

TESTOSTERONE MODULATES ATRAZINE-INDUCED CYTOTOXICITY IN TESTICULAR CELL LINES

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Received: 05-08-2023

Accepted: 16-08-2023

<https://dx.doi.org/10.4314/sa.v22i2.22>

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Journal Homepage: <http://www.scientia-african.uniportjournal.info>

Publisher: *Faculty of Science, University of Port Harcourt.*

ABSTRACT

Testosterone greatly influences healthy growth and development of male sex organs. Testosterone has been demonstrated to possess antioxidant and anti-cytotoxic effects in cells. The present study examine the protective effect of testosterone on ATZ-induced cytotoxicity using TM3 Leydig and TM4 Sertoli cell lines as testicular in vitro models. TM3 Leydig and TM4 Sertoli cell lines were treated with atrazine (ATZ) 232 µM with varying concentrations of testosterone (0.1, 1, 10, 20 nM) for 6 h, 12 h and 48 h. Administration of testosterone increased the viability of the cell lines for 12 h and 48 h exposure compared to ATZ values ($p < 0.005$). Furthermore, testosterone reduced TM3 cell and TM4 cell GSH levels. Exogenous testosterone intake minimizes cell death and GSH levels in testicular cell lines exposed to ATZ. However, the lowest dose of testosterone attenuates the ATZ-induced increase of GSH levels in both TM3 and TM4 cells ($p < 0.005$).

Keywords: Atrazine, Testosterone, cytotoxicity, Glo-GSH

INTRODUCTION

In adult males, testosterone is a vital hormone that is required for the proper growth and development of the male genitalia. In addition, it is recognized as a crucial component of lipid metabolism and glucose homeostasis (Saad and Gooren, 2009). More recently, testosterone synthesis in mouse Leydig cells was shown to be stimulated by gonadotrophin-releasing hormones I and II (Lin et al., 2008). Leydig cells in the testes produce and secrete testosterone in response to luteinizing hormone, which is then counter-regulated by testosterone and its metabolites (Hwang et al., 2011). According to several studies (Palomar-

Morales et al., 2010, Hwang et al., 2011, Ballantyne et al., 2013), testosterone has been shown to exhibit protective qualities against cytotoxicity in several cell lines. Leydig and Sertoli cells are two important types of cells found in the testes of males (Zhang et al., 2015). These cells play an important function in the male reproductive system and have been used to investigate the effect of exogenous toxicants on testis function (Krishnamurthy et al., 2001). For spermatogenesis to continue through meiosis and for mature spermatids to be released at stage VIII in rats, testosterone is necessary. In fact, the withdrawal of testosterone causes; a severe deterioration in the blood-testis-barrier's integrity (Willem et

al., 2010), the transformation of round spermatids into elongated spermatids (O'Donnell et al., 1996), and the release of spermatids from Sertoli cells (Holdcraft and Braun, 2004).

The most extensively used agricultural chemical in Nigeria (Adesina et al., 2014) and one of the most widely used herbicides globally (Pathak and Dikshit, 2012) is atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine). It is used to suppress weeds in sorghum and maize fields (Moorman et al., 2001). Atrazine is a commonly used herbicide that has been shown to have toxic effects on various organisms, including mammals (Manske et al., 2004). One of the organs that is particularly sensitive to ATZ exposure is the testis. ATZ exposure has been linked to a range of effects on testicular function, including changes in hormone levels, alterations in testicular structure, and decreased sperm production (Belloni et al., 2011; Aziz et al., 2018; Rey et al., 2009). According to Swan (2006), ATZ has been shown to reduce male fertility and the quality of semen in farming environments.

According to several studies (Kniewald et al., 2000; Abarikwu et al., 2010), exposure to ATZ may cause Sertoli cells of rat testis to degenerate, disrupt the development of germ cells, reduce spermatogenesis, reduce testicular testosterone level, deplete antioxidant levels, and increase levels of lipid peroxidation. Also, studies have demonstrated that ATZ exposure can lead to decreased cell viability in various cell types, including human (Manske et al., 2004; Abarikwu and Farombi, 2015) and animal cells (Friedmann, 2002; Sagarkar et al., 2016). ATZ has been shown to induce oxidative stress in cells, leading to cell damage and death, it can also cause DNA damage and apoptosis (programmed cell

death) in cells. In addition to its direct effects on cells, ATZ can also affect cell signaling pathways, including those involved in cell growth and survival (Chen et al., 2021; Brunetti et al., 2022). ATZ has been shown to interfere with the activity of hormones such as estrogen and testosterone (Stoker et al., 2000), which can affect cell proliferation and differentiation (Warner et al., 2020).

