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Exploration of Viability of Ketorolac on Isolated Leydig Cells of Bucks (*Capra hircus*) *In Vitro*: Part I

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Abstract

The present study was conducted to clarify the effect of ketorolac (NSAID) on Leydig cells of Buck to achieve the ketorolac event through two experiments. First is to determine the survival time of Leydig cells to produce maximum viable growth; The second is to assess the viability using multiple ketorolac series concentration of 0.03-0.15 *in vitro*. The viability of proliferative cell was decreased as dose dependent due to the negative correlation of the cessation, transition and development from the G2 phase to the intermediate M phase of cell cycle, which is the stopover proliferation of cells and diminishes the viability of isolated buck Leydig cells. So the inhibition of NF-κB through ketorolac may be augmenting the chemo-sensitivity of Leydig cells to the DNA topoisomerase inhibitor, which was presumably induction of apoptosis shown by DNA mismatch repair proficiency covered disruption of cell metabolism.

Keywords: Captopril; Leukotriene B4; GnRH; Testosterone; Leydig cell; LTB4; Steroidogenic

Introduction

The medicinal analgesic agent's compromised nonsteroidal anti-inflammatory drugs diminution creation of eicosanoids derived prostaglandins through the interference with cyclooxygenase, which is the main enzyme that results in physiological yield of eicosanoids as referred mainly to prostaglandins [1]. New generations were developed in a form of carried by pharmaceutic drug store and we have very little information on their sexual effect and consequences after use. According to this fact, the ketorolac one of floating projected in highly extensive uses without wariness on the safety knowledge of side effect [2]. Ketorolac is a powerful analgesic agent of the non-steroidal, anti-inflammatory class (NSAID). It is a non-opioid type and has no known opioid receptors. Its method of activity increase the inhibition of the cyclo-oxygenase enzyme system and mentioned to the forward synthesis of prostaglandin and exhibits a minimal anti-inflammatory effect at its analgesic dose [3]. Ketorolac is potent antipyretic and anti-inflammatory. Ketorolac was used for the short term treatment of post-operative pain and different therapeutic maneuvers; it is highly selective for the COX-I enzyme [4]. The aim of this study was to assess the collection effect of the adding ketorolac on the isolated Leydig cell culture of the Buck testis on the following goals: Determine the Leydig cells viability and integrity of cell membranes and mitochondria efficiency.

Materials and Methods

Animals and testicular preparation

Ten testis samples "left and right of healthy adults (one year old, ~35 Kg) male goats were obtained from private slaughter house (Buhriz-Dyala province 45 Km east of Baghdad)" were used for this study. Premortal examination was performed on the animals before slaughter. Furthermore, it had a definite perceived vaccination program and external and internal parasite treatments list. The Buck testes were excised and transferred to cold EMDM media and transferred to the University of Baghdad, College of Veterinary Medicine- laboratory of pharmacology within 2 hours.

Steps for isolation of leydig cells

Dissociation and digestion. The Buck testes were collected for isolation and culture of the Leydig cells as follows:

De-capsulated testes from 10 Bucks and remove the epididymis: The de-capsulated testis was pre-incubated for 10 min in 100 ml of pre-chilled; freshly prepared 1:1 (Ham's F12 medium and Dulbecco's Modified Eagle's Medium "DMEM" with Sodium bicarbonate 1.2 g/L, with HEPES 15 mM, with Sodium Pyruvate 55 mg/l) at 34°C, the procedure described by Zhang and Cui [5].

Macro-section and sample preparation: The testis was cut to the right of the mid-sagittal plane, and testis pieces were further cut to smaller sections, saved in Ham's F12: DMEM medium. The pieces of the testicular tissue; 10 mg in the conical tube containing 3 ml of 10% PBS with bovine serum albumin (0.01%), streptomycin-penicillin (1%), gentamicin (0.1%), and fetal calf serum (1%). The tissues were manually flickered for 1 min.

