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Article

# The influence of dehydroepiandrosterone on effector functions of neutrophils

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Dehydroepiandrosterone (DHEA) is a steroid hormone secreted by the adrenal glands, gonads and brain. It is a precursor to sex hormones and also is known to have immune modulatory activity. However, little is known about the relationship between DHEA and neutrophils and thus our study evaluates the influence of DHEA in the effector functions of neutrophils. Human neutrophils were treated *in vitro* with DHEA and further infected with *Salmonella enterica* serovar Typhimurium. The treatment of neutrophils with 0.01 μM of DHEA increased the phagocytosis of *Salmonella* independent of TLR4 as the treatment did not modulate the TLR4 expression. Additionally, DHEA caused a decrease in ROS (reactive oxygen species) production and did not influence the formation of the neutrophil extracellular trap (NET). Steroid treated neutrophils, infected or stimulated with LPS (lipopolysaccharide), showed reduced production of IL-8, compared to untreated cells. Also, the protein levels of p-NFκB were decreased in neutrophils treated with DHEA, and this reduction could explain the reduced levels of IL-8. These results led us to conclude that the steroid hormone DHEA has important modulatory functions in neutrophils.

**Keywords:** Dehydroepiandrosterone. Neuroimmunoendocrinology. S. typhimurium. Innate immunity. Phagocytosis.

## INTRODUCTION

Dehydroepiandrosterone (DHEA) and its sulfated form (DHEAS) are the most abundant steroid hormones in humans (Ebeling, Koivisto, 1994). DHEA is an essentially human molecule produced in the reticular zone of the adrenal glands cortex, gonads and brain, and is synthesized from pregnenolone and secreted in response to the release of adrenocorticotropic hormone (ACTH) in face of a stress agent, like pain, hypothermia, hypoglycemia, hypoxia and trauma (Butcher *et al.*, 2005). Unlike other hormones secreted by the adrenal glands, such as cortisol and aldosterone, the secretion pattern of DHEA is age-dependent. The plasma concentration of this hormone declines with age (Hinson, Raven, 1999; Allolio, Arlt, 2002; Racchi *et al.*, 2003; Arlt, Hewison, 2004), in concert with an increased cortisol/DHEA ratio. Thus, the decline of DHEA levels have been associated with the development of age related diseases, including insulin resistance, obesity, cardiovascular disease, cancer, depression and immune-senescence, that are all associated with decreased microbicidal function of neutrophils and reduction of cytotoxic activity of natural killer cells (Allolio, Arlt,

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2002; Tchernof, Labrie, 2004; Maninger *et al.*, 2009; Hazeldine *et al.*, 2010).

The relationship between this neuroendocrine and the immune system has been described for more than twenty years ago and this basic bidirectional communication is necessary for the regulation of various processes in the body in both physiological and pathological contexts (Weigent *et al.*, 1990). This crosstalk is mediated by cytokines, neuropeptides, hormones and their receptors (Quintanar, Guzmán-Soto, 2013). It is known that DHEA has an important immune function through modulating secretion of cytokines including IL-2 (Suzuki *et al.*, 1991), IL-6 (Straub *et al.*, 1998), TNF-α (Ben-Nathan *et al.*, 1999) by immune system lymphocytes and monocytes. With respect to neutrophils, these polymorphonuclear (PMN) cells are the only leukocytes expressing the "organic anion-transporting polypeptide D" (OATP-D) which mediates DHEAS influx into the cell (Radford *et al.*, 2010). The treatment of neutrophils for 4 h with DHEAS (0.01-10 μM) and with the chemotactic peptide fMLP (formyl-methionyl-leucyl-phenylalanine) results in dose-dependent chemotaxis inhibition of neutrophils (Koziol-White *et al.*, 2012). However, few studies have shown the influence of this steroid on neutrophil function.

Here we evaluated the influence of DHEA in neutrophils through the analyses of phagocytosis, the expression of TLR4, IL-8 release, nuclear factor NFκB expression, ROS and Neutrophil Extracellular Traps (NET) release. PMN neutrophils were infected with the gram-negative bacteria *Salmonella enterica* serovar Typhimurium, a worldwide endemic pathogen that is usually transmitted by contaminated food and which causes diarrhea, nausea, vomiting, fever and abdominal pain. The ingested *Salmonella* spreads rapidly along the gastrointestinal tract, invading the mucosa throughout the gut, colonizing the lamina propria and Peyer's patches, where this bacterium is quickly recognized by dendritic cells, macrophages and neutrophils (Eckmann, Kagnoff, 2001). These immune cells trigger an immune response directed to the elimination of this invading pathogen. Here we demonstrated that DHEA-conditioned neutrophils can efficiently engulf bacteria, probably by an independent manner of TLR4 expression, as the DHEA treatment did not modulate the expression of this receptor. Besides increasing phagocytosis, the PMN cells treated with the steroid had a decrease in the production of ROS and other inflammatory mediators with no effect on NET release.

## METHODS

### Bacterial strain, growth and processing

The strain UK-1 (Universal Killer) of *Salmonella enterica* serovar Typhimurium χ3761 used in the experiments was kindly donated by Dr. Hebert Seixas Hanna (University of Sao Paulo, Ribeirao Preto, SP, Brazil). From a frozen aliquot of bacterial of unknown concentration, the *S. Typhimurium* were plated in solid BHI (brain heart infusion) media (Acumedia, Michigan, USA) for 18 h at 37 ºC. Next, some colonies were recovered and were inoculated in liquid BHI, incubated at 37 ºC with constant shaking, until the optical density of the bacterial suspension reached a value of 0.3 which corresponds to 3.5 x $10^8$ CFU mL-1. Bacteria were then resuspended in RPMI-1640 culture medium (Gibco, Grand Island, USA). This bacterial culturing procedure was performed for each experiment and the Multiplicity of Infection (MOI) chosen was 10 bacteria for 1 neutrophil.

### Isolation of human neutrophils

Blood samples were obtained from 15 healthy male donors, with an average age of approximately 28 years old ± 4.77. The study was approved by Ethics Committee of Faculdade de Ciencias Farmaceuticas de Ribeirao Preto, and informed written consent was obtained from all the blood donors (protocol number 1.124.032). Only male blood was used in this study due to the notorious variation in hormone circulation across the women estrous cycle, aiming to minimalize their possible influences on experiments. Peripheral blood was collected in heparin-containing Vacutainer tubes (BD Biosciences, Franklin Lakes, USA) and the blood was centrifuged to separate plasma and cellular portion of blood. Cellular portion was applied to a Percoll (GE Healthcare, Uppsala, Sweden) gradient formed by two concentrations (1.095 and 1.080 g $mL^{-1}$) and centrifuged at 600 g. The layer of PMN cells was collected from the gradient interface and washed with phosphate buffered saline (PBS). The viability and number of cells were obtained with Trypan Blue (Gibco, Grand Island, USA) exclusion dye (Strober, 2001). Is important to note that the experiments were made at different days because the number of cells harvested per donation is not sufficient to perform all the techniques with the same sample.