Therefore, this present work was designed to further evaluate the modulatory effect of testosterone on cultured cell lines from ATZ-induced cytotoxicity. We proposed that testosterone administration prevented cells from ATZ-induced cytotoxic and cell death. Using the TM3 Leydig cell line and the TM4 Sertoli cell line as *in vitro* cell models, we explored the positive effects (cell viability and GSH) of testosterone co-administration in this study.

MATERIALS AND METHODS

Testosterone powder (purity \geq 99%), catalogue No. 86500) purchased from Fluka Chemie Co. (Buchs, Switzerland) was prepared in alcohol while ATZ (Sigma-Aldrich, St. Louis, MO, USA) was prepared in dimethylsulfoxide (DMSO). Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) (Gibco, Johannesburg, South Africa), Horse Serum (Gibco), Fetal Bovine Serum (Gibco, Johannesburg, South Africa) Penicillin-Streptomycin (Sigma-Aldrich, St. Louis, MO, USA), Trypsin/EDTA (Gibco, Johannesburg, South Africa) and other reagents used are of analytical grade. The final concentration of DMSO/alcohol in the preparation was not more than 0.01%. All other reagents were commercially available and were of analytical grade.

Preparation of Chemicals and Culture of Testicular Cell Lines

TM3 cells are Leydig cell lines derived from 11-13d mouse testis (ATCC No CRL-1714, Manassas, VA, USA) and TM4 Sertoli cell lines were a gift from Dr Sylvester Omoruyi (University of the Western Cape, South Africa). Cells were cultured using standard sterile cell culture techniques, maintained using DMEM/F-12 with 5% Horse Serum, 2.5% Fetal Bovine Serum and 1% Penicillin-Streptomycin. Cells were cultured in 75 ml culture flasks and incubated at 37°C with 5% CO₂. When confluent and ready for experimental preparations, cultured cells were detached using 0.25%. Testosterone was dissolved in alcohol to achieve a 0.1 M stock solution. Aliquots of the stock solution were frozen at -20 °C until use. The stock solution was further diluted with culture medium in order to achieve 0.1, 1, 10 and 20 nM testosterone solutions. For each experiment, four concentrations (0.1, 1, 10, 20 nM) of testosterone were prepared fresh from stock solution for use. ATZ was dissolved in dimethylsulfoxide (DMSO) to achieve a 1 M stock solution and aliquots of this stock solution were frozen at -20 °C until use. For each experiment aliquots of stock solution were further diluted with the culture medium to achieve a working concentration of a stock solution of 232 µM such that the final concentration of DMSO was less than 0.01%. The dose of ATZ (232 µM) for the *in vitro* study was based on earlier report of ATZ testicular concentration that decreased testosterone levels (Friedmann, 2002) and also based on ATZ cytotoxicity *in vitro* in different cell culture models (Pogrmic et al., 2009; Pogrmic-Majkic et al., 2010; Abarikwu et al., 2011). The low dose of testosterone in the present study was reported earlier to decrease oxidative damage in TM3 Leydig cells and

TM4 Sertoli cells (Hwang et al., 2011; Zaker et al., 2022) and validated in our preliminary observations to select the range of the experimental doses that were applied in the present study.

Cell viability assay

The effects of ATZ and testosterone on TM3 and TM4 cell viability were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide (MTT) cell proliferation assay for 6 h, 12 h and 48 h period as described previously (Pearce et al., 2023). Briefly, a density of 3.0×10^3 cells per well was seeded into a 96-well plate in 100 µL of complete culture medium for 24 h. Next, cells were treated with varying concentrations (0.1, 1, 10, and 20 nM) of testosterone and 232 µM of ATZ for 6, 12 and 48 h. After the treatment, the medium was removed and MTT (10 µL) was added to each well. After 4 h incubation at 37°C, the medium was removed and 100 µL DMSO was added to solubilize the formazan crystals. The colour developed was measured at 570 nm using a multiplate reader (BMG LABTECH's POLARstar Omega multi-detection microplate reader, Ortenberg, Germany). The results were expressed as percentage viability using the following formula: % viability = (T/C) × 100%, where C = absorbance of control and T = absorbance of treated cells.

