Original Article

Effect of Atorvastatin on 7,12-Dimethylbenz (α) Anthracene and Testosterone-induced Prostatic Intraepithelial Neoplasia in the Prostate of Wistar Rats: Role of TRPM7

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ABSTRACT

Aim/Background: The current study was conducted to examine the influence of atorvastatin in experimentally induced prostatic intraepithelial neoplasia in rats. TRPM7 is a potential therapeutic target for treatment of prostate cancer. **Materials and Methods:** In this study, we investigated the effects of atorvastatin (25 and 50 mg/kg/BW p.o.) on cell proliferation of prostate induced by a sequential regimen of testosterone plus single administration of 7,12-Dimethylbenz(a) anthracene. **Results:** Results of present study revealed that the different pattern of prostatic intraepithelial neoplasia with associated High grade PIN in model control group while concurrent treatment with atorvastatin increases the effect by developing a pattern of high grade PIN with dysplasia. Furthermore, atorvastatin treatment produced elevated levels of TRPM7 protein expression with higher Ca/Mg ratio and low ratio of serum testosterone to dihydrotestosterone. **Conclusion:** The results of the present study indicate that atorvastatin has a promotive role in DMBA-testosterone-induced intraepithelial neoplasia in rats through increasing expression of the TRPM7 channel.

Key words: Atorvastatin, Testosterone, PIN, Ca^{2+}/Mg^{2+} ratio, TRPM7.

INTRODUCTION

Prostate Cancer accounts for 9% mortality burden bearing upon around 1.3 million men globally in the year 2018.[1] Prostate cancer is a heterogeneous disease with unknown etiology nevertheless related with age, race and environmental effects.[2,3] Prostatic carcinogenesis research has been hampered due to lack of reliable animal models[4] leading to a major hurdle in evaluating medicines for prostate carcinoma. However previous studies have reported combined treatment with hormone and carcinogen leads to prostatic dysplasia that mimics the human prostate cancer model.[4,5] Presently, mechanism of signal transduction pathway in prostate cancer cell lines (PC-3), specifically for the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signalling pathway is well established for prolonging survival time and enhancing quality of life of prostate cancer. Previous studies had reported that TRPM7 (Transient receptor potential melastain 7) is essential for PI3K/AKT-dependent growth signalling of lymphocyte growth and proliferation.[6,7] TRPM7 is non-selective cation channel from the largest and most diverse TRPM subfamily (TRPM1-8) of TRP superfamily.[8] TRPM7 is recognized and accepted as a key regulators of Mg homeostasis and transporter of other cations.[9,10] Changes in Mg^{2+} concentration have been shown to alter cell proliferation in various cells.[11] Sun *et al.* 2013[12] demonstrated that the TRPM7

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channel has an important role in prostate cancer and have identified that the Ca^{2+}/Mg^{2+} ratio could be essential for the initiation/progression of prostate cancer. Further, Lin reported that TRPM7 was involved in the apoptosis of PC-3 cells induced by TNF-related apoptosis inducing-ligand (TRAIL), indicating that TRPM7 may be applied as a therapeutic target for prostate cancer.[7]

Novel regimes are being developed and advances are being made between various standard treatments of prostate cancer. Increasing evidence suggests that commonly prescribed cholesterol-lowering agent "Statins",[13] independent of their effects on serum cholesterol levels, they show the differential effect on the prevention and treatment of cancer.[14] Further, *in vitro* studies, proved that statins have been shown differential effects on Prostate cancer via a number of cholesterol-mediated and non-cholesterol-mediated mechanisms that affect pathways essential for cell proliferation differentiation and apoptosis regulation[15-18] but the specific mechanisms underlying these effects are not fully understood. Indeed, Increasing findings are being published that provide innovative evidence about the use of Atorvastatin, has been linked to a lowered risk of advanced prostate cancer and improved Prostate Cancer-specific survival.[16-19] In contrast, an *in-vitro* study of atorvastatin on the proliferation of prostate cancer cells showed quite surprising results that atorvastatin and dihydrotestosterone appear to have synergistic effects on prostate cancer cell growth at high concentration.[20] Further, clinical trial in 2018 described that atorvastatin does not lower prostate cancer proliferation rate compared with placebo overall.[21]

