Parenteral Succinate Reduces Levels of Reactive Oxygen Species, but It Does Not Reduce Caspase-3 Serum and Tissue Levels.

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Abstract

Purpose: In sepsis Reactive Oxygen Species (ROS) production and apoptosis are two physiopathological processes that are increased. *Succinate* is a Krebs cycle intermediate that is reduced in Complex II of the mitochondria. This work aims to determine if succinate administration to septic rats improves serum ROS levels and serum and tissue levels of apoptosis.

Methods: Sepsis was induced with cecal ligation and puncture in 200gr Sprague Dawley rats. 4 groups were formed (control, succinate, sepsis, and sepsis + succinate). 5mml/kg of intraperitoneal succinate were administered to the succinate and sepsis + succinate groups 2 h before sepsis induction and 2 h before sample taking. ROS levels were measured with dichlorofluorescein-diacetate, and apoptosis was measured with a Caspase-3 ELISA kit.

Results: There were significant differences in serum ROS levels between the control group and the sepsis group (P = 0.012), but there were no differences in serum Caspase-3 levels (P = 0.15). The succinate administration reduced serum ROS levels in the sepsis + succinate group compared with the sepsis group in a statistically significant way (P = 0.004), but it did not reduce serum Caspase-3 levels (P = 0.39). There were no differences between groups in kidney and liver Caspase-3 levels. There was no correlation between serum ROS levels and serum Caspase-3 levels.

Conclusions: In this model, ROS levels were reduced with succinate infusion, but Caspase-3 levels were not. In addition, ROS levels and apoptosis levels are not correlated, which suggests that those processes occur at different times.

Introduction

Sepsis is a complex syndrome of concurrent pathophysiological processes [1]. Its incidence is increasing worldwide; therefore, it is essential to understand its pathophysiology correctly [2–6].

In the multiple pathophysiological processes developed simultaneously in sepsis, mitochondrial dysfunction and ROS production play an essential role [7]. ROS are a group of molecules that include oxygen radicals and non-radical oxidizing agents or readily converted into radicals [1]. In addition, there are reactive nitrogen species, both radical and non-radical [7,8]. Several definitions of oxidative stress exist, but the most common one is the imbalance between ROS production and the cellular antioxidant capacity, which can potentially damage cells and destroy tissues [9].

Another pathophysiological process described in sepsis is apoptosis, a highly regulated and conserved cell death mechanism in which the cell self-destructs [10]. It is a usual method in which multicellular organisms eliminate undesired or superfluous cells, neutralizing the potential damage caused by cells with defective DNA [10]. In recent years, interest in apoptosis in sepsis has increased. Some studies have shown that apoptosis levels are increased in different models of sepsis [11–14]. Remarkable studies have shown that apoptosis levels are increased in different models of sepsis [11–14]. In other

publications, the degree of cell death is disproportionately low compared with the degree of severity of clinical or biochemical presentation of multiple organ failure (MOF) [15]. On the other hand, some authors showed that apoptosis markers could be used as prognostic markers [16–18].

Succinate is a Krebs cycle intermediate that is reduced in mitochondrial Complex II. Different studies describe that succinate improved oxygen consumption in septic rat muscle [19], prolonged survival [20], and improved liver metabolic profile [21]. In previous studies, it has been shown that succinate reduces ROS levels in septic rats [22].

Both pathophysiological processes (ROS production and apoptosis) and others are the cause of the MOF observed in sepsis. This study aims to observe whether both markers are correlated and, thus, infer if the processes are simultaneous, what their relationship is, and, in addition, be able to associate these processes with organ failure.

Materials And Methods

Animals

Male Sprague Dawley rats weighing 200 grams, adapted to 12h light cycle for 7 days and fed *ad libitum* at standard temperatures (24°C). The current research was approved by the Institutional Animal Care and Use Committee (CICUAL) of the Faculty of Medicine of the University of Buenos Aires (EXP-UBA: 02282/2012).

There were 4 groups, with 10 rats per group:

1) Group I (control) did not undergo any intervention during the study.

2) Group II, to which intraperitoneal succinate was administered 2 hours before the surgery corresponding to groups III and IV and 2 hours before the taking of the sample.

3) Group III (sepsis group) underwent cecal ligation and puncture, as described in the corresponding section. Resuscitation was performed with 20 ml/kg of NaCl 0.9%, and antibiotic treatment with ceftriaxone (30 mg/kg) and clindamycin (25 mg/kg).

