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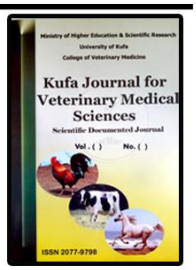

Attenuation of Ifosfamide Adverse Effect on Leydig, Sertoli and Spermatogonia Cells of Ram Testis by Liposome Technique *in vitro*

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Abstract:

Male infertility is a broad phenomenon adverse effect of the chemotherapeutic drug ifosfamide. Present warning proposes that the ifosfamide prodrug and their metabolite may contribute to this testicular cell toxicity. The present experimental study modified ifosfamide dosage form via liposome encapsulation inspected the special effects of ifosfamide and liposome carrying ifosfamide (small and uni/bi-lamellar) on certain isolated testicular cells; Leydig, Sertoli, and spermatogonia in primary culture. The three cells were isolated in percoll gradient density separation technique from 10 rams and inoculated in separately in LH, FSH and testosterone under growth factor trigger exposed to ifosfamide formulas.

The ability of the gonads-protectant cells medication liposomal entrapment ifosfamide to prevent ifosfamide-induced testicular cell injury was also assessed and prevented liberation potent metabolite of ifosfamide via trapping harmful metabolite that due to the Ifosfamide liposomal entrapped size ranged between 60-110 nm, the small size of liposome prolong t½ life and delay liberation and act as an additional compartment and prevent entry to the secondary targeted cells. Ifosfamide (300, 600, 1200, 2400 and 3600 µM) were produced dose-dependent drops viability and growth behavior, mitochondrial integrity and ATP levels, cell number and Membrane integrity, COMET DNA analysis, phagocytosis property of Sertoli cell and Leydig cell testosterone concentration, verified the IC50s.

The conclusion was exposure of the isolated cells of ram testis to liposome carrying ifosfamide protected cell via shield or delay of time exposed to harmful of unwanted effect of ifosfamide anticancer drug of all cell types, whereas, the ifosfamide produce decrease proliferative cell viability and demand energetic of cell supplier and function of certain cells as well as upset of DNA properties. This promotes an efficacious manner of augmented safer use of ifosfamide as a preliminary study.

Keywords: Ifosfamide, Liposome, tissue culture, Testosterone, Leydig, Sertoli, Spermatogonia

توهين التأثير سلبي للافوسفاميد في خلايا لايدك وسيرتولاي و ,سليفات النطف لخصية الكباش بتقنية اللايبوسوم في الزجاج

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الخلاصه:

عقم الذكور هو احد الظواهر الواسعة الانتشار ومصاحبة للتأثيرات السلبية بالعلاج الكيميائي للافوسفامايد. ان الايفوسفامايد كدواء اولي اظهر نذيرا سلبيا اسهم في سمية خلايا الخصية, الدراسه التجريبيه الحاليه حورت جرعة الافوسفامايد بتغليفة باللايبوسوم واستقصيت التأثيرات الخاصة للافوسفامايد المحمل باللايبوسوم ذو الطبقه الاحاديه او الثنائيه في بعض خلايا الخصية المعزولة خلايا ليدك وسيرتولاي وسليفات النطف في الزرع النسيجي الاولي لها. عزلت الخلايا الثلاثة بتقنية تمايز الكثافة للبيركول من عشر كباش وحضنت باضافة الهورمون الاصفري والهورمون المحفز الجريبي وهورمون الشحمون الخصوي تحت تأثير العامل المحفز للنمو وعرضت هذه الخلايا للايفوسفامايد بتراكيز مختلفه. ان قدرة الاستطباب بالافوسفامايد المحمل باللايبوسوم لمنع احداث الاذى الخصوي بواسطة منع تحرر ايضيات الافوسفامايد عن طريق مسك المواد المتأيضه الضاره وذلك يعود الى حجم اللايبوسوم 60-110 نانوميتر وصغر الحجم

الايبوسوم يطيل من مدة عمر النصف ويؤخر تحرير محتواه ويكون بذلك حجرة خزن اضافيه مانعا من استهداف الخلايا كأهداف ثانوية. الايفوسفامياید (003 و 006و 0021و 0042 و 0063) مايكروميتر احدث استجابه معتمد على الجرعه بانخفاض حيوية الخلايا ومنحنى صفات النمو وفعالية المتقدرات والادنوسين ثلاثي الفوسفات وعدد الخلايا وسلامة الجدار الخلوي وزيادة في الكومت لل دنا وانخفاض في قابلية البلعمه لخلايا سيرتولاي كما اضهرت اختلافا في قيمة نصف التثبيط. استنتج ان تعرض خلايا خصية الكبش المعزوله للايبوسوم المحمل بالايفوسفامايد حمى انواع الخلايا من الاذى المحدث من مضاد السرطان الايفوسفمايد الغير محمل عن طريق احاطته وتقليل وقت التعرض بينما الافوسفاميد احدث نقصان في تكاثر الخلايا وحيويتها واضر بمصادر الطاقه الخلوية ووضيفتها ومضر بخصاص الدنا. هذا عزز امان استخدام الايفوسفمايد المحمل باللايبوسوم وعدت هذه دراسة اوليه.

Introduction

The advances in the Nano practical's to use of liposomes designated to encapsulate drug as a vehicle and transporting therapeutic medicinal agent to sites of target tissue had ensued in the past 10 years (1, 2).

Liposomal ifosfamide the earlier liposomal anticancer drug was created to improve the safeness profile on proliferative and somatic normal cells. Whereas, several trails were carried out on the practical avoidance of cytotoxic adverse effect of classical ifosfamide (3), which, lately approved for the therapy of metastatic breast cancer (4). The hypothetical benefits of liposomal-encapsulated and carrier-mediated remedy are augmented solubility, prolonged duration of action and time of exposure, specified delivery of entrapped of medicinal remedy to the site of action, broadness "therapeutic index", and potentially overwhelming drug resistance attendant with the classical antitumor agent (3 and 5).

The recent formulas of liposomes containing ifosfamide with a single liposome may be improve selective toxicity in the preclinical challenge (6). In addition, ifosfamide alkylating agents may be representing rational candidates of liposomal formulations (7).

Ifosfamide as a course of numerous sessions or cycle for 21 days cycle time for more than 3-6 months as cycle scale time, the long cycle time encouragement several direct side effects and unpredicted adverse effects (8)

Ifosfamide treatment was recorded decrease of fertility. Some notions in literatures gave an attentions worried about this and the lack an efficient method of avoiding the adverse effect and increase selectivity toxicity, for this reason, the objective of this study based on encapsulation of ifosfamide to achieved protect testicular cells from occupying compatibility with the active site of these cells (1).

The present study was conducted to investigate the avoidance of ifosfamide induced testicular dysfunctions and appraise the liposome encapsulated ifosfamide on Leydig, Sertoli, and spermatogonia cells viability and functional properties of certain isolated cell culture, and evaluate some tolerated liposomal formulas doses on DNA defect and mitochondrial-ATP performance and groundwork of cell membrane integrity and safety of the exposure of liposomal ifosfamide in cell culture with growth behavior.

Materials and Methods

Animals care and testicular transportation

Ten healthy adult rams (~30 kg, ~1 year old, local breed Awassi). The testes (220-250g) were obtained from sloughed carcasses immediately; (slaughter house/Baghdad) preserved in cold DMEM media Dulbecco's Modified Eagle Medium

and transferred the testes to the College of Veterinary Medicine-Pharmacology lab after one hour in a cool box.

The epididymis and capsulate were amputated from the testes, the de-capsulated testis were immersed in 100 ml pre-chilled at 34 °C in DMEM; containing Sodium bicarbonate 3.7 g/l for 10 min (9)

Testicular tissue section: The testicular section was segmented to thin small sections ~10 mg, preserved in 1:1 (Ham's F12: DMEM medium). The segment was flickered for 1min in 3 ml of 10% PBS, bovine serum albumin 0.01%, streptomycin-penicillin 1%, gentamicin 0.1% and fetal calf serum 1% in conical tube.

Cells dissociation: The segmented tissue pellets were diluted by PBS solution 0.01%, pH 7.3, the tissue was washed and removed after centrifugation (1500 r/min) and substitute with digestion buffer collagenase I contain 6.01 IU/ mg per ml of DMEM medium (0.1% bovine serum albumin, 15 mM of HEPES, and 0.7 g/L of Sodium bicarbonate) and DNAase 1.28 mg (1.28 ml) pH 7.4. The tissue suspension was incubated for 30 min. at 38C˚ with shaking~50 C./min. under O_2:CO_2 (95%: 5%). The tissue fragments were separated via Nylon mesh filter; 100µm, and the filtrate was re suspended by 2 ml DMEM re-centrifugation; 500 r/ 5 min. to disregard the enzymes remnants, The cell was suspended in 2 ml DMEM, centrifugation 300 rpm/5min) and filtration (10).

Percoll gradient cells isolation and purification

The percoll gradient concentrations 21%, 26%, 37%, and 60% in DMEM (v/v) were separated cells layers of percoll media in a conical tube 15 ml size; The gradient percoll-was centrifuged at 3000 rpm for 30 min, thenthe interface fraction between 21% - 26% was collected the Spermatogonia cells, between 26% - 37% was collected the Leydig cells and in the surface of 60% of percoll gradient was collect the Sertoli. Each type of cells re-centrifugation 500 rpm for 7 min and the pellets were washed with the DMEM 15ml medium for percoll elimination of the twice time. The cells isolate were diluted with DMEM and incubated in 37Cº (11, 12).

Viability and Purity

Leydig, Sertoli, Spermatogonia Cell Viability (trypan blue)

The cell viability generally was assayed according to Guoxin *et al*. (2010). The protocol of technique was conducted on counting unstained brilliant cells and unviable blue cell proportional to total cells. Cells counted of the large grids on the hemocytometer (16). Trypan blue solution 0.1ml (0.4% PBS of, pH 7.4) was mixed with cell isolate, the stained cells was counts by hemocytometer under light microscope 200X.

$$\text{Viable cells } \% = 1\frac{\text{Number of stained cells}}{\text{Number of total cells}} \times 100$$

The calculation of the viable cells number per ml of culture well, the below formula was used for correction the dilution.

$$\text{Number of viable cells} \times (10^4 \times 1.1) = \text{cells per ml culture}$$

Leydig cell 3β-HSD

Stained with 3β-HSD using 1 g/ml etiocholanolone; enzyme substrate was witnessed to be reach 82% purity (13).

Oil Red O of phagocytosis of Sertoli cells

The treated Sertoli cells with Ifosfamide and liposome carrying Ifosfamide and control were washed in phosphate buffer saline twice time. The Sertoli cells were fixed with 10% formalin saline for 30 min. The cells were stained with ORO (Sigma) solution; ORO-saturated solution in isopropanol: water, 3:2) 15 min exposure. The cells were rinsed with 70% hydro-alcohol for 10 second removes background staining. The Sertoli cells were rinsed in tap water; the slides were stained with Harris hematoxylin for 20 sec., and mounted in glycerol-phosphate buffer saline (9:1). The lipid droplets area of Sertoli cells were quantified by a light microscope expressed the nucleus as reference for correction as nucleus area to lipid droplets ratio that refer to phagocytosis activity (14).

