EFFECT OF CHRONIC TREATMENT WITH A CYCLOOXYGENASE INHIBITOR ON REPRODUCTIVE PARAMETERS IN MALE RAT

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Background: Indomethacin is a member of non-steroidal anti-inflammatory drugs (NSAIDs) commonly used for treatment of gout, arthritis, and other inflammatory conditions. It has been shown to inhibit ovarian prostaglandins synthesis in mammals, birds, fish and reptiles. However, the effects of its chronic administration on male reproductive functions remain largely unknown. Using rat as a model, we studied the effect of chronic treatment with indomethacin on the male reproductive system. **Methods:** Testosterone was measured in the serum, testicular tissue, and testicular interstitial fluid by radioimmunoassay. Moreover, we also studied the direct effect of indomethacin *in vitro* on luteinizing hormone stimulated testosterone secretion from the Leydig cells isolated from various treatment groups. **Results:** Indomethacin treatment for 50 days caused a significant but reversible decrease in prostate weight, epididymal sperm reserves and sperm motility score compared with control rats ($p<0.05$). *In vitro* stimulation of Leydig cells isolated from treated rat's testes with luteinizing hormone (250 µIU) produced significantly reduced testosterone compared with cells from control groups ($p<0.05$). Furthermore, stimulatory effect of luteinizing hormone on the control Leydig cells was significantly reduced when these cells were challenged with luteinizing hormone in the presence of indomethacin, ($p<0.05$). Testosterone concentration in the testicular tissue and testicular interstitial fluid reduced after indomethacin treatment ($p<0.05$). **Conclusion:** Due to its significant inhibition of key reproductive hormones, indomethacin effectively inhibits reproductive functions if used on a long-term basis. In his study, we have identified potential risks in the long-term use of cyclooxygenase inhibitors.
Keywords: Cyclooxygenase inhibitor, reproductive functions, male rats

INTRODUCTION

Prostaglandins (PG) play substantial roles in regulating reproductive activity in both male and females.[1,2] Prostaglandins are abundantly found in the male reproductive tract.[3] Prostaglandins E and F play an important role in sperm metabolism and its functions[4,5], and also increase the contractility of the epididymal tubule smooth muscle layer[6]. Moreover, systemic administration of PGs increases circulating levels of Prolactin (PRL)[7,8], Follicle Stimulating Hormone (FSH)[7] and Luteinizing Hormone (LH),[9,10]

It is clear that, chemical inhibition of the PGs have dramatic effect on female reproduction.[11] Indomethacin has been shown to inhibit ovarian prostaglandins synthesis in mammals[12], birds[13], fish[14], and reptiles[15]. Inhibition of PG biosynthesis by treatment with indomethacin or other NSAIDs during pro-oestrus, estrus and metestrus phases, prevents ovulation in rats[16] *In vitro* and *in vivo* treatment with indomethacin has shown inhibitory effect on basal and GDF-9-stimulated progesterone in pre-ovulatory Granulosa cell culture system.[17] Moreover, if administered at the time of implantation in rabbits indomethacin decreases plasma progesterone concentration and the number of viable embryos.[18] Prolonged treatment with indomethacin also reduces fertility in female animals.[19] We have demonstrated previously[20] that chronic treatment of male rats with indomethacin effectively inhibits steroidogenesis at the

level of testis, which leads to rise in plasma luteinizing hormone (LH).

Indomethacin, a non-steroidal anti-inflammatory drug (NSAIDs), inhibits both isoezymes, COX1 and COX2.[21,22] Inhibition of COX-1 may result in adverse effects on the gastro-intestinal tract and the renal functions. COX-2 inhibition leads to anti-inflammatory effect[23] Indomethacin is prescribed for alleviation of pain, tenderness, inflammation, and stiffness caused by gout, arthritis, and other inflammatory conditions.[17] Despite its well-known role as a PG inhibitor, indomethacin is used for the long-term treatment of several inflammatory conditions, the effects of its chronic administration on male reproductive functions remains largely unknown. In this study, we have investigated the effect of chronic treatment with a cyclooxygenase inhibitor (indomethacin) on reproductive functions. Moreover, we looked at direct *in vitro* effects of indomethacin on LH stimulated testosterone secretion from Leydig cells.

MATERIAL & METHODS

Twenty adult virgin male rats (Wistar, average weight 171±23 g) about 90 days old were randomly allocated to two groups of 10 each. These rats were obtained from AKU animal facility where they were maintained under standard conditions of 14 hr light and 10 hr dark cycle at 23 °C. The rats had free access to food and water.

