ESCOLA BAHIANA DE MEDICINA E SAÚDE PÚBLICA
CURSO BIOMEDICINA

RAVENA SENA OSÓRIO CORDEIRO

Influência do Cloridrato de Pioglitazona na Regulação da Resposta Inflamatória de Pacientes com Leishmaniose Cutânea Causada por *L. braziliensis*

Salvador – BA
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Trabalho de Conclusão de Curso apresentado à Escola Bahiana de Medicina e Saúde Pública, como parte dos requisitos para obtenção do título de Bacharel em Biomedicina.

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INFLUÊNCIA DO CLORIDRATO DE PIOGLITAZONA NA REGULAÇÃO DA RESPOSTA INFLAMATÓRIA DE PACIENTES COM LEISHMANIOSE CUTÂNEA CAUSADA POR *L. BRAZILIENSIS*

Este trabalho de conclusão de curso foi julgado adequado à obtenção do grau de Bacharel em Biomedicina e aprovada em sua forma final pelo Curso de Biomedicina da Escola Bahiana de Medicina e Saúde Pública.

Salvador – BA, 09 de Novembro de 2019.

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Um líquido é um estado da matéria sem formato específico. Ele muda facilmente e se molda ao seu recipiente.

O corpo humano é 70% água.

SUMÁRIO

1. ARTIGO CIENTÍFICO

PPAR RESEARCH

INFLUENCE OF PIOGLITAZONE HYDROCHLORIDE ON THE REGULATION OF INFLAMMATORY RESPONSE IN PATIENTS WITH CUTANEOUS LEISHMANIASIS CAUSED BY *L. braziliensis.*

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ABSTRACT

Cutaneous leishmaniasis (CL) caused by *Leishmania braziliensis* is an inflammatory disease in which the development of skin ulcers is associated with the presence of mononuclear cells and high levels of inflammatory cytokines. Recently was demonstrated that activation of PPAR-γ by anti-diabetic drugs decreased cytokine production in many types of inflammatory diseases. We hypothesized that activation of PPAR-γ by pioglitazone hydrochloride could regulate the inflammatory response observed in CL. In this article, we show that pioglitazone hydrochloride at different concentrations downregulated TNF, IL-6, IL-1β and IL-10 production after TLR4 activation in monocytes of healthy subjects. However, we have shown that peripheral blood mononuclear cells from *Leishmania* antigen-stimulated CL patients had decreased TNF levels after treatment with this drug at a low concentration for a short time of stimulation. This observed immunomodulatory effect, was modified by exposing these cells to a higher concentration of pioglitazone hydrochloride for a longer time. In addition, macrophages infected with *L. braziliensis* showed reduced parasitic load when treated this drug. These results suggest that pioglitazone hydrochloride in low concentration may have benefits in adjuvant CL therapy.

INTRODUCTION

Cutaneous Leishmaniasis (CL) caused by *Leishmania braziliensis* is an infection disease characterized for one or more ulcers with raised borders and few parasites. CL lesions present an intense inflammatory reaction with the predominance of lymphocytes, mononuclear phagocytes [1,2]. Although the inflammatory response is necessary to control parasite replication, the exaggerated production of inflammatory cytokines such as TNF [3,4] and IL-1β [5,6] contribute to tissue damage and development ulcers in skin. Regarding the treatment of patients in endemic areas of Brazil, the Ministry of Health indicates the use of pentavalent antimonial (Sb^v) as the drug of first choice. However, this drug presents high toxicity to the patient and high therapeutic failure reaching 70%. [7,8].

Previous studies have shown that the association between Sb^v and immunomodulatory drugs has beneficial effects on the healing of CL patients, just like the topical use of GM-CSF (macrophage and granulocyte colony stimulating factor) associated with standard doses of Sb^v decreases cure time in refractory patients [9]. Another drug association is the oral Pentoxifylline (drug that decreases TNF production), there showed to be more effective - accelerating the healing process of cutaneous and mucosal leishmaniasis [10,11,12].

