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Exploration of Steroidoginic Activity of Ketorolac on Isolated Leydig Cells of Goat (*Capra hircus) In Vitro*: Part II

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Abstract

In the present thesis was conducted to clarify the effect of ketorolac (NSAID) on the Leydig cells of goats to achieve the ketorolac event, by the evaluation steroidoginic activity of ketorolac series on Leydig cell concentrations *in vitro*. Mitochondrial defects reduction of functional processes of sterodoginc activity reduction of testosterone levels via mitotracker probe indicator, ketorolac may be affected on the steroidogenic acute regulatory (STAR) protein and translocator protein (TSPO). Ketorolac might mutilation and impair the cells were via the uncoupling of oxidative phosphorylation in the cells followed by uncouple mitochondrial respiratory chain as well as ATP depletion. Testosterone level displays in all treated maneuvers LH, LH-GnRH and GnRH in ketorolac challenge decrease testosterone levels at a concentration of ketorolac from 0.6-0.15 mM with the exception in GNRH – ketorolac maneuvers result showed an increase in testosterone level in 0.03-0.06 mM, then fall up down other maneuvers of treatment. The testosterone synthesis in Leydig cells of mammalian is achieved almost motivated by the binding of luteinizing hormone (LH) to the plasma membrane of Leydig cell receptors efficaciously, that lead to creation of c.AMP Inhibition of LTB4 that due to direct inhibition of lipoxygenase action, and had a direct effect to reduce LTB4 and may be various eicosanoids also 12 (S) - hydroxyeicosatetraenoic acid and 15 (S) - hydroxyeicosatetraenoic acid binds to and activates LTB.

Introduction

NSAIDs are common usage drug for, chronic (long term) such as arthritis (rheumatoid arthritis, osteoarthritis and others) and lupus, sharing the broad worse health endpoint [1]. New generations were holding on pharmaceutics drugs store and not have a little notion on their sexual consequence. According to this fact the ketorolac one of floating projected in highly extensive uses without wariness on the safety knowledge of side effect [2]. Both *in vitro* and *in vivo* revisions and reports have established that numerous NSAIDs classes might modulate endocrine function with developed dramatic progression of reduced fertility of male [3]. Ketorolac is a powerful analgesic agent of the non-steroidal anti-inflammatory class (NSAID). It is a non opioid type and has no known belongings on opioid receptors. Its method of activity increases 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 [4].

The aim of this study was to assess the collection effect of the adding ketorolac on the Leydig cell culture of the buck testis on the following goals:

Evaluate steroidogenic activity of *in vitro* isolated Leydig cells, and Explore the contribution of leukotriene B4 on mode of action Ketorolac Tromethamine. This study performed to explore the activity of ketorolac in Leydig cells to achieve this idea the parameters are cell function's parameters; Leydig cell membrane integrity and mitochondrial integrity and hormonal, and biochemical parameters, testosterone LTB4 and ATP concentration.

Material 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. Post-mortem 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. The ethical programs was followed by the Al-Bayati and Khamas.

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

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(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 cycles/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]. 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

2:3:1 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 stand 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 laid 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].

LH induction of proliferation and steroidogenesis

The cells were incubated in a different setting for ketorolac challenged the steroidogenic activity and Leukotriene's concentration. The protocol of culture and growing of cell display as follows:

a) 10×10^6 Leydig cells in 24 whole plates with Ham's F12/DMEM medium+Charcoal- stripped fetal bovine serum 10% v/v+penicillin-streptomycin 1% (Total 1.2 ml).

b) Ketorolac add in treating cultures and Incubation with hCG-GnRH hormones 2.5×10^{-10} mol/ml and hatch in O_2:CO_2 (95:5%), 38°C, 24 hours

c) Harvested the culture Leydig cells with culture media.

d) Centrifugation 2300 g, 15 min.

e) The supernatant was separated and Incubate in water bath 80°C, 5 min.

f) Centrifugation 2300 g, 15 min and kept frozen for Assay testosterone.

Testosterone radioimmunoassay

The assessment of testosterone levels and plotted as:

- Time vs concentration responses in primary of Leydig cell culture served as standard curve firstly,

- Challenged of ketorolac concentration versus testosterone concentration secondly.

