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ORIGINAL ARTICLE

Distribution of cyclooxygenase-1 and cyclooxygenase-2 in the mouse seminal vesicle

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Summary
Cyclooxygenase is the enzyme responsible for the production of prostaglandins (PGs). This cyclooxygenase exists in two isoforms: cyclooxygenase-1 (COX-1) and cyclooxygense-2 (COX-2). In humans and primates high levels of COX-2 are detected in the seminal vesicle. Further, the main source of PGs in the semen of these species is from the seminal vesicle. In rodents, the source of PGs in semen is from the vas deferens and abundant levels of COX-2 are detected. A direct relation is thought to exist between COX-2 levels and the source of PGs in semen. Moreover, the role of COX-1 and COX-2 in the seminal vesicle of rodents is obscure. The present study aims at localizing COX-1 and COX-2 in the seminal vesicle of mice. Immunohistochemical staining and COX activity assay revealed COX-1 as a dominant isoform in the mouse seminal vesicle. On treatment with nimesulide – a preferential COX-2 inhibitor - no change in staining intensity and COX activity was observed. The total PG levels also appeared to be unaltered following nimesulide treatment. This confirms that nimesulide had no effect on COX-1. The results presented here suggest COX-1 is the dominant isoform in the mouse seminal vesicle and is responsible for PG synthesis.

Key words: cyclooxygensase-1 – cyclooxygenase-2 – mice – nimesulide – prostaglandins – seminal vesicle

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INTRODUCTION

Nephrotoxicity and hepatotoxicity are commonly reported adverse effects with respect to the use of cyclooxygenase-2 (COX-2) selective inhibitors (Balasubramaniam 2000, Montensinos et al. 2001). The reason for such toxicity following suppression of COX-2 suggests a physiological role for COX-2 in these tissues (Morham et al. 1995). This COX-2, which is one of the isoforms of the enzyme cyclooxygenase (COX), is thought to be induced only

during inflammation, whereas cyclooxygenase-1 (COX-1), the other isoform, is constitutively expressed in most of the tissues and serves the 'house keeping' function of fluid electrolyte balance (Botting 2006). Recent studies have reported the constitutive expression of COX-2 in brain, kidney, uterus and ovary. Several functions for COX-2 are proposed with respect to these tissues (Simmons et al. 2004).

The role of COX-1 and COX-2 with respect to male reproductive organs remains obscure and differs from species to species. In humans and monkeys, high levels of COX-2 are found in the seminal vesicle while COX-1 is the dominant isoform in the testis and epididymis (Kirschenbaum et al. 2000, Lazarus et al. 2004). In rodents, high levels of COX-2 but not COX-1 are detected in the vas deferens as well as in the caudal part of the epididymis (McKanna et al. 1998). This constitutive expression of COX-2 along with COX-1 is required for prostaglandin (PG) synthesis in male reproductive organs. The source of PGs in human semen is from the seminal vesicle while in rodents, the vas deferens serves this purpose.

The seminal vesicle is an important accessory reproductive organ whose secretions form the bulk of sperm. PGs in semen were originally thought to be synthesized from the prostate gland, but later the seminal vesicle was found to be the source (Von Euler 1936). In the present study, the authors tried to localize COX-1 and COX-2 in the mouse seminal vesicle in order to understand which isoform is intensely expressed. Presently, COX-2 inhibitors are preferred when compared to COX-1 and nonspecific COX inhibitors. The toxicity of COX-2 inhibitors is also widely reported and added recently to their toxic profile is the increased risk of cardiovascular events. For the above reason, Nimesulide – a preferential COX-2 inhibitor was chosen to suppress COX-2. Through this, it is possible to delineate which isoform of COX is predominantly involved in the synthesis of PGs. In addition, any adverse effects of nimesulide on this particular organ can be ruled out.