### Phenotypic analysis of neutrophil

After isolation of PMN cells, phenotypic analysis was performed. 5 x $10^5$ cells were re-suspended in a solution of PBS containing 2% fetal bovine serum (FBS – Gibco, Grand Island, USA). Monoclonal antibodies against CD11b, CD16b and CD32 (*BD Biosciences, San Diego, CA*) were added to the cells, followed by a 25 minute-incubation at 4 ºC, in the dark. Then, the cells were washed and re-suspended in a solution of PBS with 1% formaldehyde for analysis by flow cytometry on a FACS Canto II flow cytometer (*BD, Biosciences, San Diego, USA*).

### Treatment with DHEA

For the treatment of neutrophils, one stock solution of 50.000 µM of DHEA (Trans-Dehydroandrosterone, Sigma-Aldrich, St. Louis, MO, USA) diluted in dimethyl sulfoxide (DMSO – Macron Chemicals, Center Valley, USA) was prepared. Next, this solution was diluted in DMSO to stock solutions of 500 µM and 5 µM and was used in the experiments at the final concentration of 1 and 0.01 µM, respectively. In addition, a dose of 100 µM also has been used in some experiments. The neutrophils were treated with DHEA for 1 h, at 37 ºC, in 5% $CO_2$ before the infection with *S. Typhimurium*.

### Cell death analysis

Neutrophils (5 x $10^5$) from eight different donors were plated in 24-well plates and the cells were pre-treated with DHEA at concentrations of 100 µM, 1 µM and 0.01 µM, for 1 h. The control cells, neutrophils without treatment or neutrophils in 0.2% DMSO were used in the assay. The cells were washed with PBS 1x, and were transferred flow cytometry tubes. Viability, apoptosis and necrosis rates were determined by flow cytometry analysis of annexin V/propidium iodide (PI), according to manufacturer's instruction (BD Biosciences, San Diego, USA). The cells were analyzed on a FACS Canto II flow cytometer (BD, Biosciences, San Diego, USA). The percentage of viable cells (annexin V-/PI-), necrosis (PI+) and apoptosis (annexin V+/PI-) were noted.

### Phagocytosis assay

Neutrophils (2 x $10^5$) from eleven different donors were distributed in a 96-well plate and pre-treated with DHEA and then incubated with *S. Typhimurium* (MOI=10) for 15 min, at 37 ºC, in 5% $CO_2$. After 15 min of culture (Van Bruggen *et al*., 2007), the non-phagocytosed bacteria were washed away, and the cells were lysed with 0.05% w/v of saponin (Sigma-Aldrich, St. Louis, USA), for release of the intracellular bacteria. The isolated bacteria were serially diluted (1:10) and 100 µL of the dilution was plated in solid BHI for CFU counting. The plates were kept on 37 ºC, overnight to allow the colony growth. Too choose a dilution factor, colonies must range for 30-300 CFU in agar medium. The data were expressed as CFU/mL as described below:

CFU/mL=(number of colonies x dilution factor) x 1000/100

### ROS production assay

After DHEA treatment at 100 µM, 1 µM and 0.01 µM, the ROS production by neutrophils was analyzed in response to opsonized *S. Typhimurium* or phorbol 12-myristate-13-acetate (PMA – Sigma-Aldrich, St. Louis, USA). For the opsonization process, the bacteria were incubated with 20% normal human serum (NHS) for 30 min, at 37 ºC. The bacteria were washed twice and placed in culture with the neutrophils (10:1 MOI). As a positive control, PMA ($10^{-7}$ M) was used. As a positive control, PMA (10-7M) was used. Non-stimulated cells were used to determine the basal production of ROS. The chemiluminescence reaction was monitored in the luminometer (AutoLumat Plus LB 953 EG&G Berthold), for 1 h, at 37 ºC in the presence of the 100 µM luminol (Sigma-Aldrich, St. Louis, USA), and data were recorded as photon counts per minute (cpm). Neutrophils from sixteen donors were used for control and DHEA (0.01 µM) treatment, and for the conditions with DHEA (100 and 1 µM) a total of ten donors were recruited. The results were expressed as area under the curve of chemiluminescence.

### TLR4 expression by qPCR

After treatment of neutrophils (from seven different donors) with DHEA in the concentrations of 0.01 µM, the cells were infected with *S. Typhimurium* (MOI=10) for 1 h. Next, RNA was extracted using a PureLink RNA Mini Kit (Life Technologies, Carlsbad, USA), according to manufacturer's instruction. The extracted RNA was quantified by a fluorescent probe Qubit kit (Life Technologies, Carlsbad, USA) and

readings were performed on a Qubit® 2.0 fluorometer (Invitrogen, Calsbad, USA). For the real-time PCR, the mRNA was translated into cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) according to manufacturer's recommendations. For the reaction of qPCR, 50 ng of cDNA was used per reaction, and the predesigned primers for TLR4 and endogenous internal control ACTB (**β**-actin) (Integrated DNA Technologies –IDT, Coralville, Iowa, EUA) were used. The analysis was made using the GoTaq-Green Master Mix (Promega), with a final reaction volume of 20 µL and then, quantitated using StepOnePlusTM equipment (Applied Biosystems, Foster City, USA), with amplification conditions: Denaturation of the DNA followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. $2^{-\Delta\Delta Ct}$ method was used for the analysis of the RT-PCR data (Livak, Schmittgen, 2001).

**Detection of IL-8.**

The production of IL-8 was analyzed in the supernatant of neutrophils from six different donors, stimulated with lipopolysaccharide (LPS – Sigma-Aldrich, St. Louis, USA) or *S. Typhimurium*. The cells ($2 \times 10^5$), previously treated with DHEA, were cultured with *S. Typhimurium* (MOI=10) for 24 h, at 37 ºC, in 5% $CO_2$ in the presence of 49.5 µM of bacteriostatic chloramphenicol. Alternatively, neutrophils were stimulated with 1 µg/mL LPS, for 18 h, at 37 ºC, in 5% $CO_2$. The supernatants were collected and the IL-8 was quantified by Enzyme Linked Immunosorbent Assay (ELISA). This interleukin was quantified using specific antibodies and cytokine standards, according to the manufacturer's instructions (R&D Systems, Minneapolis, USA). The reading was performed in a spectrophotometer at 450 nm, where the IL-8 concentrations in the samples were calculated according to absorbance values of the samples using the standard curve.

**NFκB analysis by Western blotting**

After treatment of PMN (from three different donors) with DHEA 100 µM and 0,01 µM, the cell protein extract was performed using CelLytic Mammalian Cell Lysis/Extraction Reagent (Sigma-Aldrich, St. Louis) with phosphatase and protease inhibitor cocktails (Sigma-Aldrich, St. Louis). The quantification of protein was made using Bradford protein assay (Bio-Rad Laboratories, USA). After this, 20 micrograms of proteins were separated by 10% dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE Healthcare, Germany). The membrane was blocked with solution of 5% of non-fat dry milk in Tris-buffered saline containing 10% of Tween 20. Primary antibodies against NFκB (sc-372; Santa Cruz), phospho-NFκB (sc-101752; Santa Cruz) and Histone H3 (9715S; Cell Signalling) were used. The reaction was developed using ECL Western blotting system (GE Healthcare, Germany).