The incubated Leydig cells isolate at a concentration of approx. 100×10^6 cells/ml was incubated in Falcon culture plates, activated steroidogenic function by LH 100 ng/ml the incubated Leydig cell culture was harvested and centrifuged at 100 g for 5 min. The supernatant was separated and stored frozen at -20°C for both testosterone and Leukotrienes [8] Testosterone had assayed by radioimmunoassay (RIA) [9].

Determination of LTB4 by ELISA

The LTB4 ELISA kit (Abcam) is a competitive immunoassay for the quantitative determination of LTB4 in biological fluids. "The Assay Designs Leukotriene B4 Enzyme Immunoassay", described briefly; a specified polyclonal antibody was utilized for LTB4 binding, the LTB4 in the sample containing LTB4 covalently binding attached directly composed complex. The incubation time 2 hours 25°C, to achieve excessive reaction development with reagents with substrate is added. The yellow color creates and give the sign of reaction stopping, the complex reaction was measured by reader at 405 nm. Both stander curve and ketorolac concentration relationship was a plot. The strength of the yellow color was inversely relative to the LTB4 concentration in both standards and treatment. The assay recovery was 97.3%.

Mitochondrial and cell membrane integrity of leydig cells

Mitochondria and membrane integrity were assayed as follows:

Preparation of Mitotracker stock solution: Diluted 1 mM of Mitotracker stock in DMSO; 25 μg CMXROS at 47 μl, and the stock stain solution was stored in deep freeze.

Tissue preparation: The Leydig cell cultures were adjusted until reach a density 1×10^6 Leydig cell per ml. MitoTracker were added into the culture media at final concentrations of 1 μl to 5 ml Leydig cell suspension of tissue culture. The Leydig cells were incubated for 30 min at 38°C, the cells were examined under fluorescence microscopy "red filter" emitting red fluorescent light at 576 nm wavelength, and green for membrane integrity.

Image J software was used to determine the intensity of fluorescence Leydig cell culture. The summation of these values directly indicated the total mitochondrial mass (green) and activity (red).

Leydig cells ATP concentration: The protocol of determination of ATP concentration in the Leydig cells was consisted triple fused parts composed of the following:

Leydig cells isolate was scratched and centrifuged at 300 rpm for 5 min and settled tablet was separated and adjusted the cell concentration to 1×10^6 and stored in -18°C until intended to ATP triple parts of ATP determination.

Leydig cell wash: The stored pellets of Leydig cells were thawed, the cells were suspended by normal saline 1% 5°C and let the isolated cells settle and separated, then re suspended and repeated the settle cell and finally, in separated for preparation of extraction.

ATP extraction: The liberation of ATP from the cells was followed as:

a) 0.1 ml of 0.6 M at 5°C Perchloric acid was merged with washed Leydig cells for 20 min; the cell suspension was transferred to conical tube and centrifuged 5 min, at 80000 rpm.

b) The cells at the bottom of the cells was separated and suspended with 50 μl 3.5 M of K_2CO_3, and centrifugation for 5 min at 8000 rpm.

ATP analysis: The supernatant of lysis Leydig cells was assayed via add the following:

