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Cytotoxicity of methamphetamine exposure on Sertoli cells: a pilot study with implications for male infertility

David Fisher^{a,b} , Omar Zabida^{a†}, Sahar Abdul-Rasool^a  and Chontrelle Willemse^a 

^aDepartment of Medical Bioscience, Faculty of Natural Science, University of the Western Cape, Bellville, South Africa; ^bSchool of Health Professions, University of Missouri, Columbia, MO, USA

ABSTRACT

Methamphetamine (Meth), a psychoactive drug, has been shown to reduce testicular weight and decrease sperm count, indicating its potential role in contributing to male infertility. We therefore assessed Meth's effects (0.1–100 μM) on TM4 Sertoli cell viability, toxicity, and proliferation (trypan blue exclusion assay), mitochondrial activity (MA) (XTT assay), while transepithelial electrical resistance (TEER) was used to examine monolayer permeability. The acute study (only 24-hour Meth exposure) mimics recreational users and the chronic study, the Meth addicts who require daily doses (24–96 hours). Acute Meth treatment had minimal impact on TM4 Sertoli cell viability and toxicity, while chronic exposure resulted in reduced cell viability and increased toxicity in a dose-related manner. Acute exposure suppressed cell division at 72 hours, while chronic exposure suppressed cell division at both 72 and 96 hours. Long-term suppression of MA was observed for both acute and chronic Meth exposure (20 μM and 100 μM). Both acute and chronic Meth exposure affected permeability across the blood–testis barrier (BTB), which persisted for up to 96 hours. Given the pivotal role of Sertoli cells in spermatogenesis, our findings provide a two-pronged mechanism for Meth-induced male infertility and indicate that short-term exposure may have long-term effects on the germinal epithelium.

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Introduction

The universal misuse of methamphetamine (Meth) has resulted in its well-documented psychostimulant and neurotoxin effects (Daiwile et al., 2022; Ferrucci et al., 2019). As Meth continues to dominate global trafficking, it results in an increase in deaths among the drug variants as stipulated by the World Health Organization (WHO) (UNODC, 2018). Han et al. (2021) indicated that overdose deaths within the United States alone increased from 5526 in 2015 to 15 489 in 2019. This underscores the substantial impact of Meth on the global illicit drug trade (UNODC, 2022). Men are highlighted to be the predominant users of Meth (Daiwile et al., 2022) and as male fertility has been decreasing over the past years (Ravitsky & Kimmins, 2019), this psychoactive drug may contribute to male infertility (Hamed et al., 2023).

Reports of animal studies indicated that Meth treatment resulted in reduced glycolysis which was correlated with the inhibition of spermatogenesis in rats (Yang et al., 2019). These authors also showed that Meth exposures caused decreased bilateral testicular weight, with significant decreases in the testicular index (testicular index uses body weight as a normalizing variable), as well as decreased sperm counts (Yang et al., 2019). These Meth-induced low sperm counts were also

characterized by a dose-related decrease in progressive sperm motility (Yang et al., 2019). Another animal study has shown that Meth consumption results in Meth-induced DNA damage which is capable of initiating autophagy signaling that leads to progressive apoptosis in the testicular tissue (Peirouvi & Razi, 2022). A human, postmortem study on testicular tissues linked Meth addiction to spermatogenesis disruption (Aryan et al., 2022). Correspondingly, these authors reported that Meth addicts had a decreased number of Sertoli cells and also a decreased number of round and elongated spermatocytes. These are testicular variables that would indicate an ensuing decrease in sperm concentration, and hence an increased risk of male infertility (Aryan et al., 2022). It is therefore crucial that more investigation is required to understand how Meth affects male infertility at the level of the Sertoli cell. The Sertoli cell functions are indispensable for the survival of the germ cells and fertility, and Sertoli cell numbers are directly correlated to sperm production (Thumfart & Mansuy, 2023).

Sertoli cells establish specialized tight junctions between them to form the blood–testis barrier (BTB) (Thumfart & Mansuy, 2023). The BTB resides primarily at the level of the Sertoli cells which form the seminiferous tubules of the testis. Tight junctions between these adjacent Sertoli cells divide

CONTACT David Fisher  dfisher@uwc.ac.za

[†]Posthumous.

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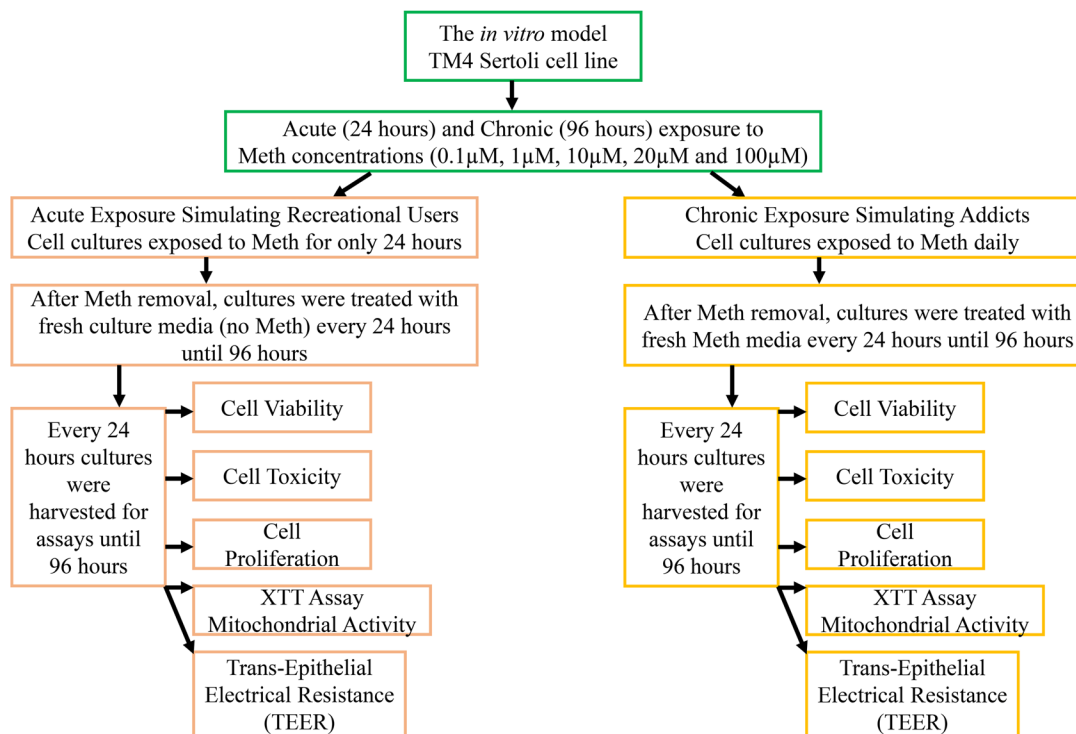