Spermatogonia UCHL staining marker

Fixed spermatogonial cell culture was dehydrated alcohol-Xylene multi-dilutions. Both primary and secondary antibodies were diluted in PBS with 1% bovine serum albumin (Sigma). Fixed cells were blocked with 15% fetal bovine serum (Gibco), incubated with polyclonal rabbit anti-UCHL-1 (DAKO 1:500) at 4°C for 12 hours, washed twice times with PBS, incubated with 3% H_2O_2 for 15 minute, washed twice times with PBS, incubated with goat anti-rabbit antibody (Calbio-chem; 1:200) for 20 minute at 37°C, dip 5 times with PBS, incubated for 5 minute in DAB substrate kit (Vector Laboratories), stained with hematoxylin, mounted in Vectamount (Vector Laboratories) and examined under a microscope (15).

Leydig cells steroidogenesis and Sertoli, spermatogonia proliferation

The cells were incubated with Ifosfamide for 24 hours concentrations "300, 600, 1200, 2400, and 3600" for test viability and steroidogenic function. The cells 8×10^6 in 96 well plate with Ham's F12/DMEM medium + Fetal Bovine Serum 10% v/v + penicillin-streptomycin 1% (Total 1.2ml) + "hCG hormone 2.5×10^{-10} mol/ml for Leydig cells only and (FSH) for Sertoli cell only 2.5×10^{-5} IU and (Testosterone) hormone 2.5×10^{-10}mol/ml for spermatogonia". O_2:CO_2 (95:5%), 38C˚, 24 hours. Ifosfamide and liposome carrying Ifosfamide was added treated cultures. The cells harvested of the culture with culture media, centrifugation 2300 g, 15 min. The cells pellet was incubate in water bath 80C˚, 5 min. Re-suspended by DMEM 1.2ml. Centrifugation 2300 g, 15 min., kept frozen, measure testosterone levels. The supernatant of Leydig cells was assayed for testosterone concentration, "The Testosterone level was measured by radioimmunoassay (RIA) in Lab Radio-isotope/ Baghdad-Iraq (23)". Cells pellet form of cells measure ATP, Mitochondrial integrity, DNA Quantity and deformity, cell membrane integrity, MMT test for viability (10, 17, and 19).

MTT Viability Assay

The cell viability was checked in different concentrations of Ifosfamide, The culture was scraped to detach the adherent cells, and the harvested cells were suspended into DMEM

media (2ml) and flicked. The suspended cells were incubated for 2 hours. The cell numbers were standardized to 4 x 10^6 of Leydig cells, Sertoli cells 2 x 10^6, and Spermatogonia 6 x 10^6 and rinsed in DMEM (50 ml) (20). Different dilutions of tested cell types including: 1x10^4, 1 x 10^2, and 1 x 10^6 cells/ml, of Leydig cells, Sertoli cells, and spermatogonia cells respectively. The isolated cells were incubated for 24 hours, MTT substrates 200 µl was added to the wells of incubated treated cells the cells incubated for 2 hours for stain development, the absorbance was checked at 570 nm (21, 33).

Mitochondrial and cell membrane integrity of cells

Mitotracker green stain stock (1mM) was diluted with diluent mixture of Dimethyl Sulfoxide (DMSO) 25µg Chloromethyl-X-rosamine (CMXROS) at 47µl, which genitally shaked and used (24). The cell cultures 2×10^6 cells/ml was mixed with 1 µl Mitotracker stain. The stained cells were incubated at 38C° for 30 min. The cells were examined in fluorescence microscopy 576 nm. Cells were checking illuminated tenacity and number "red filter" emitting red fluorescent light mitochondrial activity and green for membrane integrity (25).

ATP concentration in cultured cells

The cultured cells were centrifuged at 300 rpm for 5 min separated then adjusted the cell concentration was adjusted to 0.5×10^6 and stored in liquid nitrogen -196 C° (26,27).
Cell wash: Cell pellets were thawed suspended by normal saline (1%, 5 C°), the cells were settled, separated and re-suspended for three times, to separate the cells.
ATP Extraction: The ATP released from the cells was possessed by adding 0.1ml of 0.6M perchloric acid at 5C° and was mixed gently with cells for 20 min. Cells suspension was centrifuged at 80000 rpm for 5 min. The cells were separated and suspended with 50µl, 3.5M of K_2CO_3, and then centrifuged for 5 min at 8000 rpm.
ATP analysis: The supernatant of lysis cells was assayed by analysis media; (100µl) nicotinamide adenine dinucleotide NADH linked enzyme, as stock (1mg/ml); 20µl glucose 6 phosphate dehydrogenase, hexokinase as stock (1mg/10.5ml); 60µl glucose as stock 9mg/10.5ml; 50µl Nicotinamide adenine dinucleotide phosphate NADP, dinucleotide phosphate 10mg/10.5ml; 0.1 N of 800µl telomeric repeat amplification protocol (TRAP buffer) pH 7.6. The ATP concentration was assessed at 340 nm of spectrophotometer the value was calibrated from standard curve of ATP, as previously prepared for multi dilutions (27, 28).

COMET assay of DNA

The COMET was detected via "single-cell gel electrophoresis technique" (29, 30).

COMET procedure the prepared agarose slides were dipped in a lysing media(NaCl 2.5 M, Na_2EDTA 100 mM, Tris pH 10+10% DMSO 10 mM, Triton X-100 1%) for 12 hours.

Slides were dipped in 10 mM dithiothreitol for 20 min inside lysis media. The slides were transferred to the tank of horizontal gel electrophoresis buffer, alkalization COMET assayed for denaturation of DNA, 10 min at 4 C° in alkaline electrophoresis buffer: NaOH 300 mM, Na_2EDTA 1mM and HCl pH Modifier amounts.

Electrophoresis migration (27 V: 0.8 V/cm by 300 mA at 4 C° for 5 min). The slides were incubated in 0.3 M Sodium acetate-ethanol base for 30 min. and dried in 99% ethanol for 1 hours and 70% ethanol for 5 min. The slides were air-dried at (25±2C°), and stained with 12 mg/ml ethidium bromide. Fluorescent microscope was used to detect the slides at X 200. Scoring the DNA/100 cells and quantifies tail length percentage of damaged DNA. (31)

The concept of comet calculation is based on DNA fragment percentage,

$$\text{Tail} = \text{Tail length} \times \text{DNA in the tail}$$

Statistic and calculations

Real examination of the control and treated assembling were subjected to examination of variance (ANOVA) two courses analyses .A probability of $p < 0.05$ was required to mean a significant contrast .Each group comprised of in any event sex reproduces . A correlation was made with both IC_{50} and maximal tension stress (32).

Results

Liposomal preparation parameters

The physical characteristics of Liposome are shown in Table 1. The size of Liposome was 60-120 µm ranged 91.36 µm and the lamellar is commonly unilamellar to bilamellar (1-2) lamellar. The entrapment percent of Liposome was 85.46±6.51% and entrapping efficiency 92.75. The osmolarity of entrapped Liposomal displayed tolerance stability there number of Liposome in 0.375 mol ml and 1.58 M as with wideness range.

Table 1: Type of Liposome based on physical and pharmacological characteristics

Size		Lamellar		Entrapment %
Range	mean	range	Mean	
60 - 120	91.36	1 - 2	1.79± 0.03	85.46 ±6.51

Photo micrographic of light and electron (Trans and scan) microscope with high contrasted phase of Liposome

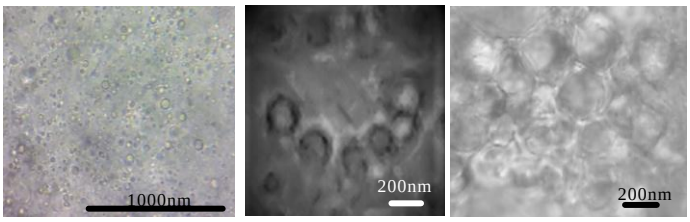


Figure 1: photomicrographic of Liposomal preparation in light microscope with contrasted phase.

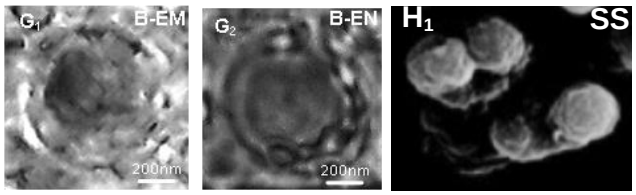


Figure 2: Photomicrograph of transmission electronic microscope of Liposome; unilamellar Liposome encapsulated Ifosfamide drug.

Tissue Culture results

Leydig, Sertoli, and Spermatogonia cells capability were proliferate to inoculated for 0, 2, 4, 6, 12, and 48 hours and influence of hCG (2.5×10-10 mol/ml), FSH (2.5× 10-5 IU), and Testosterone (2.5×10-10mol/ml) hormones respectively act as motivation of growing in DMEM modified media of each Leydig, Sertoli, and Spermatogonia cells at different times. The viable percentage of each Leydig, Sertoli, and Spermatogonia cells was displayed no significant (p<0.05) value between 24 and 48 hours, figure 2.

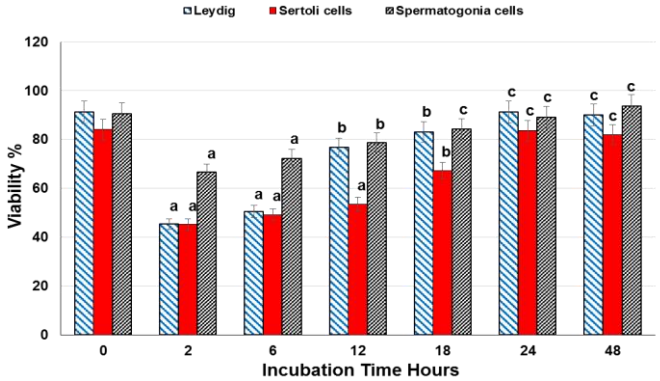


Figure 2: The time effect on viability of ram Leydig, Sertoli, and Spermatogonia cells ***in vitro*** culture. The letters denoted differences within cells groups

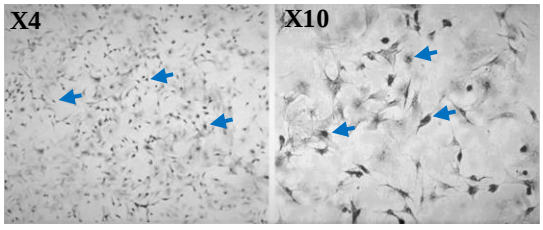


Figure 3: Leydig cells primary culture with modified DMEM media

Growth and growth behavior

Growth behavior of Leydig, Sertoli, and Spermatogonia cells exhibited variant behavior with significant p<0.05 shallow Spermatogonia growing as limiting primary proliferative sequence and the shape of growing colonies in figure 3.