**Quantification of NETs**

Neutrophils ($2 \times 10^5$) from four different donors previously treated with DHEA, were stimulated for NET release by PMA (0.162 µM) or *S. Typhimurium* (MOI=10), for 4 h at 37 ºC in 5% $CO_2$. As negative control, for inhibition of NETs formation, the cells were treated with 100 U/mL DNaseI (Invitrogen, California, USA) prior to stimulus. After the 4 h of cell culture, the supernatants were treated with 20 U/mL of the restriction enzymes EcoRI and HindIII (New England BioLabs, Ipswich, USA). Next, the extracellular DNA was quantified using the commercial kit Quant-iT™ Picogreen® dsDNA (Molecular Probes, Oregon, USA), according to the manufacture's instruction. Briefly, the DNA standard provided by the kit, was also treated with the restriction enzyme, and after, dilutions were made for the construction of standard curves. At the samples and the standard, the Picogreen fluorescent reagent was added, the plates were read at fluorimeter (excitation of 480 nm, emission 520 nm) and the concentrations were calculated using the standard curves.

**NET visualization**

The culture of neutrophils was made above glass coverslips of 13 mm diameter (Knittel Glass, Braunschweigi, Germany), treated with 0.01% of poly-L-lysine (Sigma-Aldrich, St. Louis), in 24-well flat-bottom culture plates. The cells were stimulated with PMA (0.162 µM) or *S. Typhimurium* (MOI=10) for 4 h at 37 ºC in 5% $CO_2$. Some cultures were treated with DNaseI (100 U/mL) (Invitrogen, California, USA) before the bacteria infection in

order to inhibit NET formation. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, USA) at room temperature for 10 min and then were permeabilized with 0.5% Triton X-100 v/v (Sigma-Aldrich, St. Louis, USA), for 5 min. Next, the neutrophils were incubated for 30 min with 3% w/v of bovine serum albumin (BSA – Sigma-Aldrich, St. Louis, USA), and then NETs were stained with rabbit anti-Neutrophil elastase antibody and mouse anti-histone H1 antibody (Abcam, Cambridge, USA) for 18 h at 4 ºC, followed by incubation with donkey anti-rabbit IgG Alexa Fluor 488 and rat anti-mouse IgG2 Alexa Fluor 647 secondary antibodies (Abcam, Cambridge, USA), for 1 h in the dark at room temperature. Additionally, DAPI (Life Technologies, Eugene, USA) was added to stain DNA (10 min, at room temperature) and the slides were mounted using mounting medium (DAKO, California, USA). The slides were analyzed using a confocal laser scanning microscope (Leica, TCS SP8), and the software LAS (Leica Application Suite) was used for the image acquisition.

### Statistical analyses

All statistical analyses were made using GraphPad Prism software, version 5.0. Statistical significance was assessed using paired Student's t test, one-tailed, comparing cells without treatment and with treatment. $p \leq 0.05$ was considered to be statistically significant.

## RESULTS

### Isolation and treatment of neutrophils with DHEA did not affect the viability of cells

The purity and the phenotypic characteristic of isolated neutrophils were analyzed by flow cytometry. As exhibited in figure 1A, after the use of density-gradient centrifugation for purification, 93.9% of the cells obtained were CD32+CD16+ (Q2) and 96% were CD11b+C16+ (Q2-1) showing that the population of cells used in the following experiments was, in fact, polymorphonuclear neutrophils.

Viability was also tested and the neutrophils were shown to be 80% viable as shown at figure 1B. Also, control neutrophils have an approximately 15% basal rate of apoptotic death after the purification, as showed in figure 1D. The treatment of cells with 0.2%, DMSO reduces the viability of neutrophils by about 7.5%, probably because the rate of apoptosis was increased, as showed in figure 1D. This profile was not seen in the cells treated with 100 μM, 1 μM or 0,01 μM DHEA. With regard to necrotic cells (figure 1C), no differences were observed amongst the treatments, and the rates of necrotic cell death were lower than deaths due to apoptosis.

### DHEA enhanced the phagocytic activity rates of neutrophils

As depicted in Figure 2, the number of bacteria phagocytosed by DHEA pretreated neutrophils was significantly higher when compared to untreated neutrophils, suggesting that DHEA increases the phagocytic capacity of neutrophils against *S. Typhimurium*.

### DHEA reduced the production of ROS by neutrophils stimulated with PMA

We observed that non-opsonized *S. Typhimurium* was not able to stimulate ROS production (data not shown). Thus, bacteria were opsonized using 20% normal human serum and incubated with neutrophils and the probe luminol. As shown in Figure 3, the opsonized bacteria were able to stimulate the production of ROS, but these amounts were less than those seen with PMA stimulation. With respect to the treatment with DHEA, the results showed that the hormone reduced the production of ROS by neutrophils. The concentration of 100 μM cause the highest reduction of ROS production, regardless the stimuli (bacteria or PMA). The concentration of 0.1 μM and 0.01 μM DHEA also caused a reduction of ROS production, albeit on a smaller scale, in both cells infected with *S. Typhimurium* or in neutrophils treated with PMA.