4) Group IV (Sepsis + succinate group) in which sepsis was induced as described and which was administered *succinate(5 mmol/kg)* 2 hours before surgery and 2 hours before the sample taking. In addition, resuscitation was performed with 20 ml/kg of NaCl 0.9%, and antibiotic treatment with ceftriaxone (30 mg/kg) and clindamycin (25 mg/kg).

Between the surgery and the sample taking 24 hours elapsed. The study is outlined in Figure 1.

Cecal Ligation and Puncture

The procedure was performed under anesthesia with 100 mg/kg of intraperitoneal Ketamine and 2.5 mg/kg of intraperitoneal Xylazine. Using the traditional technique [23–28], skin and aponeurosis incisions, and a midline laparotomy were performed. A plane-by-plane dissection was performed, and once the abdomen was entered, the cecum was identified, and 1 cm was ligated. Then, both sides of the ligated cecum were punctured with a 25 x 8 needle, and, subsequently, a layered suture was performed.

Succinate

5 mmol/kg of succinate, from a 0.4 M solution were administered intraperitoneally, as specified above. The solution was prepared with succinic acid (Sigma Chemical Co.), adjusted to pH 7.4 with NaOH, and filter sterilized with a 0.2 µm filter (Ministart®) in a laminar flow cabinet. The chosen dose corresponds to that described in the literature [29].

Serum Sample Taking and Euthanasia

A blood sample was taken through the cardiac puncture 24 hours after the surgery of the respective groups. This process was done under anesthesia with 100 mg/kg of intraperitoneal Ketamine and 2.5 mg/kg of intraperitoneal Xylazine. Later the animal was euthanized by sectioning the great vessels and removing the heart. Blood was centrifuged in dry tubes at 3000 rpm. Then, the serum was separated and frozen at -75 ° C until it was time to make the different determinations. The measurements were made within 60 days of the sample taking.

Tissue Sample

The animal was euthanized immediately after. A sample of the liver, which was perfused with 30 ml of physiological solution through the portal vein, was collected and prepared in cold water. In the same way, a sample of the left kidney, which was perfused through the renal vein, was obtained. After the samples were taken, they were frozen at -20 ° C.

Subsequently, tissue homogenization was performed in glass/Teflon Potter-Elvehjem homogenizers to break cell membranes. In this process a homogenization buffer containing protease and phosphatase inhibitors was used (10 mM Hepes, 2 mM EDTA, 15 mM KCl, 0.2 M sucrose, 0.5 mM DTT, 0.5 mM PMSF, 1 MNaF, 200 mM Na3VO4, 2.5 mM benzamidine, 10 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, pH = 7.6). The crude homogenate was used for determinations of Caspase-3 measurement activities. This homogenate was centrifuged at 1500g, and the supernatant was used for the described determinations.

Determination of Serum Biochemical Variables

Levels of creatinine, urea, total bilirubin, and lactic acid were determined in mg/dl and processed on a Vitros 5600 Ortho Clinical Diagnostics analytical platform, using the dry chemistry method.

Measurement of Serum ROS

The measurement of serum ROS was obtained using dichlorofluorescein-diacetate. (DCFH). 12 µl of serum were incubated for 10 minutes, in 1000 µl of TE buffer solution and 10 µl of NaOH were added to separate the diacetate and, thus, activate the dichlorofluorescein. Emitted fluorescence was measured with Jasco FP770 equipment. An emission spectrum between 500 and 550 nm was used with each sample. The expressed value is that of the emission at 525 nm. since this is the emission peak of DCFH (see annex). This fluorophore (dichlorofluorescein diacetate) needs the diacetate to be separated, activated, and emit a signal at 525nm. This step takes place within the cells due to the presence of esterases. This technique is also described for measurements in extracellular fluids [30–33]. In this case, it is necessary to previously separate the diacetate through prior incubation with NaOH, without the need for esterases [30–33].

Determination of Serum and Tissue Caspase-3

This measurement was taken using the ELISA technique, with a MyBioSource Rat Caspase 3 ELISA Kit (Catalog # MBS763727 Lot #R0143D032), following the manufacturer's specifications. The animal serum and the processed tissue supernatant were used as described above. Samples were measured in an EMP M201 Microplate Reader.