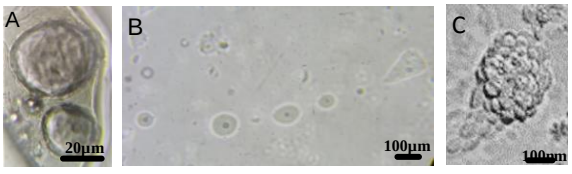


Figure 4: Growth of a colony of Leydig cells A; Sertoli cell B; and Spermatogonia cells C. in culture Growth behavior of testicular cultured cells

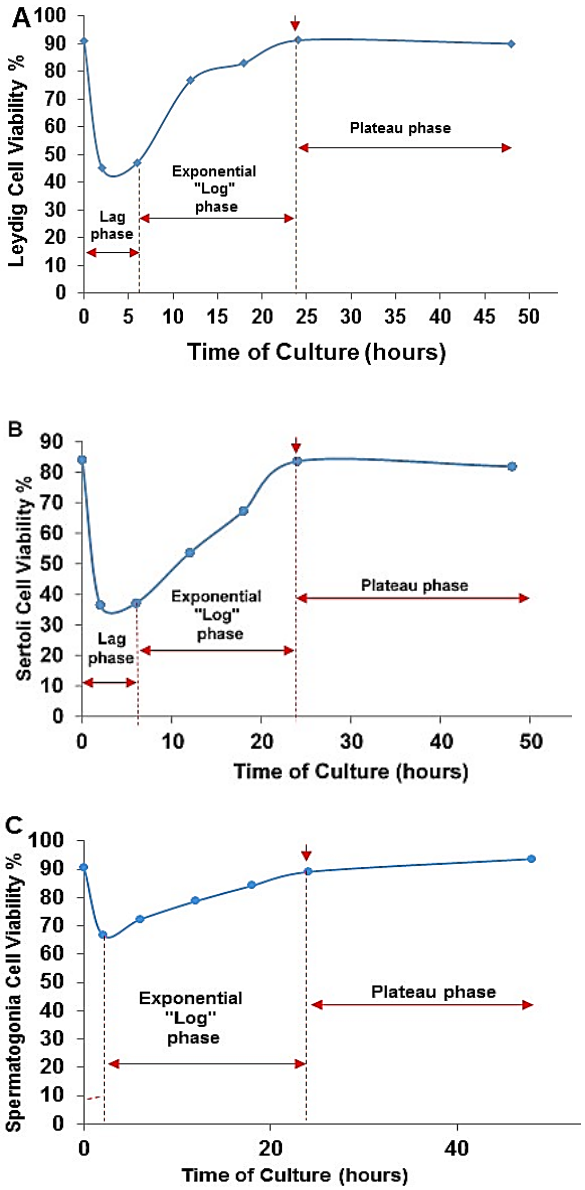


Figure: 5: showed the growing behavior depiction of three types of cells. A. Growth curve and culture maintenance, the plot of Leydig cell viability concentration% versus time hours from subculture, show the lag phase 6.258±0.172, exponential phase 18.157±2.16, and plateau started 24.529, and indicates times which subculture and feeding should be performed. B. The plot of Sertoli cell viability concentration % versus time hours from subculture shows a lag phase of 7.599±1.003, exponential phase 19.843± 4.067, and plateau of 22.195±3.115, and indicating times which subculture and feeding should be performed. C. The plot of Spermatogonia cell viability concentration% versus time hours from subculture, scheme display the behavior of growth task; the lag phase 2.702±0.045, exponential phase 22.212± 3.74, and plateau started 23.101±3.015, and indicating times which subculture and feeding should be performed.

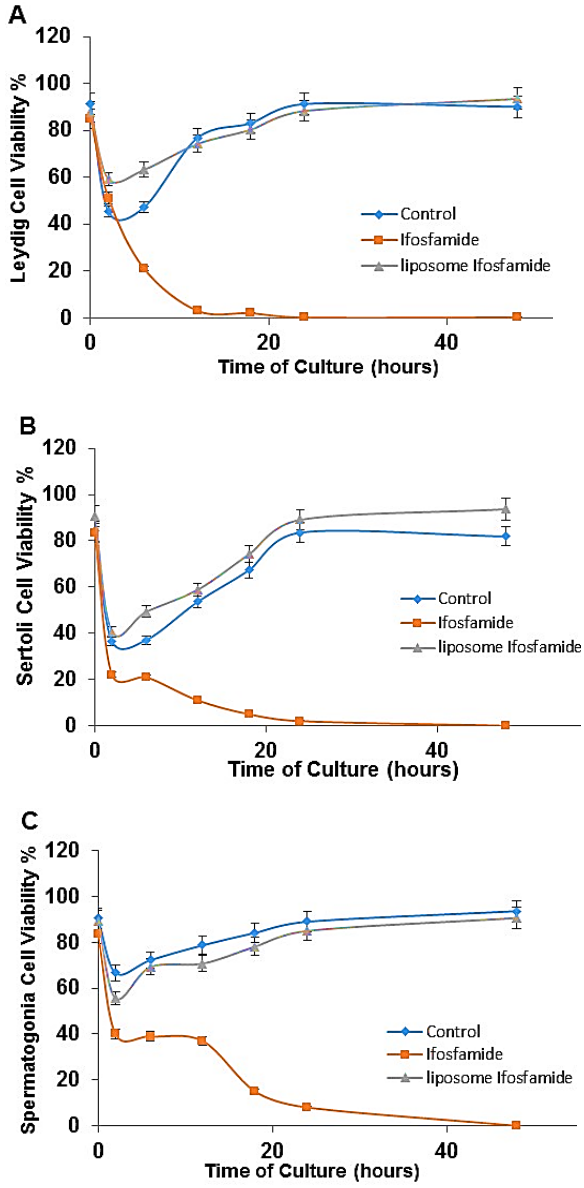


Figure 6: reveals the growth curve expression of the viable cell density versus the time spent in culture.

The cells cultured in media containing Ifosfamied and other liposome carrying Ifosfamide in culture usually proliferate following a different behavioral deviation of standard growth pattern (fig. 6).

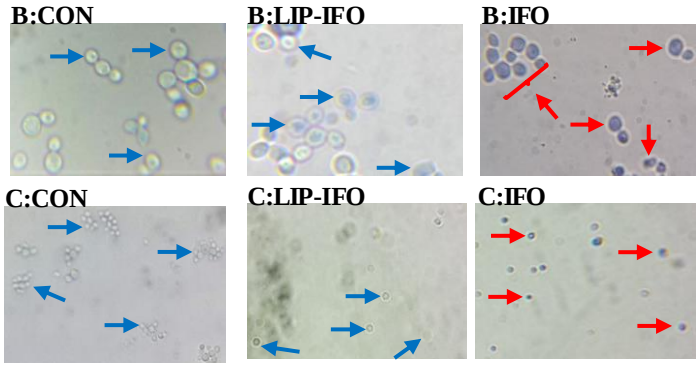


Figure 7: The effect of **IFO** Ifosfamide, **LIP-IFO** Liposome carrying Ifosfamide, on the ram Leydig cells **A**; Sertoli cells **B**; and Spermatogonia cells **C**. compared with **CON** control; the arrows and refer to the live and dead cells, respectively. X40

MTT absorbance

a cell calibration curve was created from serial dilution of primary Leydig, Sertoli, and Spermatogonia cells suspension of ram and MTT absorbance was measured at 570 nm (Fig. 8).

Absorbance data were corrected by subtracting the absorbance value of the blank from cultured media on MTT standard bio regent. The calibration curve indicates a linear response between viable cell number and absorption. These data showed a direct proportional correlation between culture cell of Leydig, Sertoli, and Spermatogonia viability and dilution.

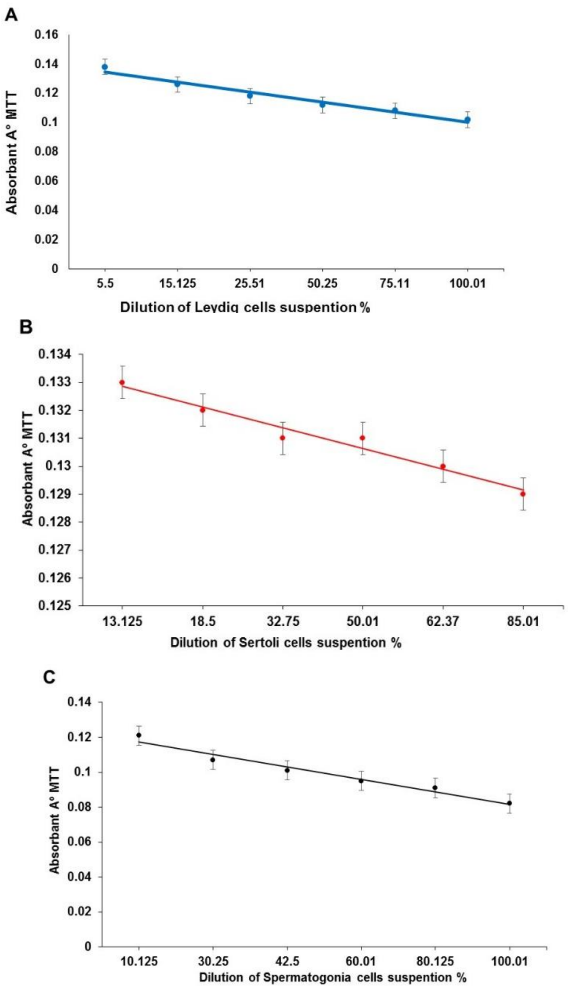


Figure 8: The calibration curve of viable cells MTT versus dilution series of ram Leydig **A**; Sertoli **B**; and Spermatogonia cells **C**. suspensions

The result is graphed as scatter in fig (9) and graphically represented in correlation regression higher r_2=(0.9238, 0.8935 and 0.8974) of Leydig, Sertoli, and Spermatogonia cell respectively. The viability percentage increased significant ($p<0.05$) with the increase of concentration of Leydig, Sertoli, and Spermatogonia cell number. These final standardized the culture cells before treatment of effect exploration figure (11. A, B, C).