a) 100 μl NADH linked enzyme as stock 1 mg/ml

b) 20 μl glucose 6 phosphate dehydrogenase, hexokinase as stock 1 mg/10.5 ml,

c) 60 μl glucose as stock 9 mg/10.5 ml

d) 50 μl NADP-nucleotide, dinucleotide phosphate 10 mg/10.5 ml

e) 0.1 N of 800 μl TRAP buffer pH 7.6

The ATP concentration was measured via 340 nm of a spectrophotometer and applied to a standard curve of ATP prepared before for multi dilution.
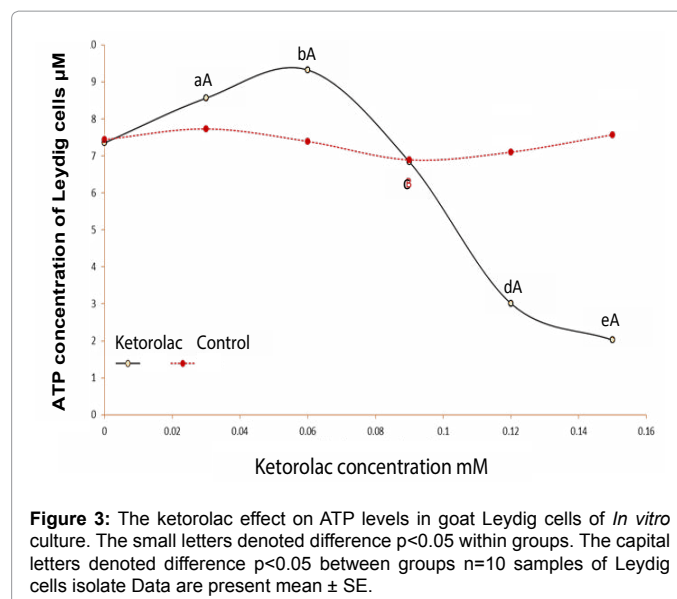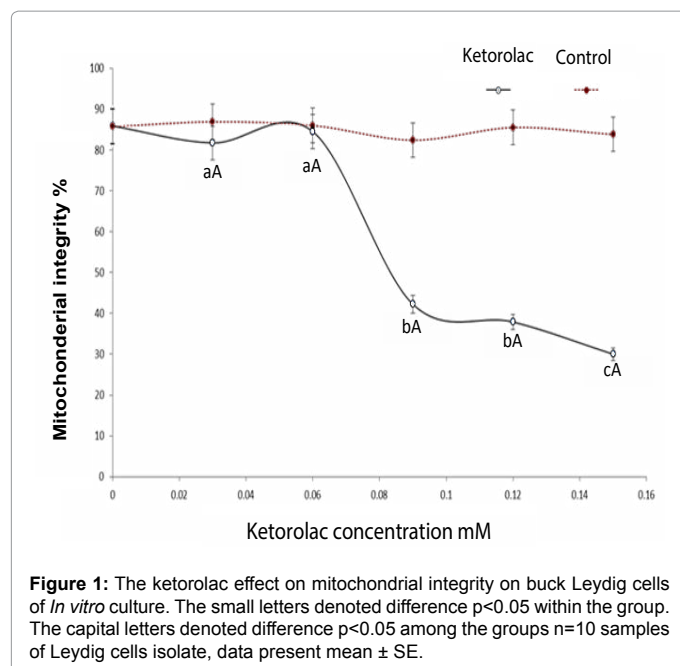
Statistical analysis: The real examination of the control and treated assembling were subjected to examination of analysis of variance (ANOVA) two way analyses. A probability of <0.05 was required to indicate a significant difference. Each group comprised in any event six repeats. LSD test was used for comparison between groups. A correlation between responses was made. The best fitted curve was managed by the following equation y=bm × x.

Results

To establish a dose-response curve for mitochondrial integrity of Leydig cells isolates treated a ketorolac and were ordered various concentrations (0.03, 0.06, 0.09, 0.12 and 0.15 μM) for 24 hours incubation. In these tests, the lower concentrations of ketorolac 0.03 and 0.06 μM were displayed no significant p>0.05 changes in mitochondrial integrity as compared with control Leydig cell while the other concentration were lesser than control and other treated Leydig cell isolates significantly p<0.05. The IC_{50} was 0.08215 μM (Figure 1).

In Figure 2, Specified the ability of ketorolac to inhibit mitochondrial activity in Leydig cells and to confirm whether its altered mitochondrial morphology. Leydig cells cultured *in vitro* stained the mitochondria with the probe Mitotracker appeared in Green and Red filter. This result displayed there were reduced in mitochondrial morphology (mass) in high and moderate concentration 0.03 and 0.15 respectively of ketorolac treated Leydig; green. Furthermore, analysis of Leydig cells labeled with Mitotracker; Orange and ultrastructural analysis of incubated Leydig cells resulted in similar observations. In Figure 3, the dose-response curve for the levels of ATP concentrations at which a putative high associated with mitochondrial functions would be noticeable. This result also recorded the IC_{50} of ATP in the presence hCG in the Leydig cells 0.0872. The ATP – ketorolac concentrations displayed increased the ATP levels in 0.03 and 0.06 μM significantly p<0.05 as compared with control and other proliferative Leydig cells, meanwhile the growing Leydig cells showed a significant p>0.05 reduction in ATP levels as compared with control isolates.