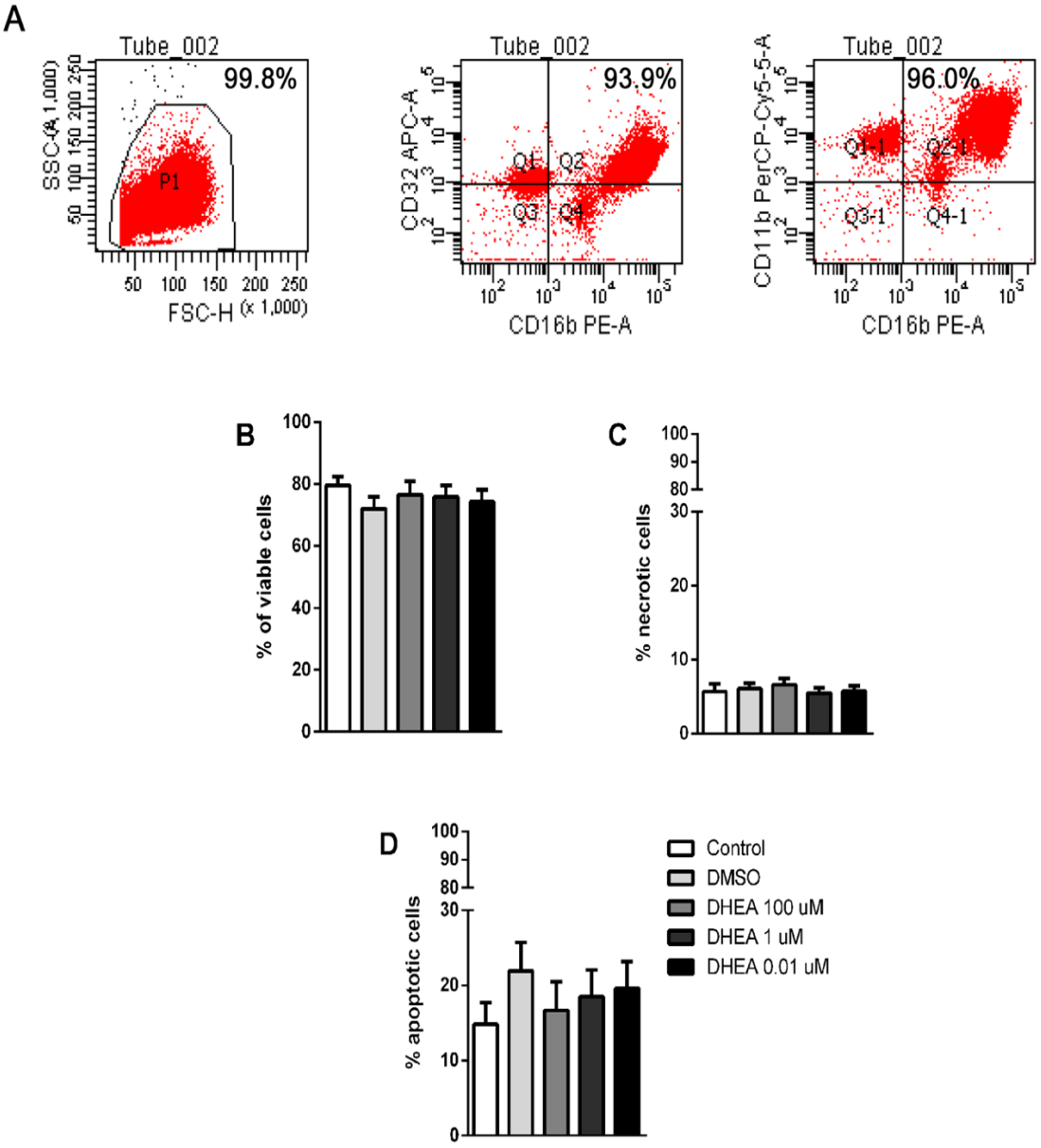


**FIGURE 1 –** Purity and viability of neutrophils. PMN were isolated from peripheral blood of healthy men using a Percoll density gradient. (A) After separation, the isolated cells (5 x 105) were incubated with antibodies against CD11b, CD16 and CD32 and analyzed by flow cytometry to verify the purity. Once isolated, neutrophils were treated with DHEA in the concentrations of 100 µM, 1 µM and 0.01 µM for 1 hour. (B) The percentage of viable cells (anexin-/PI-), (C) necrotic cells (PI+) and (D) apoptotic cells (annexin+/PI-) were analyzed by flow cytometry. Data express the Mean (SD) of 4 independent experiments performed on different days, with cells from 8 different donors.

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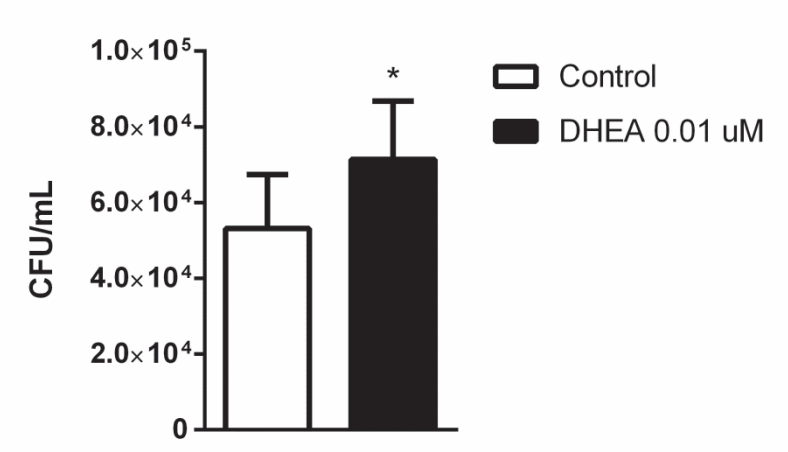


**FIGURE 2 –** Phagocytosis by neutrophils infected with *S. Typhimurium* is increased by DHEA treatment. Neutrophils isolated from healthy men were treated *in vitro* with 0.01 μM of DHEA for 1 hour and then the cells (2 x 105) were infected with *S. Typhimurium* (MOI=10) for 15 min. Non-phagocytosed bacteria were washed out, and cells were lysed with 0.05% saponin (w/v) to release the intracellular bacteria. The suspension of bacteria was subjected to serial dilutions (1:10), plated in BHI medium and the number of CFU was determined. Data express the Mean (SD) of 8 independent experiments performed on different days, with cells from 11 different donors.

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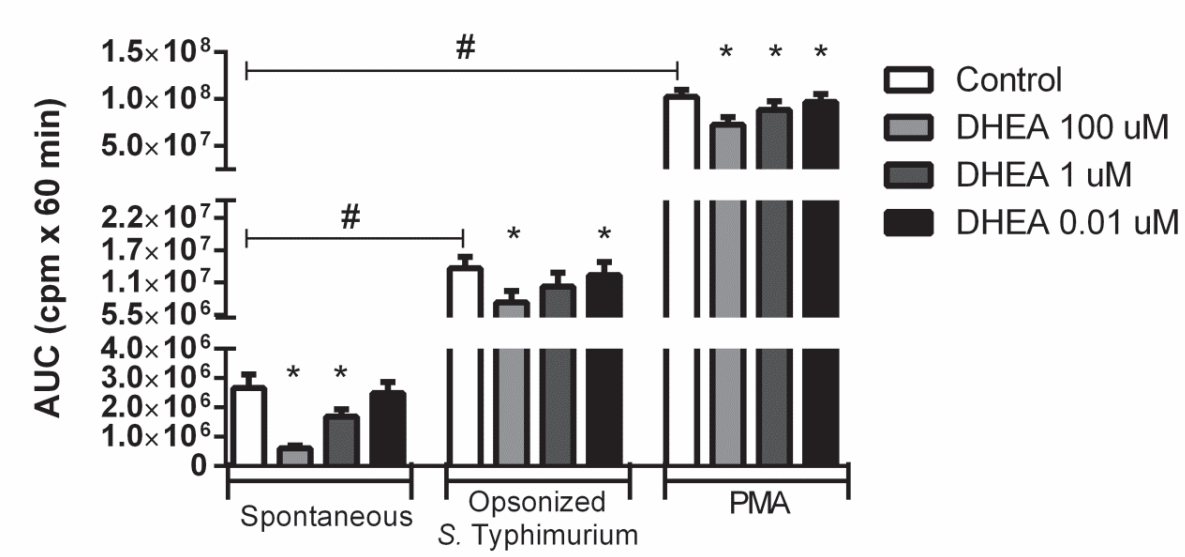


**FIGURE 3 –** DHEA can modulate the oxidative burst of neutrophils.

Neutrophils, treated or not with DHEA, were stimulated with PMA (10-7 M) or with *S. Typhimurium* (MOI=10), previously opsonized with 20% normal human serum, in the presence of luminol (10-4 M). The reaction was monitored for 1 hour in luminometer and recorded in photons per minute. The graph expresses the Mean (SD) of area under the curve (AUC) of chemiluminescence of 11 independent experiments for Control and DHEA (0.01 μM) with 16 different donors, and 5 independent experiments for DHEA (100 and 1 μM) with 10 different donors. *$p<0.05$ vs control with the same treatment; #$p<0.05$ vs spontaneous neutrophil with opsonized bacteria and with PMA.