MATERIALS AND METHODS

Animals and drug treatment

Adult male albino mice of Swiss strain with body weight 25 ± 2 g and approximately 90 days old bred in the central animal house (Rajah Muthiah Medical College, Annamalai University, Tamilnadu, India) were used in this study. All the animals were fed on standard pellet diet (Agro Corporation Private Limited, Bangalore, India). The pellet comprised 21% protein, 5% lipids, 4% crude fibre, 8% ash, 1% calcium, 0.6% phosphorous, 3.4% glucose, 2% vitamin and 55% nitrogen-free extract (carbohydrates), thus providing metobolizable energy of 3600 kcal/kg. Water was available *ad libitum*. The animals were housed in plastic cages under controlled conditions of 12 h light/12 h dark cycle, 50% relative humidity and at a temperature of 30±2 °C. They were maintained in accordance with the guidelines of the National Institute of Nutrition (Indian Council of Medical Research, Hyderabad, India) and the study was approved by the Animal Ethical Committee, Annamalai University (proposal number: 299).

Nimesulide was purchased from Sigma chemicals (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) which served as a vehicle. The final concentration of DMSO in water was 0.1%. All the drugs were administered orally; the animals were divided into the following 5 groups. Group 1 served as the control which received only 0.1% DMSO. Group 1 was maintained for 45 days and sacrificed along with group 5. Group 2 and 3 received a single oral dose of nimesulide 12 mg/kg body weight and maintained for 3h and 6 h respectively. Group 4 and 5 received nimesulide 12 mg/kg twice a day for 15 and 45 days respectively.

Immunostaining for COX-1 and COX-2

Immunostaining was carried out according to Neeraja et al.(Neeraja et al 2003). Briefly, the seminal vesicle was dissected free of the connective tissue and immediately fixed in Bouin's solution, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax. 7µm thick sections were cut and mounted on poly-L-lysine coated slides. The sections were dewaxed in xylene and rehydrated in descending grades of ethanol. The slides were microwaved at 95 °C for 5 min for antigen retrieval. Non-specific binding sites were blocked with 5% normal goat serum, incubated with rabbit anti-COX-1 antibody (Cayman Chemicals, USA, Cat. No. 160109) overnight at 4 °C and after 1:5000 dilution in 0.1% bovine serum albumin (BSA), rinsed in phosphate buffered saline (PBS) followed by incubation in a secondary antibody (biotinylated goat antirabbit IgG). Immunoreactivity was visualized by treating with avidin-biotin-horseradish peroxidase complex (ABC) for 60 min at 22 °C with diaminobenzidine as the chromogen and counterstained with Harris haematoxylin. The slides were dehydrated in ascending grades of ethanol and mounted with Mount-Quick, coverslipped and photographed using a Nikon microscope. The above mentioned procedure was followed exactly for COX-2 immunostaining using the rabbit anti-COX-2 antibody affinity purified (Cayman Chemicals, USA, Cat. No.160126). The staining intensity was determined by a staining index

as follows: – no staining, + mild staining, ++ moderate staining, +++ intense staining.

COX activity assay

COX activity was measured by using an enzyme immuno assay kit (Cayman Cat., No.760151). The kit measures the peroxidase activity of cyclooxyganase. The peroxidase activity was measured colorimetrically by monitoring the appearance of oxidized N,N,N´,N´-tetramethyl-p-phenylenediamine (TMPD). According to the manufacturer's instructions animals were perfused with tris-HCl, the seminal vesicle was removed and homogenized in a cold tris-HCl buffer followed by centrifuging at 10,000 × g for 15 min at 4 °C and the supernatant stored at –80 °C until assayed. The samples were assayed in triplicate and each sample was assayed for total COX activity (TA) as well as COX-1 and COX-2 activity using isospecific inhibitors supplied with the kit. The percentage of COX-1 or COX-2 present in the sample was calculated using the following formula:

$$\frac{\text{Total activity of the sample - Total activity of inhibitor treated sample}}{\text{Total activity of the sample}} \times 100$$

Prostaglandin levels assay

Tissue samples were prepared as mentioned above. Total PGs in the tissue samples were quantified using an enzyme immuno assay kit (Cayman kit Cat. No.514012). The assay was acetylcholinesterase (AChE) competitive enzyme assay. The PG-AChE conjugate (PG tracer) competes with the PG in the sample in binding to the PG antiserum which in turn binds to the antibody in the well. The amount of PG tracer bound to the well is inversely proportional to the amount of free PG present in the well during incubation.