After the tissue settled in the bottom of the tube, the PBS solution was removed and replaced with digestion buffer collagenase I; 6.01 IU/mg per ml of M199 medium (g/L of Hepes, 0.1% bovine serum albumin, 25 mg/L of trypsin inhibitor, and 0.7 g/L of sodium bicarbonate) and DNAase 1.28 mg (1.28 ml) pH 7.4. The mixture was incubated for 30 min at 38°C with shaking ~ 100 cycle/minute under O_2:CO_2 (95%:5%). The digestion maneuvers were repeated after tissue settled to the bottom, and the supernatant was removed.

After digestion, filtration; nylon mesh (100 μm) to separate tissue fragments, and centrifugation 500 rpm for 5 min to remove the enzyme. The filtered tissue was re-suspended in 2-3 ml of DMEM media, centrifugation 300 rpm for 5 min to complete removal of the digestive media [6].

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Leydig cell purification on a multiple gradient of Percoll, the tissue as pellets on the bottom of the conical tube were initially separated on a multiple gradual gradient of Percoll which involved 21%, 26%, 37%, and 60% (v/v) in DMEM medium as described by another investigator or state Sharma et al., and keep the number [7]. The gradient Percoll-was centrifuged at 3000 rpm/min for 30 min, the interface fraction between 37%-60% was collected, re-centrifugation 500 rpm/7 min., and the pellet was washed twice with the DMEM 15 ml medium for Percoll removal. The Leydig cells isolate were diluted with DMEM following the procedure described by name the researchers and keep the number [8].

Purity and viability

The Leydig cells purity was determined by histochemical technique stain with 3β-HSD using 1 mg/ml etiocholanolone; enzyme substrate was witnessed to be reached 82% purity. The trypan blue assay for the viability of the cells was greater than 96% [9]. According to the following procedure, the Leydig cell viability was determined:

Leydig cell viability was calculated as the number of viable cells divided by the total number of Leydig cells in the large grid square on the hemacytometer. The cells colored trypan blue, which take up the stain; they were measured as dead cells "unavailable" cell.

Procedure

- Trypan blue 0.4% as stock solutions in PBS buffered solution, pH 7.4.
- 1 ml of Leydig cell isolate was stained by trypan blue 0.1 ml stain solution.
- The hemacytometer was loaded by stained Leydig cell suspension and Leydig cells counted using light microscope 20 X.
- The viable Leydig cells were calculated by the following:

$$\text{Viable Leydig cell}\,\% = 1 - \frac{\text{Number of stained Leydig cells}}{\text{Number of total Leydig cells}}$$

Methodology

Tetrazolium (MTT) leydig cell viability assay: The preserved, purified culture of Leydig cells was challenged with varying dosages of Ketorolac at 2, 6, 12, 18 and 24 hours within series of different doses 0.03, 0.06, 0.09, 0.012 and 0.015 μm. The Leydig cell viability was assayed as follows [10].

1 Leydig cell was harvested and flicking the suspension of cells to scrape the adherent cells.

2 Cell culture incubation for 2 to 4 hours

3 Re-suspend Leydig cells at 2×10^6 per ml using 50 μl DMEM media

4 Descending serial dilutions of Leydig cells from 1×10^6 to 1×10^2 cells/ml (five)

5 Incubation of the Leydig cells for 24 hours

6 200 μl of MTT Reagent was added to each dilution, including negative controls (blank) and positive control for the standard curve

7 Cell culture incubation for 2 to 4 hours and stained at room temperature

8 Microscopic detection of clear purple participates stain

9 The absorbance was measured in Leydig cell culture of five dilutions and five replicate dilutions, including the blanks, at 570 nm in spectrophotometer.

The Plot absorbance was depicted against number of cells/ml of control dilution the number of cells to use in ketorolac treated culture assay had lay within the best fitted linear of the curve portion of the plot depiction.