In Figure 4, the effect of ketorolac on membrane integrity displayed significant p>0.05 reduction in membrane stability as compared with control in all time of incubation for 24, 36, 48 and 48 hours, the fitting curves presented IC_{50} as follows 24 hours 0.0927 μM, 36 hours 0.0748 μM, 48 hours 0.0509 μM 72 hours 0.0478 μM. The curve of ketorolac-LTB4 levels showed significant p<0.05 fall in all treated media of Leydig cell growing as compared with control, in addition the behavior

Figure 1: The ketorolac effect on mitochondrial integrity on buck Leydig cells of *In vitro* culture. The small letters denoted difference p<0.05 within the group. The capital letters denoted difference p<0.05 among the groups n=10 samples of Leydig cells isolate, data present mean ± SE.

Figure 2: Analysis of mitochondrial morphology (Mass) and activity Goat Leydig cells. (A and D) Control, mitochondrial activity and mass high density of mitochondria, red filter; white arrow, and green filter high fluorescence area indicated to the mass of mitochondria. (B and E) 0.03 µm of ketorolac treated cultures, moderate mitochondrial activity, and mass. (C and F) 0.15 µm of ketorolac treated cultures, low mitochondrial activity, and mass. Bars, 10 µm.

Figure 3: The ketorolac effect on ATP levels in goat Leydig cells of *In vitro* culture. The small letters denoted difference p<0.05 within groups. The capital letters denoted difference p<0.05 between groups n=10 samples of Leydig cells isolate Data are present mean ± SE.
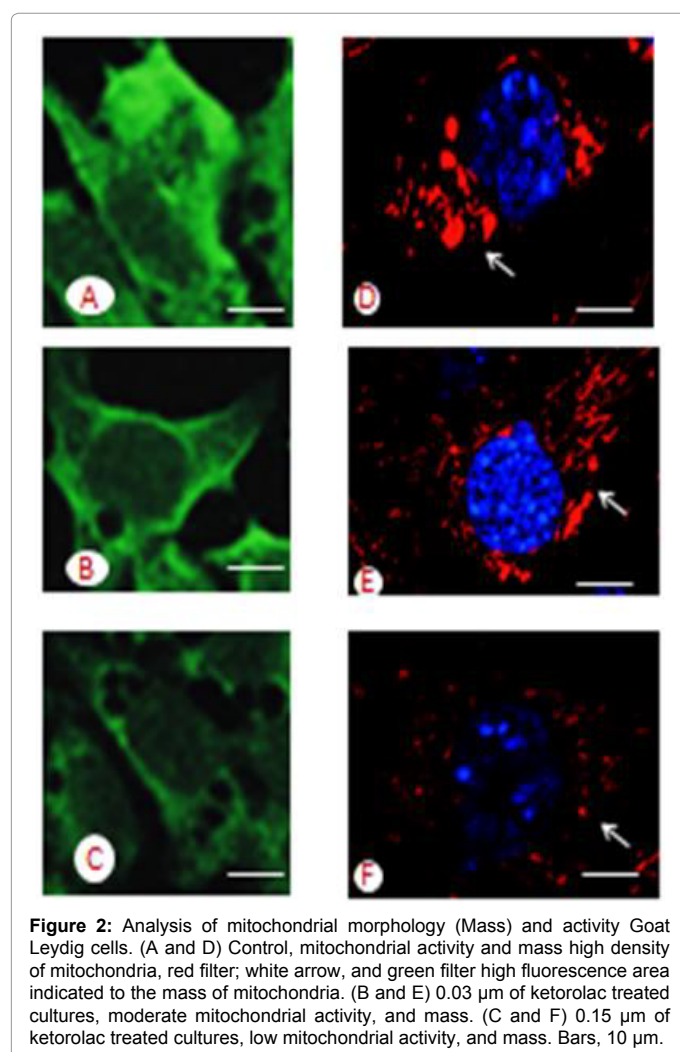
of GnRH curve displayed superior LTB4 levels in low concentration of ketorolac as compared with other ketorolac treated media (Figure 5). The IC_{50} of ketorolac in each LH containing media 0.0412 µM, GnRH containing media 0.0465 µM and LH-GnRH containing media 0.0.0254.

The dose response curve of testosterone hormone levels showed significant p<0.05 decrease testosterone levels generally as compared with control groups under the effect of ketorolac treatment in all triggered media LH, GnRH and LH-GnRH. On the other side the Leydig cells in LH containing media displayed increase as compared with control at 0.03 and 0.06 µM as compared with all groups (Figure 6). The IC_{50} of each triggered and stimulated media were as follows; LH containing media 0.1181 µM, GnRH containing media 0.09644 µM and LH-GnRH containing media 0.1195.