Lipid extraction and fatty acid analysis

Lipid extraction was carried out according to the method described earlier (Folch et al. 1957). Tissues samples were homogenized in a chloroform:methanol (2:1 v/v) mixture. The final filtrate was allowed to evaporate to dryness. The fatty acid methyl esters were extracted as described earlier (Kates 1972) and analyzed by gas a chromatograph (Shimadzu GC 17A) equipped with a flame ionization detector (250 °C) and a BCX 70 column (30 m × 0.25 mm). The carrier gas was nitrogen (1 ml/min). The oven temperature was programmed to rise from 180 to 220 °C at the rate of 2 °/min. Retention time and peak areas were automatically computed by an integrator (GC solutions). Fatty acid peaks were identified with standard fatty acid methyl esters.

Histological analysis

Tissues were fixed in Bouin's solution. After adequate fixation, tissues were dehydrated in graded alcohol, cleared in xylene and embedded in paraffin wax. Sections were cut at 7µ thickness and stained with haematoxylin and eosin.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) using the statistical package of the social sciences (SPSS) version 10.0 for windows. The values are mean ± SD for six samples in each group. P values <0.05 were considered as the level of significance.

RESULTS

Immunostaining for COX-1 and COX-2

Immunostaining of the control group seminal vesicle with the COX-1 antibody showed an intense cross reactivity in the epithelial cells (+++) of the control group. The cytoplasm was darkly stained but no cross reactivity (!) was found either in the nucleus or in the submucous or smooth muscle layer (Fig. 1a). No increase or decrease in staining intensity was observed in the 3h, 6h and 15 days nimesulide treated groups (figure not shown). The staining intensity for COX-1 was the same (+++) even after 45 days of nimesulide treatment (Fig. 1b). Immunostaining with the COX-2 antibody showed nil cross reactivity (!) in the epithelium as well as in the smooth muscle layer of the control group seminal vesicle (Fig. 1c). Nimesulide administration had no effect on COX-2 immunostaining (Fig. 1d).

COX activity assay

The TA of the seminal vesicle homogenates was around 660 ng/mg/min in the control group for which OX-1 activity was around 490 ng/mg/min. This contributes to 75% of TA. COX-2 activity was around150 ng/mg/min. This accounted for only 25% of the TA. No significant change in COX activity was observed following nimesulide administration and appeared to be same as that of control group (Fig. 2).

Total prostaglandin levels

The total PG levels in homogenates of the mouse seminal vesicle was around 150 pg/mg tissue. No

further increase or decrease in PG levels was observed in any of the nimesulide treated groups (Fig. 3).

Fatty acid levels

The levels of most mono- as well as poly-unsaturated fatty acids (PUFAs) of total lipids were not altered in any of nimesulide treated groups. They appeared be same as that of control group (Table 1).

Histological analysis

The histology of the control group mouse seminal vesicles showed normal histological features having a lobulated pattern with a uniform low cuboidal epithelium bearing a distinct nucleus (Fig. 4a). The epithelium was thrown in to short folds which did not reach the lumen and the lumen filled with secretory material which was eosinophilic in nature (Fig. 4b). No histopathological changes were observed in the mucous or submucous layer in any of nimesulide treated groups (Fig. 4c).

Table1. **Fatty acid composition (ng/100 mg) of total lipids of control and nimesulide treated seminal vesicle.**