### DHEA did not modulate the expression of TLR4 on neutrophils

In order to investigate if the DHEA can modulate the expression of the receptor TLR4, we performed real time PCR, using β-actin as an endogenous internal control. Figure 4 shows that the treatment of neutrophils with DHEA, did not cause an augment in the expression of TLR4. As expected, after infection of the neutrophils with *S. Typhimurium*, the expression of this receptor increased, but the values were not statistically significant.

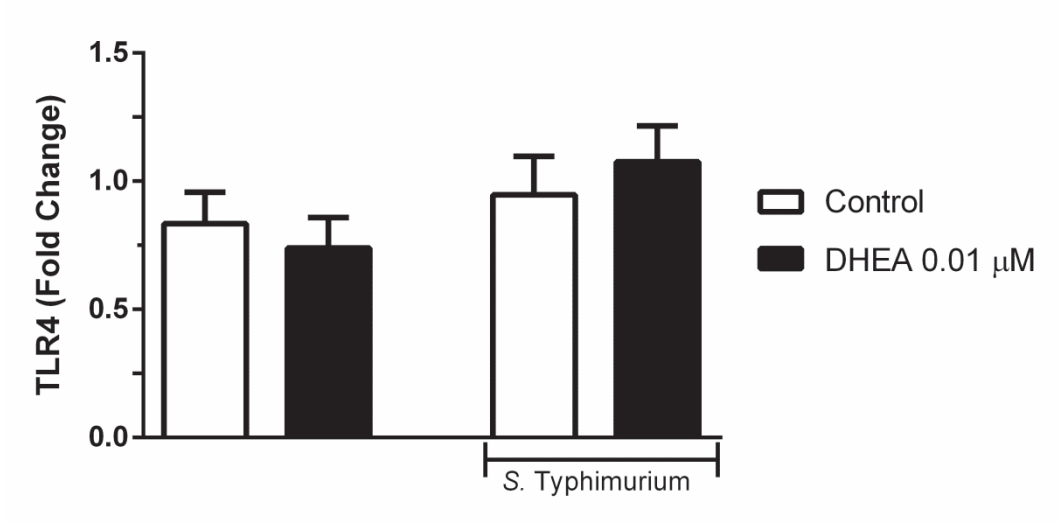


**FIGURE 4 –** DHEA treatment did not not modulate the expression of TLR4 by neutrophils.

Neutrophils (1 x $10^6$) were treated with the concentration 0.01 μM of DHEA for 1 hour, then were infected with *S. Typhimurium* (MOI = 10) for one hour. Data express the Mean (SD) of fold change expression of TLR4 relative to the ACTB house-keeping gene analyzed by Real-time PCR.

### DHEA can modulate the response of neutrophils infected with *S. Typhimurium* reducing IL-8 production

Since the mediators released by neutrophils during an immune response interfere in the outcome of bacterial infections, we next investigated the involvement of DHEA in the control of IL-8 production. As shown in Figure 5, the infection of neutrophils with *S. Typhimurium* results in an increase of the production of the chemokine IL-8, but, when cells are treated with DHEA, the production was reduced. The same behavior was seen for neutrophils stimulated with LPS. The LPS stimulates the production of IL-8 by neutrophil, but, the treatment with DHEA resulted in significant reduction of the IL-8 secretion. Is important to note that another cytokines (IFN-γ, IFN-α, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12 p70, IL-17A, TNF-α, GM-CSF, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES) were also analyzed here, but after this period of culture, the production was not considered significant since all reached the cutoff of detection.

### DHEA 0.01 μM reduces the phosphorylation of NFκB

In order to understand how DHEA modulates the production of some cytokines, we performed a Western blot to analyze the transcription factor NFκB. Figure 6 shows that the infection of neutrophils with *S. Typhimurium* stimulates the phosphorylation of NFκB. In contrast the treatment of neutrophils with DHEA at the concentration of 0.01 μM resulted in a reduced phosphorylation of this transcription factor.

### DHEA had no effect in the production of NETs

In order to analyze whether DHEA influences the release of NET, we incubated neutrophils with *S. Typhimurium* or PMA, and quantified the extracellular DNA in the culture medium. Figure

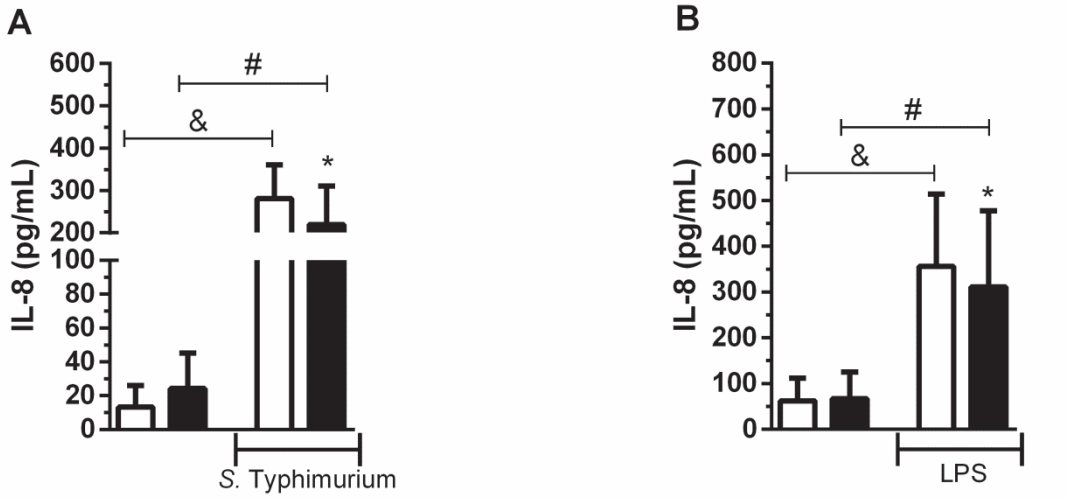


**FIGURE 5 –** DHEA reduces the IL-8 production by neutrophils infected with *S. Typhimurium* or stimulated with LPS.

Cells (2 x 105) were treated with 0.01 μM DHEA for 1 hour and infected with *S. Typhimurium* (MOI=10) for 24 h in the presence of 49.5 μM of a bacteriostatic concentration of cloramphenicol or, were stimulated with 1 μg/mL of LPS for 18 hours The IL-8 were quantified in the supernatant using ELISA for IL-8. Data express the Mean (SD) of 3 independent experiments with cells from 6 different donors.