Figure 1. The schematic outlines the experimental design to investigate the effect of acute exposure to Meth (24 hours) on cultures of Sertoli TM4 cells. In parallel, in the chronic study, we investigated the effects of daily exposure to Meth for 96 hours.

the seminiferous epithelium into two compartments, basal and adluminal compartments, the latter being a specialized environment (inside the BTB) which is essential for post-meiotic spermatogenesis (Bu et al., 2022; Chen et al., 2022; Cheng & Mruk, 2012). Development of the secondary spermatocytes, round spermatids, and elongated spermatids takes place in the specialized environment of the adluminal compartment. Thus, the regulation of the BTB to maintain this specialized luminal environment is critical for spermatogenesis and forms a crucial role in the functioning of the germinal epithelium of the testis (Wang et al., 2020; Zhou & Wang, 2022). The germinal epithelium is made up of two cell types, the germ cells, and the Sertoli cells. The Sertoli cells are essential for regulating the BTB permeability as well as maintaining the specialized environment essential for the process of spermatogenesis (O'Donnell et al., 2022). Sertoli cells are vital for nursing developing spermatids and the number of Sertoli cells has a direct relationship to the number of spermatozoa produced. Therefore, any substance that negatively affects the physiology of the Sertoli cell will have a detrimental effect on the production of sperm and therefore male fertility.

The objectives of this *in vitro* study were to investigate the effect of Meth on a 2D TM4 Sertoli cell model after short-term (24 hours) and long-term exposure (96 hours) using selected physiological parameters. These included the effects on Sertoli cell viability, toxicity, the rate of cell division, mitochondrial activity (MA), and Sertoli cell monolayer permeability over 96 hours. Thus, studying the effect of Meth on Sertoli cells will not only provide insight into the processes of the maintenance of the BTB, but also into the functional capacity of the seminiferous tubule to generate spermatozoa.

Materials and methods

Experimental design

The study was designed to investigate the Meth (Sigma-Aldrich, Cat no. M8750, Cape Town, South Africa) effect on an immortalized mouse Sertoli cell line TM4 (ATCC® CRL-1715) as this cell line is deemed useful as a model for testing male reproductive toxicity (Zheng et al., 2018). The TM4 cells were exposed for 24 hours indicative of the acute study and in the chronic study, TM4 cells were exposed daily for 96 hours (Figure 1). The rationale for treating cell cultures of TM4 cells for 24 hours was to mimic Meth exposure for the recreational user, who typically would only be exposed to Meth for a period of 24 hours. After 24 hours, Meth would be removed from the cultures, but we continued to monitor these cultures for 96 hours. The chronic study attempts to mimic the Meth addict who requires daily doses of Meth. In these experiments, the TM4 cell cultures were treated with Meth daily for 96 hours. In both the acute and chronic studies, mitochondrial function was assessed (XTT assay) as well as to monitor permeability across monolayers of confluent TM4 Sertoli cells using the transepithelial electrical resistance (TEER) technique. All experiments were carried out in triplicate at a minimum ($n = 3$) and duplicated to ensure repeatability.

Cell culture and treatment

The murine Sertoli TM4 cell line was cultured in complete DMEM/F12 media: Dulbecco's modified Eagle medium: nutrient mixture F12 with HEPES (DMEM/F12 ratio 1:1)

(ThermoFisher, Cat no. 31330038, Johannesburg, South Africa) supplemented with 2.5% fetal bovine serum (FBS) (Celtic Molecular Diagnostics/Biowest, Cat no. S181G-500, Cape Town, South Africa), 5% horse serum (Celtic Molecular Diagnostics/Biowest, Cat no. S0910-500, Cape Town, South Africa), and 1% penicillin/streptomycin (Whitehead Scientific, Cat. no. DE17-602E, Cape Town, South Africa). For harvesting of the cells, trypsin 0.25% EDTA (ThermoFisher, Cat no. 25200072, Johannesburg, South Africa) was used. The TM4 cells were cultured in the supplemented DMEM/F12 media in a humidified 5% CO₂ incubator at 37°C until they reached 70% confluence and then sub-cultured for the specified assay. Only 24 hours after seeding, the TM4 cells were exposed to Meth. Plasma Meth concentrations of addiction ranged between 0.13 and 11.1 μM and higher Meth values (up to 84 μM) were found in postmortem subjects, as such values most likely result in Meth toxicity and death (Melega et al., 2007). These plasma variables were instructive in designing relevant *in vitro* experiments. The TM4 cells were acutely (only exposed for 24 hours) and chronically exposed (24–96 hours daily) at selected Meth concentrations of 0.1 μM, 1 μM, 10 μM, 20 μM, and 100 μM. A working stock solution was prepared in sterile phosphate-buffered saline (PBS) solution and filtered using a GVS filter (0.2 μm) (Bio-Smart Scientific, Cat no. SF-0.2-CA, Cape Town, South Africa). The treatment Meth concentrations were then added accordingly to the supplemented DMEM/F12. In the acute study, after the first 24 hours of exposure, the treatment media were then removed and replaced with fresh media. For the chronic study, cell culture media containing the selected concentrations of Meth was replaced every 24 hours. All the controls received fresh, complete cultured media (0 μM Meth) daily until 96 hours. It is important to note that the supplemented growth culture media and treatment media were replaced daily to ensure Meth concentration continuity in the treatment process.

Cell viability, toxicity, and proliferation assessment

Cell proliferation in the TM4 cells was determined by calculating the increase in the cell number using the trypan blue (TB) dye (Sigma-Aldrich, Cat no. T6146, Cape Town, South Africa). In this assay, the TM4 cells were seeded in six-well plates at a density of 2×10^4 cells/well. Each experimental group was seeded in triplicate, and the volume of the media was 1.5 ml per well. The plates were incubated in a humidified 5% CO₂ incubator at 37°C for 24 hours allowing the attachment of the cells. An inverted microscope (Eclipse-Ts2-Ls, Nikon, Amsterdam, The Netherlands) was used to monitor the attachment of the TM4 cells. After 24 hours, the growth medium was removed and replaced with the untreated (controls) and treated Meth media. The control TM4 cultures were treated with the completed DMEM/F12 media. The cells were incubated for 24, 48, 72, and 96 hours. At each time interval, cultured TM4 cells were washed with PBS and harvested using trypsin 0.25% EDTA. After centrifugation (2500 rpm for 5 min), the pellets were resuspended in complete DMEM/F12. Cells were then immediately prepared for cell counting by mixing 20 μl of the 0.4% TB solution with 20 μl of the cell suspension ensuring a 1:1 ratio. A 10 μl cell suspension 0.4%

TB mixture was added to the Neubauer improved counting chamber (Lasec Group, SKU: GLAS2C26M0610030, Cape Town, South Africa) (Neubauer hemocytometer) to count the cells. A manual count of live cells and dead cells was done using an inverted microscope (Eclipse-Ts2-Ls, Nikon, Amsterdam, The Netherlands). The total cell count, percentage cell viability, and cell toxicity were then respectively determined.