MTT cell viability assay: After the cells were preserved with variable dosages of captopril, leukotriene B4, LH and GnRH for 2, 6, 12, 18, 24 hours, the viability of cells were assayed as follows [9]

1. The culture medium was filtered out and the cells were harvested,
2. Re-suspended in 200 μL 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) stock solution; 5 mg/ml methyl thiazolyldiphenyl-tetrazolium bromide in phosphate-buffered saline, then incubated for 2 hours at 37°C.
3. The converted dye was solubilized with 3 ml acidic isopropanol (0.04 M HCl in absolute isopropanol).
4. The absorbance of which was measured at a wavelength of 570 nm with a background subtraction at 650 nm [8].

DNA quantity: The comet assay "single-cell gel electrophoresis technique" had been considered as DNA damage detector and DNA concentration in single cells [11].

The strength comet tail under florescent microscope close related to the head like emittances reflection DNA damage. The single Leydig cells isolate were suspended in normal saline and adjusted until achieving 1×10^6 cells/ml density for "Single Cell Gel Electrophoresis (SCGE)". Concurrent comet slides set which contain slides, negative only adjuvants and positive control "self-control".

Data scoring comets were expressed as comets number/slide/culture, treated isolates of Leydig cells; approaches for the measurements of concentration of normal and or damaged DNA criteria [12].

Comet procedure: The prepared slides were dipped in a lysing media for 12 hours contain. This media contains: Lysing media compound 2.5 M, Na_2EDTA 100 mM, Tris pH 10 and 10% DMSO 10 mM and Triton X-100 1%. Slides were dipped in 10 mM dithiothreitol for 20 min in lysis media [13]. Transfer the slides to the tank of horizontal gel electrophoresis buffer. Alkaline comet assayed for denaturation of DNA, 10 min at 4°C in alkaline electrophoresis buffer Alkaline electrophoresis buffer: NaOH 300 mM, Na_2 EDTA 1mM and HCl pH Modifier amounts.

Electrophoresis migration was done at the following tenets; 27 V: 0.8 V/cm by 300 mA at 4°C for 5 min. The slides were transferred to 0.3 M Sodium acetate-ethanol base incubated for 30 min. Slides gels were dried in 99% ethanol for 1 hours and followed by dipping in 70% ethanol for 5 min.

Air-dried slides (25 ± 2°C) were stained using 12 mg/ml ethidium bromide; the slides were checked by fluorescent microscope using a 20X lens and analyzed by "Image J" program (use NIH as a reference and list at the end). Score the amount of DNA/100 cells and quantify the tail percentage of damaged DNA. The comet calculation based on migration length and the migrated DNA percentage, and worked within the concept [13].

Leydig cell chromatin/DNA evaluation: DNA integrity was examined for chromatin condensation through cyto-chemical detection techniques. The three protocols of staining were:

a. Acridine orange test (AO)

b. Aniline blue (AB)

c. Toluidine blue (TB) [14].

Aniline blue (AB) test: Revealing of chromatin condensation anomalies. Aniline blue stains "lysine-rich selectively" were linked to histones residue.

Toluidine blue test: DNA fragmentation of chromatin condensation. Toluidine blue was a metachromatic colorant, fragments of DNA bind with free site phosphate in the chromatin strands [15].

Acridine orange test (AO): Single-double-stranded DNA differentiate and grade. Acridine orange was a fluorescence probe metachromatic colorant of double-stranded DNA green fluorescent and single-stranded DNA red fluorescent.

Statistical analysis: The real examination of the control and treated assembling were subjected to examination of analysis of variance (ANOVA) two way analyses. A probability of <0.05 was required to indicate a significant difference. Each group comprised in any event six repeats. LSD test was used for comparison between groups. A correlation between responses was made [16]. The best fitted curve was managed by the following equation y=bm × x.

Results

The capability of Leydig cells to proliferate and grow in culture media was inspected in 0, 2, 4, 6, 12, 18, 24 and 48 hours Leydig cell media were treated with hCG (ng/ml). The viable Leydig cells were motivated at different times of culture. The number of viable Buck Leydig cells increased significantly p<0.01 during 24 hours in culture media compared with untreated cells and other incubation of proliferation times (Figure 1). Under our culture conditions, Bucks Leydig cells were able to survive in the primary culture after isolation and purification at zero time.