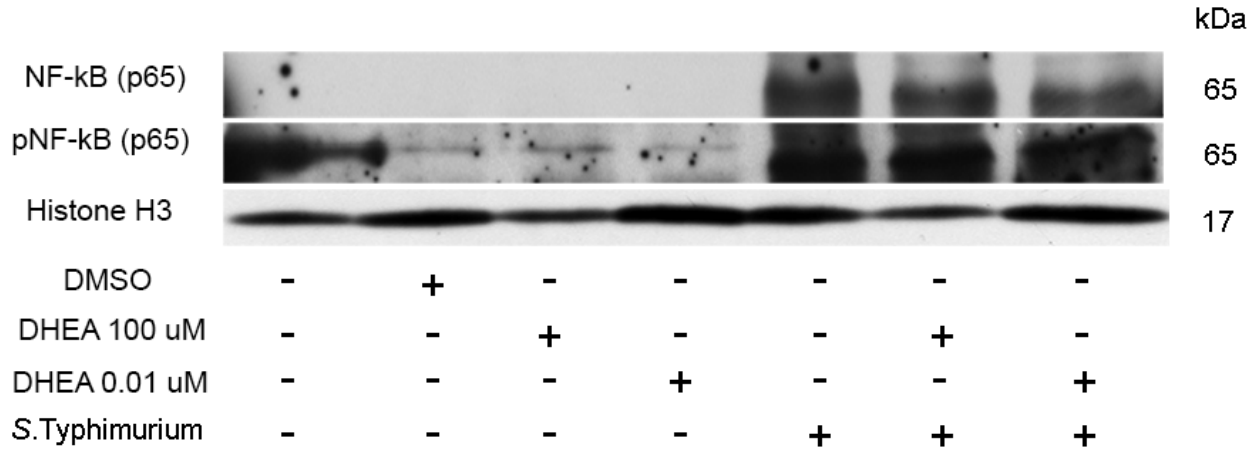


**FIGURE 6 –** DHEA can modulate the phosphorylation of NFkB.

Neutrophils (5 x $10^6$) were treated with 100 μM and 0.01 μM DHEA and infected with *S. Typhimurium* for 1 h. Then, cells were washed and the proteins were extracted with CelLytic Mammalian Cell Lysis/Extraction Reagent using phosphatase and protease inhibitor cocktails. The proteins were quantified using the Bradford method and 20 μg of protein were separated using SDS-PAGE. The protein was transferred to a nitrocellulose membrane, and anti-NFκB and anti-p-NFκB antibodies were used for detection. In this experiment, a pool of protein derived from cultures of neutrophils isolated from 3 individuals was used.

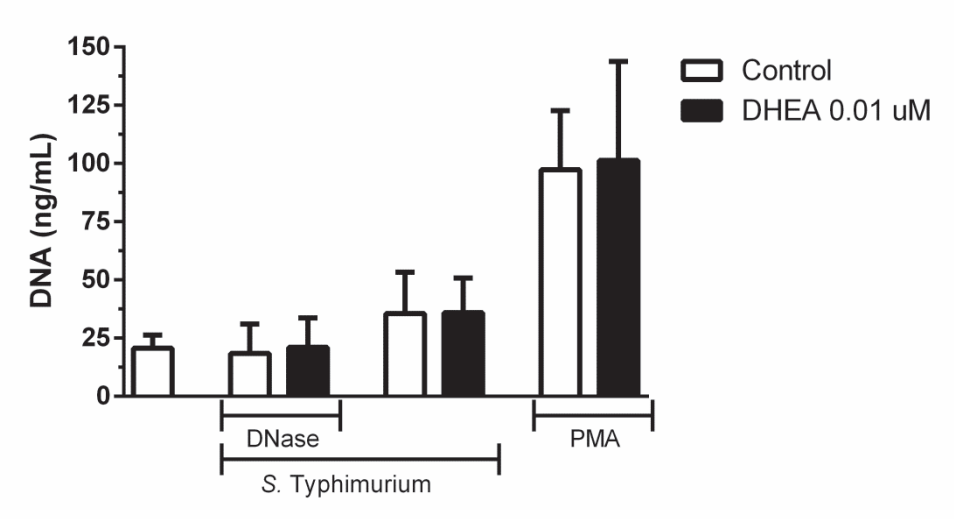


**FIGURE 7 –** DHEA have no effects on NET production.

Neutrophils (2 x 105) were treated or not for 1 hour with 0.01 μM DHEA, then were incubated with PMA (0.162 μM) or *S. Typhimurium* (MOI=10) for 4 h, and the supernatants were collected for quantification of fragmented DNA released by cells. As a negative control the enzyme DNaseI (100 U/mL) was used 30 min before infection. For quantification, the supernatants were treated with 20 U/mL of the restriction enzyme EcoRI and HindIII, according to the manufacturer's directions. NETs were quantified using the Quant-iT™ Picogreen® dsDNA kit. Data represent the Mean (SD) of 4 independent experiments performed on different days, with cells from 4 different donors.

7 showed that the PMA was able to trigger NET production, while DHEA had no effect. Although not statistically significant, the neutrophils infected with *S. Typhimurium* also produced NETs, with the rate of 35.6 ng/mL of extracellular DNA compared to 20.7 ng/mL produced by non-stimulated neutrophils.

To confirm the occurrence of the above NET results we used confocal microscopy for visualization of this fibrous structure. As depicted in Figure 8A and Figure 8B, *S. Typhimurium* and PMA stimulate neutrophils to release the NETs, but the steroid hormone DHEA had no influence in this phenomenon. As a negative control, when the enzyme DNaseI was added to the culture, the NETs were degraded and it was not possible to visualize the structures and, as expected, DHEA did not influence the formation of NET in the presence of DNaseI (Figure 8C).