Discussion

Mitochondrial defect reduction, functional processes of steroidogenic activity was confirmed this suggestion by the result of mitochondrial integrity was reduce as one state with reduction of testosterone levels via mitotracker probe indicator (Figures 1 and 2) [11] as well as the NSAID, including ketorolac may be affecting on the steroidogenic acute regulatory (STAR) protein [12] and translocator protein (TSPO) [13], that may be led to critical drop for mitochondrial cholesterol transfer; termed the transduceosome [14].

The above finding facilitated the concept result in a Figure 2a suitable correlation between testosterone in GnRH media production under influence GnRH to LTB4 for steriodogenic reduction of testosterone in otherwise both testosterone under LH and both LH-GnRH displayed (2a and b) very low correlation value with LTB4 several reports the LTB4 had a proliferative effect of Leydig cells [15] and steriodogenic ability that documented effect of ketorolac on major cell viability. The intensively deleterious effect of ketorolac within time of exposure gradual decrease showed in Figure 4 impact as increase machinery of programmable cell death [16] and manifested as impaired the metabolic processes recovered cells Glutathione-free radical metabolic [17] as well as exhausted the enzymatic factor km and saturation delay of clearance unwanted substance in cells under ketorolac, that give an impression long term treatment with ketorolac
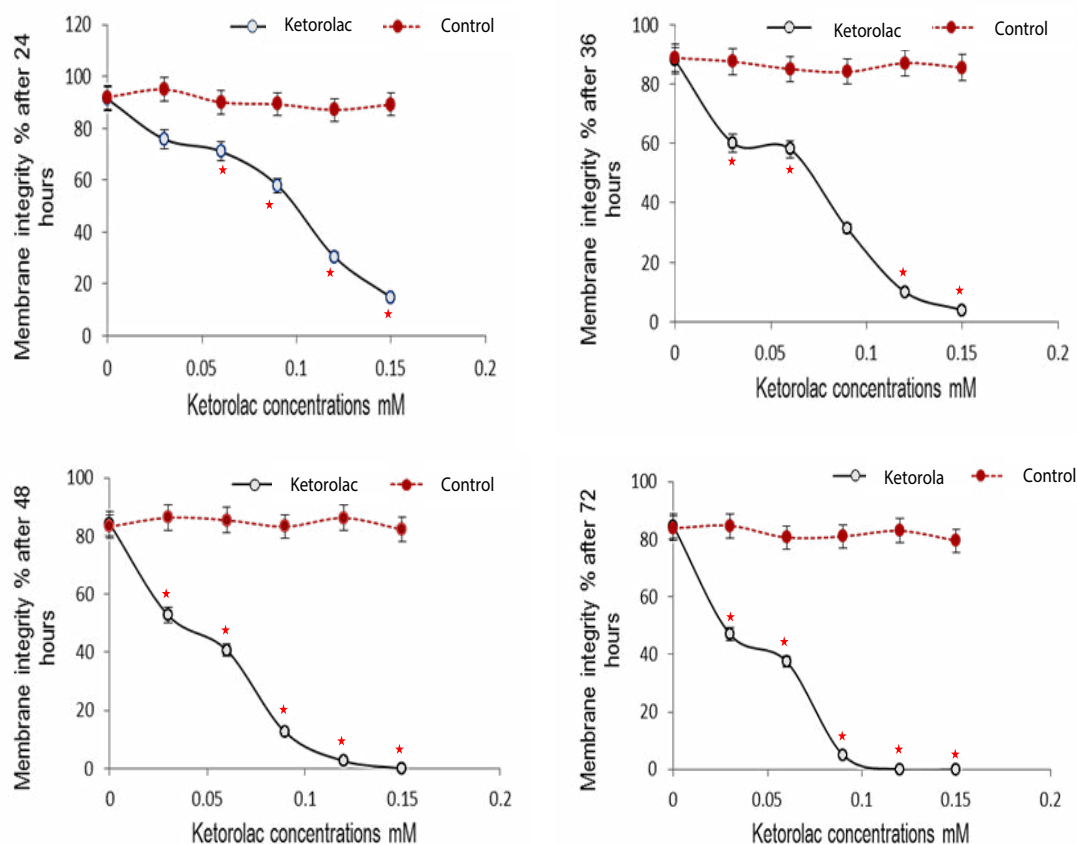
Figure 4: The ketorolac effect on membrane integrity in goat Leydig cells at 24, 36, 48 and 72 hours of *In vitro* culture. The stars denoted difference p<0.05, n=10 samples of Leydig cells isolate. Data are present mean ± SE.
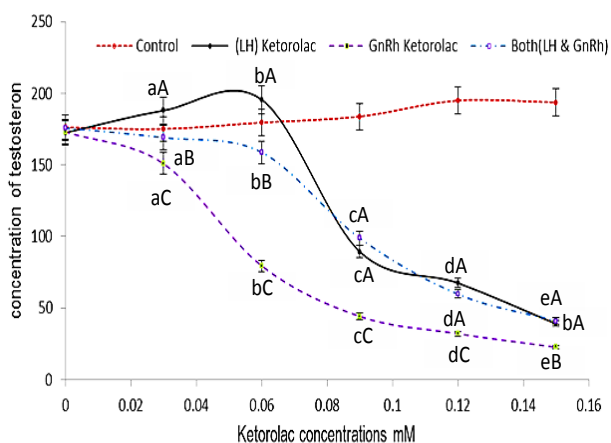