GSH assay

Furthermore, the effects of ATZ and testosterone on TM3 and TM4 on intracellular GSH level was determined using the GSH-Glo™ GSH Assay from Promega, Madison, WI. Briefly, a density of 3.0×10^3 cells per well was seeded into a 96-well plate in 100 µL of complete culture medium for 24 h. Next, cells were treated with varying concentrations (0.1, 1, 10, and 20 nM) of testosterone and 232

μM of ATZ for 48 h. After the treatment, the medium was removed, cells were treated with Glo-GSHTM reagent and incubated for 30 min in accordance with the manufacturer's procedure, then treated with a luciferin detection reagent. In the presence of glutathione, a luciferin derivative changed into luciferin. A BMG LABTECH's POLARstar Omega multi-detection microplate reader (Ortenberg, Germany) was used to detect luminescence.

Statistical analysis

RESULTS

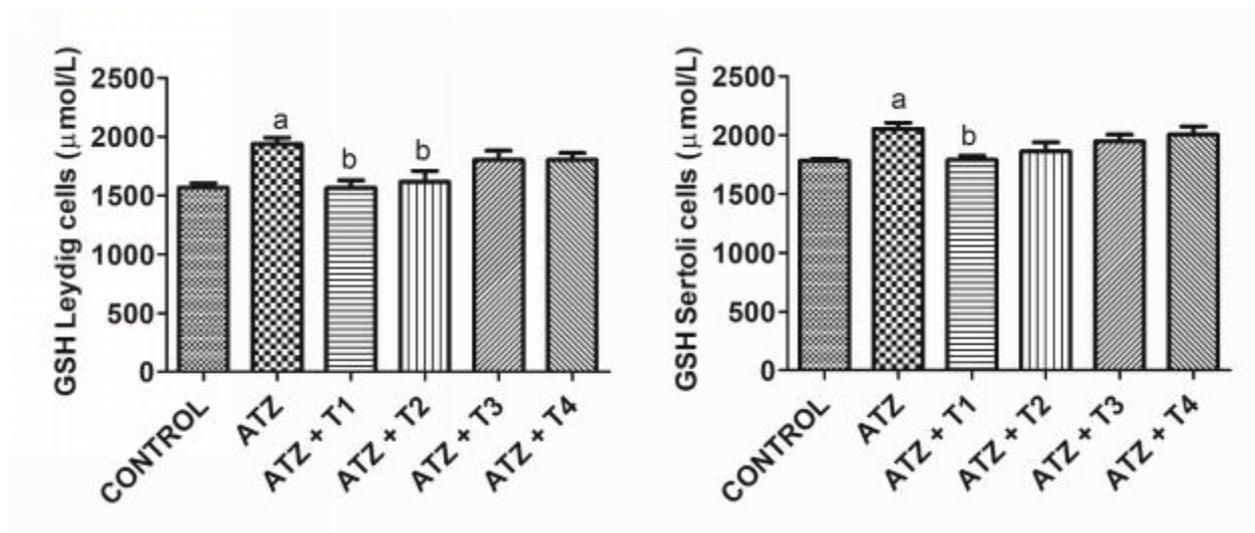


Figure 1: Testosterone effect on GSH level in ATZ exposed TM3 and TM4 cells lines using Glo-GSH assay kit. Data are represented as the mean \pm SD (N = 5). CONTROL= Normal control; ATZ= Atrazine; ATZ+ T1= Atrazine and Testosterone 0.1 nM; ATZ+ T2= Atrazine and Testosterone 1 nM; ATZ+ T3= Atrazine and Testosterone 10 nM; ATZ+ T4= Atrazine and Testosterone 20 nM.