Prior to assessing cell samples, the Leydig cell isolated assay was calibrated using viable cells standardized using a cell number dilutions standard curve (Figure 2). The cell number - dilution curve is a series of six Leydig cells dilutions (from 1.00 to 3.125 check it), the curve was fitted by linear regression analysis. Figure 3 shows how the Leydig cell counts from the isolation and purification assay correlate directly with dilution of cell concentration values. The results were the correlation coefficients of the cell number dilution linear regressions high, lower cell counts combined with an increased slope for the cell dilution.

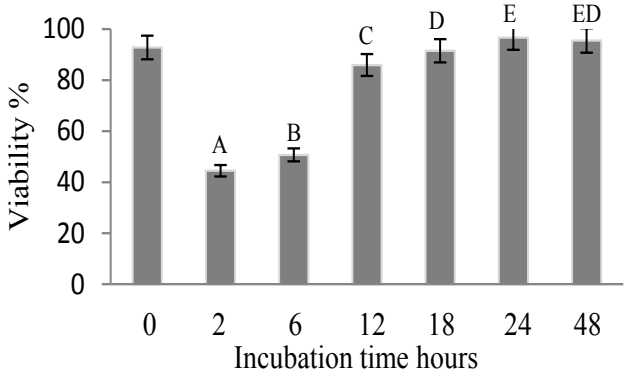
Results are graphed as scatter in Figure 4 and graphically represented in correlation regression with higher r^2=0.9853. The percentage viability was found to be significant p<0.05 increasing with increasing concentration of Leydig cell numbers. These final standardized the Leydig cell line before treatment effect exploration.

As seen in Figure 5, incubation of Buck Leydig cells with Ketorolac 0, 0.03, 0.06, 0.09, 0.12 and 0.15 µM for 24 hours inhibited the Leydig cell number in proliferation period significantly (p<0.05) as, compared to control: with concentrations of 0.06-0.15 µm of incubation, the IC_{50} 0.0937 µm value was calculated under hCG. On the other hand, the low concentration of ketorolac 0.03 µm did not show significant changes in the Leydig cell number or in maximum growing time.

A dose response of the Ketorolac effect on Leydig cells is exhibited in Figure 6. The cell calibrated curve was processed to create a ketorolac dose response curve showing the classic sigmoidal form associated with cell viability percent. The IC_{50} was calculated to be 0.0931 µM under hCG. The dominant result compares well with the control indicating ketorolac upset viability in Buck Leydig cell lines with concentrations in the higher range (0.09-0.15 µM), as explored by growth inhibitions MTT assays. Whereas, the low concentration of ketorolac displayed significant p<0.05 increase in Leydig cell viability percentage as compared with control and other treated groups.

Curve-fitting was performed for DNA deformity using COMET versus Ketorolac treatment. The dose-response curves were shown in Figures 7. A DNA tail incidence and B DNA tail Length. The results showed significant p<0.05 increase of both incidence of tail and tail length with increase ketorolac concentrations as compared with the control. The IC_{50} of ketorolac in both tail incidence and tail length were 0.0731 µM and 0.0676 µM respectively.

The ketorolac-DNA abnormality fitting curves showed significant p>0.05 increase in each AO, BO and TB indices generally, whereas, the AO and BO displayed unchanged in low concentration 0.03 and 0.06



The capital letters denoted difference p<0.05 among the group; n=10 samples of Leydig cells isolate; Data are presented mean ± SE

Figure 1: The time effect on viability of Buck Leydig cells isolate *in vitro* cultures.