Regardless of the innovative research, there is inadequate evidence which fuelled this dichotomous fact about an enigmatic effect of atorvastatin. Hence, this study sought to investigate whether orally administrated atorvastatin affects DMBA and testosterone-induced prostatic intraepithelial neoplasia in the prostate of Wistar rats. We also aimed to evaluate whether the effect, if present, is mediated through TRPM7 channel.

MATERIALS AND METHODS

Animals

10-12 weeks old healthy male Albino Wistar rats of weighing between 250-300 grams were used after one week of adaptation. Rats were housed in sanitized polypropylene cages containing paddy husk and kept under controlled room temperature (25 ± 2°C), relative humidity (55 ± 10%) in a 12 h light-dark cycle. They were permitted free access to drinking water and fed with a controlled standard rat pellet diet throughout the experimental period. The experiment was approved by the Institute Animal Ethical Committee (20180749).

Experimental design of the studyInduction of prostate tumor

Induction of prostate tumor was carried out by a modified protocol derived by Bosland *et al.* 1990[22] and Sharmila *et al.* 2014.[5] First, each rat received daily 50 mg/kg body weight testosterone undecanoate (TU) injections via intraperitoneal (IP) administration for 21 consecutive days. One day after the final dose of TU at 23rd day, rats were received daily 100 mg testosterone undecanoate per kg body weight for 3 days via subcutaneous injection. A day after the final dose of testosterone undecanoate at day 27, rats injected with a single IP injection of 30 mg/kg body weight of DMBA (7,12-Dimethylbenz(α) anthracene) dissolve in corn oil at 10 mg/ml. After three days DMBA injection, rats received an intraperitoneal injection of 4 mg testosterone undecanoate per kg body weight alternatively for 24 weeks.

Experimental Design

A total of 40 rats were divided into four groups and each group consisted of 10 rats. In Group I (NC), rats that received vehicle alone by IP injection, considered as controls. In Group II (TD), rats were induced prostate cancer by using carcinogen (DMBA) plus hormone (testosterone). In Group III (TDA25) and Group IV (TDA50), rats were induced prostate cancer with simultaneous supplementation of Atorvastatin 25 mg/kg and 50 mg/kg body weight, respectively once daily for 20 wk through oral gavage (Figure 1). Atorvastatin supplementation was begun on day 27, one hour before carcinogen treatment and continued until the termination of the study (24 wk). Body weights were recorded every week and Prostate Weight Index (PWI) was calculated. At the end of the experiment (24 weeks), animals fasted for 18 hr and then they were anaesthetized and blood samples were collected from retro-orbital plexus and allowed to clot for 30 min at room temperature. It was then centrifuged at 5000 rpm for 15 min. The serum obtained was used for estimation of testosterone level, DHT level, calcium and magnesium level in serum. After that, rats were sacrificed prostate was dissected and washed several times with cold phosphate buffer saline and blotted and then weighed accurately. The prostate tissues were then fixed with 10% formalin for histological analysis.

Tissue processing for biochemical analysis

Preparation of (100 mg/ml) tissue homogenate: A 100 % (w/v) organ in 0.1 M phosphate buffer (pH 7.4) was homogenized with tissue homogenizer at a speed

of 5000 rpm for 10 min in the ice-cold surrounding environment. The homogenates were centrifuged for 10 min at 10,000 rpm using high-speed cooling centrifuge.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from prostate tissue according to the instructions of the TRIzol Reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.). RT−PCR was performed using the Super Script One-step RT-PCR system (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. There were 35 cycles of 90°C for 35 sec, 56°C for 30 sec and 72°C for 30 sec. β-actin served as an internal control.