Figure 5: The ketorolac effect on testosterone levels in goat Leydig cells with LH, GnRH, LH-GnRH of *In vitro* culture. The small letters denoted difference p<0.05 within groups. The capital letters denoted difference p<0.05 between groups. n=10 samples of Leydig cells isolate. Data are present mean ± SE.

may increase risk of lost cell integrity. Whereas, ATP is required for several steps in Leydig cell mitochondrial energetics and steroid synthesis using progenitor cells or adults Leydig cells [18-20]. The

additional mechanism by which NSAIDs might mutilation and impairs the cells were via the uncoupling of oxidative phosphorylation in the cells followed by uncouple mitochondrial respiratory chain as well as the endpoint is ATP depletion [21]. Several NSAIDs had been revealed to uncouple mitochondrial respiration, consequently that presumably decreased consistent cell functions including steroidogenesis. Attributed depletion of ATP to The NSAIDs motivated uncoupled of basal respiration, ATP synthesis reduction figure (3:3), joined with the membrane potential collapse of mitochondria.

Testosterone levels (5) displays in all treated maneuvers LH, LH-GNRH-GNRH in ketorolac challenge decrease testosterone levels at a concentration of ketorolac from 0.6-0.15 mM with the exception in GNRH – ketorolac maneuvers result showed an increase in testosterone level of 0.3-0.6 mM, then fall up down other maneuvers of treatment. The testosterone synthesis in Leydig cells of mammalian is achieved almost motivated by the binding of luteinizing hormone (LH) to the plasma membrane of Leydig cell receptors efficaciously, that lead to creation of c.AMP [22].

Ketorolac effect on steroidoginic tissue culture bioviability of testosterone. This may be attributed to same found influence LH and both LH-GnRH as promoting formation Leydig cells cluster enrich cell in hyperchromic granules of the testosterone formation. This finding approved the ketorolac effect exerted on cell viability for production testosterone at the level of biosynthesis more than cell
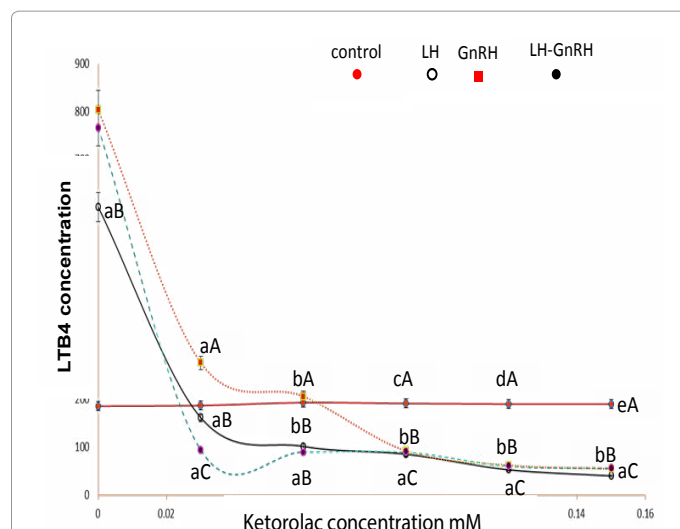
Figure 6: The ketorolac effect on LTB4 concentrations on buck Leydig cells of *In vitro* culture. The small letters denoted difference p<0.05 within groups. The capital letters denoted difference p<0.05 between groups n=10 samples of Leydig cells isolate Data are present mean ± SE.

proliferation dynamic influenced by GnRH craft, but they ketorolac diminished sterodiogenic actively diminished by reduction of viable cells and directly in the blocker activity particulate element presence of testosterone production [23,24].