The XTT assay

The TM4 cells were seeded at a density of 4×10^3 in 100 μl/well of complete DMEM/F12 media in a 96-well microtiter plate (SPL Life Sciences, Cat no. 30096, Pocheon-si, South Korea) and were incubated for 24 hours. After 24 hours, the culture media (supplemented DMEM/F12) was removed and the cells were treated with the selected Meth concentrations of 0.1, 1, 10, 20, and 100 μM for both acute (24 hours) and chronic (treating daily for 96 hours) treatments. The cells were incubated with XTT reagent for 4 hours in a 5% CO₂ incubator at 37°C, resulting in the formation of formazan crystals which were quantified by determining the absorbance reading at a wavelength of 450 nm, using a 96-well microplate reader (POLARstar Omega B.M.G. Labtech, Ortenberg, Germany). The XTT assay (Roche, Cat no. 11465015001, Basel, Switzerland) was prepared according to the manufacturer's protocol: 50 μl of yellow XTT solution was added to each well making up a final volume of 150 μl/well. Formazan formation was determined by measuring the absorbance that was read at a wavelength of between 450 and 600 nm. The percentage of MA was then determined using a standard formula.

Transepithelial electrical resistance

The TM4 Sertoli cells were seeded on mixed cellulose esters culture inserts (Millipore/Merck, Cat no. PIHA01250, Darmstadt, Germany) in a 24-well plate at a cell density of 5×10^4 cells/well/insert, and cultured at 37°C and 5% CO₂ ($n = 3$; day = 0). The total volume of media in each well was 800 μl (300 μl inside the insert where the cells are seeded and 500 μl in the well, which contained the untreated or treated Meth media). The TM4 cells were allowed to attach to the insert overnight and divided to confluence. The effects of Meth treatment were bio-electrically investigated for 24, 48, 72, and 96 hours, by recording TEER readings for both treatments (acute and chronic). Before measuring TEER, each of the 24 hours well plates was removed from the 37°C incubator and was allowed to acclimatize to ambient temperatures for 20–30 min in the laminar flow cabinet. Aseptic strategies were utilized to maintain culture sterility. The electrodes were sterilized by placing them in ethanol for 15 min and followed by immersing the electrodes in supplemented media for 15 min, which served as an electrolyte solution used for the stabilization of the electrode interface. For the control, TM4 cells were exposed to complete media only. In addition, blank inserts, which contained only complete media with no TM4 cells, were used to subtract the electrical resistance of the cellulose membrane (blank

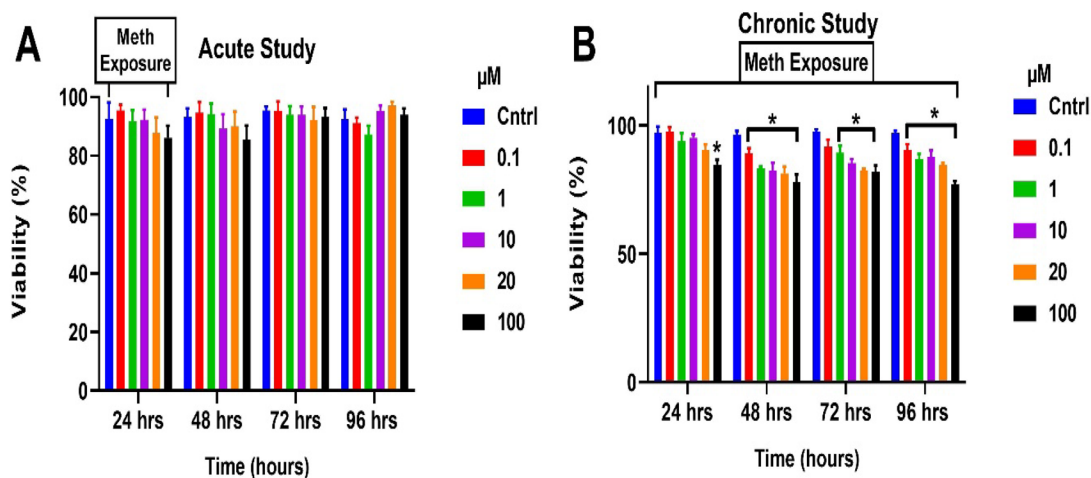


Figure 2. The effect of acute Meth exposure (24 hours) (A) and chronic Meth exposure (96 hours) (B) on the viability (%) of TM4 Sertoli cells over the experimental period of 24–96 hours using the trypan blue exclusion assay. Results were displayed as mean \pm SEM ($n = 3$). *Statistical significance ($p < 0.05$) between experimental samples as compared to controls by using ANOVA.

reading). TEER was measured using the EVOM TEER measurement system (EVOM: American Laboratory Trading Inc., East Lyme, CT). Measurement was determined by placing the electrodes on either side of the cell monolayer measuring the resistance of the TM4 monolayer by holding the electrode until the meter indicated a stable resistance reading (experimental reading). TM4 cells were only exposed to the fresh media after the TEER measurements were recorded to prevent the influence of the fresh media on the TEER readings across the TM4 cell monolayer.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 8, GraphPad Software, San Diego, CA). Data were expressed as mean \pm SEM. The differences between the groups were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. Statistical significance was accepted at $p < 0.05$ for a 95% confidence interval.

Results

The effect of Meth on the TM4 cell viability

To investigate the short-term effects of Meth on cultured TM4 Sertoli cells, we exposed the cultures to Meth for 24 hours (acute study) and then removed the Meth containing media and replacing it with fresh media but continued to monitor and collect data between 48 and 96 hours (Figure 2(A)). Although Meth treatment resulted in a slight non-statistical suppression of viability at 100 μ M (at 24 and 48 hours), none of the Meth concentrations were statistically significant from the control (Figure 2(A)). Overall, after short-term exposure to Meth across 96 hours, viability (%) was not statistically different from the controls. However, in the chronic study, where cell cultures of TM4 Sertoli cells were exposed daily over 96 hours to the selected Meth concentrations, resulted in a dose-related suppression of viability from 48 to 96 hours (Figure 2(B)).