Statistical Analysis

The mean with standard deviation (SD) and mean with 95% confidence interval (CI 95%) were indicated for all results. To analyze whether there were significant differences in continuous variables between the 2 groups, the Student test, and Wilcoxon Rank Sum were used. On the other hand, to analyze continuous variables among 3 or more groups, the ANOVA or the Kruskal-Wallis test were used. The Pearson or Spearman tests were used to correlate 2 variables. In all cases, a statistical significance of $P < 0.05$ was assumed. Statistical analyses were performed using: EPIinfo 7.0, Statistix 7.0, and Graph Pad Prism8.0.2

Results

Serum ROS levels were measured in rats from all 4 groups using the DCFH technique. Means and SD for the groups were the following: Control group = 0.0373 (0.012); Succinate group = 0.0393 (0.014); Sepsis group = 0.0835 (0.027); Sepsis + Succinate group = 0.0623 (0.001) (Figure 2). There were significant differences in ROS levels among the 4 groups ($P = 0.017$) when using the ANOVA test. However, there were no significant differences in ROS levels between the Control group and the Succinate group ($P = 0.29$), nor between the Control group and the Sepsis + Succinate group ($P = 0.18$). In addition, there were significant differences between the Control group and the Sepsis group ($P = 0.012$). Furthermore, the administration of succinate reduced ROS levels in the Sepsis + Succinate group compared with the Sepsis group, showed a statistical significance ($P = 0.004$).

Caspase-3 serum levels were measured. Means and SD stated in pg/ml were as follows: Control group = 16.96 (6.28); Succinate group = 17.55 (7.76); Sepsis group = 16.65 (4.25); Sepsis + Succinate group = 15.03 (4.68) (Figure 3). There were no significant differences among the 4 groups ($P = 0.35$) when using

the ANOVA test, nor were there significant differences between the Control group and the Succinate group (P = 0.26), the Sepsis group (P = 0.15), and the Sepsis + Succinate group (P = 0.22). Additionally, there were no significant differences between the Sepsis group and the Sepsis + Succinate group (P = 0.39) regarding serum levels of Caspase-3.

Caspase-3 levels were analyzed at the tissue level in the liver and kidney. Means and SD measured in the liver are stated in pg/ml and were as follows: Control group = 261.14 (44.72); Succinate group = 239.72 (26.75); Sepsis group = 205.73 (20.52); Sepsis + Succinate group = 251.51 (8.45). On the other hand, levels measured in the kidney were as follows: Control group = 226.83 (34.93); Succinate group = 212.87 (47.93); Sepsis group = 203.57 (46.48); Sepsis + Succinate group = 269.26 (47.56) (Figure 4). There were no differences among the 4 groups in the liver (P = 0.17) when using the ANOVA test. Moreover, there were no significant differences in Caspase-3 levels in the kidney (P = 0.93) among the groups. There were no differences in tissue levels of Caspase-3 in the liver (P = 0.17) and the kidney (P = 0.27) between the Control group and the Succinate group. There were no differences between the Control group and the Sepsis group in the liver (P = 0.11) and in the kidney (P = 0.29) either, nor between the Control group and the Sepsis + Succinate group in the liver (P = 0.35) and the kidney (P = 0.4). Furthermore, there were no differences between the Sepsis group and the Sepsis + Succinate group in the liver (P = 0.14) and the kidney (P = 0.45).

The values of the means and SD for the serum creatinine levels, stated in mg/dl, were as follows: Control group = 0.31 (0.1); Succinate group = 0.309 (0.1); Sepsis group = 0.38 (0.16); Sepsis + Succinate group = 0.43 (0.25) (Figure 5). The ANOVA test was used, and no significant differences were found in serum creatinine levels among the 4 groups (P = 0.3). There were no significant differences between the Control group and the Succinate group (P = 0.44), between the Control group and the Sepsis group (P = 0.1), or between the Sepsis group and the Sepsis + Succinate group (P = 0.14) either. However, a significant increase in creatinine levels can be observed between the Control group and the Sepsis + Succinate group (P = 0.009).