Recently more attention has been focused on anti-diabetics drugs in various types of inflammatory and infectious diseases [13,14,15,16] including CL due to ability to regulate inflammatory response [17]. Pioglitazone is an anti-diabetic drug from the family Thiazolidinediones (TZDs) are that function as insulin sensitizers in peripheral and hepatic tissues by binding to and activating nuclear peroxisome proliferator-activated receptor γ (PPAR-γ) [18]. PPAR-γ is also expressed in immune response cells such as monocytes and macrophages and its activation for pioglitazone can trigger anti-inflammatory actions such inhibition of NF-κB [19,20, 21,22].

The aim of the present study was to evaluate the role of pioglitazone hydrochloride in modulating the inflammatory response in patients with cutaneous leishmaniasis. We documented that pioglitazone hydrochloride a potent PPAR-γ agonist in low concentration decrease TNF levels without modify the levels of IL-10, but in high concentration this profile is modified and increased IL-1β production in PBMC from CL patients. In addition, pioglitazone hydro-

chloride enhances the killing from *L. braziliensis* by macrophages in low or high concentrations.

MATERIALS AND METHODS

Subjects

The sample was composed of 9 individuals with CL from the *L. braziliensis* transmission area of Corte de Pedra, Bahia, Brazil and 10 HS living in an area where *Leishmania* species are not endemic. Diagnosis of CL was made based on the presence of typical skin ulcer associated with a positive PCR result as previously described [23,24]. All participants in this study, did not have diabetes, obesity or in using for anti-inflammatory drugs.

Ethics Statement

The present study was approved by the Ethics Committee of the University of the State of Bahia (License number 2.471.185) and informed written consent was obtained from all study participants. All participants were adults.

Cell viability

PBMCs obtained from health subjects and culture for 24 and 48 hours in presence or absence of LPS (10ng/mL), pioglitazone hydrochloride 1µM, 10µM and 100µM (Sigma, St. Louis, MO). Unstimulated cells were used as a positive control representing 100% of viable cells, while cells stimulated with PFA were used as negative control. After these time points the cell viability was by the MTT assay technique as previously described [28].

Soluble *Leishmania* antigen (SLA)

SLA was prepared with an isolate of *L. braziliensis* as previously described [26]. Briefly, promastigotes resuspended in lysis solution (Tris, HCl, EDTA, and leupeptin) were immersed in liquid nitrogen and thawed at 37°C. After freezer-thaw procedure, they were sonicated, and the disrupted parasites were centrifuged at 14,000g. The supernatant was filtered and assayed

for protein concentration, tested for endotoxin using the Limulus amebocyte lysate test, and used at a concentration of 5µg/mL.

Culture of PBMCs

PBMCs were isolated from heparinized venous blood by FicollPaque (GE Healthcare) gradient centrifugation and after washing steps in saline, the cell concentration was adjusted to $3x10^6$ cells in 1mL of RPMI-1640 (Gibco) supplemented with 10% FBS (Gibco), 100UI penicillin/mL, and 100µg streptomycin/mL. PBMCs were dispensed into 24-well plates and incubated at 37°C, 5% CO2 for 24 and 48 hours in the presence or absence of SLA (5µg/mL), LPS (10ng/mL), pioglitazone hydrochloride 1µM, 10µM and 100µM (Sigma, St. Louis, MO).

Determination of Cytokine Levels

Levels of the cytokines TNF, IL-6, IL-1β and IL-10 were evaluated in the supernatants of PBMC, according to the manufacturer's information (BD Pharmingen, San Diego, CA). The results were expressed in pg/mL.

Parasite culture

Isolate of *L. braziliensis* (MHOM/BR/LTCP11245) was obtained from a skin lesion of a CL patient and identified as *L. braziliensis* by multilocus enzyme electrophoresis [25]. The parasites selected for this study had not been previously passaged in liquid culture medium. After selection, the parasites were expanded in Schneider's medium (Sigma, St. Louis, MO) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Gibco, Waltham, MA), 100UI penicillin/mL, and 100µg streptomycin/mL.