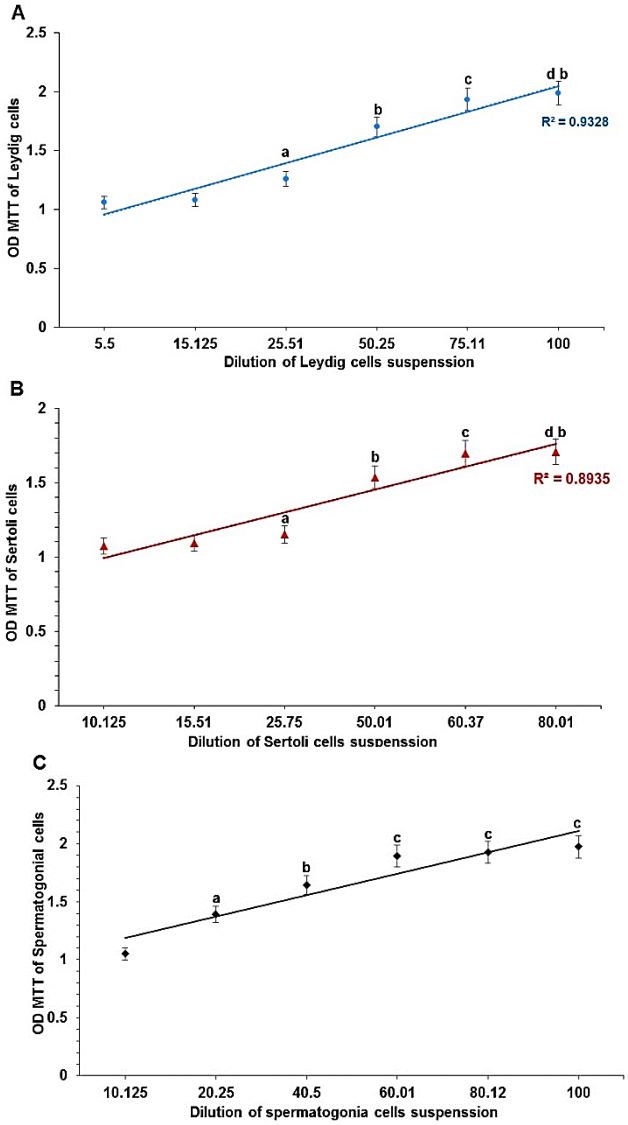


Figure 9: The correlation between the viability of Leydig **A**, Sertoli **B**, Spermatogonia cells **C**, MTT with the same cells numbers of ram isolation

The small letter denoted difference $p < 0.05$ within group
n = 10 samples of culture cells
Data percent mean ± SE

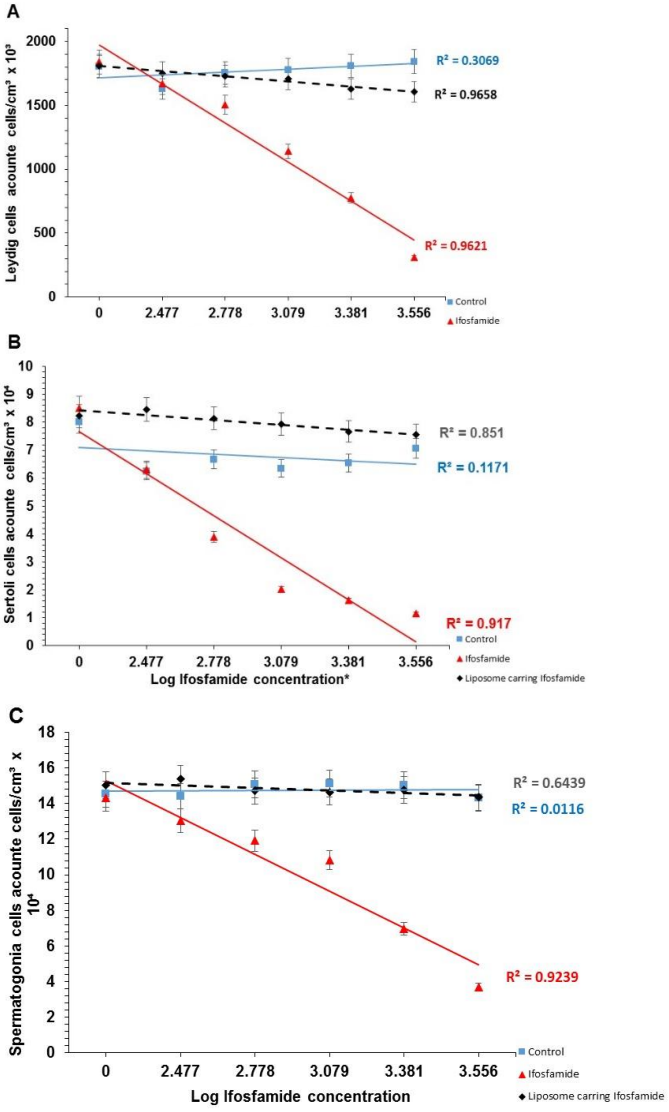


Figure 10: The Ifosfamide and Liposome carrying Ifosfamide effect on number of ram Leydig **A**; Sertoli **B**; Spermatogonia cells **C**, *in vitro* culture

The effect of Ifosfamide, Liposome carrying Ifosfamide on Number of Cells

The effect of increasing concentration of Ifosfamide on cell count treated Leydig, Sertoli, and Spermatogonia cells inoculated for 24 hours was studied, the harvested cell count displayed significant ($p<0.05$) decrease in cell number and deprived cell growth as compared with control (Figure 11. A, B, C).

On the other hand, all harvested cells treated of Ifosfamide-Liposomal formula inoculation had study growth and proven normal cell counting as compared with control. The hazardous effect was determined by IC_{50} show.

The effect of Ifosfamide, Liposome carring Ifosfamide on viability of cells with MTT assay

Effect increasing concentration of Ifosfamide on cell viability treated Leydig, Sertoli, and Spermatogonia cells incubated for 24 hours was investigated, the harvested cells showed significant reduction ($p<0.05$) and diminished of the viable cell count as compared with control growth cell Fig. (12) and (13). The Liposome carrying Ifosfamide displayed no significant ($p>0.05$) difference in comparison with control. The Liposome formula upset the topical effect of Ifosfamide and act of a cell.

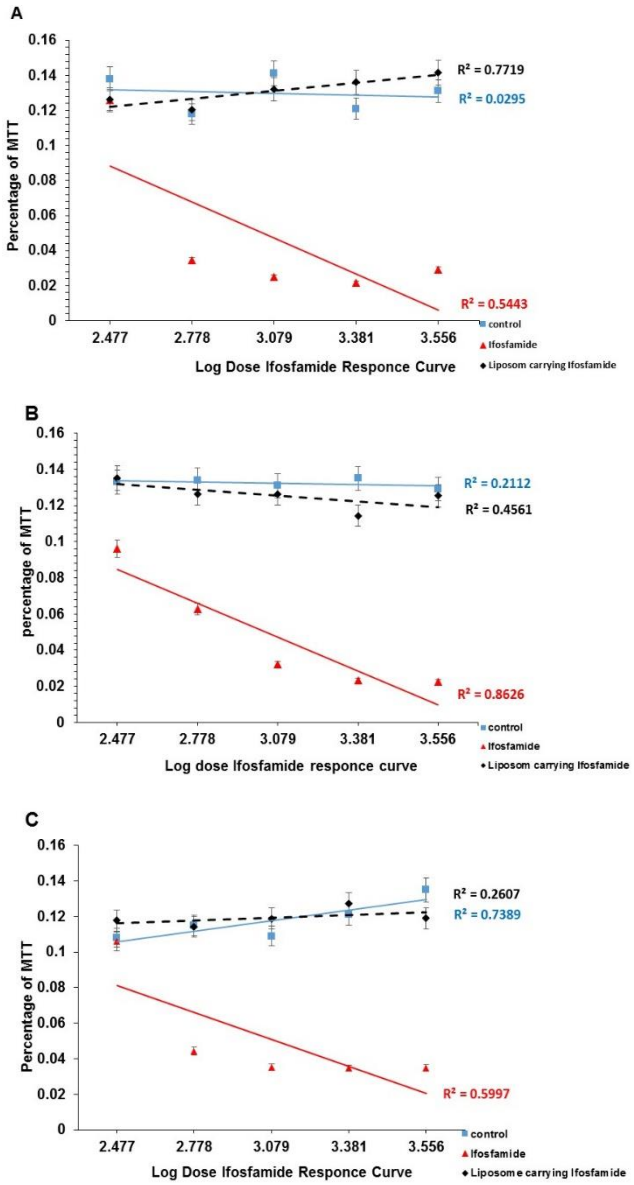


Figure 11: The Ifosfamide and Liposome carrying Ifosfamide effect on viability of Ram Leydig **A**; Sertoli **B**; Spermatogonia cells **C**, "MTT" *in vitro* culture

Data presented are mean ± SE
n = 10

The effect of Ifosfamide and Liposome carring Ifosfamide on steroedogenesis

The testosterone is produced under LH trigger. With previous incubation Ifosfamide in Leydig cells (fig. 14), showed significant reduction in testosterone level in culture media as concentration dependent manners and compared with control as well the Liposome – Ifosfamide formula showed normalized of testosterone level as in control showed no significant (p>0.05) on the otherwise the bad prognosis performance degree of Ifosfamide rated as IC_{50} = 3.049 lower than liposome formula. IC_{50} = 3.507 produce more significant (p<0.05) Liposomal type and a chive protect cell from Ifosfamide toxic effect.

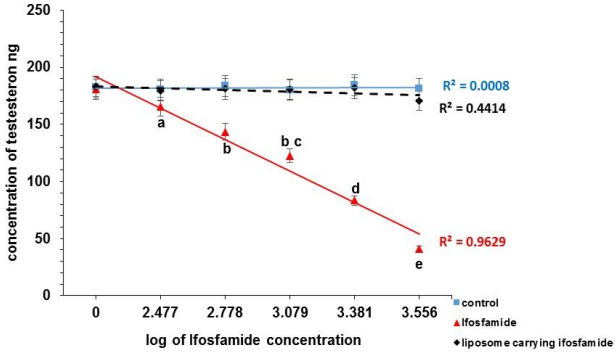


Figure 12: The Ifosfamide and Liposome carrying Ifosfamide effect on testosterone level in ram Leydig cells *in vitro* culture

Morphology of Leydig, Sertoli and Spermatogonia cells

Normal Leydig cells showed more granulated and irregular shape and basic large nuclei. The borders of cultured Sertoli cells appeared wrinkled and irregular with spaces or concavities similar to *in vivo* conditions, with less cytoplasmic area per cell. Nucleus size appeared larger than gonadal Sertoli cells the active form. Spermatogonia cells seemed aggregated with small and large round regular shape (figure 15. A, B, C).

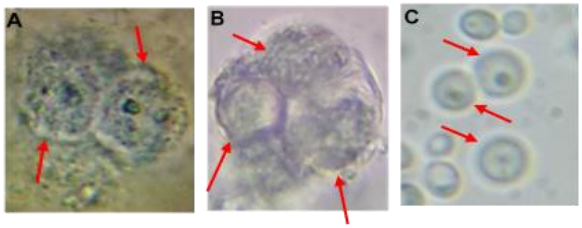


Figure 13: Morphological appearance of Leydig A, 100µm; Sertoli B, 150µm; and Spermatogonia C, 300µm cells.