Lastly, the values of means and confidence intervals from total serum bilirubin levels, stated in mg/dl, were as follows: Control group = 0.12 (CI 95%: 0.075-0.16); Succinate group = 0.15 (CI 95%: 0.059-0.24); Sepsis group = 0.13 (CI 95%: 0.075-0.19); Sepsis + Succinate group = 0.14 (CI 95%: 0.092-0.18) (Figure 6). The Kruskal-Wallis test was used, and there were no differences in total serum bilirubin levels among the 4 groups (P = 0.61). There were no significant differences between the Control group and the Succinate group (P = 0.59), nor between the Control group and the Sepsis + Succinate group (P = 0.21). Furthermore, there were no differences between the Control group and the Sepsis group (P = 0.49), nor between the Sepsis group and the Sepsis + Succinate group (P = 0.67).

The Pearson test was used to analyze the correlation between serum ROS and Caspase-3 levels in animals in all 4 groups (Figure 7). There was no correlation between the levels of both variables. A *scatter plot* was made with the values of both variables (R = -0.17; P = 0.3) (Figure 6). There was no correlation

when analyzing only the groups that did not receive Succinate (R = -0.16; P = 0.5), nor the groups that received Succinate (R = -0.25; P = 0.32).

The correlation between creatinine, urea, bilirubin levels, serum Caspase-3 levels, and ROS levels was assessed using the Pearson test. No correlation was found among them (Tables 1 and 2).

The correlation between tissue apoptosis levels and organic functions was also assessed. The association of liver Caspase-3 levels with total serum bilirubin levels was analyzed using the Spearman test, and there was no correlation (R = 0.23; P = 0.36). Also, there was no correlation between apoptosis levels in the kidney and creatinine levels. The correlation between Caspase-3 levels in the kidney and creatinine levels was analyzed, and there was no association (R = 0.0046; P = 0.98).

Discussion

The aim of the administration of succinate to the rats was to provide a substrate for Complex II of the electron transport chain and reducing ROS production. Thus, it was determined that systemic ROS levels are elevated in septic rats when compared with the Control group and that the administration of parenteral succinate reduces the production of these species in septic rats. In contrast, parenteral succinate administered in rats that did not undergo cecal ligation and puncture does not cause any changes regarding the Control group, suggesting that succinate does not affect non-septic rats. In previous studies, it has been documented that succinate reduces serum ROS levels, but it does not improve creatinine levels in septic rats [22], but the present research aimed to find a correlation between serum ROS levels and serum apoptosis markers, also, the correlation between apoptosis markers and organ dysfunction markers as bilirubin and creatinine.

There are controversial publications regarding succinate levels in pathological situations, and one of them is hypoxia-reoxygenation. Chouchani et al. demonstrated an accumulation of succinate during hypoxia-reoxygenation cycles in mice [34]. Through various tests, glucose, palmitate, glutamate, and GABA do not contribute to its accumulation. Instead, the cause of the accumulation would be fumarate. On the other hand, Wijemars et al. analyzed biopsies of transplanted kidneys and observed a drop in tissue succinate accumulation [35]. However, hypoxia-reoxygenation is not the only pathophysiological mechanism in sepsis.

Several studies evidenced changes in the processes that occur within the mitochondria during sepsis, among which the electron transport chain is affected. Literature is not conclusive regarding the activity rank of the different complexes at this level. Lorente et al. demonstrated decreased action in the electron transport chain in Complex IV [36], while Brealey et al. found decreased Complex I activity but found no differences in Complexes II, III y IV [37–39]. Furthermore, the same group demonstrated that the activity of Complexes II and III remained unchanged both in the muscles and the liver in septic rats [37–39]. Contrariwise, in those organs, Complex I activity seemed to increase with the severity of sepsis in rats.

In another study done on septic rats, using the cecum ligation and puncture technique, the infusion of dimethyl succinate improved the survival of the rats [20]. On the other hand, it was demonstrated that, in rats that were administered LPS, dimethyl succinate infusion improved ATP levels and the ATP/ADP ratio, which would suggest a recovery in the activity of the electron transport chain [21]. A different study determined that Complex I activity was decreased in the soleus muscle of rats with moderate/severe sepsis caused by the intraperitoneal administration of a fecal matter preparation. In contrast, there were no changes in Complex II activity in control group rats. In addition, the administration of malate and glutamate (as Complex I substrates) and succinate (as Complex II substrate) improved muscle oxygen consumption compared with the administration of malate and glutamate alone. Furthermore, this improvement was more pronounced in rats with moderate/severe sepsis than in rats with mild sepsis [19].