Ten rats received daily subcutaneous injections of indomethacin suspension (Sigma Chemical Co., St Louis, MO, USA) at a dose of 2 mg/Kg body weight in

200µl saline for 50 days. This dose was selected after preliminary tests with different doses. Control (n=10) received the same volume of saline. Treatment plan is given in the figure-1. At the end of the treatment rats were euthanised. Blood, was collected and accessory reproductive organs (testes, prostate, seminal vesicle, and epididymes) were dissected, removed and weighed. A small piece of testes was cut and fixed in 5% gluteraldehyde for histological studies.
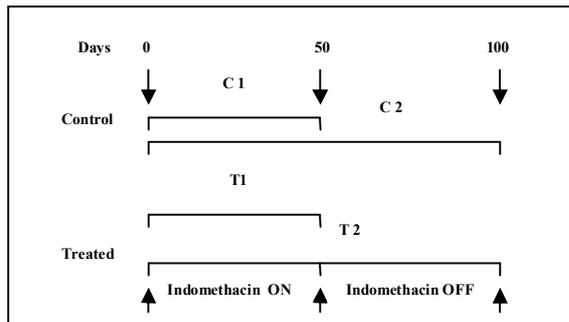

Figure-1: Treatment Plan

Leydig cell isolation was carried our according to the procedure described previously[20] Cell viability (>80% viable) was determined by counting number of cells excluding trypan blue on a haemocytometer.[24]

The Leydig cells from each rat in all groups were incubated with LH and indomethacin, alone or in combination (no stimulation, LH 250 µg or LH250+indo 40 µg). LH dilutions were made in the incubation medium and indomethacin was dissolved in the organic solvent dimethylsulfoxide (DMSO, Sigma, 2 mg/ml). LH (NIDDK-hLH-B-SIAFP2) was a gift from National Hormone and Pituitary Programme California, USA (potency 6100 IU/mg in terms of WHO IRP 68/40).

TIF was collected as previously described with some modifications.[25–28] Briefly, one testis was used to collect TIF and it was given small 2 to 4 mm incisions at the caudal ends of the testes capsules. The testis was suspended 1 cm above 20 µl of 0.1 M acetic acid in glass tubes overnight (18–20 hr) at 4 ºC. The TIF volumes were then carefully measured and stored at -70 ºC till testosterone was measured in them by radioimmunoassay.

One epididymis from each rat was removed to determination the epididymal sperm reserve by a technique modified from.[29] Each epididymis was homogenized using a tissue homogeniser as described by.[30] Homogenate was diluted 1:1 and the epididymal sperm reserve was calculated by using a haemocytometer.

Sperm motility score was determined through a technique described by.[30] Sperm samples from the contralateral vas deferens were used for epididymal sperm reserve assessment. Each sample was diluted in 0.5 ml of M-199 (Sigma) at 37 ºC for 10 min, and the percentage of motile sperm and the degree of their motility (expressed in percent) were determined by phase contrast microscopy.

Testosterone was measured in the incubation medium directly by a highly sensitive RIA according to WHO protocol, using ^{3}H labelled testosterone, as tracer and a highly specific antiserum for testosterone from Guildhay UK. Serum and testicular testosterone was measured in extracted samples, whereas RIA reagents were directly added to tubes containing incubation medium. After addition of all the reagents, tubes were incubated for 30 min. at 4 ºC. The bound and unbound fractions were separated by the addition of 0.1% charcoal. Radioactivity was measured in a liquid scintillation counter. Testosterone concentration was calculated by logit-log transformation.[31]

The sensitivity of testosterone assay was 0.0125 ɳg and the intra-assay coefficient of variation was <10%. The levels of testosterone in the media were expressed as ɳg/ml.

Testicular tissue was prepared for histology by epoxy resin sectioning. Briefly, a small piece of testes was fixed in 100 mM cold gluteraldehyde and cacodylate buffer *p*H 7.2. Ascending grades of acetone were used to dehydrate the tissue. Tissue was infiltrated by the mixtures of acetone and ACM mixture and embedding was done in ACM II mixture. One micron thick sections were cut in Nova Ultra tome and stained with 1% toluidene blue in 1% borax.

All The data obtained were expressed as Mean±SD. The data was analysed for differences among treatment and from its withdrawal, using Student's *t*-test. A '*p*-value' of 0.05 or less was considered significant.