Human macrophage culture and infection with *L. braziliensis*

Monocyte-derived macrophages from HS subjects were prepared following a method previously shown [27] by our laboratory to yield 99% macrophages characterized by flow cytometry as CD14-positive, CD3-negative, CD19-negative. Briefly, 1,25x106 PBMC were dispensed into 4-well plates (Nunc Labtek) and monocytes were separated by adherence. After 6 days of culture in RPMI-1640 (Gibco) supplemented with 10% FBS (Gibco), 100UI penicillin/mL, and 100µg streptomycin/mL the adherent cells displayed characteristics of macrophages. After diferention, these cells were infected with *L. braziliensis* stationary phase pro-

mastigotes at a 10:1 ratio during 2 hours and uninfected macrophages were used as controls. After incubation, the remaining extracellular parasites were removed by gentle washing and cells incubate with pioglitazone hydrochloride. The percentage and the number of amastigotes were determined from two independent observers.

Statistical Analysis

Data were analyzed using the program GraphPadPrism v8.0 (GraphPad Software, San Diego, CA, USA). All results are presented on mean with standard desviation (SD) and were statistically analyzed by paired t test. The results were considered statistically significant when show $p < 0.05$.

RESULTS

Cell viability after exposure at different pioglitazone hydrochloride concentrations

To evaluate the cell viability, a dose and time response curve was constructed. We observed that LPS and none of the pioglitazone hydrochloride concentrations used showed any toxic effects, as shown in figures 1a and 1b, respectively.

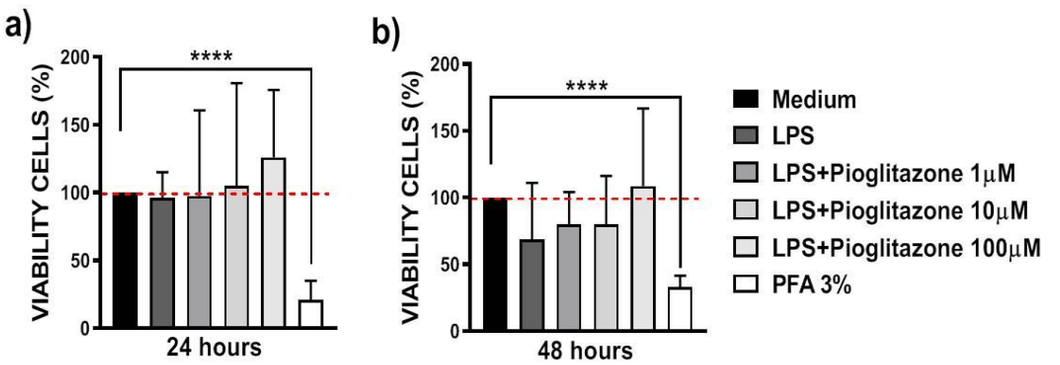

Figure 1: Cells Viability. PBMC from HS (n=10) were cultured in the presence and absence of LPS (10ng/mL), pioglitazone hydrochloride (1, 10 and 100μM) and PFA 3% for 24 hours (a) and 48 hours (b). After each time point the mitochondrial activity was evaluated by the MTT technique. The results are presented as a percentage with mean and SD, and paired t test was used for statistical analysis **** $P < 0.0001$.

Ability of pioglitazone hydrochloride to regulate cytokine production in response to LPS

Recently, it has been shown that monocytes infected with *L. braziliensis* exhibit high expression Toll like 4 receptor expression (TLR4) followed by high levels of TNF and IL-10 [29]. To evaluate whether pioglitazone hydrochloride could breaking the production for cytokines by monocyte, we induced TLR4 activation in PBMC from HS with LPS for 24 and 48 hours. In this study, we show that pioglitazone hydrochloride in low concentration (1µM) downregulated TNF (Figure 2a) and IL-1β (Figure 2b) production in response for LPS after 24 and 48 hours of treatment. However, as pioglitazone hydrochloride concentrations were increased (1µM, 10µM and 100µM), the levels of IL-6 (Figure 2c) and regulatory cytokine IL-10 (figure 2d) decreased in compared for cells stimulated with LPS at both times. These results demonstrate that pioglitazone hydrochloride downregulate the secretion of inflammatory cytokines in the initial phase of cell activation via TLR4, suggesting this drug acts to inhibit NF-κB.