Leydig cell marker 3*β*-HSD

Catalyzeation specific enzyme (3β-Hydroxysteroid dehydrogenase/Δ5-4 isomerase) (3β-HSD) use to converts the oxidative pathways of biosynthesis of classical steroidal hormones by blocking conversion of androstenediol to testosterone as a specific function of Leydig cell (figure 16)

Figure 14: Leydig cells with specific 3β-HSD enzyme. 20µm

Oil Red O of Sertoli phagocytosis cells

Evaluation of the phagocytosis function evaluate of Sertoli cells activity *in vitro* observed through detecting lipid droplets formation after the phagocytosis of apoptotic cells by using Oil Red O (ORO) staining. Using this method, the investigation of Sertoli cells phagocytic function of Sertoli cells at different development stages after treated culture

cells with Ifosfamide and Liposome carring Ifosfamide compared with control showed in figure 15.

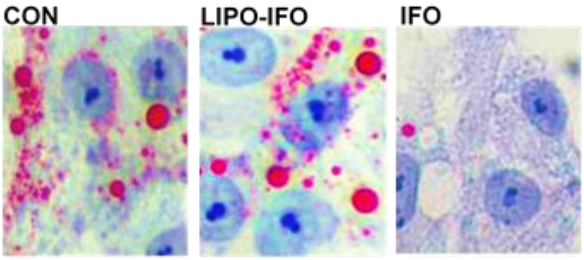


Figure 15: The specific detection and effect of "high dose" of Ifosfamide **IFO;** and Liposome carrying Ifosfamide **LIPO-IFO**, by using Oil Red O phagocytosis of Sertoli cell function and comparison with control **CON**.

Spermatogonia (UCHL) marker

In vitro, spermatogonia cells frequently underwent asymmetric divisions, which characterized by unequal segregation of anti-ubiquitin C-terminal hydroxylase UCH-L1. Also, expression level of UCH-L1 in the immature testis, where spermatogenesis was not complete affected by the location of germ cells relative to the BM. Whereas UCH-L1-positive spermatogonia were exclusively located at the basement membrane BM in the adult testis. Asymmetric division of spermatogonial stem cells SSCs appeared to be affected by interaction with supporting somatic cells and extracelluar matrix. These findings at the first time provide direct evidence for existence of asymmetric division during spermatogonial stem cells SSCs self-renewal and differentiation in mammalian spermatogenesis.

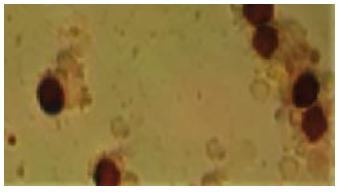


Figure 16: UCHL marker of Spermatogonia cells in culture media. 20µm

Mitochonderial Integrity

In addition the ATP changes in treated groups of culture cells were declain the mitochondrial activity. (fig. 19), and (table 2) Leydig, Sertoli, and Spermatogonia cells respectively displayed in Log dose-Mitochondria activity behavior in growing cell challenge against Ifosfamide and Liposome carrying Ifosfamide formula treatment. Increasing concentration of Ifosfamide to testes cells resulted in decreased mitochondrial activity in a concentration dependent manner the correlations as r^2 = (0.978), (0.9419), and (0.7984) significantly ($p< 0.05$). Liposomal encapsulated Ifosfamide worked as a covering protector to the inoculated cells from disruption of mitochondrial integrity.

Furthermore, the IC_{50} of Ifosfamide was (2.851 ± 0.058, 3.142 ± 0.168, and 3.010 ± 0.209) for Leydig, Sertoli, and Spermatogonia cells, respectively which is lower than liposomal type, which significantly ($p<0.05$) become out of IC_{50} (3.444 ± 0.187, 3.500± 0.207, 3.474 ± 0.158) for Leydig, Sertoli, and Spermatogonia cells respectively compromised Ifosfamide worked effect.

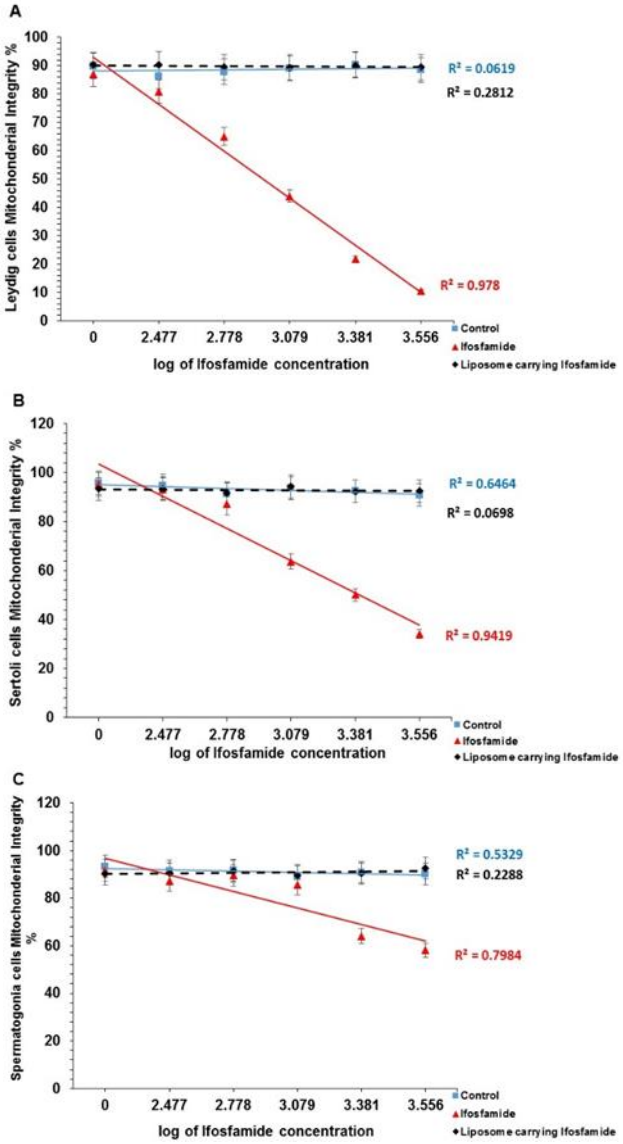


Figure 17: The Log Concentration– Mitochondrial Integrity linearity curve of serial dilutions of ordinary Ifosfamide formula and liposomal form carrying Ifosfamide in ram Leydig **A**; Sertoli **B;** and Spermatogonia cells **C**, *in vitro* growth cells culture.

Mito-tracker

The ability of Ifosfamide and Liposome carrying Ifosfamide to effective mitochondrial morphology and activity (ATP concentration synthesis) in Leydig, Sertoli, and Spermatogonia cells and to confirm its altered mitochondrial morphology was described in figure 18. The cells cultured *in vitro* stained the mitochondria with the probe Mitotracker observed in Green and Red filter. The result showed that was reduction in mitochondrial morphology (mass) of Ifosfamide treated Leydig, Sertoli, and Spermatogonia cells in (green filter) and activity in (red filter) compare with control and Liposome carrying Ifosfamide.

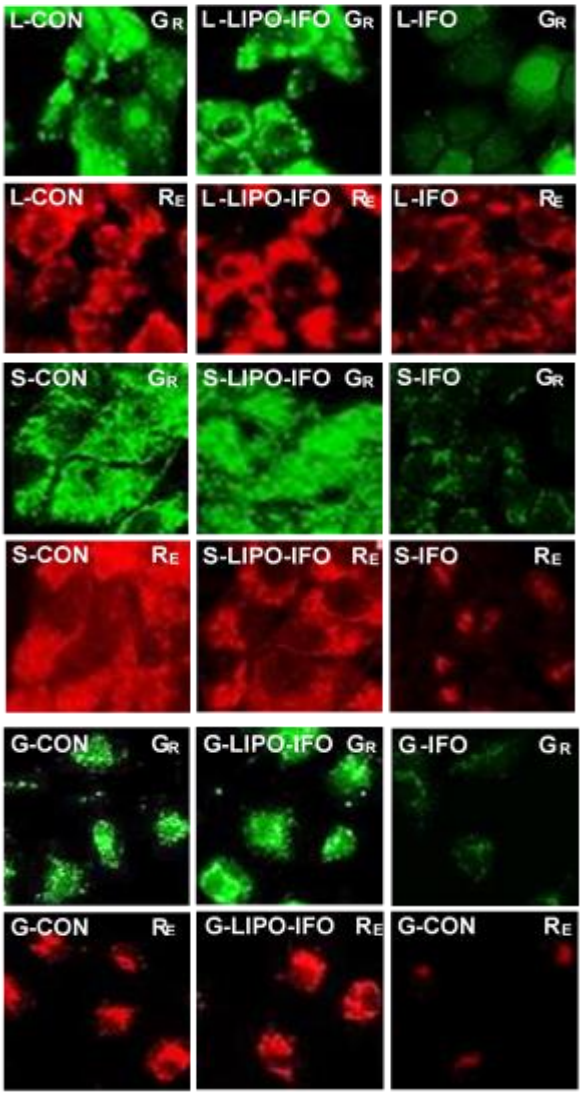


Figure 18: The effect of Ifosfamide **IFO**; and Liposome carrying Ifosfamide **LIPO-IFO**; in Mitotracker filter "green $\mathbf{G_R}$ membrane display; and red $\mathbf{R_E}$ mitochondrial activity"; compared with control **CON**; on culturing of ram Leydig **L**; Sertoli **S**; and Spermatogonia cells **G**.

Membrane Integrity

The membrane integrity affected by Ifosfamide and Ifosfamide carrying Liposome in Leydig, Sertoli, and Spermatogonia cells. The Ifosfamide formulation is presented of membranous integrity in Leydig, Sertoli, and Spermatogonia cells were showed in (fig. 19).

The marked reduction in the percentage of membrane integrity gradually proportioned with concentration increase r^2 = (0.927, 0.9803, and 0.9393), whereas the liposome carrying Ifosfamide formula displayed a significant ($P<0.05$) protection of cell membrane. Percentage of damaged integrity was not significant ($p>0.05$) in cells treated with Ifosfamide groups relative to the Liposomal formula and control groups.