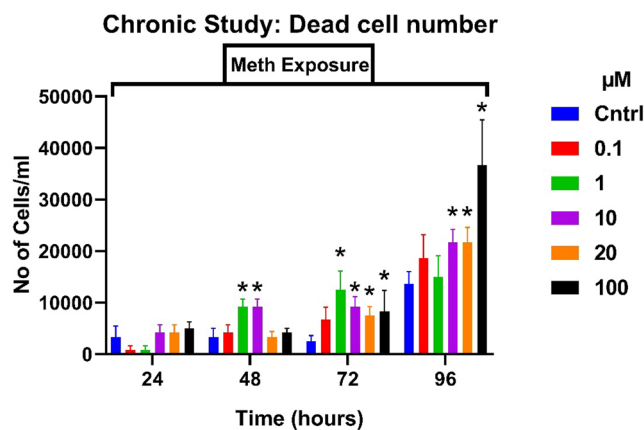


Figure 3. The effect of daily Meth exposure on levels of dead cells (chronic study). From 48 to 96 hours, Meth tended to increase the number of dead cells compared to control TM4 cell cultures. Results were displayed as mean \pm SEM ($n = 3$). *Statistical significance ($p < 0.05$) between experimental samples as compared to controls by using ANOVA.

The effects of Meth on TM4 cell toxicity

The effect of exposing the TM4 cells for 24 hours (acute study) to the selected Meth concentrations had no statistically significant toxicity when compared to the levels of toxicity in the control TM4 cell cultures. This indicated that the level of dead cells in the control TM4 cultures did not differ significantly from levels of dead cells in the TM4 cell cultures groups that were exposed to Meth for 24 hours. However, in the chronic study, where cells were exposed to Meth for 96 hours, the levels of dead cells (toxicity) statistically increased from 48 to 96 hours when compared to the control cell cultures of TM4 cells (Figure 3).

The effect of Meth on cell proliferation

The effects of acute and chronic treatment of Meth on TM4 Sertoli cell division were investigated by comparing the number of live cells in culture over a period of 96 hours. In the acute study, where cells were only exposed to Meth for

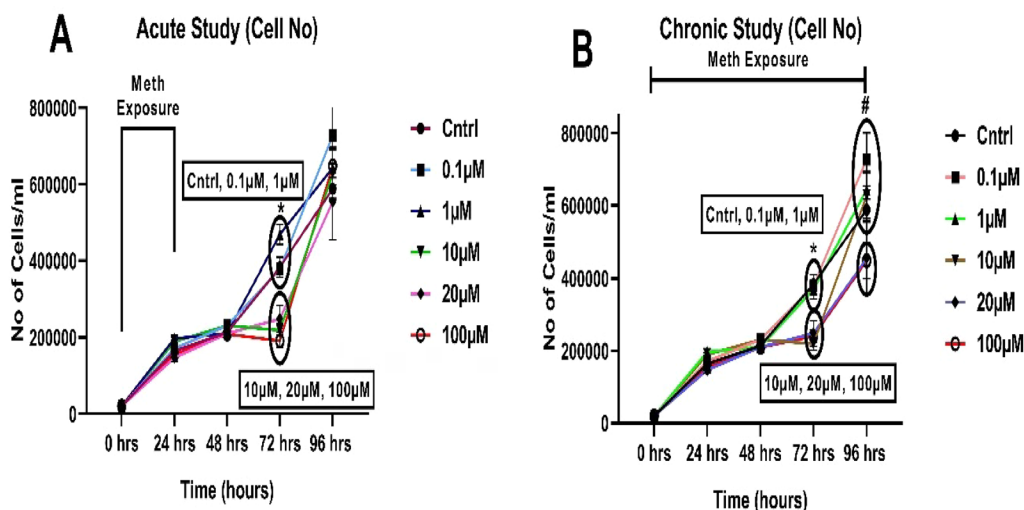


Figure 4. The effect of acute Meth exposure (24 hours) (A) and chronic Meth exposure (96 hours) (B) on the proliferation of TM4 cells over the 24–96 hours experimental period. *A statistically significant difference at 72 hours between experimental groups 0.1 µM, 1 µM, and controls, where the number of live cells in groups 10, 20, and 100 µM was significantly decreased ($p < 0.05$) (A, B). #In the chronic study, cells exposed for 96 hours to 0.1, 1, and 10 µM Meth were statistically no different from controls. However, exposure to 20 and 100 µM Meth significantly decreased ($p < 0.05$) the number of live cells compared to controls at 96 hours (Figure 4B). Data were displayed as mean \pm SEM ($n = 3$).