Effects of testosterone on ATZ-cytotoxic increase in reduced glutathione in Sertoli and Leydig cell lines

We examined the protective effects of testosterone against ATZ-induced GSH level in TM3 and TM4 cells. As shown in Figure 1, ATZ-induced increase in glutathione level significantly and testosterone protective effect was observed using Glo-GSH kit in TM3 cells

Statistical analyses of the acquired data were done using GraphPad Prism version 6.0 software. (GraphPad Software, Inc., San Diego, CA, USA). Values of the measured parameters were expressed as mean \pm standard deviation (S.D.). For comparison between the different groups, one-way analysis of variance followed by Tukey multiple comparisons test was used. The results were considered statistically significant when p -value was less than 0.05.

and TM4 cells. ATZ increased GSH level by 14.18% on TM3 cells and 12.35% on TM4 cells compared to control (Figure 1). However, treatment with testosterone at 0.1, 1, and 10 nM doses reduced GSH concentration by 14.43%, 12.49% and 6.73% for TM3 cell line and 12.54% and 8.49% and 5.167% for TM4 cell lines compared to ATZ respectively. Hence, restoring GSH levels in ATZ-treated cells.

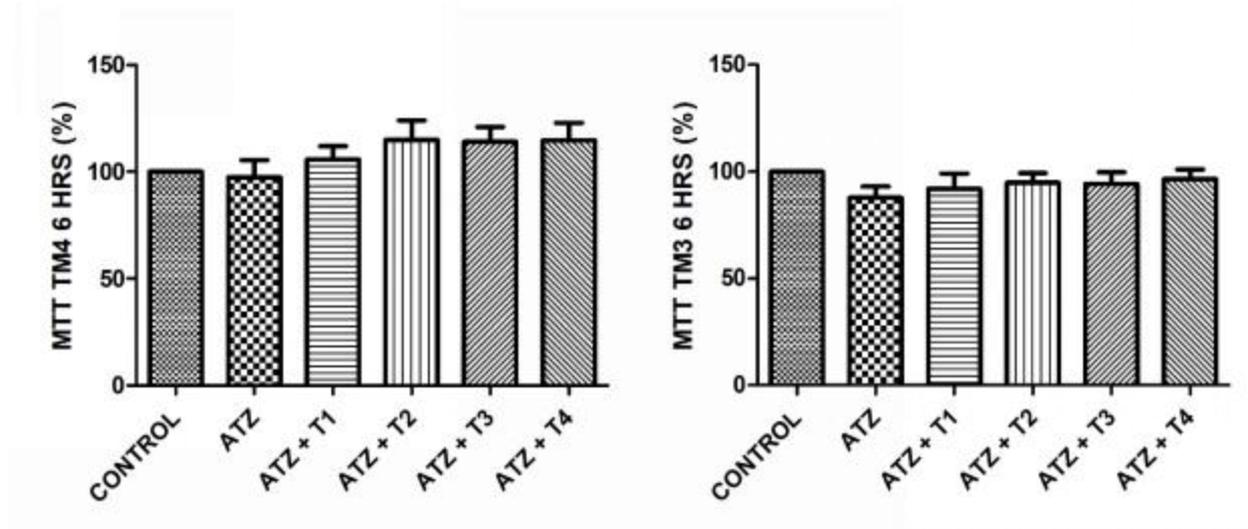


Figure 2: Testosterone effect on cell viability (%) of ATZ-exposed TM3 and TM4 cells for 6 hrs using MTT assay. Data are represented as the mean \pm SD (N = 5). CONTROL= Normal control; ATZ= Atrazine; ATZ+ T1= Atrazine and Testosterone 0.1 nM; ATZ+ T2= Atrazine and Testosterone 1 nM; ATZ+ T3= Atrazine and Testosterone 10 nM; ATZ+ T4= Atrazine and Testosterone 20 nM.