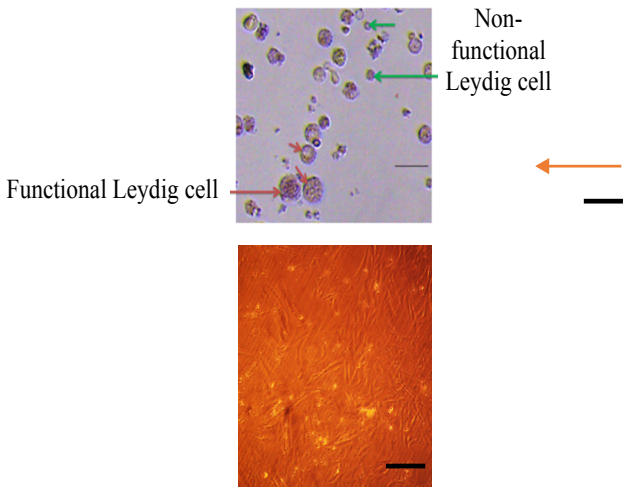
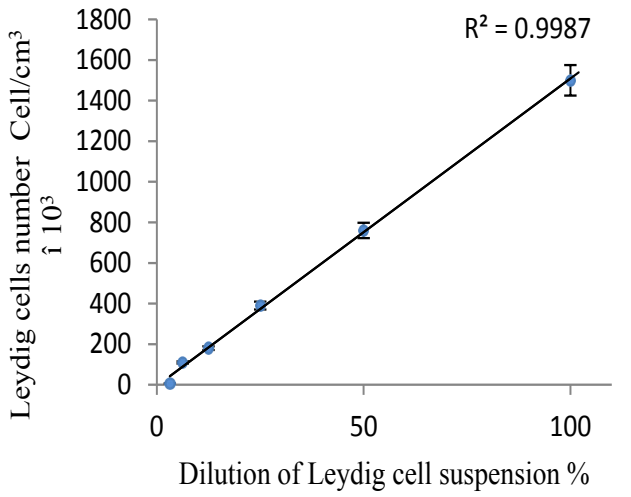
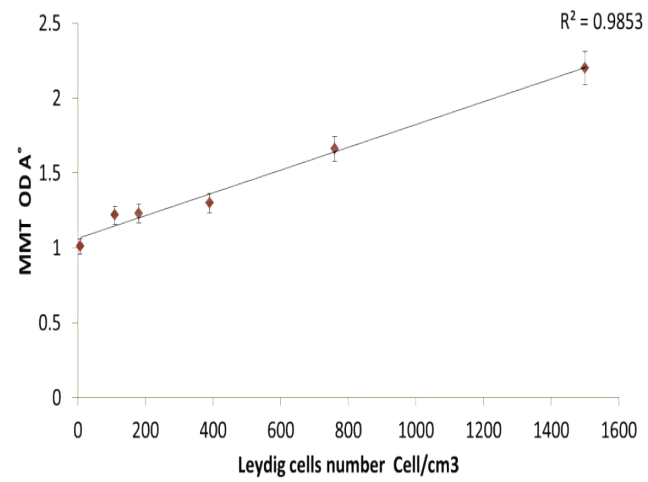


Figure 2: Leydig cell primary isolate Bars, 20 µm.



The small letters denoted difference p<0.05 within the group; n=10 samples of Leydig cells isolate; Data are presented mean ± SE

Figure 3: The calibration curve of viable cells numbers versus series dilution of Buck Leydig cell suspensions.



The small letters denoted difference p<0.05 within the group; n=10 samples of Leydig cells isolate; Data are presented mean ± SE

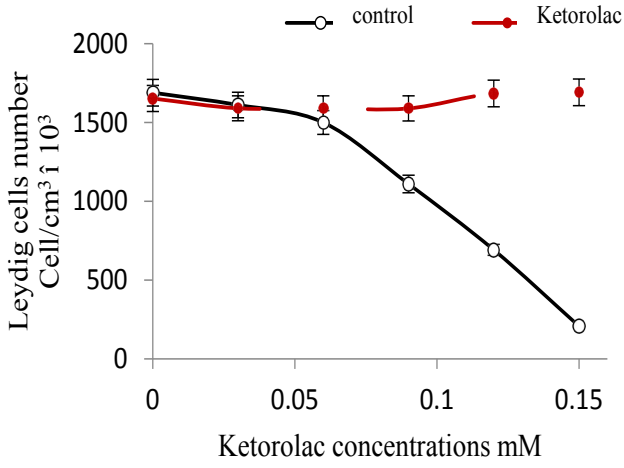
Figure 4: The correlation between viability of Leydig cells MTT and Leydig cell numbers of Buck isolate.