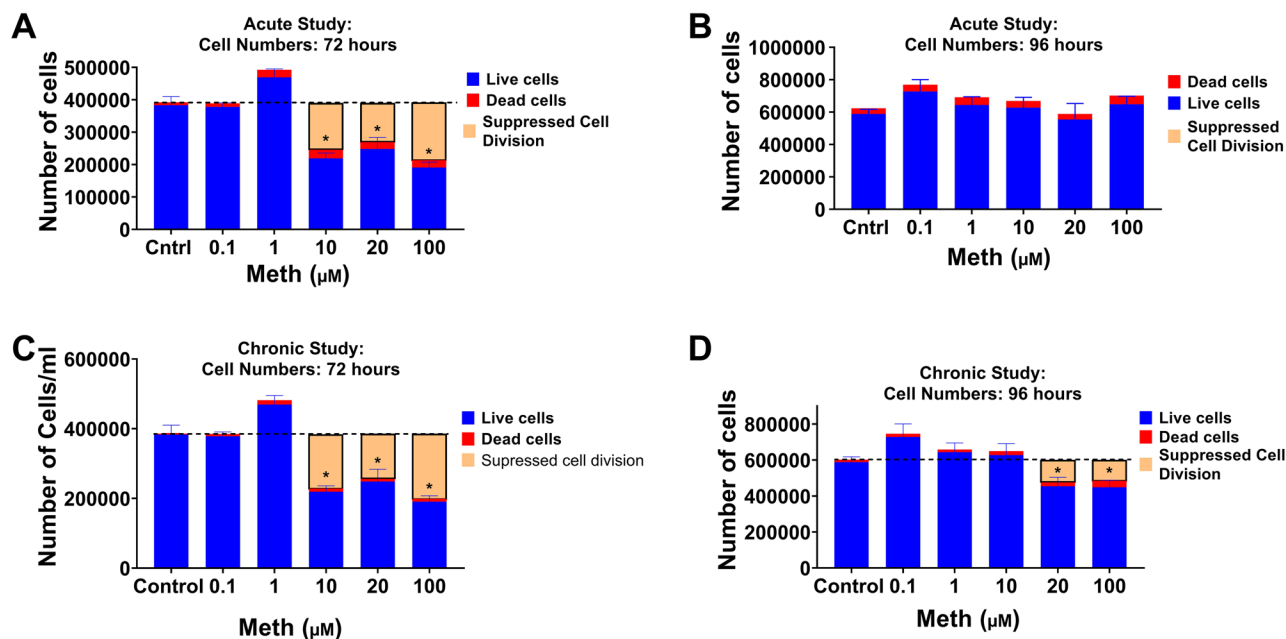


Figure 5. Suppression of cell division by Meth: (A, B) the acute study (Meth exposure for 24 hours) shows the suppression of cell division at 72 hours but not at 96 hours. (C, D) The chronic study (daily exposure to Meth) showed higher levels of suppression of cell division at 72 hours at concentrations of 10–100 µM, while less suppression of cell division was observed at 96 hours (at 20–100 µM). Data were displayed as mean \pm SEM ($n = 3$), * $p < 0.05$.

24 hours, cultures exposed to 0.1 and 1 µM were not found to be statistically different from controls throughout the 96 hours. However, cells exposed to 10, 20, and 100 µM Meth were found to have their proliferation significantly suppressed at 72 hours (Figure 4(A)). In the chronic study (Figure 4(B)), cells exposed for 96 hours to 0.1 and 1 µM Meth were found statistically no different from the proliferation of controls. However, 10, 20, and 100 µM Meth significantly suppressed the division of TM4 cells compared to controls at 72 hours, but at 96 hours, only 20 µM and 100 µM remained suppressed (Figure 4(B)).

Was the number of dead cells responsible for the suppression of cell division?

It was important to ascertain whether the levels of cell death could account for the suppression of cell proliferation shown in Figure 4(A,B). A simple analysis was conducted to compare total cell numbers (live and dead cells) in control TM4 cell cultures to total cell numbers of TM4 cells that had been treated with Meth. We expected that the number of dead cells would account for the decrease in total cell number. In the acute study, while there was no cell suppression at

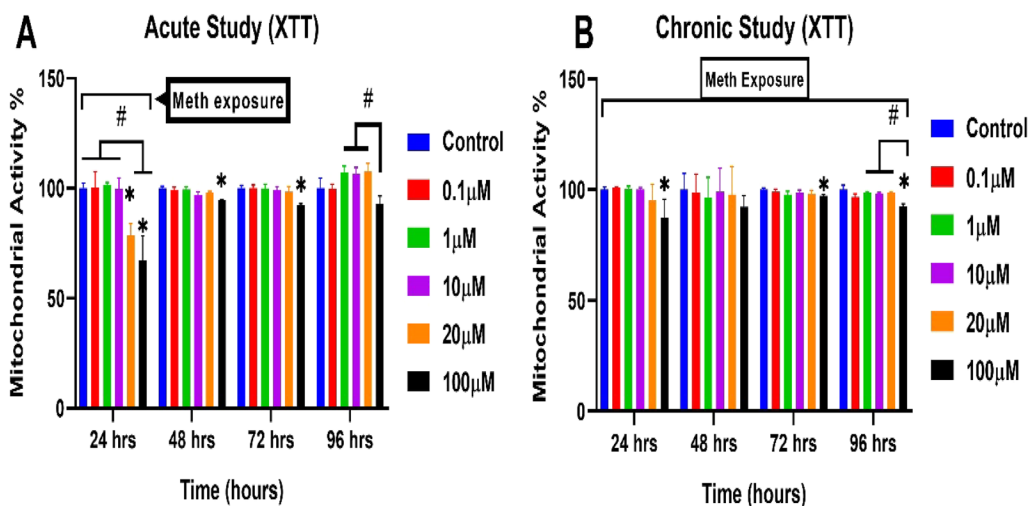


Figure 6. The effect of acute Meth exposure (24 hours) (A) and chronic Meth exposure (96 hours) (B) on the mitochondrial activity (%) of the TM4 Sertoli cells in comparison to controls (not exposed to Meth) at selected time intervals. *The statically significant differences between experimental samples compared to the control at ($p < 0.05$), and #the significant differences between the treatment groups ($p = 0.05$). Data were represented as mean \pm SEM ($n = 5$) by using ANOVA.

24–48 hours; however, at 72 hours Meth (10, 20, and 100 μ M) significantly suppressed cell division (Figure 5(A)), as the number of dead cells could not account for the decrease in cell numbers. However, at 96 hours, TM4 cells that had been exposed for 24 hours to Meth had recovered to control concentrations of total cell numbers, and therefore, had overcome suppression of cell division (Figure 5(B)).

In the chronic study (daily exposure to Meth), we also observed no cell division suppression between 24 and 48 hours; however, at 72 hours, Meth (10, 20, and 100 μ M) significantly suppressed cell proliferation (Figure 5(C)), and in contrast to the acute study where at 96 hours, cell division was no longer suppressed, Meth concentrations of 20 and 100 μ M continued to display suppression of cell division, but the lower Meth concentration of 10 μ M recovered to control levels of total cell numbers (Figure 5(D)).

XTT: the effect of Meth on the mitochondrial activity

Given the sensitivity of cell division to mitochondrial function, we investigated whether the suppression of cell division after Meth exposure was related to the function of the mitochondria. Hence, we investigated the effects of acute (24-hour exposure to Meth) and chronic Meth exposure (daily over 96 hours) on MA. Acute Meth exposure suppressed MA at 20 and 100 μ M only at 24 hours, but even after the removal of Meth, only 100 μ M Meth was able to suppress MA (Figure 6(A)). Chronic exposure to Meth only suppressed the MA at 100 μ M throughout the 96 hours, but significantly at 24, 72 and 96 hours (Figure 6(B)).

TEER: the effect of Meth on the Sertoli monolayer permeability

Sertoli cells are the primary regulatory cellular component of the BTB. Their transepithelial regulation of permeability is crucial for the process of meiosis. We measured the TEER across

confluent monolayers of Sertoli TM4 cells, as an indication of the transepithelial permeability, after both acute and chronic exposure to Meth. In the short-term, acute Meth had a dose-related effect in suppressing TEER at 24 hours (significantly at 20 and 100 μ M). However, at 48–96 hours Meth showed a dose-related non-statistical trend of increased TEER (significant at 100 μ M at 72 hours) (Figure 7(A)). Chronic exposure caused a dose-related suppression of TEER across the 96 hours of Meth exposure (significant at 20 and 100 μ M (24 and 96 hours) and significantly different from controls at 100 μ M (at 48 and 72 hours) (Figure 7(B)).