Cells types	Ifosfamide IC_{50}	Liposome carrying Ifosfamide IC_{50}
Leydig	2.851	3.444
Sertoli	3.142	3.500
Spermatogonia	3.010	3.474

Table 2: IC50 of Cell mitochondrial activity parameter

Cells types	Ifosfamide IC_{50}	Liposome carrying Ifosfamide IC_{50}
Leydig	3.095	3.440
Sertoli	2.897	3.325
Spermatogonia	3.104	3.269

Table 3: IC50 of membrane integrity parameter

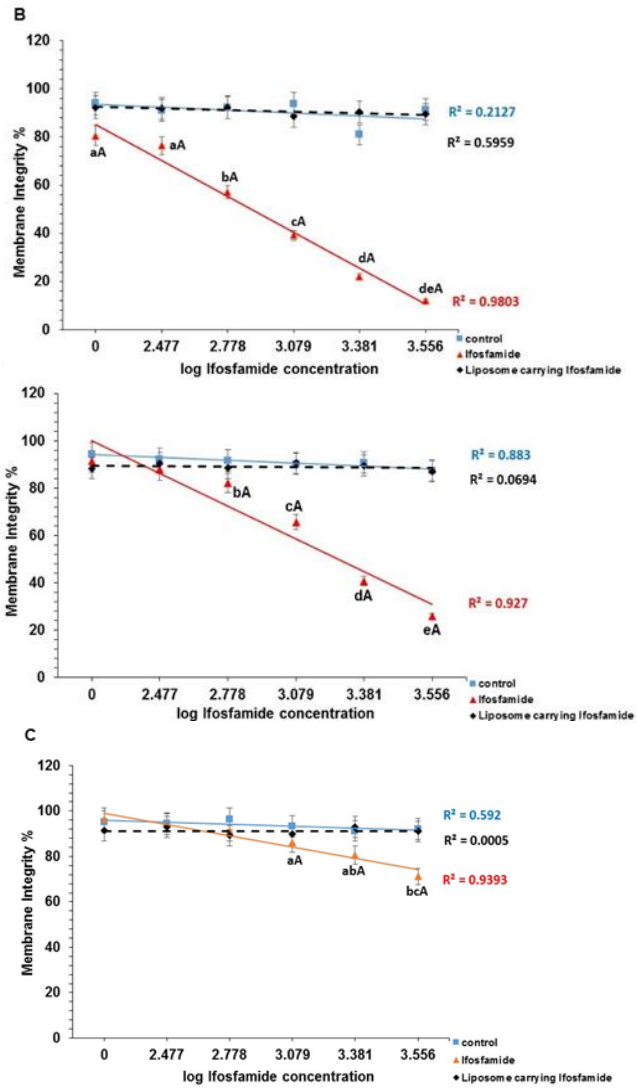


Figure 19: The Log concentration – Membrane Integrity linearity curve of serial dilutions of ordinary Ifosfamide formula and liposomal form carrying Ifosfamide in ram Leydig **A**; Sertoli **B**; and Spermatogonia cells **C,** *in vitro* growth cells culture

The small letter denotes difference ($p<0.05$) with treated concentration of culture cell

The capital letter denotes difference ($p<0.05$) between Ifosfamide formulas treated concentration of culture cell

Data are presented as mean ± SE

The cell membrane integrity was evaluated in Leydig, Sertoli, Spermatogonia cells previously incubated with Ifosfamide or Ifosfamide encapsulated Liposome and exposed to increased concentrations of Ifosfamide (300, 600, 1200, 2400, and

3600) µM.

However, the addition of Ifosfamide to the incubated medium did cause reduction in membrane integrity in a concentration – dependent manner (figures 12, A, B, and C) a significant (p<0.05) decrease in the integrity was observed with above second Ifosfamide concentration.

The addition of Liposome carrying Ifosfamide to the incubated culture medium of certain testicular cells (Leydig, Sertoli, and Spermatogonia cells) did not result in a significant difference (p>0.05) in integrity of membrane as compared with control group. *Toxic* effect of Ifosfamide is dominancy of potency IC_{50} than Liposomal-Ifosfamide formula (table 3) and proved resistance of cell exposure to Liposome – Ifosfamide formula.

ATP Concentrations

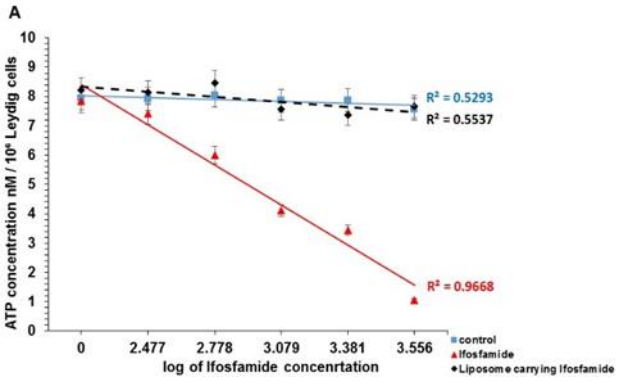
The effect of Ifosfamide and Ifosfamide formulated Liposome on ATP levels was evaluated in the presence or absence of Ifosfamide with vehicle of drugs showed in (Fig. 20).

The addition of increasing concentration of Ifosfamide to Leydig, Sertoli, and Spermatogonia cells separately. (300, 600, 1200, 2400, and 3600) resulted a decrease in the ATP level in a concentration and dose dependent manner (r^2= 0.9668, 0.8427, and 0.9661) to Leydig, Sertoli, and Spermatogonia cells, respectively. Whereas the effect of Ifosfamide formulated Liposome showed quietness variable no significant (p<0.05) between concentrations as compared with control and followed their softness state growth behavior.

Cells Types	Ifosfamide IC_{50}	Liposome carrying Ifosfamide IC_{50}
Leydig	3.175	3.510
Sertoli	2.807	3.551
Spermatogonia	2.762	3.467

Table 4. IC_{50} of ATP concentration % parameter

On the otherwise the IC_{50} of Ifosfamide in Leydig, Sertoli, and Spermatogonia cells was (2.986±0.056, 3.181±0.058, 2.975±0.062) respectively which was more potent than Liposomal formula to same cells IC_{50} (3.333±0.172, 3.476±0.147, 3.046±0.0995). This suggested that the Liposome capsulated drug is the main drug to protect cells, by reducing toxic effect of isolated cells.



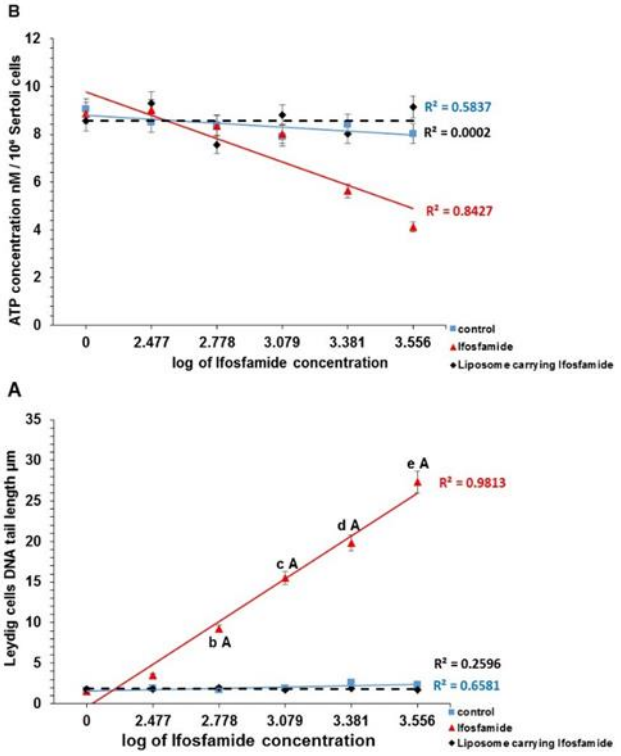


Figure 20: The Log Concentration-ATP concentration nM / 10^6 linearity curve of serial dilutions of ordinary Ifosfamide formula and liposomal form carrying Ifosfamide in ram Leydig **A**; Sertoli **B**; and Spermatogonia cells **C.** *in vitro* growth cell culture

DNA Quantity "COMET assay

following 24 hours of incubation time, DNA tail length was evaluated by COMET assay in Leydig, Sertoli, and Spermatogonia cells in the absence (control) and previously incubated with Ifosfamide and Liposome carrying Ifosfamide formula at concentration (300, 600, 1200, 2400, and 3600 µM) increasing significant (p<0.05) tail fragment length of DNA as concentration dependent. Whereas the DNA tail length did not change following the incremental addition concentrations of Liposome carrying Ifosfamide as compared with control incubated cells. (Fig. 21), and (Fig. 22)

Exposure of cells to The Ifosfamide was more toxic than Liposome formula. When studying IC_{50} to distinguished tonic potencies sorted severity.

Cells types	Ifosfamide IC_{50}	Liposome carrying Ifosfamide IC_{50}
Leydig	2.986	3.333
Sertoli	3.181	3.476
Spermatogonia	2.975	3.046

Table 5: IC_{50} of DNA tail length parameter

Cells types	Ifosfamide IC_{50}	Liposome carrying Ifosfamide IC_{50}
Leydig	2.900	3.289
Sertoli	2.952	3.545
Spermatogonia	2.801	3.479

Table 6: IC_{50} of DNA tail number parameter

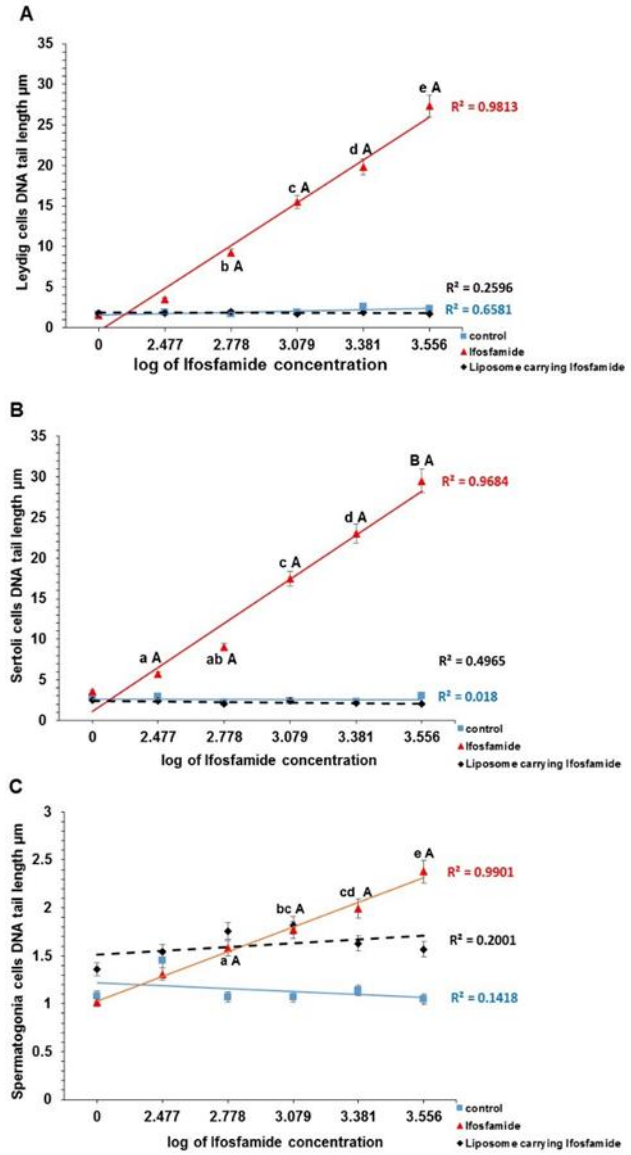


Figure 21: Effect of Ifosfamide and Liposome carrying Ifosfamide on DNA tail length of ram Leydig **A**; Sertoli **B**; and Spermatogonia cells **C**. *in vitro* culture

The small letter denotes difference $p<0.05$ with treated concentration of culture cell
The capital letter denotes difference $p>0.05$ between Ifosfamide formula treated concentration of culture cell
Data are presented as mean ± SE

To further underline the impact of Ifosfamide and Liposome carrying Ifosfamide on different tested testicular cells including: Leydig, Sertoli, and Spermatogonia cells the evaluated Ifosfamide effectively when facing pre-incubated cells were significant ($p < 0.05$) increase DNA Tail Incidence percent as compared with control and Ifosfamide-Liposome formula exposure and deal with protectant effect of Liposome technique to the cell there were not have significant ($p > 0.05$) as compared with control incubated cells. The protect event through Liposome was more obvious represented IC_{50} as fallows.