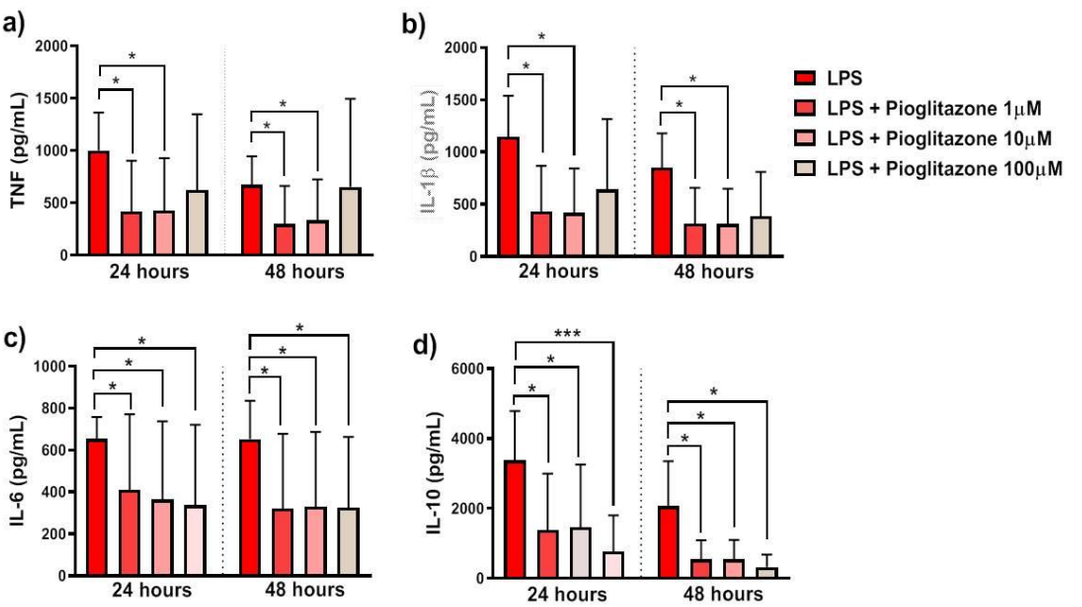

Figure 2: Ability of pioglitazone hydrochloride to regulate cytokine production in response to LPS. PBMC from HS (n=10) were cultured in the presence and absence of LPS (10ng/mL) and pioglitazone hydrochloride (1, 10 and 100µM) for 24 hours and 48 hours. After each time point the production of TNF (a), IL-1β (b), IL-6 (c) and IL-10 (d) was evaluated by the ELISA technique. The results are presented as mean and SD and paired t test was used for statistical analysis *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Pioglitazone hydrochloride induces pleiotropic responses in LC

Previously we demonstrate that pioglitazone hydrochloride presented anti-inflammatory effects after one strong activation of TLR4 with LPS. We investigated whether PBMC from CL

patients stimulated with SLA would respond well to pioglitazone hydrochloride. Similar to the results shown above (Figure 2) pioglitazone hydrochloride at 1μM decreased TNF production after 24 hours of stimulation, but after 48 hours this effect was eliminated (Figure 3a). Although IL-1β (Figure 3b) and IL-6 (Figure 3c) levels were lower in presence at 1μM of pioglitazone hydrochloride, we not none observe any statistically significant difference. Curiously, these cells in presence the high concentration of pioglitazone hydrochloride (100μM) increased production of IL-1β (Figure 3b) after 24 and 48 hours. Regarding at IL-10 levels we observed to decrease after 24 hours with 100μM and this effect was more after 48 hours in all concentrations tested (Figure 3d).