Discussion

Stereotypically, there are two types of Meth users, recreational users who expose themselves to Meth occasionally, and those individuals who are addicted to daily use of Meth. In this study, we simulated the effects of occasional Meth use by exposing TM4 Sertoli cells for 24 hours (acute exposure experiments) and monitored any short- or long-term effects on the Sertoli cells, while daily (chronic) treatment of Sertoli cell cultures to Meth would simulate individuals who are addicted and require daily exposure ('fix') to Meth. The study aimed to determine whether acute and chronic Meth exposure to TM4 Sertoli cells would have differential effects on the viability, proliferation, MA, and permeability of TM4 cell monolayers over 24–96 hours.

The effect of Meth on the TM4 cell viability

In our acute study on viability, cells were exposed to Meth for 24 hours, and then monitored for viability over 96 hours. We found that acute Meth exposure produced no statistically significant effects over the 48–96 hours compared to control cell viability. However, when exposing cells to Meth on a daily basis (chronic study), viability was suppressed at the 100 μ M concentration at 24 hours, and thereafter, in a dose-related

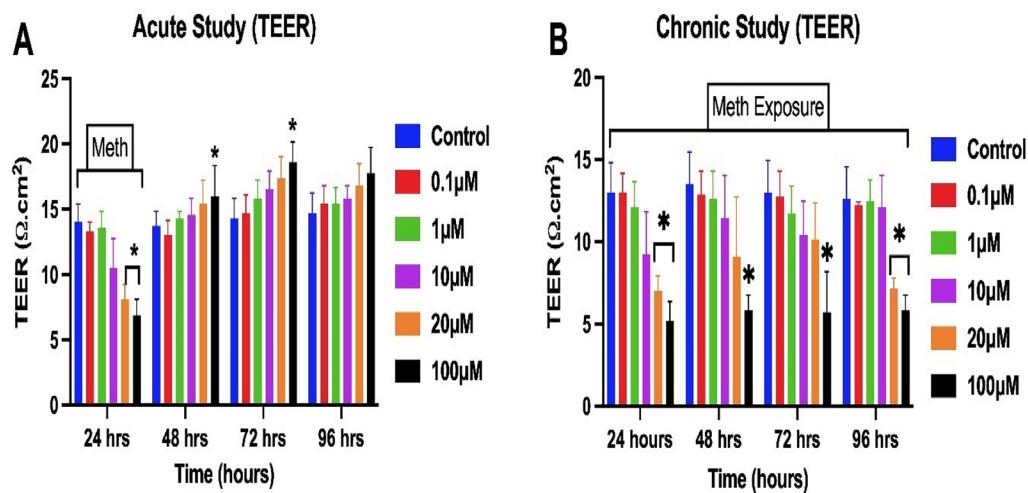


Figure 7. The effect of acute Meth exposure (A) and chronic Meth exposure (B) on TEER across TM4 Sertoli cell monolayers compared to controls at selective time intervals. Data are presented as mean \pm SEM ($n = 3$). *Statistically significant differences in the TEER over time between experimental samples compared to the controls. Statistical significance was designated at $p < 0.05$ by using ANOVA.

manner at 48–96 hours. This indicated that although the viability of TM4 Sertoli cells was fairly resistant to an acute episode of Meth exposure, Sertoli cell cultures were sensitive to repetitive exposure (chronic) to Meth. Furthermore, the analysis of the data showed a dose-related effect which further indicates that at higher doses the negative effect of repeated exposure to Meth has more serious effects on Sertoli cells. Other acute studies on other cell types (Martins et al., 2013; Zhang et al., 2009) endorsed our data in that concentrations of Meth up to 100 μM do not produce significant toxicity in cells. These studies (Zhang et al., 2009) used human brain microvascular endothelial cells (HBMECs), and found no effects on cell viability at Meth concentrations of 100 μM , 500 μM , and 1 000 μM for 24 hours. Only at supraphysiological concentrations of Meth (2500 μM , 5000 μM , and 10 000 μM) where cell viability significantly decreased, by eliciting a dose-related suppression of viability. This trend was observed in our acute study at 24 hours, where the 100 μM produced the largest effect, albeit only a small decrease in viability (see Figure 2). At 24 hours, in the chronic study, this concentration of 100 μM just produced a small statistical decrease in viability. In support of our data, when HBMECs were used as an *in vitro* blood–brain barrier (BBB) model only the supraphysiological concentration of 5000 μM significantly decreased cell viability, whereas the lower concentrations of 100 μM and 1000 μM did not affect the cell viability (Hwang et al., 2020). When Martins et al. (2013) studied rat brain vascular endothelial cells, no cell toxicity was observed when exposed to 0.1 μM , 1 μM , 10 μM , 30 μM and 100 μM of Meth. Furthermore, in a chronic exposure study by Fisher et al. (2015), the concentration range of Meth (0–1000 μM) on brain endothelial cells (bEnd5) had no significant effect on cell viability. A chronic study done by Kong et al. (2022) found that the cell viability of the immune cell lines Jurkat, NK-92, and THP-1 was mildly suppressed at low concentrations of 0–250 μM Meth, while, cell viability was significantly decreased at the supra-physiological concentration of 2000 μM Meth. Although these studies support our data regarding the effect of Meth on Sertoli cell viability (Hwang

et al., 2020; Martins et al., 2013; Zhang et al., 2009) providing evidence that both the endothelial cells of the BBB and the Sertoli cells of the BTB have a high tolerance for Meth, immune cells appear to be more sensitive to Meth exposure. It is, therefore, clear from the literature that different cell types appear to have different sensitivities to Meth exposure.