µM. The IC_{50} of AO, BO and TB were 0.0619, 0.1131 and 0.0739 µM respectively (Figure 8).

Discussion

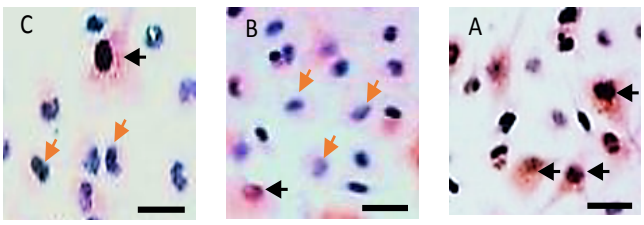
The MTT test revealed to Ketorolac upheld Buck Leydig cell proliferation and viability in a dose-dependent manner. The MTT data absorbance was recorded with best time obtain of highly Leydig cells isolate (Figure 3) the MTT data covered time of growing and proliferation scales showing highly yeid viable leydig cell at 24 and 48 hrs (Figure 3), and compared viable Leydig cells with cells dilution by regression Figure 3 display highly concentration r^2=0.9976 that calibrated with total number of Leydig cell verses dilution and finally (Figure 4) and given an impression-highly correlated r^2 in graph depiction r^2=0.998 of viability versus cell concentration (numbers) act as identifier of true growing cell, (viable) in certain number. Meanwhile, in Figure 5 showed a significant p<0.05 low viable cells in the range (0.09-0.15) µM. Ref. [17] confirmed the NSAIDs might be having the aptitude and ability to perturb DNA overhaul pathways and this may have vital implications for the Leydig cell response to NSAID agents. It is likewise inflammation may probably repress through "epigenetic mechanisms, DNA mismatch repair" and is one of causal reduction of viability.

Furthermore, NSAIDs do not only upset cyclooxygenase action: NSAID conduct to knowingly modify gene expression and thus NSAIDs can be reasonably described as having Pleiotropy, besides the prolonged NSAID loading dose mostly inhibited genes as genotypic adversity [18]. This is given as an indication of DNA results increased abnormality (Figure 9) best tittered dose response curve and micrograph of DNA defect types display gradual increase with along concentration increase in three ideal part on it is two concentration and comet indices endpoint Figure 6A and 6B with Ketorolac concentration increased, dose depended made an impressions of direct contributions with presumably causal attribution of reduce the Leydig cells counts and viability express MTT test. In addition, other transcriptional DNA pathways affected by NSAID treatment validate NF-κB signaling, this involved in modifiable apoptosis [19-21]. Ketorolac may be presumably antagonized the NF-κB pathway. Name of the authors [22] explore