The primers were as follows:

For TRPM7 [(GenBank, AC000071)

Forward, 5'-CTG AAGAGGAATGACTACAC-3'

Reverse: 5'-ACAGGAAAAAGAGAGGGAG-3'

For β-actin (GenBank, AC000080)

Forward: 5'−TGAGCTGCGTGTGGCCCCT-GAG-3'

Reverse: 5'-GGGGCATCG GAACCGCTCATTG-3'

PCR products were visualized in agarose gels and results were expressed as TRPM7 expression normalized to β-actin expression for semiquantitative RT-PCR experiments.

Statistical ANALYSIS

The results are expressed as Mean ± SEM. The results were analysed for statistical significance using one-way ANOVA followed by Tukey's *post hoc* test. The value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Atorvastatin on Prostate weight index

As shown in Figure 2 the prostatic weight index was significantly increased in TD (0.26 ± 0.014) as well as simultaneous atorvastatin treatment (0.29 ± 0.012; 0.32 ± 0.009) as compare to NC (0.11 ± 0.004).

Effect of Atorvastatin on Testosterone to Dihydrotestosterone ratio

Illustrated results in Figure 3, indicates that the significantly ($p < 0.05$) low value of T/DHT ratio in TD, TDA25 and TDA50 group, with respect to Group I (NC).

Effect of Atorvastatin on Calcium/Magnesium ratio

Group II i.e. TD showed significant increment in Calcium to Magnesium ratio as compared to NC. In addition, Atorvastatin treatment lifts it to a considerably higher level in TDA25 and TDA50 groups. (Figure 3)

Effect of atorvastatin on the expression of TRPM7 in tissue homogenate

The expression of TRPM7 mRNA and protein were significantly up-regulated in atorvastatin group (TDA25 and TDA50) compared to NC and TD group, as shown in Figure 4.

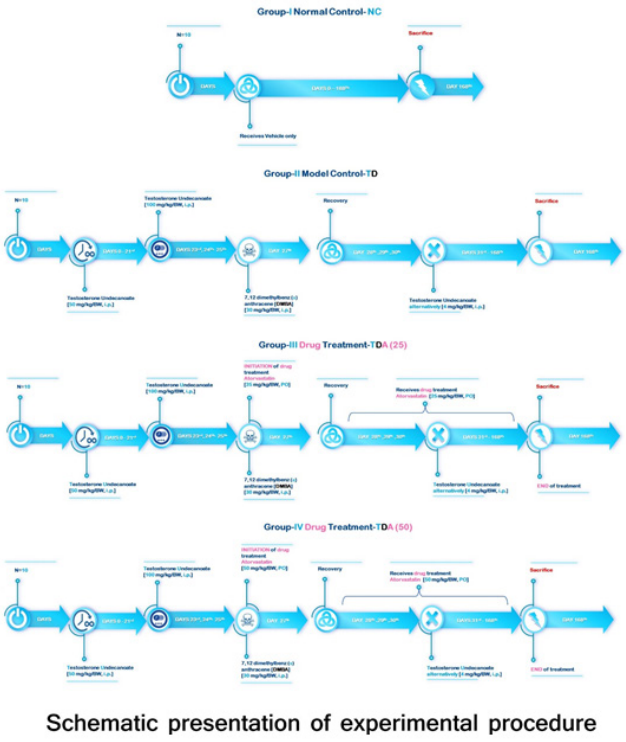


Figure 1: Schematic presentation of experimental procedure.