RESULTS

Table-1 shows the body weights and the various reproductive parameters. There was a no significant difference in the body weight between CI and TI. Indomethacin treated rats did not show any significant effect on testicular and relative weight of seminal vesicles but this treatment significantly reduced relative prostrate weight, compared to their respective control. In the recovery group, there was a significant difference ($p<0.05$) between body weights of control CII and its respective treated group TII. In these groups, there was a significant difference ($p<0.05$) between the testicular weights and in the weights of seminal vesicles.

Conversely, no significant difference was noted between weights of the pituitaries in either treatment or the reversal groups compared with their respective controls.

Relative epididymal weights in the reversal group treated animals, TII were significantly ($p<0.05$) different compared with their controls, CII. Treatment

with indomethacin and its termination, significantly ($p<0.05$) altered the epididymal sperm reserves compared to their respective controls.

The mean sperm motility score of male rats administered indomethacin for 50 days decreased significantly ($p<0.01$) compared with the control animals. Reversal group, TII showed significant ($p<0.01$) restorative effect on mean sperm motility score, compared with TI. However, this score was still significantly lower ($p<0.01$) compared with the respective control group CII.

Results of the *in vitro* stimulation of Leydig cells from control animals and treated rats are given in the Table-2. *In vitro* stimulation of Leydig cells from control animals, with LH 250 µIU resulted in significantly higher ($p<0.05$) testosterone secretion compared with control un-stimulated cells from control animals. Stimulation of Leydig cells from indomethacin treated animals, with 250 µIU LH produced significantly ($p<0.05$) less testosterone as compared with its control group. In order to investigate effect of short-term *in vitro* treatment of Leydig cells with indomethacin on testosterone secretion, Leydig cells from the control and the treated rats were stimulated with LH 250 µIU with or without indomethacin 40 µg.

Treatment with indomethacin significantly ($p<0.05$) reduced testosterone secretion in response to stimulation with LH 250 µIU. However, when Leydig cells from indomethacin treated rats were stimulated with LH 250 µIU, their steroidogenic response was significantly ($p<0.05$) reduced compared with LH stimulated Leydig cells from the control animals. In the group TII, *in vitro* treatment with LH 250 µIU showed a significant ($p<0.05$) stimulatory effect compared with control. Although statistically at a non-significant level, LH 250 stimulated increase in the secretion of testosterone was inhibited both in TI and TII groups by indomethacin treatment *in vitro*.

Table-3 shows the concentration of testosterone in testicular tissue, TIF and serum. The testicular tissue testosterone concentration significantly decreased ($p<0.05$) in the treated group compared with control. Similarly, TIF testosterone concentration in indomethacin treated animals was significantly lower ($p<0.05$) compared with controls. Testosterone concentration in testicular interstitial fluid in TII group was significantly higher ($p<0.05$) compared with the treated animals. Serum testosterone in the treated group was significantly lower ($p<0.05$) compared with control. However, there were no significant differences in serum testosterone levels between CII and TII or TI and TII.

Table-1: Effect of Chronic Treatment With Indomethacin on Some Reproductive Parameters in the Male Rat

Treatment (n =5)	Body Weight (g)	Relative Testes wt. (g/100g of b.w)	Relative Prostate wt. (g/100g of b.w)	Relative Seminal vesicle wt. (g/100g of b.w)	Relative Epididymis wt. (g/100g of b.w)	Relative Pituitary wt. (g/100g of b.w)	Epididymal Sperm Reserve 10-6	Sperm Motility Score
CI	370±50	0.42±0.02	0.19±0.01	0.1±0.01	0.13±0.01	2.0±0.2	5.3±0.55	1730±180
TI	374±32	0.47±0.02	0.15±0.01*	0.1±0	0.13±0	2.2±0.16	3.8±1.1*	465±60*
CII	436±12‡	0.35±0.02‡	0.18±0.04	0.07±0‡	0.11±0‡	1.8±0.2	7.3±0.7	1860±147
TII	334±44†	0.42±0.05†	0.18±0.05	0.1±0.0†	0.15±0.01†§	2.0±0.3	9.2±1.1†§	1109±199†§

*Significant difference between CI & TI, †Significant difference between CII & TII, ‡Significant difference between CI & CII, §Significant difference between TI & TII. **Key:** CI (Saline treated), TI (Indomethacin treated), CII (Saline discontinued), TII (indomethacin discontinued)

Table-2: Effect of Indomethacin on LH Stimulated Testosterone secretion from the Leydig Cells

