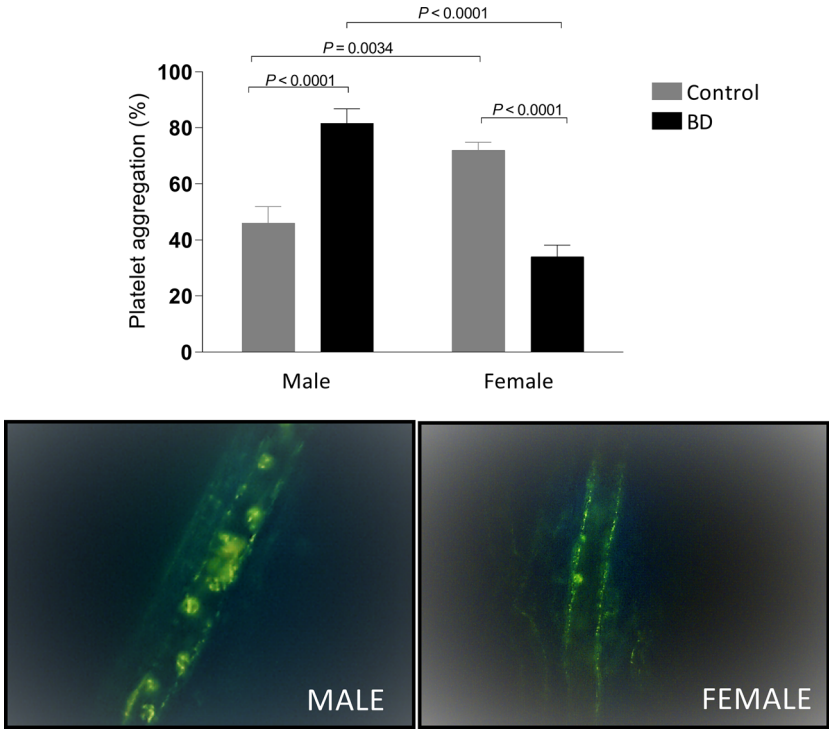


Figure 3 Platelet aggregation. Platelet aggregation percentage (n = 7 per group) and photomicrography showing platelets marked with FITC in the mesenteric microcirculation of control rats (non-manipulated animals) and brain death groups (rats subjected to brain death). Data are presented as mean ± SEM.

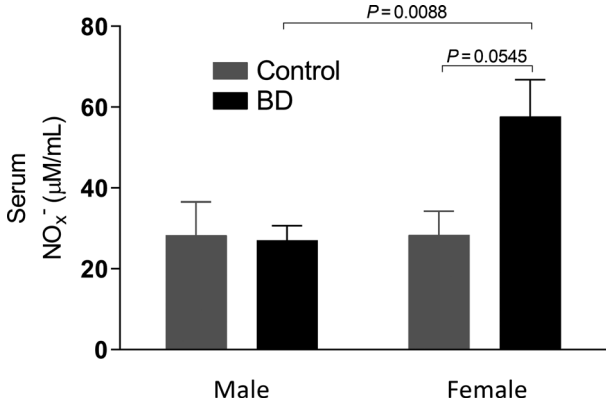


Figure 4 Concentration of total nitrites (NO_x^-) in the serum samples after brain death (BD). NO_x^- was determined using the Griess method after previous incubation with nitrate reductase in control rats (non-manipulated animals) and BD groups (rats subjected to brain death; n = 7 per group). Data are presented as mean ± SEM.

comparison to the control female group ($P = 0.015$); FIBTEM: In this pathway, the BD group female decreased α-Angle in comparison to the control female group ($P = 0.023$) and BD male group ($P = 0.009$). In the EXTEM pathway, clot formation rate (CFR) analysis (angle between the baseline and the tangent at the maximum slope), the BD female group presented a smaller angle than the other groups, but without statistical difference. The same response was found in the INTEM pathway, but with a difference in relation to the control female group ($P = 0.022$), the FIBTEM pathway showed this reduction in comparison with control female ($P = 0.018$) and BD male groups ($P = 0.028$).