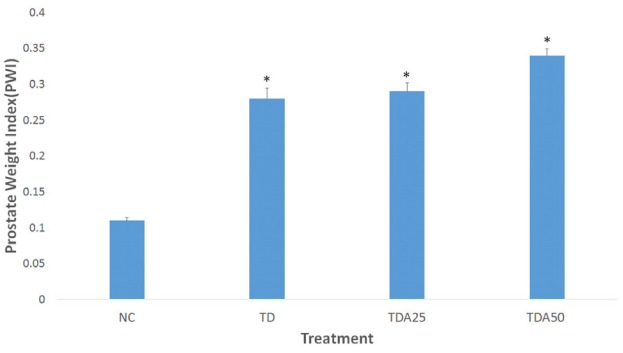


Figure 2: Effect of atorvastatin on prostatic weight index Prostate Weight Index(PWI) =Prostate weight/Body weight * 100 n = 10, * P <0.05 as compared to Normal Control (NC) group, one way ANOVA followed by Tukey's post hoc test NC: Normal Control TD: Model Control TDA25: Atorvastatin (25 mg/kg) TDA50: Atorvastatin (50 mg/kg).

Histopathology

Vehicle Control rats showed normal prostate architecture, while rats treated with DMBA-testosterone treated group shows Prostatic intraepithelial neoplasia (PIN) architectural pattern (Figure 5) i.e. loss of basal epithelial cells, increased chromatin content and increased cell density with sparse cytoplasm as seen in human High-grade prostatic intraepithelial neoplasia (HGPIN). Fully developed PIN with loss of basal epithelial cells was observed in model control and test groups. Groups treated with low 25 mg/kg atorvastatin showed enraged secretory cells, vary in size, slightly increased chromatin content with almost intact basal cell layer (Figure 5). While animals treated with 50 mg/kg atorvastatin showed High grade prostatic intraepithelial neoplasia i.e. dysplastic cells going from the periphery towards the centre, the nuclei become smaller and the nucleoi become less apparent with disrupted basal cell layer (Figure 5).

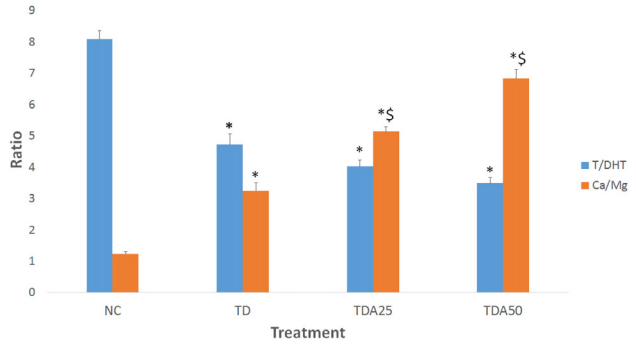


Figure 3: Effect of atorvastatin on serum Testosterone/Dihydrotestosterone (T/DHT) Ratio and Calcium/Magnesium (Ca/Mg) Ratio n = 10, * *P* <0.05 as compared to Normal Control (NC) group, one way ANOVA followed by Tukey's *post hoc* test $ *P* <0.05 as compared to TD group, one way ANOVA followed by Tukey's *post hoc* test NC: Normal Control TD: Model Control TDA25: Atorvastatin (25 mg/kg) TDA50: Atorvastatin (50 mg/kg).

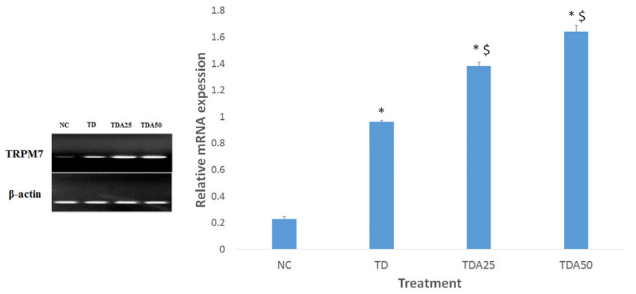
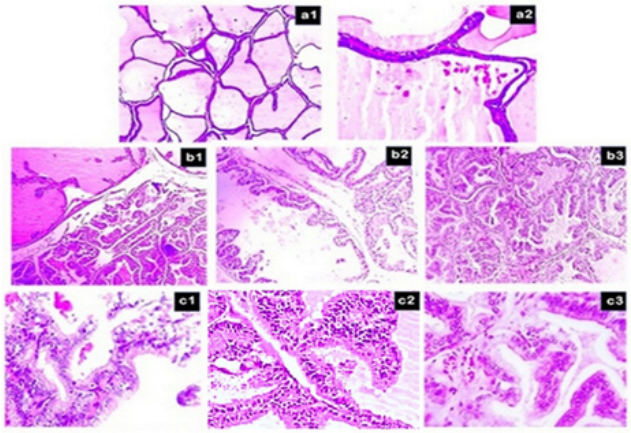