The effect of Meth on the Sertoli cell proliferation

As the viability data showed that TM4 cell cultures were mildly affected by chronic exposure to Meth, we investigated the effects of Meth on TM4 cell proliferation over a period of 96 hours. Proliferation is a measure of the rate of cell division while in culture. In both the acute and chronic studies (Figures 4(A,B)), the proliferation of Meth-exposed TM4 Sertoli cells was not statistically different for 48 hours. However, in both the acute study and the chronic study suppression of proliferation was observed after 48 hours: this indicated a delayed response to the effects of Meth. In the acute study, at 72 hours, 10 μM , 20 μM , and 100 μM exhibited suppression, but recovered by 96 hours. The chronic study showed a similar trend at 72 hours. At 96 hours, both 20 and 100 μM Meth chronic treatment continued to bring about decreased proliferation rates in TM4 Sertoli cells; however, TM4 Sertoli cells treated with 10 μM Meth recovered back to control levels. Given the scarcity of Meth studies on Sertoli cells, we compared our results with other cell types. Our chronic data were similar to an *in vitro* study done by Anari et al. (2022) on adipose tissue stem cells indicated that Meth exposure at various concentrations (0.6, 1.2, 6, 12, 40, 60, and 130 μM) over seven days also significantly, reduced cell proliferation and viability. The data in this study (Anari et al., 2022) were supported by similar data reported by Mohammadzadeh et al. (2022) on Wharton's jelly stem cells. This may be indicative that the Sertoli cell line could be more robust in comparison to the other cell lines as TM4 Sertoli cells exposed to Meth concentrations similar to the average of addict's plasma Meth concentration (Melega et al., 2007), recovered by 96 hours.

Toxicity is minimally responsible for decreased Sertoli cell numbers

In Figure 3, the rate of cell death increased statistically with increased Meth concentrations. However, the increase in cell death was maximized at approximately 35 000 cells/ml at 96 hours, which could be considered low at 5% of total cells. For the acute study (Figure 4(A)), we observed that at 10, 20, and 100 μ M (72 hours), we have a decrease in cell numbers relative to controls (Cntrl: 390 000 cells/ml; 100 μ M Meth: 200 000 cells/ml). Thus, Meth induced a decrease in the live cell number of approximately 190 000 cells. The number of dead cells for the acute study did not differ from controls at 72 hours at around 5000 cells/ml. Therefore, the number of dead cells could not account for the decrease in cell number. The only alternative to account for the decrease in cell number is the suppression of Sertoli cell division. At 96 hours, in the acute study, cell division was observed to recover to control levels.

In the chronic Meth study (Figure 4(B)), we observe a similar decrease in cell numbers at both 72 (10, 20, and 100 μ M) and 96 hours (20 and 100 μ M), and once again cell death could not account for the decrease in cell numbers (maximum cell death is in the range of 35 000 cell/ml), whereas the suppression of cell division (Cntrl: 600 000 cell/ml compared to Meth treated cells: 400 000 cells/ml), resulting in a decrease of 200 000 cells per/ml.

We show this suppression in cell division in Figure 5. Thus, the rate of Meth induced cell toxicity only plays a minor role in causing the decrease in cell numbers, while suppression in cell division plays a much larger role (compare the red columns (dead cells) to pink columns suppressed cell division) (Figure 5). Thus, although the data showed an increase in 'statistical' toxicity in Figure 3, this is not necessarily physiologically relevant. Our data show that Meth induced toxicity does not play a major role in the decrease of TM4 Sertoli cell numbers, further indicating that TM4 Sertoli cells are remarkably resilient against a chronic Meth insult. The robust nature of the BTB to Meth toxicity (Hau et al., 2020) is thus not unexpected in view of the need for developing germ cells to require protection against toxic blood-borne substances while developing into spermatozoa.

The effect of Meth on the mitochondrial activity

Given that cell division is an energy-intensive process, any compromise in mitochondrial function may have triggered the suppressed cell division. Throughout the experimental time-frame of 96 hours, in both the acute and chronic study, TM4 cells exposure to Meth concentrations of 0.1–20 μ M were statistically not different from controls, indicating that the MA of the TM4 Sertoli cells was surprisingly unaffected at the lower concentrations. Significantly, the highest concentrations of Meth (100 μ M) in the acute study had a long-term effect, suppressing MA for up to 96 hours. MA is a measure of mitochondrial capacity and its ability to generate ATP. The Sertoli cell's ability to carry out its physiological functions, namely, facilitating spermatogenesis and maintaining the integrity of the BTB are dependent on the relationship between MA and ATP. Therefore, the mechanism whereby

Meth suppresses Sertoli cell proliferation does not appear through impairing ATP production. This is contrary to a study by Ajjimaporn et al. (2005) who reported that the effects of chronic Meth on dopaminergic SK-N-SH cells showed a dose-dependent decrease in cell viability which was linked to a decrease in MA (as per a decrease in ATP production) at 10 μ M (20% decrease) and at 100 μ M (32% decrease), of Meth at 24 hours. Other studies reported that neurodegeneration within catecholamine cells (PC12) was influenced by a dose-dependent Meth concentration that significantly influences specific mitochondrial structural damages (Lenzi et al., 2022). However, these effects of low doses of Meth seem to be peculiar to neural tissue, which appears to have mitochondrial mechanisms which are more sensitive to Meth exposure than for Sertoli cells.

The effect of Meth on the Sertoli monolayer permeability

When culturing TM4 Sertoli cells on inserts they grow into monolayers that express tight junctions, which allows for the study of the *in vitro* BTB model and the measurement of TEER (Gye, 2003; Mentor et al., 2022). Measuring the electrical resistance across Sertoli cell monolayers (TEER) is an assessment of the permeability function of the *in vitro* BTB model (Carette et al., 2013). The significance of TEER data is underpinned by the understanding that a decrease in TEER represents an increase in permeability across the monolayer and *vice versa*.

For acute Meth exposure, the TEER data displayed a decrease after 24 hours at 20 and 100 μ M. After the removal of Meth, a statistical increase in TEER at 100 μ M occurred between 48 and 72 hours, while returning to statistical control levels at 96 hours. The phenomenon within the acute exposure group of increased TEER at 100 μ M after Meth withdrawal, can be attributed to the TM4 Sertoli cell monolayer recovery to the increased permeability caused by Meth exposure. After the 24 hours Meth withdrawal, the TM4 Sertoli cells responded by reestablishing control levels of monolayer (transepithelial) permeability. Statistical significance for increased TEER, however, was only evident at the highest concentration (100 μ M until 72 hours), whereas, at the lower concentration groups (0.1–20 μ M) no statistical significance to controls was observed, signifying the TM4 Sertoli cell's recovery to control levels. However, between 48 and 96 hours at the acute exposure, at the concentration of 100 μ M Meth, the cellular permeability overshoot to lower levels of permeability (higher TEER) across the monolayer is often seen in physiological systems, and we observe that at 96 hours, this overshoot (higher TEER) decreased to control levels.