Clot firmness parameters

The clot firmness parameters are represented in Figs 5–7. The EXTEM evaluation, illustrated in Fig. 5a, shows a decrease in maximum clot firmness (MCF) in the BD female group, compared with the control female group ($P = 0.025$) and BD male group ($P = 0.016$). The same response was observed in the INTEM pathway ($P = 0.042$) and ($P = 0.026$; Fig. 6a). The FIBTEM pathway, however, revealed no differences among the groups (Fig. 7a).

There was clot firmness reduction at all the time points in the BD female group when compared with the other groups. The EXTEM pathway (Fig. 5b) and FIBTEM pathway demonstrated similar response, with a drop at all the time points, ($p < 0.05$; Fig. 7b). The INTEM pathway showed a reduction at some time points, with a statistical difference between BD female and the control female group ($p < 0.05$; Fig. 6b).

Table 1. Coagulation activation and clot polymerization parameters.

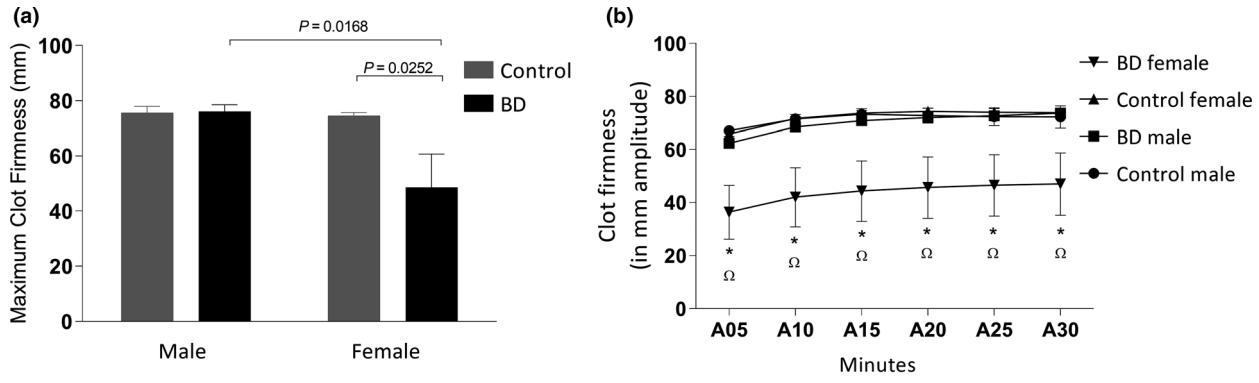
	Control male	BD male	Control female	BD female	*P* SEX	*P* BD
EXTEM						
CT (s)	55.2 ± 8.0	46.0 ± 3.4	51.6 ± 3.6	74.0 ± 25.31	0.344	0.609
CFT (s)	29.5 ± 3.3	38.2 ± 2.0	30.2 ± 2.2	103.8 ± 31.22*,†	0.024	0.006
α-Angle (°)	84.1 ± 0.6	82.3 ± 0.5	84.0 ± 0.6	64.5 ± 9.93*,†	0.049	0.022
CFR (°)	84.7 ± 0.8	83.2 ± 0.5	84.8 ± 0.3	67.5 ± 10.06	0.109	0.055
INTEM						
CT (s)	117.1 ± 10.8	130.1 ± 37.5	130.3 ± 24.1	239.2 ± 69.12	0.106	0.107
CFT (s)	41.6 ± 5.3	105.3 ± 67.6	29.6 ± 3.1	374.2 ± 198.2*	0.161	0.030
α-Angle (°)	82.7 ± 1.0	80.1 ± 2.1	84.3 ± 0.5	58.6 ± 12.3*	0.080	0.015
CFR (°)	85.0 ± 0.6	82.7 ± 1.1	85.1 ± 0.5	65.1 ± 10.15*	0.068	0.022
FIBTEM						
CT (s)	40.6 ± 4.6	34.3 ± 3.6	56.0 ± 11.2	55.3 ± 9.19†	0.021	0.633
CFT (s)	1819.5 ± 1117.6	1031.4 ± 590.8	982.0 ± 663.9	6787.6 ± 1958.4	0.057	0.053
α-Angle (°)	80.0 ± 1.3	79.14 ± 1.8	76.4 ± 5.4	45.8 ± 11.6*,†	0.009	0.023
CFR (°)	81.00 ± 1.2	80.8 ± 1.6	82.0 ± 0.5	53.5 ± 10.8*,†	0.028	0.018

CFR, clot formation rate; CFT, clot formation time; CT, coagulation time; EXTEM, extrinsic screening test; FIBTEM, fibrinogen screening test; INTEM, intrinsic screening test; α-Angle, the angle between the baseline and a tangent to the clotting curve through the 2 mm point.