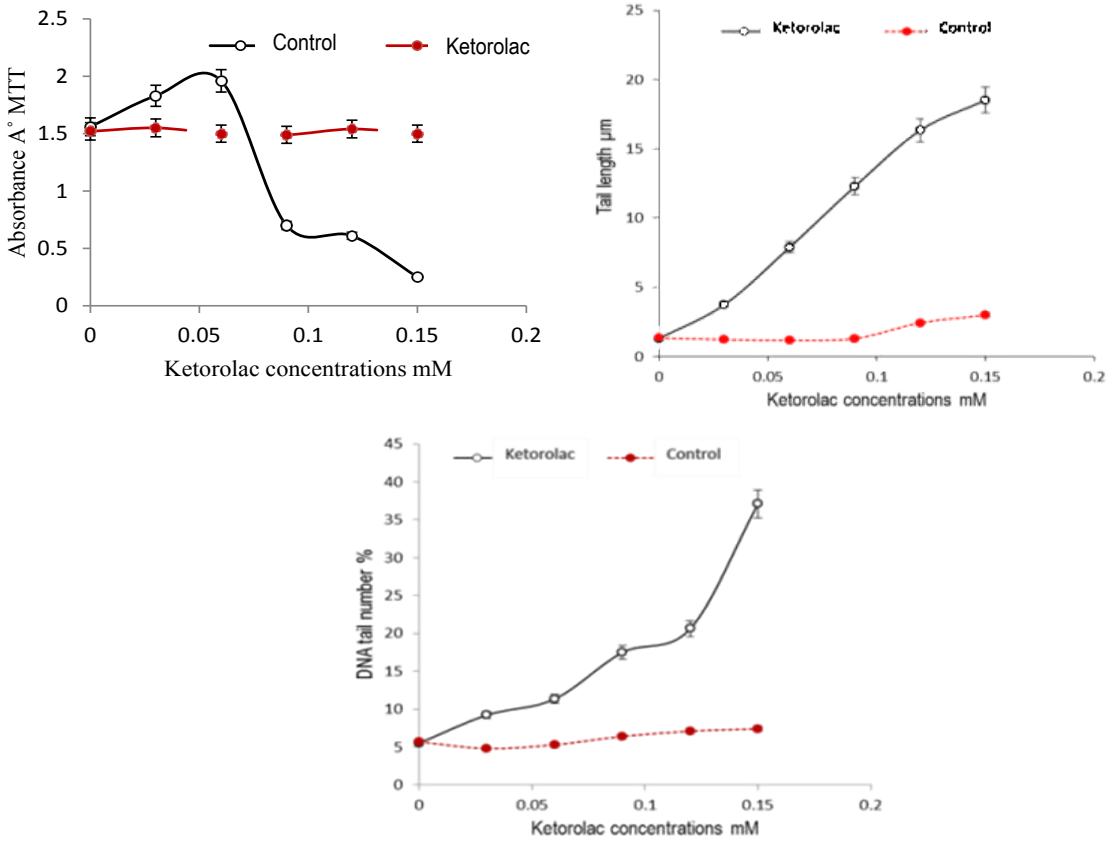
The small letters denoted difference p<0.05 within the group; The capital letters denoted difference p<0.05 between groups; n=10 samples of Leydig cells isolate; Data are presented mean ± SE.

Figure 5: The ketorolac effect on numbers of Buck Leydig cells of *in vitro* culture.



Control Normal (A) morphology was observed with many Leydig cells and (B) low Ketorolac concentration 0.03µm few dead Leydig cells were observed (C) 0.15 µm Ketorolac a notable increase in the number of dead Leydig cells was observed with loss of Leydig cell structure. Bars 50 µm. H and E. Normal Leydig cells, and refers to abnormal cells .

Figure 6: Representative light micrographs of buck testicular Leydig cells cultured for 48 hours.



The small letters denoted difference p<0.05 within the group; The capital letters denoted difference p<0.05 between groups; n=10 samples of Leydig cells isolate; Data are presented mean ± SE

Figure 7: The ketorolac effect on DNA deformity "comet" DNA tail incidence percent and DNA tail length in Buck Leydig cells of *in vitro* culture.

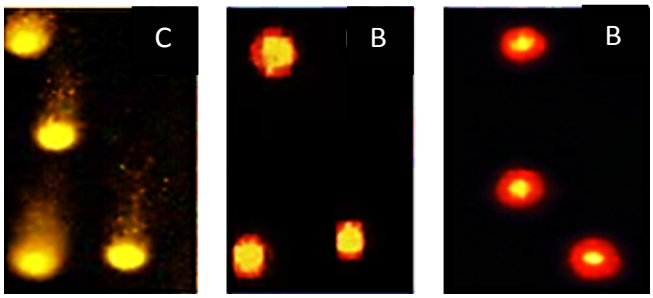
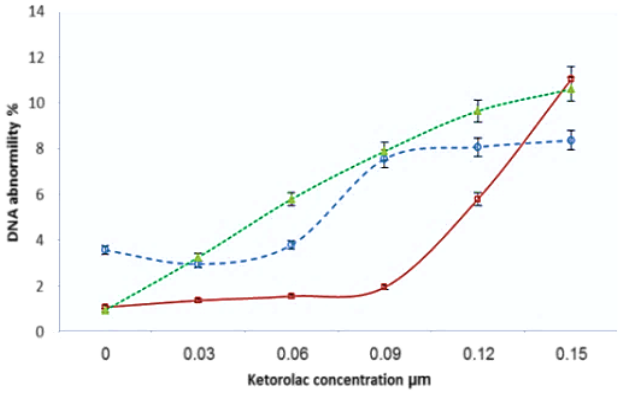