## DISCUSSION

The mutual communication pathways between the immune and neuroendocrine systems is critical for maintaining body homeostasis. This interaction plays an important role in the modulation of susceptibility and resistance to infectious and inflammatory diseases. Among the major neuroendocrine axes, the hypothalamic-pituitary-adrenal has aroused great interest in the last years (Sternberg, 2001). This axis is responsible for providing an interface between the internal and external environment of the body, ensuring the ability of the organism to respond to different cognitive (such as stress) or non-cognitive stimuli (such as antigens) (Buckingham *et al.*, 1996), through the release of several hormones including glucocorticoids, catecholamines and DHEA, and each of these have the ability to modulate the response of immune cells, including lymphocytes and macrophages (Heffner, 2011). There are few studies demonstrating the role of DHEA in neutrophils, highlighting the relevance of our research that evaluated the role of the steroid DHEA in neutrophils infected with *S. Typhimurium*. We demonstrated that treatment of neutrophils with DHEA resulted in increase of phagocytosis activity during *S. Typhimurium* infection, but this increase probably does not occur via TLR4, since DHEA did not modulate the expression of this receptor. Furthermore, we demonstrated that DHEA modulates the secretion of IL-8, and treatment with 0.01 μM DHEA resulted in

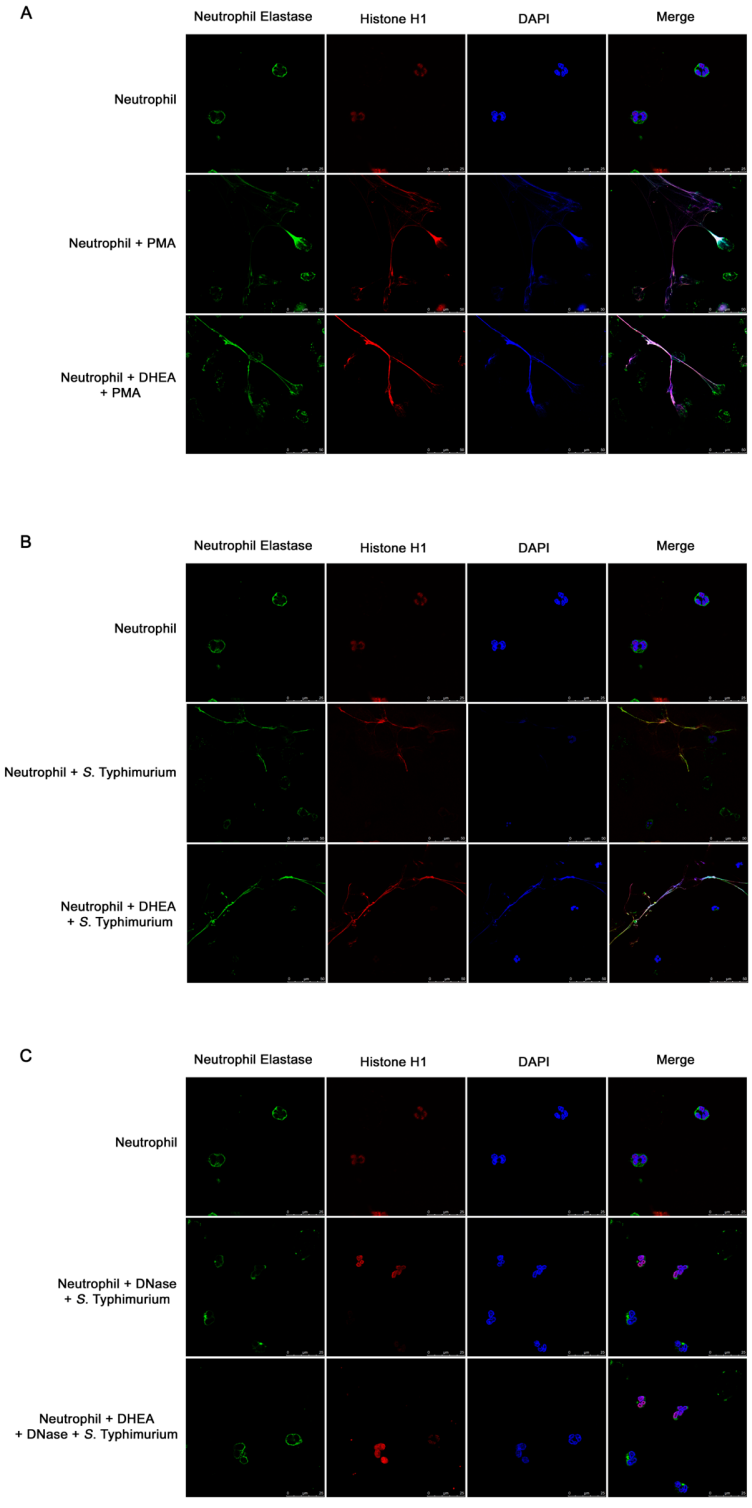


**FIGURE 8 –** *S. Typhimurium* and PMA promote NET formation by neutrophils.
Neutrophils (7 x 104) were treated or not with 0.01 µM DHEA for 1 h, (A) and stimulated with PMA (0.162 µM) for 4 h or (B) *S. Typhimurium* (MOI=10). As a negative control the cells were treated with the enzyme DNaseI (100 U/mL) 30 min before the infection (C). The neutrophils were subjected to nuclear staining with DAPI (blue), for neutrophil elastase (green) and for histone H1 (red). The cells were analyzed with a confocal laser scanning microscope, and the co-localization of the markers shows the formation of NET. Magnification: 630 x.

lower phosphorylation of the transcription factor NFκB. Further, the production of ROS by neutrophils treated with DHEA and stimulated with bacteria or PMA was lower than ROS production seen in untreated neutrophils. Finally, our study was the first to evaluate the influence of DHEA in the production of NET, demonstrating that this steroid did not influence the release of these structures by neutrophils.

We also characterized the effects of PMN on the viability of neutrophils. We observed 80% viability both with and without any treatment. However, when neutrophils were treated with 0.2% DMSO a higher rate of apoptotic death was observed, but the viability of these cells was approximately 72%, allowing to us to perform our experiments. We performed a treatment with 3 different concentrations of DHEA for some experiments, but the main concentration chosen was 0.01 μM, because this concentration is closer to the physiological concentration in the human body (Barkhausen *et al.*, 2006).

The increase in the phagocytic activity due to the treatment with DHEA has been described in the literature (Bongiovanni *et al.*, 2015) with different models of infection and cells, but the mechanism by which DHEA enhances phagocytosis is still unknown. Since the immune cells use surface receptors for the recognition of invading pathogens and this recognition is critical for the development of the immune response, we hypothesized that the ability of DHEA to modulate phagocytosis was related to the differential expression of pattern recognition receptors after hormone treatment. Indeed, mice subjected to polymicrobial sepsis induced by CLP (cecal ligation and puncture) have reduced mRNA expression for TLR2 and TLR4 on macrophages isolated from spleen, but when animals were treated with DHEA prior to CLP, the mRNA expression of TLR2 and TLR4 increased significantly (Matsuda *et al.*, 2005). However, in our study, the mRNA for TLR4 was not altered by the hormone supplementation, although the presence of bacteria alone stimulated the expression of this receptor. Along with the recognition of LPS in gram-negative bacteria, TLR4 also plays a role in the elimination of the phagocytosed pathogen. It was shown that inhibition of TLR4 leads to an inhibition of NADPH oxidase and a consequent increase of *S. Typhimurium* survival capacity within the neutrophil (Van Bruggen *et al.*, 2007), showing that the activation of the NADPH oxidase complex is critical for the elimination of intracellular *Salmonella,* and in addition, the activation of this enzyme complex requires signaling through TLR4. Based on this information, we tested whether DHEA interferes in ROS production and showed a reduction in these reactive species after DHEA treatment and neutrophil stimulation. A possible explanation for this reduction is that DHEA is a non-competitive inhibitor of the enzyme glucose-6-phosphate dehydrogenase (G6PD) of mammals, a protein with limiting function in the pentose phosphate pathway, whose major products are ribose-5-phosphate and extramitochondrial NADPH (Gordon *et al.*, 1995; Stanton, 2012). The latter compound is an electron donor essential for the production of ROS in the enzyme complex NADPH oxidase. This same anti-oxidant property of DHEA has been described in the literature (Perner *et al.*, 2003; Izumo *et al.*, 2009), showing that DHEA probably causes a modification in the metabolic profile of the neutrophils, changing the amount of ROS produced, given that this fact may contribute to a modulation of the immune response of PMN cells towards a less inflammatory Th2 phenotype, despite the increased phagocytosis rate.