BD groups, rats that were subjected to brain death (n = 7 per group); Control rats, non-manipulated animals. Data are presented as mean ± SEM.

*In relation to the respective control (control).

†In relation to male BD.



(c)

	Control Male	BD Male	Control Female	BD Female	*P* Sex	*P* BD
MCF-t (sec)	1276.0±164.58	1865.2±150.53	1334.8±95.57	2499.3±372.12*	0.124	0.001
ACF (mm)	65.8±6.98	74.5±3.90	65.3±2.75	46.2±11.50	0.059	0.479
MCE	324.8±37.38	347.5±45.03	297.3±16.84	148.2±47.40Ω	0.008	0.120

Figure 5 Sex differences in thromboelastometry (EXTEM) clot firmness parameters. Maximum clot firmness (a), clot firmness in amplitude (b) and complementary data on firmness (c) in control rats (non-manipulated animals) and brain death (BD) groups (rats subjected to brain death; n = 7 per group). Data are presented as mean ± SEM. $^{*}P < 0.05$ in relation to the respective control (control); $^{\Omega}P < 0.05$, in relation to male BD. ACF, actual clot firmness; EXTEM, extrinsic screening test; MCE, maximum clot elasticity; MCF-t, maximum clot firmness.

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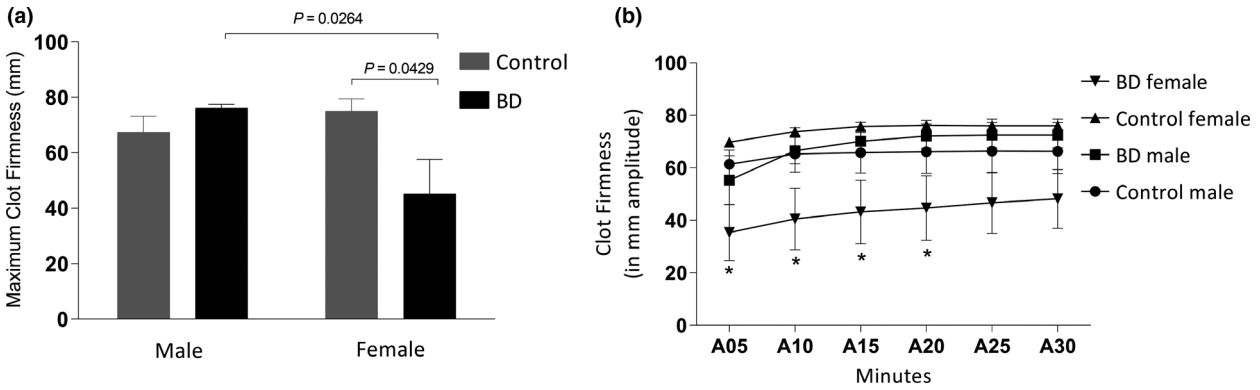
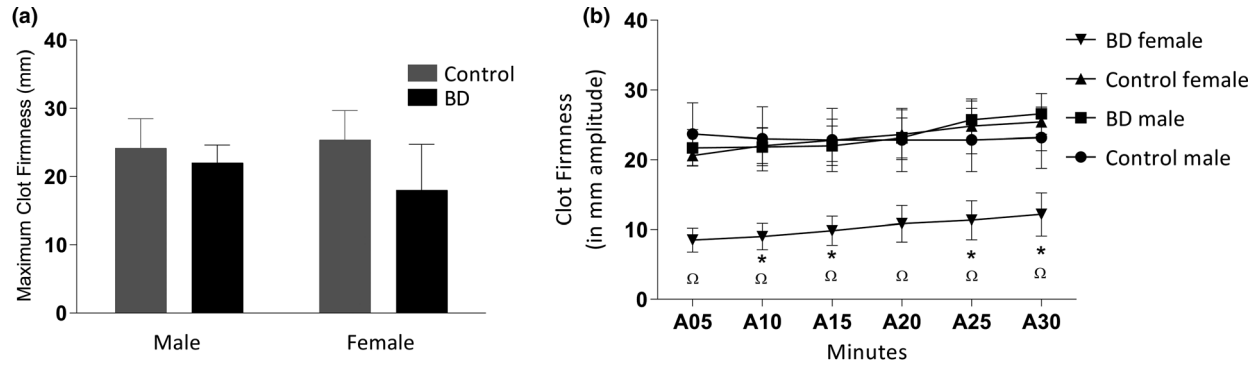