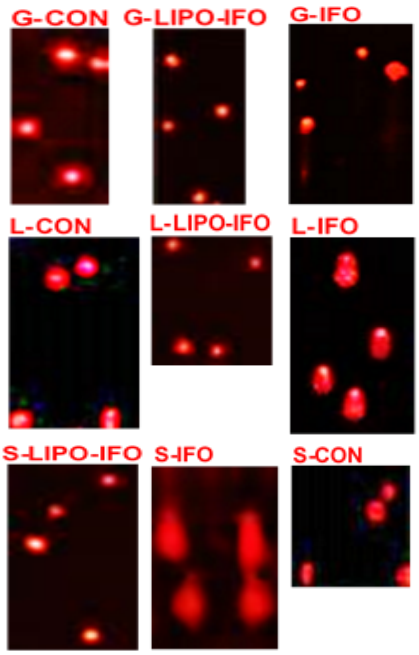


Figure 22. Effect of Ifosfamide **IFO**; and Liposome carrying Ifosfamide **LIPO-IFO**; on the DNA defect level in ram Leydig **L**; Sertoli **S**; and Spermatogonia cells **G**; compared with control **CON**, by using gel electrophoresis technique; COMET.

Discussion

The pharmaceutical manufacturing affords newfangled of different cytotoxic medicinal agents the incidence of adverse effects still rising. It has been stated these unwanted hazardous effects that cause a several predicted and/or unpredicted reproductive performance upset, wide worse effect. The practical use of the standardized medicinal remedy with "conventional chemotherapeutic agents" is abundant and may consequence in several limitations (34). Obtaining desired therapeutic bioavailability in target tissues is the aim to achieve, however the side effects are the main problems of today's chemotherapy. Targeting exploration skills and methodological pharmaceutics techniques to acquiring developed drug carriers for improving the selective toxicity criteria increase alongside approved wideness of therapeutic index of the medication agents that a priority (35).

Liposomes alongside other structures are ideal medicament carrier suggested to use liposomes in anticancer therapy. The interest of liposomes is steadily increasing based on controlled drug release and uptake by normal cells with a release profile matching the pharmacodynamics of the drug (36); fundamental conditions prevent unwanted effects on testicular cell functional properties in this objective of the project.

At The beginning, the liposomal features give a new modern model as additional compartment with a delaying

offer of drug release as well as the geometrical size of liposomal entrapped Ifosfamide in an aqueous medium had to be designated to enable potential applications of liposome programmed as drug carriers (37). Generally followed the vesicle size and composition are a critical parameter for determining the circulation half-life of liposomes, the prepared liposomes were designed uni-laminar and bilaminar. Liposomal size 60-120 µm influences the degree of drug encapsulation in liposomes table 1.

The small size of liposomes composed part of prolonged t½ (38, 39). The Ifosfamide liposomal entrapped 92.75±5.49, that attribution may be prolonged t½ in culture media through provide a non-direct dispensable to the culture cells (40), which were act one of protect growth curve from modifications or troubled of the maintenance growing cell culture behaviors of Leydig, Sertoli and Spermatogonia cells in contrast decline of cells in ifosfamide-treated cells compared with liposomally entrapped ifosfamide and control cells, that mirrored to cells viability percent complain of the loss of exponential Log phase as feeding concept in cell growth after 6, 12 and 24 hours of Sertoli spermatogonia and Leydig cells respectively. On otherwise the Ifosfamide carrying liposome formula may be presumably blocked the unspecified socket groups of the common binding site of these cells.

Nekkanti and Kalepu, 2015 (39) were approved the protective care of liposome through achievement potent therapeutic medicine with a "narrow therapeutic index" mostly require a targeted cell or binding site specific delivery. This can be attained primarily by using a ‘carrier-mediated drug delivery system' that's including liposomes.

Furthermore, the small unilamellar liposome carrier was amplified safety as well as efficacy, highly improved stability. The encapsulation efficiency and releasing drug kinetics are influenced by the number of Liposome Lamellar. The liposome uptake and intercellular fate are affected by the lamellarity (41), that means the small size of the liposome is more stable and drug become less toxic indicated by cells viability, It's counted one of the protective technique of Leydig, Sertoli, and Spermatogonia cells from hazardous effects of Ifosfamide (40,41,42).

The liposome may be also working a transporter drug, artificial barrier allocated in transmembrane facilitated transport and active transporters or pumps coupled with hydrolysis of ATP and the resulting free energy to the movement of molecules across membranes against a chemical concentration gradient (44, 45).

ATP-binding cassette (ABC) transporters are efflux pumps that derive the energy needed for Ifosfamide extrusion from the hydrolysis of ATP, the ABC transporter residing in pharmacological barriers. The accommodation of steeply decline viability in growth curve outside all or none category for cell death behavior may be attributed to qualifying the enzymatic limiting rating of ATP of duel Walker A and a Walker B motif of ABC transporter active transport, ATP decreased; The active transporters or pumps couple the hydrolysis of ATP and the resulting free energy to movement of molecules across membranes against a chemical concentration gradient (46, 47). For this, the drop of cell viability was may be actually affected in limiting orchestrating system. The further informative provider may be playing a role in deprived Ifosfamide cell entry through ABC transportation actively (48).

There was determinately scored the aggressively of alkalization Ifosfamide effects on growing curve pattern and viability. The drop of a growth curve is accelerated their rate was presumably Ifosfamide it's an alkylating anticancer mode of action. Through the active forms alkyl adducts with DNA act as an ifosfamide aziridinium intermediate metabolite. The alkylation of DNA produced DNA damage, and finally cell death (49) and diminished growing behavior curve.

Ifosfamide may be exerted their DNA alkylation harmful effects the dual pathways first convert to 4-Hydroxyifosfamide transports the plasma membrane of the cell and spontaneously forms Aldo ifosfamide as a reversible reaction Aldo ifosfamide can decompose into acrolein and ifosfamide mustard. The second pathway is ifosfamide exhibits in mediated by the metabolite chlororacetaldehyde has its own cytotoxic profile and was at least as effective as the supposed main metabolite 4-Hydroxy-IFO (50, 75).

Ifosfamide, a pro drug that requires bioactivation the cytochrome P450 (CYP) system to exert its adverse effects (51). The initial cell activation step of ifosfamide to its pharmacologically dynamically active molecule is 4-hydroxylation to 4-hydroxyifosfamide. Activation of ifosfamide to 4-hydroxyifosfamide is catalyzed by the cytochrome P450 CYP isoforms both CYP3A4 and CYP3A5 is mainly accountable for the transformation of ifosfamide into active form through autoinduction, These CYPs isoforms were detected in testicular cells and encoded the metabolic pathway for the xenobiotic as well as may be attributed the adverse effect of Ifosfamide metabolite adverse effects. Reasonably, Ifosfamide "autoinducers" its own biotransformation by activating of PXR xenobiotic receptor act as coded gene by NR1I2, That mediates auto-induction through transcriptional upregulation of YP3A4 (52).

Furthermore, the effect extended to presume the ifosfamide ability to "autoinducer" suggests that its increase metabolism rate over time of exposure. Ifosfamide metabolism by CYP3A4 led to more toxic metabolites (53). Once formed, 4- hydroxy ifosfamide is unstable and rapidly interconverts with its cells, aldoifosfamide, or is oxidized by alcohol dehydrogenase into 4-keto-4-hydroxyifosfamide (54).

In the nucleus, ifosfamide mustard is converted into the chemically reactive Carbonium ion through Imonium ion in alkaline or neutral pH. This compound may be direct bind by N7 of the guanine DNA residue forming a covalent link. The second arm in phosphoramide mustard may be direct interact with a second guanine moiety in an opposite DNA strand or in the same strand to form cross-link, presumbly the cell genetic code disrupted and uncoupling biosynthesis, as well as promotion pro-cell death sign and progress necrosis via the DNA strand, breaks result may in an inability to synthesize DNA, and led to cell death mediated via the caspase cascade (55).

Ifosfamide treatment interaction with caspase cascade as mediated cell death. Ifosfamide is an activation and increases gene expression of each caspase 3, 8, and 9. Whereas, it was presumably suggested diminutions the expression of BCL2, as a caspase inhibitor and belonging blocking the release of cytochrome c from the mitochondria. On the otherwise Ifosfamide may be activated BAX and BAK, which are pro-cytochrome c releasers (56, 57).

The bifunctional alkylation of DNA is supposed to be the chief mechanism of Ifosfamide mediated anticancer effect (58). Which were suggested the primer causes of reduction of viable cell and scale of time Ifosfamide exposure suppress and overturn growing curve and lost the feeding pattern in depict.

The Ifosfamide metabolite may be augmented with several harmful effects proposed on cultured cells showed as IPM may be proof interacts with proliferation in dramatic cascade pathways by both modifying MAP kinase signaling by downregulating expressing genes associated essentially with proliferation control and downregulating of key fact protein "heat shock protein" activity (59, 60).

Furthermore, Ifosfamide-IPM metabolic system was presumably accelerated the redox and reaction produced free radical may be through downregulates TXNRD1 in the NRF-2 pathway, responsible for oxidative stress response, Associated with reduction of thioredoxin reductase activity by ifosfamide, the cells was become incompetent to either eradicated removal the increment of oxidative stress or transcribe proteins involved in the proliferation pathways (61, 62).