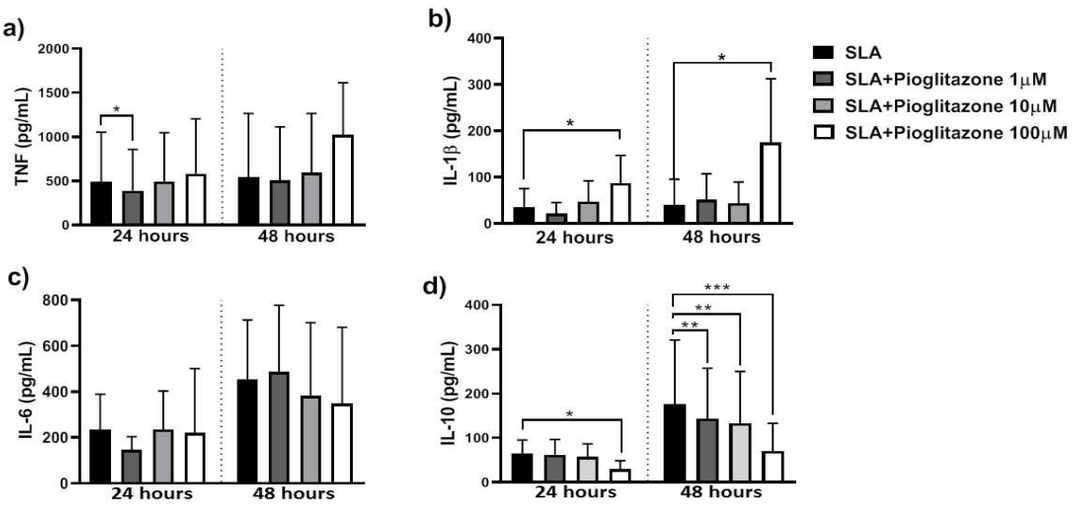

Figure 3: Ability of pioglitazone hydrochloride to regulate cytokine production in response to SLA in CL patients. PBMC from CL patients (n=9) were cultured in the presence and absence of SLA (5μg/mL) and pioglitazone hydrochloride (1, 10 and 100μM) for 24 hours and 48 hours. After each time point the production of TNF (a), IL-1β (b), IL-6 (c) and IL-10 (d) was evaluated by the ELISA technique. The results are presented as mean and SD and paired t test was used for statistical analysis *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Pioglitazone hydrochloride enhanced killing of *L. braziliensis* by human macrophage

To evaluated whether pioglitazone hydrochloride plays any role on parasite killing or survival in CL we infected human macrophages with *L. braziliensis* in presence or absence of pioglitazone hydrochloride and assessed parasite counts by microscopy in two time points after infection. We observed that pioglitazone hydrochloride at 100μM decreased the percentage of infected cells and the number of parasites after 24 (Figure 4a) and 48 hours of infection (Figure 4b). However, by an unknown factor the infection percentage was reduced in the presence of 1μM pioglitazone hydrochloride after 48 hours of infection (Figure 4a).

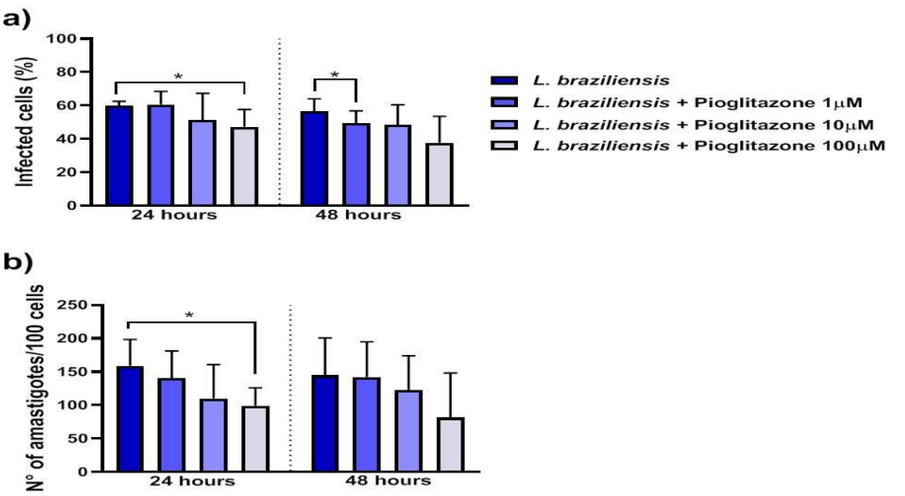

Figure 4: Effect of pioglitazone hydrochloride on the control of *L. braziliensis* infection (n=5) after stimulation with different concentrations of pioglitazone hydrochloride. HS macrophages were infected with *L. braziliensis* (10:1) for 2 hours. Following infection, cells were cultured in the presence and absence of pioglitazone hydrochloride at 1, 10 and 100μM concentrations for 24 and 48 hours. The percentage of infected cells (a) and the number of amastigotes (b) after 24 and 48 hours was determined by 2 observers by the optical microscopy technique. The results are presented as mean and SD and paired t test was used for statistical analysis *$p < 0.05$.