In the earlier showed in Figure 6 increase in LTB4 in Ketorolac treated Leydig cells as compared with control Leydig cells in low concentrations, the leukotrienes B4 increased peak may be due to increase arachidonic acid motivated by NSAID [25], NSAIDs mostly up-regulated 15-lipoxygenase-1 (15-LOX-I) in CRC cells; and induced expression in the DLD-I Leydig cells [26]. This high concentration results of ketorolac 0.03 and 0.06 μm reflect their effect on first; increase testosterone. The LTB4 had a direct through act a second messenger in steroidogenic process of GnRH aggravation activity [27] and indirect; as Ca^{++} dependent effect in testosterone production of Leydig cell mainly, it was provoked GnRH. The NSAID inhibited lipoxygenase but not cyclooxygenase activity produce and reduction of GnRH-LH and c.AMP dependent motivation of steroid yields and also propose a stimulatory effect of yield products of the lipoxygenase pathway of arachidonic acid metabolism in steroidogenesis [28,29].

On the other side high concentration of ketorolac treated Leydig cells in the Figure 6, showed inhibition of LTB4 that due to direct inhibition of lipoxygenase action, and had a direct effect to reduce LTB4 and may be various eicosanoids also 12 (S) - hydroxyeicosatetraenoic acid and 15 (S) - hydroxyeicosatetraenoic acid binds to and activates LTB.

Acknowledgements

References

1. Riendeau D, Percival MD, Brideau C (2001) Etoricoxib (MK- 0663) preclinical profile and comparison with other agents that selectively inhibit cyclooxygenase-2. J Pharmacol Exp Ther 296: 558-566.

2. Strom BL, Berlin JA, Kinman, JL, Spitz PW (1996) Parenteral ketorolac and risk of gastrointestinal and operative site bleeding: A postmarketing surveillance study. JAMA 275: 376-382.

3. Martini AC, Vincenti LM, Santillán ME, Stutz G, Kaplan R, et al. (2008) Chronic administration of nonsteroidal-ant inflammatory drugs (NSAIDS): effects upon mouse reproductive functions. Rev Fac Cien Med Univ Nac Cordoba 65: 47-59.

4. Johnston SA, Budsberg SC (1997) Nonsteroidal Anti-Inflammatory Drugs and Corticosteroids for the Management of Canine Osteoarthritis. Vet Clin North Am Small Anim Pract 27: 841-862.

5. Zhang L, Cui S (2009) Effects of daidzein on testosterone synthesis and secretion in cultured mouse Leydig cells. Asian-Aust J Anim Sci 22: 618-625.

6. Papadopoulos I, Tilki M, Lees S (2004) Promoting cultural competence in health care through a research based intervention. Journal of Diversity in Health and Social Care 1: 107-115.

7. O'Shaughnessy PJ, Morris ID, Huhtaniemi I, Baker PJ, Abel MH (2009) Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: data from mutant and genetically modified mice. Mol Cell Endocrinol 306: 2-8.

8. Salmon JA, Simmons PM, Moncada S (1983) The effects of BW 755C and other anti-inflammatory drugs on eicosanoid concentrations and leukocyte accumulation in experimentally induced acute inflammation. J Pharm Pharmacol 35: 808-813.

9. Taieb J, Mathian B, Millot F (2003) Testosterone measured by ten immune assays and by dilution gas chromatography-mass spectrometry sera from 116 men, women and children. Clin Chem 49:1371-1385.

10. Elston R, Johnson W (2008) Basic Biosttistics for Geneticists and Epidemiologists, A practical Approach. John Wiley & Sons, Ltd, New Jersey, USA, pp: 233-255.

11. Stocco DM (2001) StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 63: 193-213.

12. Manna PR, Dyson MT, Jo Y, Stocco DM (2009) Role of dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene 1 in protein kinase A and protein kinase C mediated regulation of the steroidogenic acute regulatory protein expression in mouse Leydig tumor cells: Mechanism of action. Endocrinology 150: 187-199.