In vitro Treatment	*In vivo* Treatment			
Treatment (n=3)	Control I	TI	Control II	TII
Control	1.39±0.6	0.8±0.3	1.2±0.5	0.7±0.1
LH 250 µIU	2.6±0.5*	1.1±0.2*†	1.8±0.8	1.1±0.2*
Indo. 40 µg	1.1±0.4	0.8±0.3	1.2±0.5	0.6±0.3
LH 250 ± Indo. 40	1.3±0.7‡	0.9±0.3	1.5±0.6	0.8±0.3

* Significant differences between Control & LH 250, †Significant difference between LH 250 in control I TI, ‡Significant difference between LH 250 & LH 250+Indo.40

Table-3: Testosterone Concentration in Serum, Testicular Tissue and Testicular Interstitial Fluid

Treatment	Testes-T (ηg/100 mg)	TIF –T (ηg/ml)	Serum-T (ηg/ml)
CI	114±33	105.7±33	5.1±1.5
TI	51±14*	51±14.6*	3.7±0.8*
CII	140±35	112.3±34.6	4.7±1.7
TII	101±33†‡	92.1±23†	3.4±2

*Significant differences between CI & TI, †Significant differences between TI & TII, ‡Significant differences between CII & TII

Figure-2 shows the transverse section of seminiferous tubule from a control rat. Note the presence of a single layer of myoepithelial cells (MC). Germinal epithelium is arranged compactly and vesicular nuclei exhibit DNA synthetic activity. Spermatogonia with dark nuclei (Sg) are present along the basement membrane. Primary spermatocytes (PS) show typical condensation of chromosomes depicting meiotic activity. Toward the lumen spermatids and sperms (Sp) are in various stages of development.
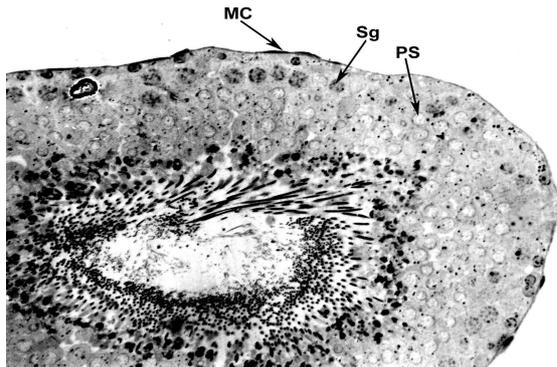

Figure-2: Control

Figure-3 shows the transverse section of seminiferous tubule after treatment. Note the presence of a single layer of MC. The organization of the germinal epithelium (GE) is disrupted. Large spaces have appeared in between germinal cells and degenerating cells are prominent. Absence of meiotic activity indicates the negative effect of treatment on the cell cycle in the lineage of spermatocytes.
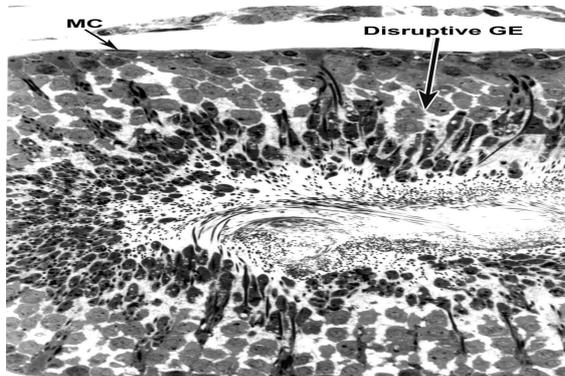
Figure-3: Treatment with Indomethacin for 50 days

The micrograph in Figure-4 shows transverse section of seminiferous tubule 50 days after termination of treatment. Note the presence of a single layer of myoepithelial cells (arrowheads). Spermatogonia (Sg) and primary spermatocytes exhibit normal nuclear profiles indicating normal cell cycle activity. Intercellular spaces in the germinal epithelium are eliminated and the luminal side of the epithelium shows maturation of spermatids and sperms.
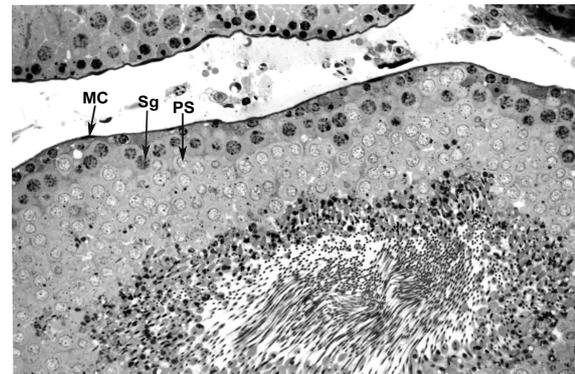