DISCUSSION

PPAR-γ is a nuclear receptor expressed in several cell types, mainly mononuclear phagocytes [19,30] and their activation is intimately involved in regulating lipid metabolism and glucose homeostasis [31,32]. However, a considerable number of studies have associated this activation by synthetic agonists with the downregulation of TLR4 and NF-κB [21,33,22]. Here, we show that PBMC from HS stimulated with LPS produce high levels of TNF, IL-6, IL-1β and IL-10 and this production is attenuated in presence of pioglitazone hydrochloride. Our results support that activation of PPAR-γ by pioglitazone hydrochloride may be involved in modulating transcription factors associated with cytokine synthesis such as NF-κB.

Previously, it has been shown that circulating monocytes from CL naturally express TLR4 [34] and this expression is increased by *L. braziliensis*, in addition, TLR4+ cells infected with *Leishmania* produce higher levels of TNF [29]. Given the importance of this signaling pathway in CL pathogenesis, we decided to evaluate whether pioglitazone hydrochloride activation of PPAR-γ could regulate the inflammatory response mediated by monocytes from CL patients stimulated with SLA. We observed that pioglitazone hydrochloride decreased TNF

levels without altering IL-10, IL-1β and IL-6 production in the initials hours of stimulated cell. However, we note that in the presence of a high concentration of pioglitazone hydrochloride IL-10 production was suppressed allowing IL-1β levels to predominate. These results suggest that parasite factors present in the SLA may be involved other signaling pathways activation than TLR4. In fact, it previously showed that lipophosphoglycan (LPG) presents in the *L. braziliensis* membrane activates TLR2 and allows NF-κB translocation culminating in the increase of TNF, IL-6 and IL-1β [35]. Therefore, simultaneous of multi pathways activation by *L. braziliensis* soluble antigens are nonspecific, complex and may be accelerating the early translocation of NF-κB, thus preventing its neutralization by pioglitazone hydrochloride-activated PPAR-γ.

Another important point that intimately implicated with PPAR-γ concerns resistance and susceptibility to infection by different *Leishmania* species. For example, peritoneal macrophages from Balb/c mice infected by *L. donovani* induce PPAR-γ expression, keeping these cells alternatively activated and thus allowing the parasites to evade inflammatory response [36]. On the other hand, PPAR-γ activation in murine macrophages infected with *L. mexicana* induced polarization to an M1 profile, with high TLR4 expression, increased TNF, IL-1β, IL-6, ROS production and decreased infection [37]. Consistent with observations the Díaz-Gandarilla et al., (2013), we show that PPAR-γ activation by pioglitazone hydrochloride, in human macrophages infected with *L. braziliensis,* showed parasite load reduced when treated with pioglitazone hydrochloride.

CONCLUSIONS

Our results suggest that pioglitazone hydrochloride improvement in the regulation of inflammation and infection observed in CL. However, more studies should be conducted to understand better the mechanisms triggered by this drug and PPAR-γ in plasticity of macrophages in *L. braziliensis* infection.

CONFLICTS OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this paper.

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2. PROPOSTA DE SUBMISSÃO

2.1 Revista: PPAR RESEARCH

2.2 Regras para Submissão:

JOURNAL TITLE

CONCISE AND INFORMATIVE ARTICLE TITLE

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[1] Department, Institute, City ZIP/Post code, Country.
[2] Department, Institute, City ZIP/Post code, Country.

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ABSTRACT

The abstract should be a single, self-contained paragraph which summarises the manuscript. Ideally it will provide a brief context for the study, before describing the scientific approach and some key results in a qualitative manner. It should finish with a sentence to describe the implications for the field. The abstract must not include references, figures or tables.

INTRODUCTION

The introduction should be succinct, with no subheadings. Limited figures may be included only if they are truly introductory, and contain no new results.

MATERIALS AND METHODS

The materials and methods section should contain sufficient detail so that all procedures can be repeated. It may be divided into headed subsections if several methods are described.

RESULTS AND DISCUSSION

Subheadings

The results and discussion may be presented separately, or in one combined section, and may optionally be divided into headed subsections.