Fatty acid	Control	3 h	6 h	15 days	45 days
C18:1n9t	269.63 ± 20.83^{b}	252.36 ± 19.21^{a}	266.04 ± 20.25^{b}	261.05 ± 19.87^{a}	258.36 ± 19.67^{a}
C18:2n6t	205.74 ± 15.66^{a}	198.04 ± 15.07^{a}	201.65 ± 15.35^{a}	200.03 ± 15.23^{a}	205.45 ± 15.64^{a}
C18:3n3	9.39 ± 0.71^{a}	8.82 ± 0.67^{b}	8.00 ± 0.60^{b}	9.65 ± 0.73^{a}	$9.32 \pm 0.71^{a,b}$
C18:3n6	$5.81 \pm 0.43^{a,b}$	$5.90 \pm 0.44^{a,b}$	5.42 ± 0.41^{a}	6.00 ± 0.45^{b}	6.22 ± 0.47^{b}
C20:0	0.94 ± 0.09^{a}	1.23 ± 0.1^{b}	1.9 ± 0.14^{d}	1.21 ± 0.10^{b}	1.66 ± 0.16^{c}
C20:2	17.39 ± 1.32^{a}	17.01 ± 1.29^{a}	18.32 ± 1.39^{a}	18.66 ± 1.42^{a}	18.22 ± 1.38^{a}
C20:3n3	156.09 ± 11.88^{a}	152.75 ± 11.59^{a}	155.54 ± 11.84^{a}	150.53 ± 11.46^{a}	157.25 ± 11.97^{a}
C20:4n6	3.50 ± 0.26^{a}	4.20 ± 0.32^{c}	$3.9 \pm 0.29^{b,c}$	$3.6 \pm 0.27^{a,b}$	$3.8 \pm 0.28^{a,b}$
C22:2	$1.22 \pm 0.1^{b,c}$	0.99 ± 0.09^{a}	1.32 ± 0.10^{c}	1.66 ± 0.12^{d}	1.15 ± 0.10^{b}
C22:6n3	12.01 ± 0.91^{a}	13.32 ± 1.01^{d}	$13.01 \pm 0.99^{b,c}$	$12.92 \pm 0.98^{b,c}$	$12.77 \pm 0.97^{b,c}$

Values are given as mean± S.D. of six experiments in each group.

DISCUSSION

PGs are end products of AA metabolism. The role of PGs with respect to female reproduction is widely reported. They are involved in the ovarian luteal function, and implantation of the fertilized ovum (Hamilton and Kennedy 1994) as well as the smooth muscle contraction of the uterus during parturition (Allport and Bennett 2001). With regard to male reproduction, PGs are thought to aid in the motility and capacitation of sperm (Shimizu et al. 1998), relaxation of the smooth muscle (Kelly 1981) and also in erection (McKanna et al. 1998). Whether the PGs required for all these functions are COX-1 derived or COX-2 derived still remains unclear. The present study demonstrates the high immunoreactivity for COX-1 and mild to nil immunoreactivity for COX-2. This suggests that the PGs in the seminal vesicle are COX-1 derived (Klein and Stoff 1987). Further, the COX activity results

confirm COX-1 as the dominant isoform. The localization of COX-1 in the apical surface of the seminal vesicle epithelium suggests a secretory role. Since the seminal vesicle is a rich source of fructose, PGs synthesized in the seminal vesicle is thought to be involved in carbohydrate metabolism. PGs in the seminal fluid are also thought to induce anaerobic respiration by inhibiting aerobic respirationduring ejaculation. This cannot be completely accepted because once the sperm enters the female reproductive tract the oxygen there is sufficient for aerobic respiration. Secondarily, PGs in semen are thought to aid in smooth muscle relaxation but even this reason does not hold good since relaxation of the uterus cannot effectively transport sperm (Kelly 1977). In rodents, high levels of COX-2 as well as COX-1 are expressed along the male reproductive tract especially in the vas deferens (Mckanna et al. 1998). The authors in a previous study have observed COX-2 is equally localized along with COX-1 in the testes of mice (Balaji et al. 2007a) whereas COX-2 is intensely localized in the mouse vas deferens and aids in motility and fertility of sperm (Balaji et al. 2007b).