Figure 8: DNA damage of Leydig cells in primary cultures of comet test (A): control Normal. (B) 0.03 µm ketorolac concentration with low comet tail (C) 0.15 µm of ketorolac concentration with high incidence of a tail comet with a long tail.

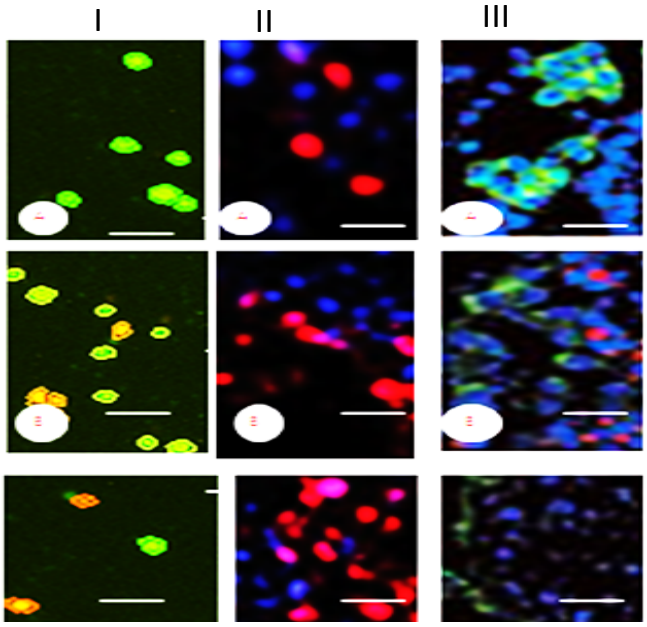


The small letters denoted difference p<0.05 within the group; The capital letters denoted difference p<0.05 between groups; n=10 samples of Leydig cells isolate; Data are presented mean ± SE

Figure 9: The ketorolac effect on DNA abnormality AO, BO and TB in Buck Leydig cells of *in vitro* culture.

NSAID inhibited NF-κB pathway-apoptosis by inhibited by direct binding with IκB kinase-B [23-25]. Inhibition of NF-κB by NSAID may be augmenting the chemo-sensitivity of Leydig cells to the DNA topoisomerase inhibitor which was presumably induction of apoptosis finished by DNA mismatch repair proficiency covered disruption of cell metabolism [26].

This indication of DNA results increased abnormality and (Figures 9 and 10) both best titer dose response curve and micrograph of DNA defect types display a gradual increase with a long concentration increase in three ideal parts on its two concentrations and comet indices endpoint and (Figures 6A, 6B and 7) with Ketorolac concentration increased, dose depended made an impressions of direct contributions with presumably causal attribution of reduction of the Leydig cells counts and viability express MTT test.



I. Acridine orange test (AO); II. Aniline blue (AB); III. Toluidine blue (TB); (A) Control Normal: green; (B) 0.03 µm ketorolac concentration with abnormal DNA; (C) 0.15 µm of ketorolac concentration with high abnormal DNA; Bars, 50 µm

Figure 10: Leydig cell chromatin/DNA test.

Meanwhile, few authors [27] suggested the *in vitro* anti-proliferative properties of the NSAIDs in Leydig cells are primarily mediated by altering the regular cell cycle phase distribution, not by apoptotic cells death induction. Nevertheless, the major outcome on the cell cycle may lead to "senescence" that reflected to indirect cell death a "slow cell death". That may be suggested occur with phenomena apoptosis resistant that are resistant to undergoing motivation of surface receptors necrosis factor receptor and growth factor [28].

Acknowledgements

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