Creatinine levels increased by 22% in septic rats, but the differences were not statistically significant compared with the Control group. In contrast, serum creatinine levels in septic rats with parenteral succinate did not decrease compared with septic rats without treatment, although they showed significant differences compared with the Control group. Creatinine is a late marker of kidney failure, and a marker such as NGAL, which would give an early account of the damage and show significant differences among the different groups, was not used [40–42].

Lastly, there was no correlation between ROS levels and creatinine, total bilirubin, and urea levels. This observation suggests that there would be no pathophysiological association between ROS levels and organ failure. This data contrasts with the previous study, where there was a correlation between ROS levels and serum creatinine levels. As previously stated, the cause and pathophysiology of MOF is multifactorial, which is probably one of the causes for the lack of correlation among the variables mentioned above. That is to say that the increase in creatinine levels would be determined by ROS status and other previously described pathophysiological mechanisms, such as pH, coagulation, endothelial, and microcirculation alterations, among others.

Total bilirubin levels in non-septic rats did not increase with succinate administration compared with the Control group. In addition, there were no differences in total bilirubin levels in septic rats with and without treatment with succinate. The only reference found in the literature regarding the effect of succinate on the liver of septic rats states that succinate infusion improves the concentration of β-hydroxybutyrate, increases ATP concentration in hepatocytes, and glucose oxidation, and also decreases the lactate/pyruvate ratio [21].

Since Caspase-3 is an apoptosis marker commonly used in the literature [12,43–46], it was measured to determine apoptosis levels [47], both at the tissue and serum levels. The aim of measuring serum Caspase-3 was to determine whether it could indicate tissue apoptosis.

There were no differences in serum Caspase-3 levels among the 4 groups, nor in tissue Caspase-3 levels in the liver and the kidney among the 4 groups. It is known that, in animal models of sepsis, apoptosis levels in different organs are increased. For example, in a study done on rats exposed to LPS, it was

observed that the activity of Caspases-3, -8, and -9, as well as TNF-α levels, increased in the left ventricle [48]. On the other hand, rats that underwent cecal ligation and puncture and were subsequently euthanized at different times showed an increase in renal apoptosis, measured using the TUNNEL technique, with a peak at 6 hours. Furthermore, renal apoptosis measured by cytokeratin 18 fragment M30 had 2 peaks, one at 6 hours and another at 48 hours [49]. In this study, the determination of markers was done at 24 hours and 48 hours, respectively. The mortality of the rats was very high and could have altered the results and, at 6 hours, it could be an early measurement, and there probably would have been no differences in serum ROS levels. Therefore, the difference between the results obtained in this research and those obtained in the above-mentioned publications could be due to the kinetics of elevation in serum of those markers.

On the other hand, immunohistochemical studies show that, in sepsis, there is a higher degree of lymphocyte and digestive tract cell death, whereas, in the kidney, liver, and lungs, cell death is lower [45,50]. In addition, in a study done on sheep (with *Escherichia coli* infusion), it was observed that there was no increase in apoptosis markers in the kidney in septic animals. However, in animals that recovered from sepsis (the infusion of bacteria was suspended, and gentamicin was administered) those markers increased in the kidney [51]. These findings coincide with the results presented in this study since the animals were septic when taking the sample.

There was no correlation between tissue Caspase-3 levels and creatinine or total bilirubin levels either. This finding would indicate that serum markers of organ failure are not associated with levels of tissue apoptosis, at least within the first hours of the event. As described in the paragraph above, as apoptosis tissue markers do not increase, this could cause the lack of increase in serum and the absence of correlation between apoptosis markers and biochemical parameters of organ failure. In addition, there was no correlation between serum Caspase-3 levels and serum ROS levels. In a previous work with septic patients, we showed that septic patients have higher levels of Caspase-3 but not higher levels of serum ROS and both markers didn´t correlate [52]. This conclusion could be since both processes are not related or do not co-occur in sepsis.

The main limitations of the study are the single time measurement of ROS and apoptosis markers which did not allowed to determinate if the lack of correlation was both processes are related but not synchronic. The other limitation is that renal function was determinated with creatinine but not N-GAL. The main strength is that serum ROS were measured and not oxidative stress, and it was correlated with apoptosis markers also with kidney and liver damage markers.