Figure 4: Effect of atorvastatin on TRPM7 mRNA Expression in prostate tissue detected by RT-PCR n = 5, * *P* <0.05 as compared to Normal Control (NC) group, one way ANOVA followed by Tukey's *post hoc* test $ *P* <0.05 as compared to TD group, one way ANOVA followed by Tukey's *post hoc* test NC: Normal Control TD: Model Control TDA25: Atorvastatin (25 mg/kg) TDA50: Atorvastatin (50 mg/kg)

DISCUSSION

Carcinoma of the prostate is one of the most formidable oncological problems in medicine and vastly dominant in elderly males. Prostate cancer is a heterogeneous disease in a heterogeneous population, the aetiology of this neoplastic disease is essentially unknown cancer.[22,23] Present study was performed to investigate the influence of Atorvastatin on DMBA-testosterone induced prostatic intraepithelial neoplasia in the prostate of Wistar rats.

The protocol for induction of prostate tumour in present investigation for model control group was based on the beginning with pre-treatment of androgen-enhanced cell proliferation in the prostate followed by promotion with higher dose of androgen (100 mg/kg/Body weight, i.p., for 3 days) results in maximum stimulation of prostatic epithelial proliferation which usually reaches a peak on the fourth day of androgen testosterone administration.[5,24,25] This sequence is continuing with a single administration of DMBA as a chemical carcinogen for enhancing the effect of cell proliferation in the targeted tissue that leads



[a1]= [VC = Vehicle Control Group] at 10x magnification, [a2]= [VC = Vehicle Control Group] at 40x magnification [b1]=[TD = Testosterone + DMBA (Model Control Group)] at 10x magnification, [b2]= [TDA25 = Testosterone + DMBA + Atorvastatin 25 mg/kg] at 10x magnification , [b3]=[TDA50 = Testosterone + DMBA + Atorvastatin 50 mg/kg] at 10x magnification, [c1]=[TD = Testosterone + DMBA (Model Control Group)] at 40x magnification, [c2] = [TDA25 = Testosterone + DMBA + Atorvastatin 25 mg/kg] at 40x magnification , [c3]=[TDA50 = Testosterone + DMBA + Atorvastatin 50 mg/kg] at 40x magnification

Figure 5: Effect of atorvastatin treatment on histology of prostate gland. a1 and a2: normal prostatic cells architecture; b1 and c1: High-grade PIN; b2 and c2: hyperplastic columnar epithelium without nuclear atypia; b3 and c3: High-grade PIN with dysplasia, epithelium thrown into papillary fronds nuclei show vesicular chromatin and prominent nucleoli.

to initiation of carcinogenesis followed by chronic exposure to testosterone treatment as a hormonal stimulation which leads to cell adaptation promotion and progression of carcinoma. The DMBA is known as pluripotent carcinogens in rats. Furthermore, it has also been claimed to induce *in situ* adenocarcinomas of the ventral prostate in F344 Rats[26,27] and dorsolateral prostate adenocarcinoma in Male Wistar Rats[26] at a dose 30 and 65 mg/kg/BW. Testosterone act as a tumour promoter by different mechanisms, one possibility is initiated prostate epithelial cells develop hypersensitivity to androgen, which could deliver them with a selective growth advantage resulting in a few of those cells progressing to malignancy. Secondly, the action of testosterone at very high dosage may be due to not solitary to androgenic effects nevertheless also to other pharmacological toxicity, owing to inflammatory infiltration with disruption of glandular structure.[28,29] Our study results showed 100% incidence of carcinomas of the prostate in TD and TDA25 group with High-grade PIN while High-grade PIN with severe dysplasia in TDA50 group were found at the end of study.