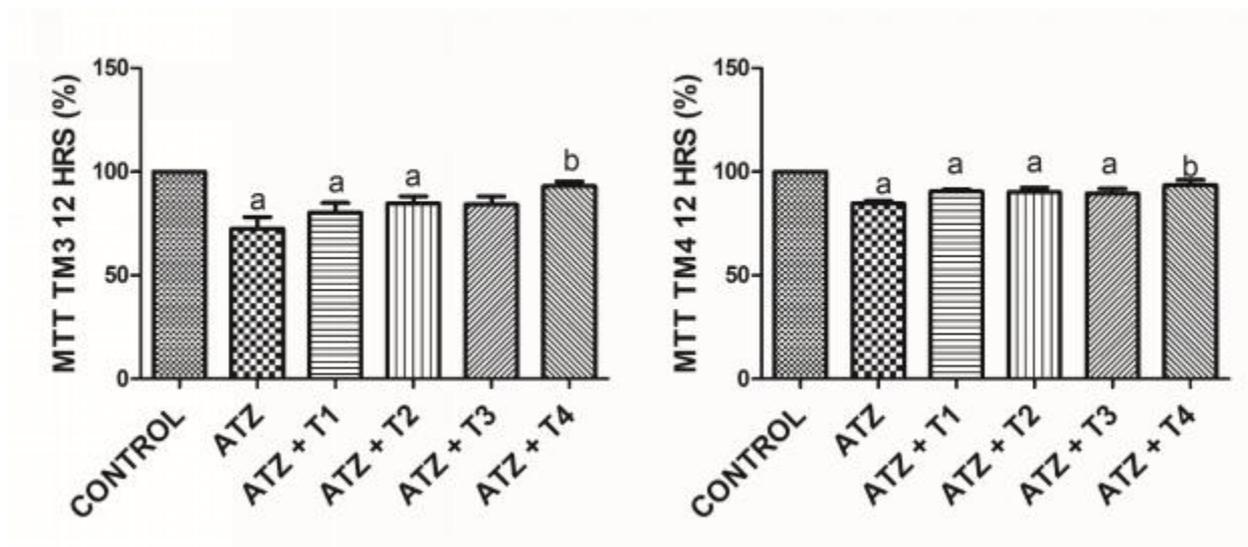


Figure 3: Testosterone effect on cell viability (%) of ATZ-exposed TM3 and TM4 cells for 12 hrs using MTT assay. Data are represented as the mean \pm SD (N = 5). ^aVersus control group, ^bVersus ATZ ($p < 0.05$). CONTROL= Normal control; ATZ= Atrazine; ATZ+ T1= Atrazine and Testosterone 0.1 nM; ATZ+ T2= Atrazine and Testosterone 1 nM; ATZ+ T3= Atrazine and Testosterone 10 nM; ATZ+ T4= Atrazine and Testosterone 20 nM.

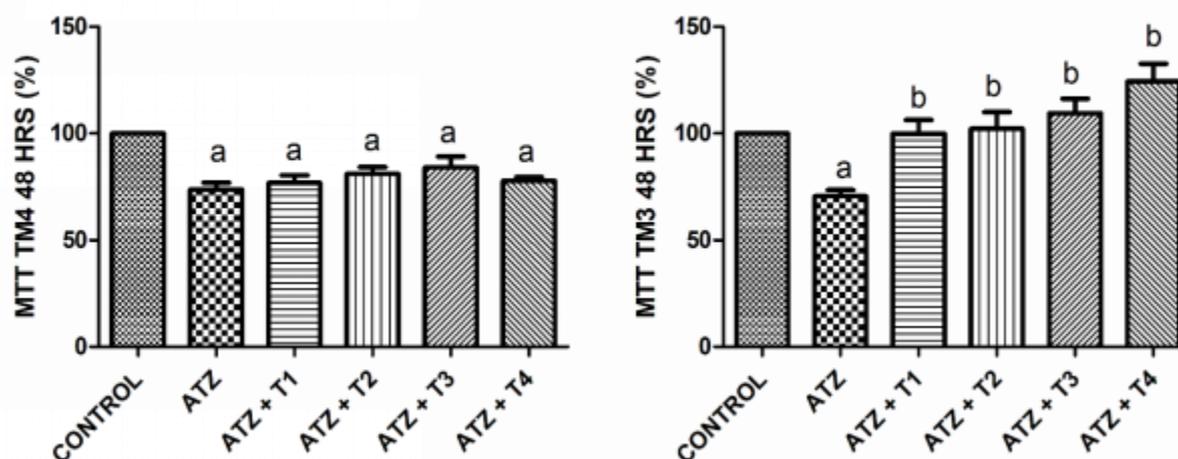


Figure 4: Testosterone effect on cell viability (%) of ATZ-exposed TM3 and TM4 cells for 12 hrs using MTT assay. Data are represented as the mean \pm SD (N = 5). ^aVersus control group, ^bVersus ATZ ($p < 0.05$). CONTROL= Normal control; ATZ= Atrazine; ATZ+ T1= Atrazine and Testosterone 0.1 nM; ATZ+ T2= Atrazine and Testosterone 1 nM; ATZ+ T3= Atrazine and Testosterone 10 nM; ATZ+ T4= Atrazine and Testosterone 20 nM.