Conclusion

Succinate reduces ROS levels in septic rats but does not reduce Caspase-3 levels as an apoptosis marker. Also, there was no correlation between ROS levels and Caspase-3 levels, suggesting that both processes are not necessarily connected. Finally, succinate didn´t reduce creatinine and bilirubin levels as markers

of organ failure and there was no correlation between the apoptosis marker and serum ROS measurement and organ failure markers.

Declarations

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Conflict of interest: Autor's declare that don´t have any conflict of interest.

Availability of data and material: The data that support the findings of this study are available from the corresponding author, Sebastián Chapela, upon reasonable request.

Author´s contributions: Sebastián Chapela contributed with the design, execution of assays and writing. Giovanna Muscogiuri contributed with the design. Luigi Barrea contributed with the writing. Evelyn Frias-Toral contributed analysing the data. Hilda Burgos contributed with the execution of the assays. María Cecilia Ricart contributed analysing the data. Alexis Muryan with the execution of the assays. Amalia Schiel with the execution of the assays. Manuel Alonso contributed with the interpretation of the data and writing. Carlos Alberto Stella directed the group and contributed with the design.

Ethics approval: CICUAL de la Facultad de Medicina de la Universidad de Buenos Aires. EXP-UBA: 0228244/2012.

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Animal Study:

All institutional and national guidelines for the care and use of laboratory animals were followed.

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Tables

Table 1. **Correlation between ROS levels and biochemical variables.** *The Pearson test was used for the analysis of creatinine and urea concentration variables, and the Spearman test was used for the analysis of bilirubin.*

Biochemical variable correlated with ROS	R	P
Creatinine (mg/dl)	0.06	0.71
Urea (mg/dl)	-0.24	0.15
Total bilirubin (mg/dl)	0.16	0.33

Table 2. Correlation between serum activated Caspase-3 levels and biochemical variables. *The Pearson test was used for the analysis of creatinine and urea concentrations. The Spearman test was used for the analysis of bilirubin.*

Biochemical variable	R	P
Creatinine (mg/dl)	0.056	0.74
Urea (mg/dl)	0.15	0.37
Total bilirubin (mg/dl)	0.17	0.31

Figures

Figure 1

Timeline of the succinate experiment in septic rats. The different steps of the experiment are represented. 4 groups were formed as described in Materials and Methods: 1- Control 2- Succinate 3- Sepsis 4- Sepsis + Succinate. 5 mmol/kg of Intraperitoneal Succinate were administered with a 0.4M solution to groups 2 and 4.

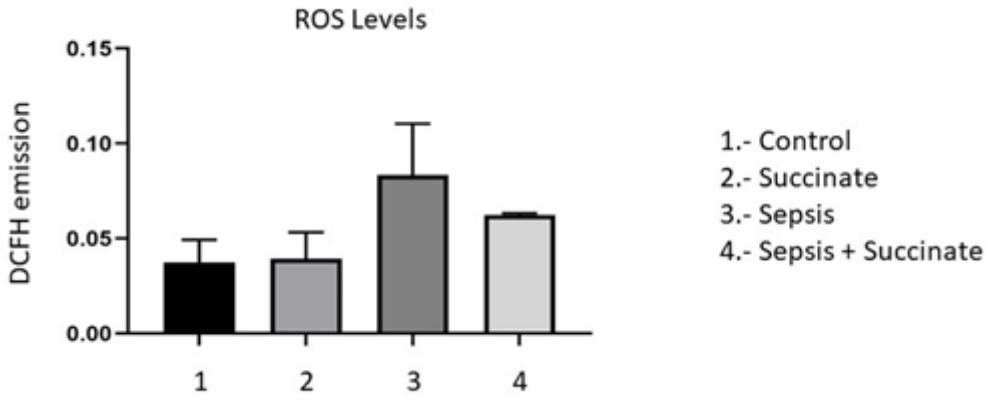

Figure 2

ROS levels in response to succinate administration. The mean values and their standard deviation of ROS (DCFH emission at 525 nm) are expressed in the 4 groups as described in Materials and Methods. There were significant differences between the Control group and the Sepsis group ($P = 0.012$), and between the Sepsis group and the Sepsis + Succinate group ($P = 0.004$).