(c)	Control Male	BD Male	Control Female	BD Female	*P* Sex	*P* BD
MCF-t (sec)	952.3±274.1	1250.0±209.0	1337.6±110.2	1749.6±300.3	0.096	0.177
ACF (mm)	62.8±7.2	69.0±4.9	72.8±1.2	47.6±10.3	0.428	0.188
MCE	271.0±73.7	290.7±49.6	368.8±6.1	131.5±46.1	0.604	0.075

Figure 6 Sex differences in thromboelastometry (INTEM) clot firmness parameters. Maximum clot firmness (a), clot firmness in amplitude (b) and complementary data on firmness (c) in control rats (non-manipulated animals) and BD groups (rats subjected to brain death; $n = 7$ per group). Data are presented as mean ± SEM. $^{*}P < 0.05$ in relation to the respective control (control). ACF, actual clot firmness; MCE, maximum clot elasticity; MCF-t, maximum clot firmness; INTEM, intrinsic screening test.



(c)	Control Male	BD Male	Control Female	BD Female	*P* Sex	*P* BD
MCF-t (sec)	207.0±48.6	195.8±48.0	404.4±257.1	1464.8±592.7$^{\Omega}$	0.032	0.115
ACF (mm)	26.7±4.1	31.4±1.7	32.8±4.3	40.8±18.6	0.439	0.523
MCE	34.5±9.1	29.6±4.5	35.8±9.2	43.5±15.9	0.470	0.894

Figure 7 Sex differences in thromboelastometry (FIBTEM) clot firmness parameters. Maximum clot firmness (a), clot firmness in amplitude, (b) and complementary data on firmness (c) in control rats (non-manipulated animals) and BD groups (rats subjected to brain death; $n = 7$ per group). Data are presented as mean ± SEM. $^{*}P < 0.05$ in relation to the respective control (naïve).

Figure 5c shows that EXTEM maximum clot firmness-time (MCF-t) of the BD female groups was higher than that of the other groups ($P = 0.001$, versus control female group). FIBTEM pathway analysis showed a time increase in the BD female group when compared with the BD male group (Fig. 7c). No differences were observed in the INTEM pathway (Fig. 6c). Regarding actual clot firmness (ACF) and maximum clot elasticity (MCE), the BD female group showed a drop in relation to the BD male group (MCE, $P = 0.008$) in the EXTEM pathway (Fig. 5c). The INTEM and FIBTEM pathways did not show differences in ACF and MCE (Figs 6 and 7c).

Clot lysis parameter

Maximum lysis detected during the run time is described as the difference between MCF and the lowest amplitude after MCF. The animals that were subjected to BD had the maximum reduction in lysis in the EXTEM pathway (Table 2).

Discussion

Previous studies have demonstrated the impairment of microcirculation after BD in male animals and suggested hemodynamic instability, which influences the perfusion of organs and compromises their viability for transplantation, as a major factor in BD donor maintenance [9,18]. It has also been shown that male and female animals differ in terms of microvascular perfusion after BD. The BD-induced hypoperfusion found in males is not present in females [12]. The associated mechanisms may involve sex hormones, as they can influence endothelial cell behaviour and the release of vasoactive elements (such as NO) [19,20]. Along with vascular tonus regulation, platelet activation and aggregation can influence perfusion [21]. In this study, we showed that microcirculatory perfusion in males and females after BD is directly linked to platelet behaviour and coagulation.