Effects of testosterone on ATZ-induced cyto-toxicity on TM3 and TM4 cell lines

To evaluate whether testosterone protects TM3 and TM4 cells from ATZ-induced cell death, we examined the direct cytotoxic effect of ATZ (232 μ M) on TM3 and TM4 cell lines in various concentrations of T (0.1 – 20 nM) for 6, 12 and 48 h. The cell viability was measured by MTT assay. As shown in Figure 2, 6 h exposure to ATZ and testosterone showed no significant difference. ATZ treatment decreased the cell viability of the cell lines at 12 h and 48 h significantly using MTT assay (Figure 3-4). However, testosterone (20 nM) significantly protects the loss of cell viability in TM3 and TM4 cells for 12 h treatment (Figure 3) while all the testosterone doses protected the loss of cell viability in TM3 cells in an increasing manner for 48 h (Figure 4). These results indicate that treatment with ATZ induces cell death and that testosterone showed therapeutic effects against ATZ-induced cell death.

DISCUSSION

In this present study, we investigated the potential protective value of testosterone on cytotoxicity induced by ATZ. ATZ co-treated with the varying concentration of testosterone was not found to hold any significant influence over Leydig and Sertoli cell lines for 6 h as demonstrated in the MTT assay. Abarikwu et al., (2012) in a preliminary study reported that about 95% of cells remained alive after 6 hours of treatment with 232 μ mol/L ATZ, which is in line with our result. Furthermore, our results showed that ATZ has deleterious effects on Leydig and Sertoli cell viability, its induced cytotoxicity was evident after 12 h and 48 h treatment as demonstrated in the MTT assay. *In vitro* studies found that exposure to ATZ alters proliferation on PC12 (Abarikwu et al., 2011), HepG2 (Powell et al., 2011), decreased CHO-K1 cells proliferation (Kmetc et al., 2008), cytotoxicity was observed in JAWSII DC cells (Pinchuk et al., 2007) and low-level and short-term exposure to normal primary cultures of human fibroblasts results in

decreased cell (Manske et al., 2004). The testosterone co-treatment of the cell lines recovered the cell viability near normal. The protective effect of low-dose of exogenous testosterone was found to be on both Leydig cells (Hwang et al., 2011) and Sertoli cells. A recent study has shown that testosterone protects female embryonic heart H9c2 cells against severe metabolic stress (Ballantyne et al., 2013). Leydig cells treated with low-dose testosterone supplementation showed cytoprotection by decreasing ROS and lipid peroxides, increasing StAR expression and relieving hypoxia stress as demonstrated by HIF-1 α stabilization (Hwang et al., 2011). The cytoprotective effect of testosterone has been reported to be sex-specific and is related to the induction of antioxidant enzyme activities in pancreatic β cells (Palomar-Morales et al., 2010).

As a major oxygen radical scavenger, GSH provides the first line of defense against oxidative stress and cell injury. GSH is an antioxidant that protects the structural and functional integrity of cell membranes (Khosravanian et al., 2014). The levels of reduced GSH in the testosterone co-treated groups gradually decreased, showing improvement in cell damage. Hence, testosterone could potentially reduce ATZ-induced oxidative stress to an extent (An et al., 2020).

CONCLUSION

According to our results, ATZ exerted its cytotoxic effect which was estimated via cell viability and also increased the GSH level on Leydig and Sertoli cell lines. In addition, we found that the co-administration of testosterone could protect the ATZ-induced cytotoxicity and also restore the GSH capacity.

Acknowledgements

Mgbudom-Okah Chidimma Juliet acknowledged the African-German Network of Excellence in Science (AGNES), German Federal Ministry of Education and Research and Alexander von Humboldt Foundation for granting a Mobility Grant in 2019 to the University of the Western Cape, South Africa, where part of this work was done.

Conflict of interest statement

Regarding the scientific content of the work and other issues, the authors are in agreement.

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