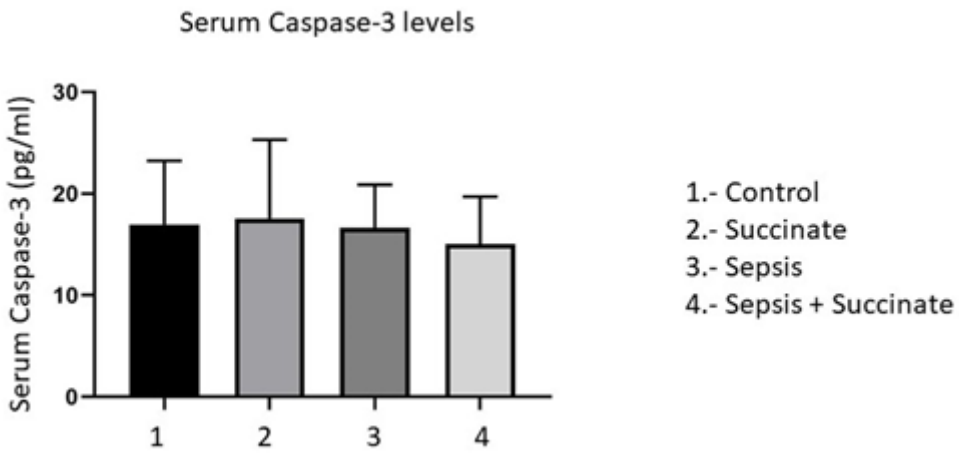

Figure 3

Levels of activated serum caspase-3 in response to succinate administration. The mean values and their standard deviation of serum activated caspase-3 are expressed in the 4 groups as described in Materials and Methods. There were no significant differences among the groups.

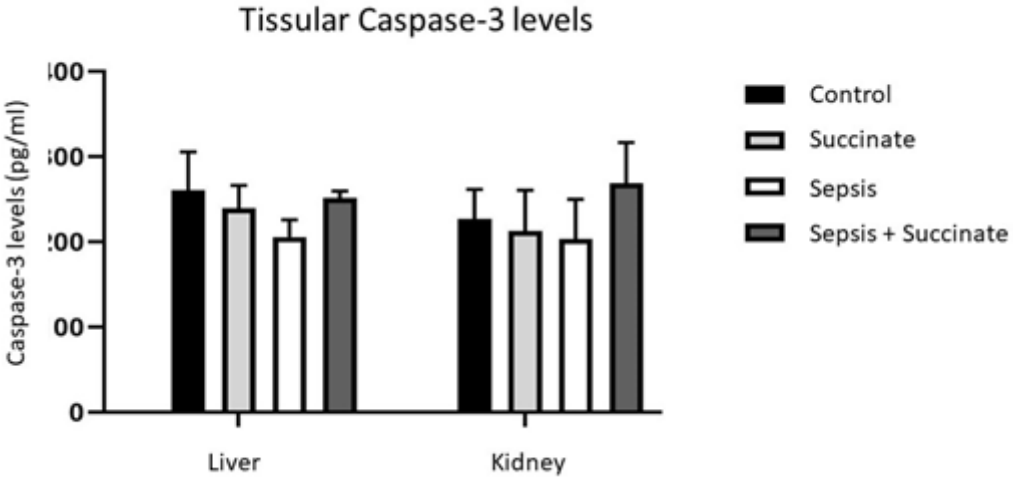

Figure 4

Levels of activated caspase-3 in tissues. Average values and their standard deviation of activated caspase-3 in liver and kidney are expressed in the 4 groups as described in materials and methods. There were no significant differences among the groups.