Along with hypoperfusion, BD leads to the activation of the inflammatory pathways and reduction in hormone synthesis and release [9,13]. BD-generated inflammatory response is higher in female animals, occurring in parallel with the acute reduction in female sex hormone concentration [12,13]. Here, in order to investigate the sex-influenced differences in the coagulation process after BD, thromboelastometry analysis was conducted and the different pathways involved in the coagulation cascade were evaluated. Experimental studies have shown that BD accelerates clot formation and triggers hypercoagulation in male animals [17]. The authors showed that such changes are in the initial phase of the BD process and normal values are restored 180 min after BD. Here, hypercoagulation in male animals was demonstrated through the reduced time required for clot formation after BD, and we found that the clot lysis capability was reduced. This reduction could contribute specially to the intravascular concentration of microthrombi in male, compromising microcirculatory perfusion. In female, the reduction of clot firmness and clot lysis capability after BD did not impact in the final perfusion, which was maintained due to the lesser clot formation. The three analysed thromboelastometry pathways, with or without the platelets, pointed to the acceleration of clot formation, and the hypercoagulability observed in the blood could potentially be linked to increased platelet activity in males. Conversely, BD female samples showed higher clotting time, regardless of the pathway analysed, indicating that in females, the coagulation process is altered.

Some studies have indicated that oestrogen may cause changes in the coagulation system [22–24] and modify platelet function and its response to stimulation/activation [25]. Despite some discordant results, sex

Table 2. Clot lysis parameters.

		Control male	BD male	Control female	BD female	*P* Sex	*P* BD
EXTEM	ML (%)	10.5 ± 3.4	3.3 ± 1.5*	12.2 ± 2.5	4.3 ± 1.4*	0.622	0.010
INTEM	ML (%)	15.8 ± 7.4	4.5 ± 0.8	23.2 ± 15.4	4.5 ± 1.0	0.666	0.086
FIBTEM	ML (%)	8.8 ± 2.2	4.1 ± 1.2	7.6 ± 2.0	5.3 ± 2.7	0.991	0.107

EXTEM, extrinsic screening test; FIBTEM, fibrinogen screening test; INTEM, intrinsic screening test; ML, maximum lysis.

BD groups, rats that were subjected to brain death ($n = 7$ per group); Control rats, non-manipulated animals. Data are presented as mean ± SEM.

*In relation to the respective control (control).

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differences in platelet behaviour have been reported and the differences point to greater aggregation of platelets in the females than in the males [26,27]. Platelets are important elements in haemostasis and inflammatory processes, and they are activated by different agonists [28,29]. Here, using adenosine diphosphate (ADP), the aggregation capacity observed in control animals confirmed oestrogen influence and pointed to higher aggregability in control female platelets. After BD, there was an inversion in this picture, with significant reduction of platelet aggregation in female rats. In this context, it is possible to suggest that, once BD leads to acute reduction in female sex hormone concentration [13], the lack of oestrogen could contribute to aggregation deficiency in females, accounting for the coagulation delay.

It is important to consider that platelet activity may be negatively modulated by substances such as NO which is released by several cells [29]. As mentioned earlier, female rats present a higher inflammatory response after BD and higher iNOS and eNOS expression than male rats [12,13]. Srihirun *et al.* [30] showed that there is an inverse correlation between nitrite levels in the blood and ADP-induced platelet aggregation. Our results indicate higher total nitrites in the female serum after BD, which is probably related to the reduction of female platelet aggregability, confirming the involvement of NO and its metabolites in coagulation and perfusion induced by BD.