Advice on Equations

Equations should be provided in a text format, rather than as an image. Microsoft Word's equation tool is acceptable. Equations should be numbered consecutively, in round brackets, on the right-hand side of the page. They should be referred to as Equation 1, etc. in the main text.

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \tag{1}$$

Advice on Figures

At the point of submission, authors may provide all figures embedded within the manuscript at a convenient break near to where they are first referenced or, alternatively, they may be provided as separate files. All figures should be cited in the paper in a consecutive order. Where possible, figures should be displayed on a white background. When preparing figures, consider that they can occupy either a single column (half page width) or two columns (full page width), and should be sized accordingly. All figures must have an accompanying caption which includes a title and, preferably, a brief description (see Figure 1).

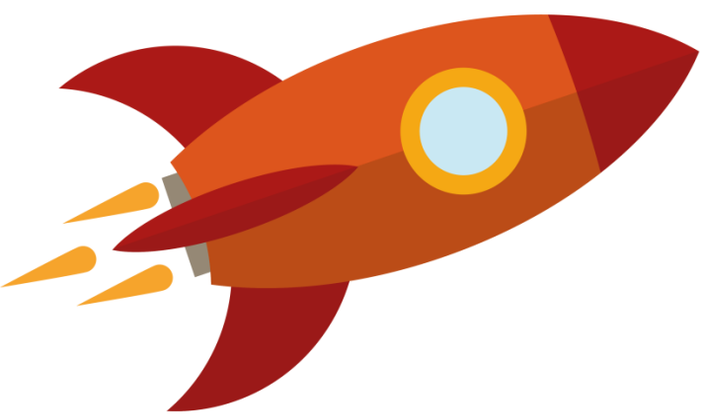

Figure 1: Basic rocket ship design. The rocket ship is propelled with three thrusters and features a single viewing window. The nose cone is detachable upon impact.

The caption can also be used to explain any acronyms used in the figure, as well as providing information on scale bar sizes or other information that cannot be included in the figure itself. Plots that show error bars should include in the caption a description of how the error was calculated and the sample size (see Figure 2).

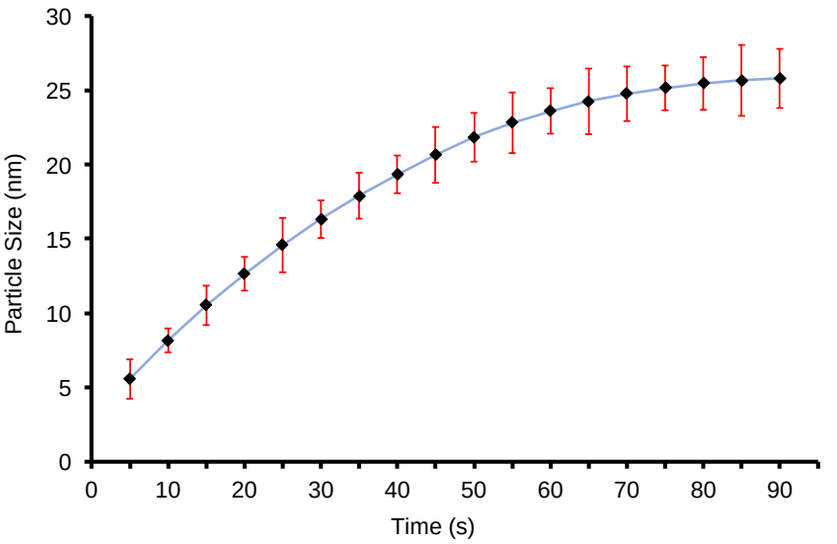

Figure 2: Plot of nanoparticle size with respect to time, recorded over a 90 s period. The error bars represent the standard deviation of measurements for 20 particles in five separate sample runs (n = 100).

If a figure consists of multiple panels, they should be ordered logically and labelled with lower case roman letters (i.e., a, b, c, etc.). If it is necessary to mark individual features within a panel (e.g., in Figure 3a), this may be done with lowercase Roman numerals, i, ii, iii, iv, etc. All labels should be explained in the caption. Panels should not be contained within boxes unless strictly necessary.