Figure-4: Reversal Group

DISCUSSION

Data from our study demonstrates that indomethacin treatment decreases prostate weight, epididymal sperm reserves and sperm motility score but cessation of treatment restores the weight of testis, relative weight of prostate, epididymes and epididymal sperm reserves, as well as sperm motility score. Epididymal tubule passage is an important process that leads to the formation of a correct milieu for sperm maturation and storage.[32] Significant difference between the epididymal sperm reserve in treated and reversal group shows a bounce back effect of removal of treatment on the spermatogenesis. The histological evidence discussed later, also supports these results.

Previous studies[20] showed an increase in plasma LH during treatment. A drop in T levels by a negative feedback system causes an elevation of LH from pituitary. This further explains that indomethacin has a direct effect at the level of testes rather than pituitary.

Sperm maturation is a complex process and prostaglandins play an important role by regulating luminal microenvironment. In basal conditions in addition to the epididymis COX-2 mRNA and protein is also expressed in a wide range of tissues like the lung, testis, brain, and prostate under.[33,34] More than 90% of the extra testicular sperms are stored in epididymis.[35] Inhibition of the COX pathway may result in disruption of epididymal microenvironment. Thus, the decrease in epididymal weight was likely due to a reduction in epididymal sperm reserves.

Testosterone concentration measured in testicular tissue and testicular interstitial fluid was reduced during treatment and recovered during the withdrawal period to almost pre-treatment levels. The same pattern was observed in serum testosterone concentration. Testosterone secretion and TIF formation are the two fundamental regulatory aspects of normal testicular function. There is evidence that AA and its metabolites are involved in testicular functions. It has been shown that LH is responsible for rapid release of AA from Leydig cells[36] related to concentration of

membrane receptors of LH-Hcg[37]. *In vitro* data from this study also supports our observation of indomethacin induced reduction in serum and TIF testosterone secretion. Leydig cells from control rats stimulated with LH in the presence and absence of indomethacin showed significant difference in testosterone production. Similarly, Leydig cells from Control group and indomethacin treated rats responded significantly different to in vitro LH stimulation.

Our histological evidence from testicular studies further supports this finding. Figure 1 shows the transverse section of seminiferous tubule from a control rat. It presents the normal architecture of the testicular tissue showing a single layer of myoepithelial cells and germinal epithelium arranged compactly with vesicular nuclei exhibiting DNA synthetic activity. Dark nucleated spermatogonia (Sg) are visible along the basement membrane and the primary spermatocytes (PS) show typical condensation of chromosomes depicting meiotic activity. Towards the lumen, spermatids and sperms (Sp) are in various stages of development. In the indomethacin treated rats, (Figure 2) organization of the germinal epithelium is disrupted. Large spaces can be seen in between germinal cells and degenerating cells are prominent. Treatment also led to a negative effect on the cell cycle in the lineage of spermatocytes, indicated by the absence of meiotic activity. This study also shows that the negative effects of indomethacin treatment are reversible. Figure 3 shows the transverse section of seminiferous tubule 50 days after termination of treatment. One can note a single layer of myoepithelial cells, and spermatogonia and primary spermatocytes exhibiting normal nuclear profiles indicating normal cell cycle activity. The germinal epithelium is without the intercellular spaces noted previously in the treated testes and the luminal side of the epithelium shows maturation of spermatids and sperms.

CONCLUSIONS

In conclusion, cyclooxygenase inhibitors undoubtedly disrupt the homeostatic processes by inhibiting the essential reproductive hormones. Indomethacin can effectively inhibit reproduction on a long-term basis. The most concerning characteristic of NSAIDs is that the concentration which is required to inhibit PG biosynthesis are usually much lower than those essential for their other pharmacological effects. The long-term use of such treatment strategies would require a better therapeutic alternate. Interestingly, the data provides insights for possibility to develop a therapeutic treatment to induce a reversible inhibition of reproductive functions. Our progress in identifying potential risks for long-term effects of indomethacin in male rats raise question about the long-term use of compounds similar to indomethacin on human reproduction but also open new avenues to look for possible contraceptives working through inhibition of cylooxygenase pathways.

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