Acting as important players during the first line of defense against invaders, neutrophils have a critical role in orchestrating the development of innate immunity and inflammatory responses by producing cytokines and chemokines. In this study, we demonstrated that neutrophils treated with DHEA and infected with *S. Typhimurium* and stimulated with LPS have reduced secretion of IL-8. We also showed a reduced phosphorylation of NFκB. It is well known that dimers of NFκB in the cytoplasm are linked to an inhibitory protein called IκB. Infectious or inflammatory stimuli induce the degradation of this inhibitory protein through the IκB kinase (IKK) complex and the phosphorylation of IκB protein results in ubiquitination and protein degradation. The dimer of NFκB, released from IκB protein, can then translocate to the nucleus and partake in gene transcription (Lawrence, 2009; Oeckinghaus *et al.*, 2011). This nuclear factor, beyond being involved in differentiation, proliferation and survival of cells, has an important regulatory role in cytokine production, including TNFα and IL-8, which were decreased in neutrophils treated with DHEA. There are reports in the literature using other models that shows treatment of cells with DHEA results in an increase of IκBα

protein and, consequently, reduced activation of NFκB and decrease of transcription of genes of cytokines regulated by this nuclear factor (Kim *et al.*, 2006; Gutiérrez *et al.*, 2007). We did not evaluate the IκB proteins, but the reduced phosphorylation of NFκB can be related to the decrease of degradation of IκB protein, and as consequence, the neutrophils treated with the steroid had a lower production of cytokines regulated by this transcription factor. Since IL-8 is considered an important chemokine for recruiting cells such as monocytes and neutrophils, are reduced after treatment with DHEA, we suggest that this hormone might be act as a regulator of the exacerbated inflammation.

Although studies have demonstrated that DHEA is a stimulator of Th1 cell polarization (Suzuki *et al.*, 1991; Straub *et al.*, 1998; Du *et al.*, 2001; Chang *et al.*, 2004), it has been shown that the addition of DHEA to spleen cells pre-activated with KLH antigen (keyole limpet hemocyanin), resulted in polarization of the immune response towards the Th2 pattern, mainly through increased IL-4 secretion with reduced IFN-γ secretion (Du *et al.*, 2001). Accordingly, Alves and colleagues (2016) showed in an experimental model of DSS-induced colitis, that the hormone replacement during the induction phase of disease resulted in decreased proinflammatory responses. The levels of IL-6 mRNA expression decreased in the colon, while the frequency of $CD4^{+}IFN\text{-}\gamma^{+}$ cells decreased in spleen. In contrast IL-13 mRNA increased in the colon and the frequency of $CD4^{+}IL\text{-}4^{+}$ lymphocytes in spleen also increased. Therefore, they suggested that DHEA might have a regulatory function that could contribute to the amelioration of colitis (Alves *et al.*, 2016). Another work evaluated the effects of DHEA on the viability, proliferation and cytokine secretion by spleen cells from mice after *in vitro* stimulation with concanavalin A (ConA) and LPS. In this context, the treatment with DHEA reduced the secretion of IL-1, IL-2 and IFN-γ. Also, higher concentrations of DHEA led to increased IL-10 secretion, but had no effect on IL-4 (Powell, Sonnenfeld, 2006). Together these data showed that DHEA might be functioning as an important regulator of the immune response, driven by the culture conditions. Our results suggest that DHEA is skewing the neutrophil response to a less inflammatory phenotype, even in the presence of bacterial infection and augmented phagocytosis.

Few studies have demonstrated a role for hormones in NET release. In order to evaluate the role of anti-inflammatory drugs in NET, Lapponi and colleagues reported that dexamethasone did not affect the formation of NETs by neutrophils stimulated with PMA or TNF-α (Lapponi *et al.*, 2013). Our study is the first to evaluate the role of DHEA in the formation of NET. We showed that 0.01 μM of DHEA had no effect on NET release, but more studies are required to evaluate if higher concentrations of the hormone change the neutrophils' response, to explore possible activation pathways, autophagy and association of neutrophil activation with membrane receptors involved in the recognition of *S. Typhimurium*.

Our work has shown that DHEA contributes to the increase of phagocytosis of *S. Typhimurium*, but the mechanism that results in this augment of ingested bacteria probably is not via TLR4 and should be further investigated. We also pointed to the ability of DHEA to drive neutrophil to a less inflammatory immune response, which could be related to tissue repair and alternatively activated macrophages that favor tissue damage control. Therefore, the data presented here can add knowledge to the discussion about inflammatory pathologies related to neutrophils' activity, including systemic lupus erythematosus, a pathology in which the beneficial role of DHEA in reducing disease remains controversial (Van Vollenhoven *et al.*, 1994; Crosbie *et al.*, 2007; Hartkamp *et al.*, 2010). During chronic HIV infection, low levels of DHEA are related to the patients AIDS progression (Kroboth *et al.*, 1999). Thus, it is reasonable to pursue the role of DHEA supplementation in these patient populations in an attempt to control the exacerbated inflammation and to boost the effector functions of immune cells in killing opportunistic microorganisms.

Finally, though the role of DHEA in modulating immune response is clear, further investigation is needed to understand the mechanisms of action of this steroid in the immune-endocrine axis. These studies could promote the development of novel hormone supplementation therapeutic strategies leading to a better control of inflammatory and infectious diseases.

## DECLARATION OF INTEREST

The authors declare that they have no commercial or other association that might pose a conflict of interest on the manuscript.

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### Author contribution statement

VSB designed and performed experiments and wrote the paper; FAZ help in the execution of some experiments; MSE and MPCN designed experiments; MKBP assisted in standardization of techniques; LSS, PMC, LJGL and CF contributed by technical assistance, standardization of techniques and advices; AML contribute with the execution of Western blotting; FGF and CRBC developed the concept; FGF designed the experiments, supervised the study, and wrote the paper.

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