Additionally, the bad end worse IPM causes an exhaustion of intracellular glutathione (GSH) function, a major antioxidant. Multiple Ifosfamide metabolites can react with GSH, resulting in the formation of various conjugates at different sites along the pathway (63). One of them, GSH conjugates with toxic metabolites, the lessening in the levels of GSH consequences in amplified harmfulness (64, 65, and 66).

The abundant information about cell resistance to drug, but less fact carrying an opinion viewing the different between cells that may be genetic resource and functional barring membrane phenotype approved kinetic bioavailability of toxic concentration of drug variable response represented as IC_{50} among the cells that directly causal fact of different of curve behavior of cell drop viability.

The mitochondrial functional properties results showed reduction of mitochondrial activity and subsequent decrease ATP in all cells in Ifosfamide treated group. This may be attributed direct effect of Ifosfamide on carnitine then abolishment ability to hydrogen schedule approved reduction in the activity and followed by diminished ATP (67, 68). On the other wise may be ROS ifosfamied derived defect complicated with organelles and specified to targeted metabolic process enzymes like lipid peroxidation and glucose-carboxylic cycle and promote directed mislaid ATP or progressed lost mitochondrial functional and disabilities of continuous properties (69).

Sprigate *et al*., 1997 (70) suggested the cell damage from Ifosamide metabolite CAA is related to glutathione depletion and mitochondrial toxicity with ATP depletion. That coincided with our results decrease in viability, mitochondrial activity and yielded ATP depletion (63, 64).

In addition may be accredited the defect of organize cells aggrieved by inhibits several Sodium-dependent transport systems as well as the sodium/proton anti-port system (68, 71). While total loss of cell viability would led to induced advance proses cell necrotizing and lost number of cell as well as lost behavioral growth curve, an unusual but devastating ifosfamide side-effect (72) that showed in (figure 18 and 19 and table 3 there were high correlation between viability and ATP and Mitochondrial activity (73).

In the present study also, exposure testicular cultured cells to Ifosfamide-CAA proposed produced reductions in cellular glutathione content. This is consistent with previous work display that ifosfamide metabolites exhaust glutathione in cells tissues (70, 74). In addition, the toxicity of Ifosfamide-CAA was enhanced in glutathione-depleted cells (75).

Therefore, the aggressive properties of Ifosfamied on cell viability more than other depended on multiple sit of action on molecular aspect of cell and then lost functional duty that seen in Leydig cells loss of testosterone production and Sertoli cells lipid formation as well as deprived spermatogonia corrected production of lactate dehydrogenase as well as the mechanism underlying this difference between cells may relate to changes in enzyme levels during exposure to parent ifosfamide drug, or might reflect changes in the activity of activating enzymes following exposure to toxic metabolites.

The evidence of drop levels of testosterone in treated Leydig cell culture treated Ifosfamide was due to decrease the viability as a total collective concentration of cells in media (76) and highly correlation compose between Leydig cell viability and testosterone.

A short test hint for a primer studies the Ifosfamide induce membrane defect and lost some properties of membrane activity (77) that may be supposed the Ifosfamide induce noteworthy reversible depolarization and decrease in conductance of membrane, consequently, loss of membrane conductance. Contribute the functional activity to demine the osmosis and the activity of testosterone synthesis, phagocytosis, and proliferative activity to Leydig cells, Sertoli cells and Spermatogonial cells respectively. Commonly, that facilitated to induce lost continuous integrity and fluidity of membrane then fated degeneration of cells. This result, in instances with the result, of mitotraker probe stain for cell membrane integrity (figure 18) the Ifosfamide treated groups suffered from membrane distractive features and partial segmented of membrane in different cells.

The Mitotraker result , of membrane integrity presumably due to lost a portion phenomenon of the cell, under Ifosfamide exposure this effect is may be through to be mediated by loss of membrane stabilization (74) Ifosfamide powerful reduction of inter cellular glutathione and redox potential, that tensely aggressive of free radical accumulation and targeted membrane protein – phospholipid . Then deprived fluidity and elasticity then

derived segmental aplasia of membrane.

The energy decrease due to oxidation of Ifosfamide generated chloroacytl-CoA; generated from chloroetaldehyde during side chain formation and prevent carnitine to transport acyl-CoA groups into the mitochondria to start the energy production (78, 79).

That agrees with the result, of mitochondrial integrity direct proportional Ifosfamide exposed concentration with reduction mitochondrial integrity in all tested cells.

Furthermore the testosterone synthesis and mobility be contingent oxidative phosphorylation NADP-NADPH system apparently mitochondrial serving activity of steroidogenesis rough endoplasmic reticulum (80) may be reduction of testosterone concentration due to Ifosfamide and their metabolite via suppress mitochondria-ATP supplier energy led to missing demined energy for steroidogenic activity belonging end point upset testosterone.

The functional properties of Sertoli cells demonstrated by ORO staining technique and challenged not accepted 3β-HSD stain and Alkaline phosphatase that displayed the phagocytosis properties specified for Sertoli cells, suspect the isolated Sertoli cells may possess the characteristic lipid droplets (81) under the employed culture conditions. Thus, the purity of Sertoli cells in these cultures was probably greater than indicated by the percentage of Oil-Red positive cells. The procedure used 37°C for enzymatic digestion. Although most of the enzymatic digestions of testicular tissue are done at 32°-34 C, it is known that Sertoli cell functions do not deteriorate at higher (37°-38°C) temperatures (82,83).

Phagocytic ability of Sertoli cells in different treated cultured Sertoli cells at certain concentrations of ifosfamide and liposomal carrying ifosfamide, expressed as area ratio of lipid droplets to nuclei

It was expected that the different functions would be expressed by Sertoli cells treated ifosfamide liposomal entrapped when compared with ifosfamide treated Sertoli cells, there is a highly differences of definitive comparative area ratio of ORO staining Sertoli cells. The analyzed the lipid-droplet formation in Sertoli cells isolated from ram of different as a reflected an effort to the phagocytic capacity of Sertoli cells in different groups, a similar phagocytic capacity of Sertoli cells at control and Ifosfamide.

The methods for *in vitro* investigation of the phagocytosis of apoptotic spermatogenic cells by Sertoli cells face a problem that it is complicated to proposed their specified functional marker of Sertoli cells phagocytosed ability categories by Sertoli cells that stage-dependent formation, also lipid droplets in Sertoli cells was associated with phagocytosis of residual bodies (84, 85, 86, 87) and apoptotic germ cells (88, 89, 90).

On the other wise it should be noted that a quantitative relationship between measures of degree phagocytic activity and amount of lipid droplets has been established for this reason the presence droplets marks for function (91, 92).

The mechanism of lipid droplet formation after phagocytosis has been explained in two different mechanisms. (93) Assumed that an accretion of lipid droplets as marks of phagocytosis of residual bodies signified the re-synthesis of lipids by Sertoli cells, whereas (85) speculated projected that the lipid droplets were last remnants of digested residual bodies. This in vitro system may provide an applied approach to approve the way of the lipid droplet formation by blocking the pathway to synthesize lipid in Sertoli cells. According to that the ifosfamide prevented formation of internal membrane system of phagocytized cells determined the formation of lipid droplets, including rich lipids, such as mitochondria and Golgi apparatus, represented in tissue culture as unviable cells during growth cells.

The ifosfamide treated Sertoli cells may be produced comparative antagonist with the detrimental site of droplet formation at ATP-binding cassette transporter 1 (ABCA1), a transporter presumably exhaustion of ATP formation that shuttled excess cholesterol and phospholipids of the cells (94), and multifunctional protein 2 (MFP-2), which was associated with peroxisomal β-oxidation (95). Thus, balances of the metabolism of lipids in Sertoli cell is disturbance and lost maintain normal activity.

Light microscopic examinations indicated that the cytoplasm and nuclei of Sertoli cells isolated - cultured from of ifosfamide treatment were smaller than those from control and Liposomal entrapped ifosfamide which were indicated to spermatogenetically active, as well as showed complex cytoplasmic processes presumably corresponding to processes that surround the germ cells *in vivo* (90,96,97).

The DNA fragmentation may be attributed to Ifosfamide alkylating effects mechanisms of cells with narrowing selective effect specified with the functional turnovers cells (98).

The result showed mostly hazardous effect of ifosfamide in COMET parameters (figure 21, 22) extensive tail incidence and length in all concentration of ifosfamide treated cells groups as compared with control and no differences see in liposomal entrapped ifosfamide and control cells cultures.

The role of Ifosfamide exposure testicular cell culture has been the subject of some controversy (99,100). While an excessive agreement of research has focused on the metabolism of Ifosfamide in relative to adverse belongings on testicular cells such as loss of functional properties of spermatogenic cells and endocrine with supported cells (101) relatively little is known concerning the relationship between pro pharmacology of ifosfamide and the drug interaction with its direct molecular cells components, that is referred to DNA. Understanding of such relationships is complicated by the fact that Ifosfamide is a pro drug.

While it would be more relevant to measure the degree of DNA damage in the tissue culture cells, this is practical in most laboratory of tissue culture cell scenarios and there are both scope view and reliable technical identified serious distinguish end point of effects associated with such measurements. DNA estimated had one perfect success applying COMET analysis to cells (30). The COMET technique used for identifies DNA strand breaks (102), Ifosfamide is thought to act by creating inter-strand cross-

links in double DNA strands cods and a process had been established to use the COMET assay technique to degree of crosslinks directly and fragmentation of nucleic acids bases compounds (103).

The COMET assay may provide useful information on the pharmacology of IFO. Fractionating, the concentrations increased of ifosfamide to results in a greater degree of DNA damage than lower concentrations of the same dose. Also, for cells treated IFO was a direct correlation between overall DNA damage and the concentration of the parent drug (103), in addition to the strand breaks as detected here.

However, the ultimate alkylating species produced by the metabolism of ifosfamide. Treatment was produced a high level of DNA strand breakage, presumably as a result of its breakdown to produce acrolein, in addition to isophosphoramide mustard. Acrolein has been shown previously to produce DNA single strand (104). Strand breaks interfere with the accurate measurement of DNA cross-linking in the comet assay (103).

Furthermore, glutathione regulated multi-drug resistance could be overcome by chloroacetaldehyde-induced depletion. These findings were confirmed by the in vitro cytotoxicity of chloroacetaldehyde observed in human stem cells, solid tumour cells and leukaemia cell lines. Beside gluthatione depletion, inhibition of aldehyde dehydrogenase could also increase cytotoxicity (or overcome multi-drug resistance), because it can metabolize 4-hydroxyifosfamide to inactive compounds (105).

The study conclude admitted using liposome encapsulated ifosfamide as protected the testicular cells from the harmful effect of free anticancer ifosfamide and attenuation the exposer to the cell culture, and attenuation the mode of action in proliferative behavior of cells.

These features of liposomal formulation of ifosfamide assist, reduction of adverse effect and expansion of indication. This leads to an improvement of the quality of fertility in cancer patients via prevent targeted of spermatogenic and somatic cells in testis.

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