Given the lack of studies in the literature on the effect of Meth on the Sertoli cells of the BTB, we compared our data to studies on the BBB. The BBB is similar to the BTB in that the paracellular pathways between the brain's capillary endothelial cells is also sealed with tight junctions proteins, occludin and claudin 5. Many of these studies reported data similar to our data for their 24 hour experiments. An acute Meth exposure study on HBMVECs indicated that Meth at concentrations of 20 μ M and 200 μ M decreased TEER causing an increase in permeability (Abdul Muneer et al., 2011).

Additionally, a study by Jumnongprakhon et al. (2016) showed that 100 μM Meth reduced TEER and significantly increased the paracellular permeability of the HBMVECs. Furthermore, a study done by Hwang et al. (2020) found that high Meth concentrations of 1000 μM for a 24-hour exposure of HBMVEC cells were able to compromise the intercellular TJs and adherent junction structures (Jumnongprakhon et al., 2016). These studies indicated that Meth affected the paracellular TJs across endothelial monolayers, and supported our findings for TM4 monolayers that were exposed to Meth for 24 hours. However, it was novel to observe that between 48 and 96 hours at the acute exposure, at the concentration of 100 μM Meth, the TM4 Sertoli cell monolayer produced an overshoot of a lower level of permeability (higher TEER) across the monolayer. This is a typical physiological feedback mechanism which is often seen in physiological systems to maintain homeostatic conditions. In support of this postulate, this overshoot (of higher TEER) decreased to control levels at 96 hours. This indicates that recreational exposure to Meth may have long-term implications for the permeability of the testicular germinal epithelium (BTB). Furthermore, the increased TEER, which is indicative of decreased permeability compared to control TM4 monolayers, suggests that Meth may seriously impair the regulatory homeostatic balance of seminiferous luminal milieu, which further may compromise spermatogenesis, and thus have implications for male fertility.

In contrast to the acute study, chronic Meth exposure showed suppression of TEER throughout the 96 hours of exposure. In the chronic exposure experiments, at the higher concentration levels (20 and 100 μM), lower TEER was observed; however, the TM4 cells show remarkable innate robustness to endure the Meth treatment (between 0.1 and 20 μM) from 48 hours to 72 hours. However, the continuous exposure to Meth eventually resulted in a significant decrease in TEER at both 20 and 100 μM at 96 hours. This indicates that continuous exposure to Meth will increase permeability across TM4 cell monolayers. Therefore, our data showed that to induce a decrease in TEER, Meth must be present, and subsequent withdrawal causes a long-term alteration in the permeability status of the BTB. The decline of the resistance across the Sertoli cell monolayers in this study might be due to Meth affecting the paracellular TJs between adjacent Sertoli cells increasing permeability in both a dose- and time-dependent manner. Studies done by Ramirez et al. (2009) showed a dose-dependent decrease of 20–50% in TEER after HBMVECs were exposed to 50 μM and 250 μM Meth for 21 hours, as a result of the partial loss of monolayer paracellular integrity. A study by Mahajan et al. (2008) investigating the effect of Meth (10, 25, and 50 nM) for longer than 24 hours using BMVECs, reported a dose-dependent decrease in TEER which was observed at 24, 48, and 72 hours. These studies, although on other cell-lines, support our chronic data study which showed that transepithelial permeability was significantly decreased by daily Meth exposure.

A key observation of this study suggests that Meth has the ability to affect the permeability across the BTB by impacting the paracellular permeability in both the acute and

chronic studies, which has negative fertility implications for both the occasional male Meth user and the addict. Exposure for 24 hours (occasional users) cause effects that persist for at least 96 hours, suggesting long-term effects for occasional Meth exposure on Sertoli cells and their barrier properties by changing the permeability properties across the BTB. This may induce dire consequences on the special luminal environment and its ionic makeup of the seminiferous tubules making this a contributing factor to Meth-induced male infertility.

Study limitations

One of the limitations of this pilot study could be that our report does not present *in vivo* data. Yet this has to be taken against the backdrop that decreases in testicular weight, Sertoli cell numbers in seminiferous tubules, and all indices of spermatogenic cells are well documented in both animal studies and human clinical studies (Allaeian Jahromi et al., 2022; Aryan et al., 2022; Farhadi et al., 2018; Peirouvi & Razi, 2022). It is known that Meth increases reactive oxygen species (ROS); however, these experiments are part of a future study. Lastly, although *in vitro* experiments cannot mimic the *in vivo* models perfectly, by focusing on the Sertoli cell, we excluded extraneous variables that may cloud the illumination of the physiological mechanisms whereby Sertoli cells contribute to Meth-induced male infertility.

Conclusions

Our data endorse the published literature that Meth use/abuse could contribute to male infertility. We showed that Meth has significant implications for Sertoli TM4 cell division and permeability, whether treated acutely or chronically. The data show that Meth addiction will have a negative impact on Sertoli cell proliferation, and should the consumption of supraphysiological concentrations occur, long-term damage could be irreversible. This corresponds to the MA data of this study, where the supraphysiological concentrations of Meth suppress the MA, consequently impacting the energy-intense physiological processes such as cell division, which has a high energy demand. The data leads us to postulate a two-prong mechanism whereby Meth affects male fertility: our TEER data provides a rationale whereby we postulate that Meth affects the permeability across the BTB by impacting the paracellular permeability between Sertoli cells with long-term effects on the meiotic mechanisms essential for spermatogenesis. Second, this mechanism is further augmented by Meth suppressing Sertoli cell proliferation, leading to a decreased apical surface of the germinal epithelium, limiting the production and 'nursing' of spermatids, emanating in a lower sperm count.

Therefore, from our data, it could be alluded that a lifestyle of Meth use, whether recreational or as an addiction might cause long-term damage to the Sertoli cells, which contributes to a negative impact on male fertility. However, additional studies to further elucidate the underlying mechanisms whereby Meth affects the Sertoli cell are necessary.

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Author contributions

Conceptualization, D.F.; methodology, C.W. and D.F.; software, D.F.; validation, D.F.; formal analysis, C.W. and D.F.; investigation, O.Z.; resources, D.F.; data curation, C.W. and D.F.; writing – original draft preparation O.Z., C.W., and S.A.R.; writing – review and editing, D.F.; visualization, D.F.; supervision, D.F.; project administration, D.F.; funding acquisition, D.F. All authors have read and agreed to the published version of the manuscript.



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ORCID

David Fisher  <http://orcid.org/0000-0001-6070-0234>
Sahar Abdul-Rasool  <http://orcid.org/0000-0002-4921-6748>
Chontrelle Willemse  <http://orcid.org/0000-0002-6081-2014>

Data availability statement

Data are available upon request. All experimental data collected are archived within the University of the Western Cape (UWC) archives and are available as per UWC data and intellectual property policy guidelines and their associated copyright protection.

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