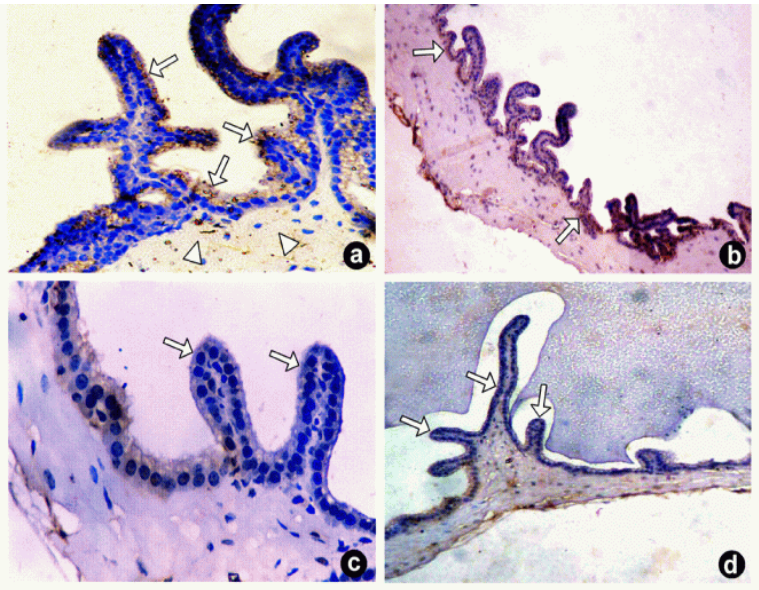

Fig. 1. *(a)* **COX-1 immunostaining of control group** mouse seminal vesicle showing intense (+++) immunoreactivity (arrow) in epithelium and nil immunoreactivity (-) in smooth muscle layer (arrow head) × 40; *(b)* COX-1 immunostaining of 45 days nimesulide treated group seminal vesicle showing intense (+++) immuno reactivity in the cytoplasm of epithelium (arrow) ×20; *(c)* COX-2 immunostaining of control group seminal vesicle showing nil (-) immuno reactivity in the epithelium (arrow) ×40; *(d)* COX-2 immunostaining of seminal vesicle after 45 days of nimesulide treated group same as that of control (arrow) ×20.

A similar finding is observed in turkeys (Kennedy et al. 2003). In humans and primates the main source of PGs is through the seminal vesicle and high levels of COX-2 are observed in the seminal vesicle of these species (Kirschenbaum et al. 2000, Lazarus et al. 2004) but the role of PGs in the seminal vesicle of rodents is not clear.

It is a well known fact that the COX-1 isoform is involved in the regulation of tissue homeostasis. In the present study, COX-2 is minimal and the dominance of COX-1 is observed in immunostaining as well as the COX activity assay. This suggests that PGs produced via COX-1 might be responsible for performing the normal physiological functions of the cell (Leung et al. 2004). Since the secretions of the seminal vesicle form the bulk of semen it is quite possible that a small number of PGs in the semen are derived from the seminal vesicle and that they might be involved in the immuno modulatory effects of the sperm in the female reproductive tract (Kelly 1995).

Nimesulide and other COX-2 selective inhibitors are widely used to suppress COX-2 in order to assess the role of COX-2 in reproductive tissues (Shafiq et al. 2004, Sakurai et al. 2003). In the present study

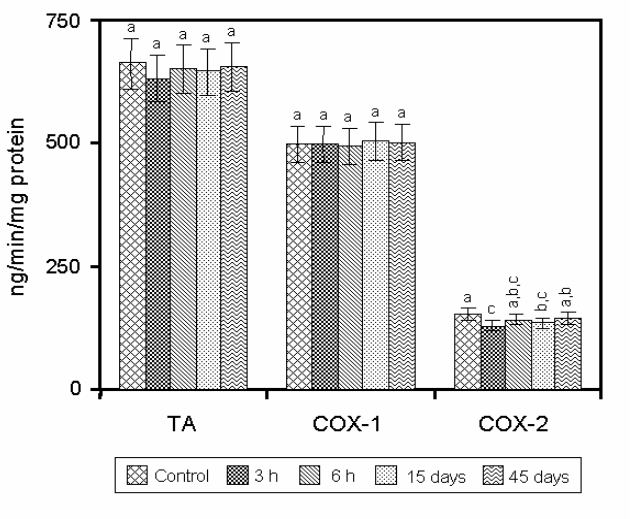

Fig. 2. **TA, COX-1 and COX-2 activity of mice seminal vesicle in experimental versus control groups.** Values are given as mean ± S.D of six experiments in each group. Bar values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT).