Previously, to identify mechanisms involved in the microcirculatory perfusion compromise after BD, we focused on vascular elements such as NO and endothelin. We showed that after BD, increase in eNOS expression can enhance mesenteric microvascular perfusion [10] and that females present higher eNOS protein expression, despite the reduction in estradiol triggered by BD, sustaining NO synthesis and controlling vascular tone [12]. BD male animals did not show significant reduction in eNOS in the mesentery [10]. Therefore, it is imperative to study the role of platelets and the coagulation process to understand other mechanisms that might be involved in BD-induced microcirculatory hypoperfusion. Our results confirmed the influence of platelets on microcirculation perfusion, with male rats presenting increased platelet aggregation associated with reduced perfusion and females presenting a reduction in platelet aggregation and maintenance of perfusion. We observed thrombus formation in male vessels through intravital microscopy, confirming that this occurrence might be responsible for organ hypoperfusion observed after BD. It is important to mention that the treatment of male BD rats with estradiol is able to restore the microcirculatory perfusion [31].

This study has some limitations. We used an investigation time of 3 h, which is usually the standard time for microcirculatory studies in this model [10,12]. However, the coagulation phenomenon is installed immediately after BD induction and the perfusion compromise is maintained [11]. Although the evaluation of the female rats only on the proestrus phase of the cycle (higher estradiol concentration) could be seen as a limitation, this approach allowed us to focus on estradiol effects in coagulation.

In conclusion, there is sex dimorphism in platelet function and clotting process, which are altered in different ways by BD. In this regard, it is possible to connect the reduction in microcirculatory perfusion in males to intravascular microthrombi formation and the maintenance of perfusion in females to a higher inflammatory response and NO synthesis. The higher inflammatory status in female organs and the knowledge of BD derived thrombus formation in male could indicate the necessity of different donor management protocols to be applied depending on donor sex.

Authorship

CJC and ACBF: designed and performed the study, analysed the data and wrote the manuscript. FYRS, RAJr, LFA and MVS: contributed to the collection and analysis of data. RSCS and MLPS: contributed to data collection. HGDL: contributed to data analysis and wrote the manuscript. LFPM: designed the study, analysed data and wrote the manuscript.