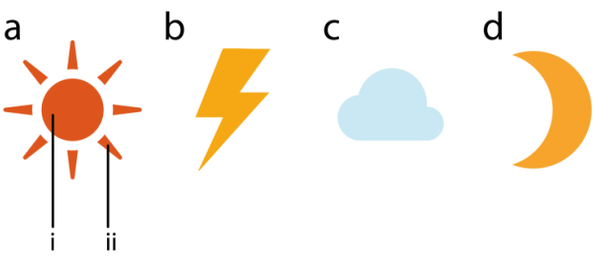

Figure 3: Representations of some common weather symbols. (a) The sun with (i) core, and (ii) rays. (b) Thunder bolt. (c) Cloud. (d) Moon.

Upon acceptance, authors will be asked to provide the figures as separate electronic files. At that stage, figures should be supplied in either vector art formats (Illustrator, EPS, WMF, FreeHand, CorelDraw, PowerPoint, Excel, etc.) or bitmap formats (Photoshop, TIFF, GIF, JPEG, etc.). Bitmap images should be of at least 300 dpi resolution, unless due to the limited resolution of a scientific instrument. If a bitmap image has labels, the image and labels should be embedded in separate layers.

Advice on Tables

Every table must have a descriptive title and, if numerical measurements are given, the units should be included in the column heading. Vertical rules should not be used (see Table 1). Tables should be cited consecutively in the text.

Table 1: Temperature and wildlife count in the three areas covered by the study.

Location	T [° C]	Turtles	Sharks	Octopuses	Starfish
Blue Lagoon	21.2	5	3	4	543
Regent's Canal	5.2	8	0	24	312
Shark Bay	12.8	4	7	9	122

CONCLUSIONS

The Conclusions section should clearly explain the main findings and implications of the work, highlighting its importance and relevance.

DATA AVAILABILITY

A data availability statement is compulsory for research articles and clinical trials. Here, authors must describe how readers can access the data underlying the findings of the study, giving links to online repositories and providing deposition codes where applicable. For more information on how to compose a data availability statement, including template examples, please visit: https://www.hindawi.com/research.data/#statement.

CONFLICTS OF INTEREST

This section is compulsory. A competing interest exists when professional judgment concerning the validity of research is influenced by a secondary interest, such as financial gain. We require that our authors reveal any possible conflict of interest in their submitted manuscripts. If there is no conflict of interest, authors should state that "The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper."

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FUNDING STATEMENT

Authors should state how the research and publication of their article was funded, by naming financially supporting bodies followed by any associated grant numbers in square brackets.

ACKNOWLEDGMENTS

An Acknowledgements section is optional and may recognise those individuals who provided help during the research and preparation of the manuscript.

SUPPLEMENTARY MATERIALS

If Supplementary Materials are provided (e.g., audio files, video clips or datasets) they should be described here. Note that authors are responsible for providing the final Supplementary Materials files that will be published along with the article, which are not modified by our production team. You should remember to reference the Supplementary Materials' contents at appropriate points within the manuscript. We recommend citing specific items, rather than referring to the Supplementary Materials in general, for example: "See Figures S1-S10 in the Supplementary Material for comprehensive image analysis."

REFERENCES

References will be reformatted in house, there is no need to adhere to a specific style at the point of submission. Authors are responsible for ensuring that the information in each reference is complete and accurate. All citations in the text must be numbered consecutively in square brackets, before any punctuation, for example, "as discussed by Smith [1]," and "as discussed elsewhere [2,3]." All uncited references will be automatically removed. The references should not contain footnotes. For your information, our citation style is:

[x] Author initials and surname, “Title in sentence style,” Journal title, vol. (volume number), no. (issue number), pp. (page numbers separated by an en-dash), Year.

For example:

[1] J. D. Watson and F. H. C. Crick, “A structure for deoxyribose nucleic acid,” *Nature*, vol. 171, no. 4356, pp. 737–738, 1953.

For articles with six or more authors, the first three authors are listed followed by ‘et al.’. When journals use only article numbers, no page numbers are necessary. For example:

[2] B. P. Abbott, R. Abbott, T. D. Abbott et al., “Observation of Gravitational Waves from a Binary Black Hole Merger,” *Physical Review Letters*, vol. 116, no. 6, Article ID 061102, 2016.