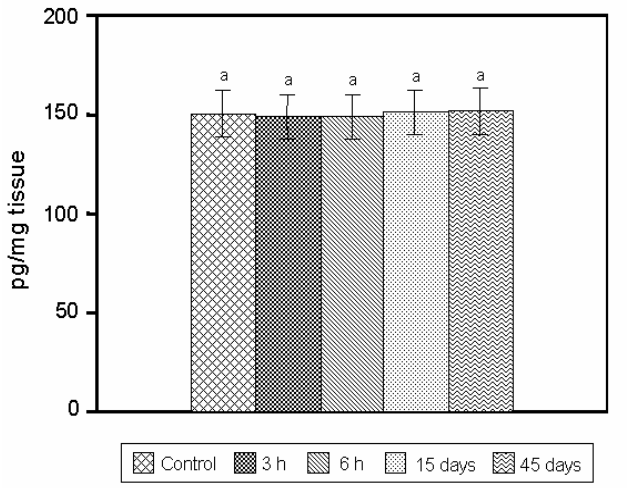

Fig. 3. **Total prostaglandin levels of mice seminal vesicle in experimental versus control group.** Values are given as mean ± S.D. of six experiments in each group. Bar values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT).

nimesulide did not alter COX-1; hence the total PG levels were not altered. This is obvious as nimesulide selectively inhibits COX-2 without disturbing COX-1 (Bernareggi 1998). It is worth mentioning that even COX-2 activity is not altered upon nimesulide treatment because COX-2 activity in the mouse seminal vesicle is minimal and nimesulide does not completely abolish COX-2 activity (Giuliana and Warner 1999).The dose used in the present study is close to the therapeutic dose. Because of this it is further confirmed that, PGs in the mouse seminal vesicle are COX-1 derived. NSAIDs, especially nimesulide, are prone to cause hepatotoxicity by inducing oxidative stress (Mingatto et al. 2002, Tay et al. 2005). NSAIDs also reduce inflammation by accumulating AA which is

cytotoxic to the cell (Monjazeb et al. 2005). No such changes such as increase in AA or other fatty acids were observed in the present study. The histological analysis of the tissues also appeared to be normal, which suggests that nimesulide induced toxicity is due to suppression of COX-2. Since COX-2 is basal in the mouse seminal vesicle no such changes were reflected in our study.

To conclude, COX-1 is the dominant isoform in the mouse seminal vesicle and PGs synthesized in the mouse seminal vesicle are COX-1 derived.

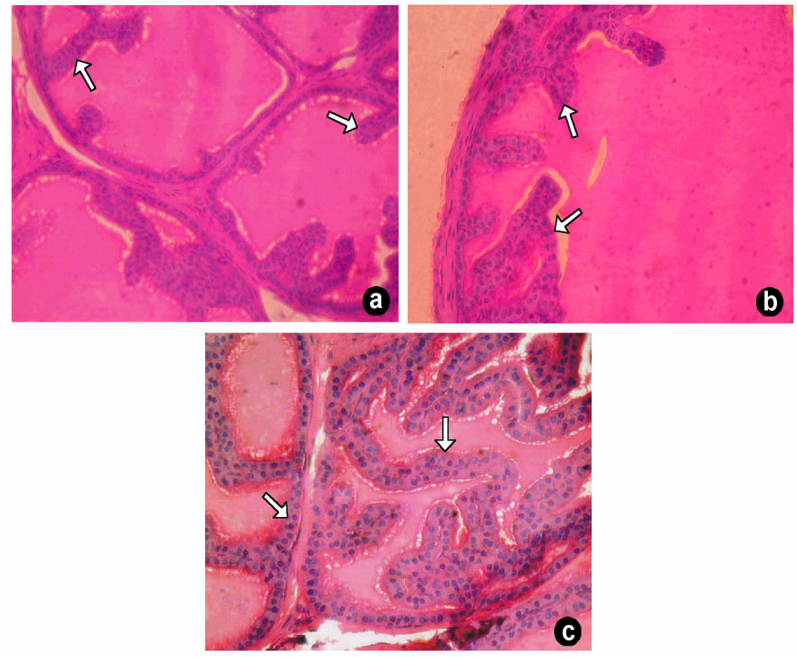

Fig. 4. *(a)* **Photomicrograph of seminal vesicle in control group** showing normal histological features of lobulated pattern with low cuboidal epithelium (arrow) × 20 H&E; *(b)* photomicrograph of control group seminal vesicle showing distinct nucleus in the epithelium (arrow) × 40 H&E; *(c)* seminal vesicle of 45 days nimesulide treated group showing distinct nucleus (arrow) in the epithelium × 40 H&E.

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