13. Selvaraj V, Nepal N, Rogers S, Manne ND, Arvapalli R, et al. (2015) Lipopolysaccharide induced MAP kinase activation in RAW 264.7 cells attenuated by cerium oxide nanoparticles. Data in brief 4: 96-99.

14. Aghazadeh Y, Rone MB, Blonder J, Veenstra TD, Hales DB, et al. (2012) Hormone-induced 14-3-3? adaptor protein regulates steroidogenic acute regulatory protein activity and steroid biosynthesis in MA-10 Leydig cells. J Biol Chem 287: 15380-15394.

15. Sullivan M, Brian A (1985) Control and production of leukotriene B4 in rat tumor and testicular Leydig cells. Biochem J 230: 821-824.

16. Morris ID (1996) Leydig cell toxicology. In: The Leydig Cell in Health and Disease. Payne AH, Hardy MP, Russell LD (eds.). Cache River, New York, USA, pp: 575-580.

17. Brealey D, Brand M, Hargreaves I, Heales S, Land J, et al. (2002) Association between mitochondrial dysfunction and severity and outcome of septic shock. The Lancet 360: 219-223.

18. King SR, Liu Z, Soh J, Eimerl S, Orly J, et al. (1999) Effects of disruption of the mitochondrial electrochemical gradient on steroidogenesis and the steroidogenic acute regulatory (StAR) protein. J Steroid Biochem Mol Biol 69: 143-154.

19. Allen JA, Shankara T, Janus P, Buck S, Diemer T, et al. (2006) Energized, polarized, and actively respiring mitochondria are required for acute Leydig cell steroidogenesis. Endocrinology 147: 3924-3935.

20. Artemenko IP, Zhao D, Hales DB, Hales KH, Jeffcott CR (2001) Mitochondrial processing of newly synthesized steroidogenic acute regulatory protein StAR, but not total StAR, mediates cholesterol transfer to cytochrome P450 side chain cleavage enzyme in adrenal cells. J Biol Chem 276: 46583-46596.

21. Mahmud T, Wrigglesworth JM, Scott DL, Bjarnason I (1996) Mitochondrial function and modi® cation of NSAID carboxyl moiety. Inflammo Pharmacology 42:189-194.

22. Puett D, Li Y, DeMars G, Angelova K, Fanelli F (2007) A functional transmembrane complex: the luteinizing hormone receptor with bound ligand and G protein. Mol Cell Endocrinol 260: 126-136.

23. Yao B, Liu HY, Gu YC, Shi SS, Tao XQ, et al. (2011) Gonadotropin-releasing hormone positively regulates steroidogenesis via extracellular signal-regulated kinase in rat Leydig cells. Asian J Androl 13: 438-445.

24. Lin T (1984) Mechanism of Action of Gonadotropin-releasing Hormone-stimulated Leydig Cell Steroidogenesis. J Androl 5: 201-205.

25. Bingham CO, Austen KF (1999) Phospholipase A2 enzymes in eicosanoid generation. Proc Assoc Am Physicians 111: 516-524.

26. Shureiqi I, Chen D, Lee J, Yang P, Newman RA, et al. (2000) 15-LOX-1: a novel molecular target of nonsteroidal anti-inflammatory drug-induced apoptosis in colorectal cancer cells. J Natl Cancer Inst 92: 1136-42.

27. Ludwig K, Andrzej F, Andreas J, Maria S, Przylipiak, et al. (1991) Production of leukotrienes in gonadotropin releasing hormone-stimulated pituitary cells: Potential role in luteinizing hormone release. Proc Natl Acad Sci USA 88: 8801-8805.

28. Mohanad Al-Bayati, Khamas W (2015) The Importance to Implement and Enforce of Standardized Guidelines For The Care And Use Of Laboratory Animals in Research And Teaching In Iraqi Scientific Institutions. TOFIQ Journal of Medical Science 2: 34-43.

29. Mohanad Al-Bayati A (2014) A remarkable Activity of Steroid Biosynthesis in Captopril Preserved Leydig Cells of Mice Implicated Leukotriene B4 and Gonadotropin Releasing Hormones *in vitro*. Am J BioSci 2: 203-210.