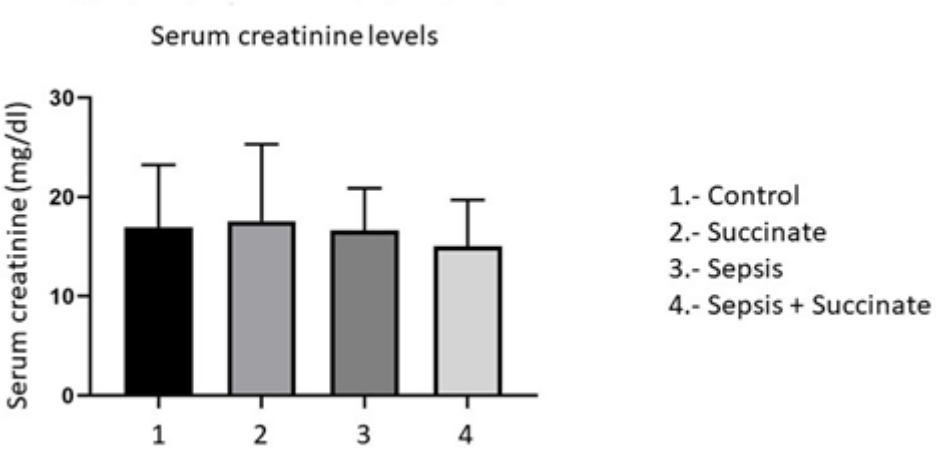

Figure 5

Serum creatinine levels in response to succinate administration. The mean values and their standard deviation of serum creatinine are expressed in the 4 groups as described in Materials and Methods. There were no significant differences among the groups.

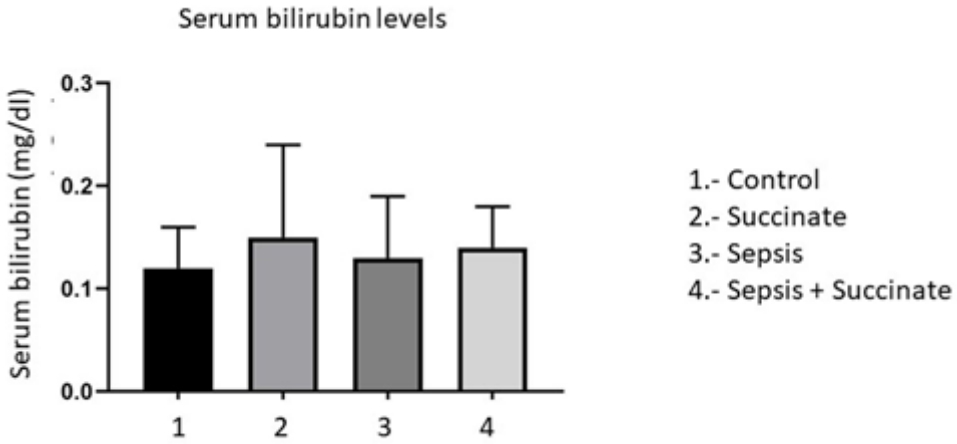

Figure 6

Total bilirubin levels in response to succinate administration. The mean values and their standard deviation of total serum bilirubin are expressed in the 4 groups as described in materials and methods. There were no significant differences among the groups.

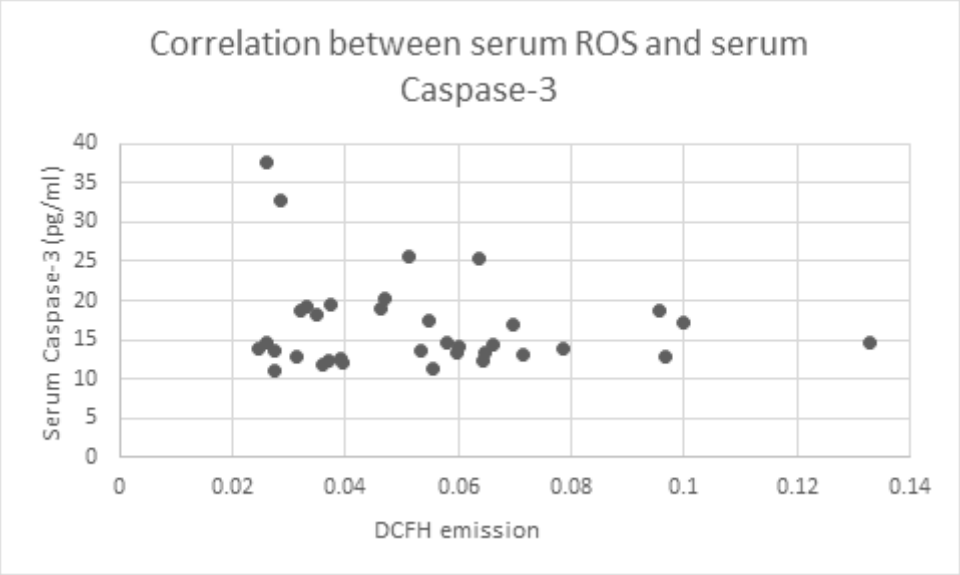

Figure 7

Correlation between systemic ROS levels and serum Caspase-3. There was no correlation between both variables (R = -0.17 P = 0.3). Emission values are expressed at 525 nm DCFH as a ROS marker.

Supplementary Files

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