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Conflict of interest

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REFERENCES

1. Kaczmarek I, Meiser B, Beiras-Fernandez A, *et al*. Gender does matter: gender-specific outcome analysis of 67,855 heart transplants. *Thorac Cardiovasc Surg* 2013; **61**: 29.
2. Demir A, Coosemans W, Decaluwé H, *et al*. Donor-recipient matching in lung transplantation: which variables are important? *Eur J Cardiothorac Surg* 2015; **47**: 974.
3. Miller AJ, Kiberd BA, Alwayn IP, Odutayo A, Tennankore KK. Donor-recipient weight and sex mismatch and the risk of graft loss in renal transplantation. *Clin J Am Soc Nephrol* 2017; **12**: 669.
4. Lai Q, Giovanardi F, Melandro F, *et al*. Donor-to-recipient gender match in liver transplantation: a systematic review and meta-analysis. *World J Gastroenterol* 2018; **24**: 2203.
5. Okamoto S, Corso CO, Nolte D, *et al*. Impact of brain death on hormonal homeostasis and hepatic microcirculation of transplant organ donors. *Transpl Int* 1998; **11**(Suppl 1): S404.
6. Okamoto S, Corso CO, Leiderer R, *et al*. Role of hypotension in brain-death associated impairment of liver microcirculation and viability. *Transpl Int* 2000; **13**: 428.
7. Obermaier R, von Dobschuetz E, Keck T, *et al*. Brain death impairs pancreatic microcirculation. *Am J Transplant* 2004; **4**: 210.
8. Yamagami K, Hutter J, Yamamoto Y, *et al*. Synergistic effects of brain death and liver steatosis on the hepatic microcirculation. *Transplantation* 2005; **80**: 500.
9. Simas R, Sannomiya P, Cruz JW, *et al*. Paradoxical effects of brain death and associated trauma on rat mesenteric microcirculation: an intravital microscopic study. *Clinics (Sao Paulo)* 2012a; **67**: 69.
10. Correia CJ, Armstrong R Jr, Carvalho PO, *et al*. Hypertonic saline solution reduces microcirculatory dysfunction and inflammation in a rat model of brain death. *Shock* 2019; **51**: 495.
11. Simas R, Zanoni FL, Coutinho e Silva RS, Moreira LFP. Brain death effects on lung microvasculature in an experimental model of lung donor. *J Bras Pneumol* 2020; **46**: e20180299.
12. Ferreira SG, Armstrong-Jr R, Kudo GK, *et al*. Differential effects of brain death on rat microcirculation and intestinal inflammation: female versus male. *Inflammation* 2018; **41**: 1488.
13. Breithaupt-Faloppa AC, Ferreira SG, Kudo GK, *et al*. Sex-related differences in lung inflammation after brain death. *J Surg Res* 2016; **200**: 714.
14. Levi M, van der Poll T. Coagulation and sepsis. *Thromb Res* 2017; **149**: 38.
15. Barklin A. Systemic inflammation in the brain-dead organ donor. *Acta Anaesthesiol Scand* 2009; **53**: 425.
16. Lisman T, Leuvenink HG, Porte RJ, Ploeg RJ. Activation of hemostasis in brain dead organ donors: an observational study. *J Thromb Haemost* 2011; **9**: 1959.
17. Hvas CL, Fenger-Eriksen C, Høyer S, Sørensen B, Tønnesen E. Hypercoagulation following brain death cannot be reversed by the neutralization of systemic tissue factor. *Thromb Res* 2013; **132**: 300.
18. Simas R, Kogiso DH, Correia Cde J, *et al*. Influence of brain death and associated trauma on solid organ histological characteristics. *Acta Cir Bras* 2012b; **27**: 465.
19. Ross RL, Serock MR, Khalil RA. Experimental benefits of sex hormones on vascular function and the outcome of hormone therapy in cardiovascular disease. *Curr Cardiol Rev* 2008; **4**: 309.
20. Novella S, Dantas AP, Segarra G, Medina P, Hermenegildo C. Vascular aging in women: is estrogen the fountain of youth? *Front Physiol* 2012; **6**: 165.
21. Secor D, Li F, Ellis CG, *et al*. Impaired microvascular perfusion in sepsis requires activated coagulation and P-selectin-mediated platelet adhesion in capillaries. *Intensive Care Med* 2010; **36**: 1928.
22. Moro L, Reineri S, Piranda D, *et al*. Nongenomic effects of 17beta-estradiol in human platelets: potentiation of thrombin-induced aggregation through estrogen receptor beta and Src kinase. *Blood* 2005; **105**: 115.
23. Lemini C, Jaimez R, Franco Y. Gender and inter-species influence on coagulation tests of rats and mice. *Thromb Res* 2007; **120**: 415.
24. Del Principe D, Ruggieri A, Pietraforte D, *et al*. The relevance of estrogen/estrogen receptor system on the gender difference in cardiovascular risk. *Int J Cardiol* 2015; **187**: 291.
25. Nakano Y, Oshima T, Ozono R, *et al*. Estrogen replacement suppresses function of thrombin stimulated platelets by inhibiting Ca(2+) influx and raising cyclic adenosine monophosphate. *Cardiovasc Res* 2002; **53**: 634.
26. Leng XH, Hong SY, Larrucea S, *et al*. Platelets of female mice are intrinsically more sensitive to agonists than are platelets of males. *Arterioscler Thromb Vasc Biol* 2004; **24**: 376.
27. Otahbachi M, Simoni J, Simoni G, *et al*. Gender differences in platelet aggregation in healthy individuals. *J Thromb Thrombolysis* 2010; **30**: 184.
28. Murugappa S, Kunapuli SP. The role of ADP receptors in platelet function. *Front Biosci* 2006; **11**: 1977.
29. Almeida Cardelli NJ, Elisa Lopes-Pires M, Bonfitto PH, Ferreira HH, Antunes E, Marcondes S. Cross-talking between lymphocytes and platelets and its regulation by nitric oxide and peroxynitrite in physiological condition and endotoxemia. *Life Sci* 2017; **172**: 2.
30. Srihirun S, Sriwantana T, Unchern S, *et al*. Platelet inhibition by nitrite is dependent on erythrocytes and deoxygenation. *PLoS One* 2012; **7**: e30380.
31. Vieira RF, Breithaupt-Faloppa AC, Correia CJ, *et al*. 17β-estradiol as a new therapy to preserve microcirculatory perfusion in small bowel donors. *Transplantation* 2020; **104**: 1862.