A significant increased in prostate weight index (PWI) were observed in DMBA-testosterone induced prostatic intraepithelial neoplasia. The rise in PWI might be due to DMBA that after a single dose along with constant stimulation of testosterone persuades proliferation of fibromuscular tissue and squamous epithelium of the prostate.[30] While, simultaneous treatment with atorvastatin caused increased tumor incidence with an associated increase in PWI suggests the promotive effect of atorvastatin in the initiation process.

Development, maturation and normal function of the prostate are depending on androgen homeostasis. Prostate cancer always accompanied by the conversion of testosterone into dihydrotestosterone (DHT), the more potent form of testosterone with the help of 5α-reductase which increases the cancer cell growth. DHT has a potent effect owing to its higher affinity to the Androgen Receptor (AR)[31] which in turn binds to Androgen Receptor Elements present in the promoter regions of many genes involved in cellular proliferation.[31] The higher expression of 5α-reductase activity is an indication of a higher conversion rate of testosterone into dihydrotestosterone. Interestingly when taking a ration of T/DHT, which represents the status of the 5α-reductase activity.[32] In the case of group TD, TDA25 and TDA50, the ratio of T/DHT is lower as compared to the normal control group, which is an indication of a higher conversion rate of testosterone into DHT suggesting significant augmentation in the 5α-reductase activity.

Recent studies established that TRP channels are involved in maintaining tissue homeostasis via proliferation, differentiation and apoptosis.[33] TRPM7 can permeate divalent and monovalent cations as a result of the ion channels characteristic.[9,10] TRPM channels are widely expressed in cells including prostate tissues.[34] It has been recently demonstrated that reducing extracellular Mg^{2+} and rising Ca^{2+}/Mg^{2+} ratio increase cell proliferation in prostate cancer cells.[12,35] TRPM7 have been shown to be permeable to both Ca^{2+} and Mg^{2+} cations.[12] Furthermore, the Ca^{2+} to Mg^{2+} ratio, which facilitates Ca^{2+} entry, was increased in cancer cells and led to an increase in cell proliferation and inhibiting TRPM7 activity can limit cell proliferation.[12] Increasing Ca^{2+} and Mg^{2+} influx promotes the proliferation of prostate cancer cells through activating TRPM7.[12,36] In consistence with this, findings from the current study showed significant enhancement in Ca^{2+}/Mg^{2+} ratio and highest level was produced by Atorvastatin treated TDA25 and TDA50 groups. In addition groups treated with atorvastatin showed raised expression of TRPM7 in prostate tissue homogenate compare to TD group. Further oral administration of atorvastatin has been reported to increase in serum calcium level.[37] Overall, the results presented here suggest that in Atorvastatin might promote cell proliferation via TRMP7 channel indicating its promotive effect on prostate cancer.

The promotive effect of atorvastatin was affirmed by histological analysis in prostate tissue. This histological changes closely share the similar features found in human prostatic intraepithelial neoplasia (PIN). PIN is a widely accepted premalignant condition of prostate cancer. The DMBA-testosterone treated animal shows PIN architectural pattern as seen in human HGPIN.[5]

CONCLUSION

To conclude, the present *in vivo* study established that administration of atorvastatin leads to cell proliferation of prostate glands by up regulating TRPM7 channel.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

TRP channels: Transient Receptor Potential channels; **TRPM7:** The transient receptor potential ion channel subfamily M, member 7; **DMBA:** 7,12-Dimethylbenz[a] anthracene; **PWI:** Prostatic weight index; **DHT:** Dihydrotestosterone; **PIN:** Prostatic intraepithelial neoplasia; **HGPIN:** High-grade Prostatic intraepithelial neoplasia; **TU:** Testosterone undecanoate.

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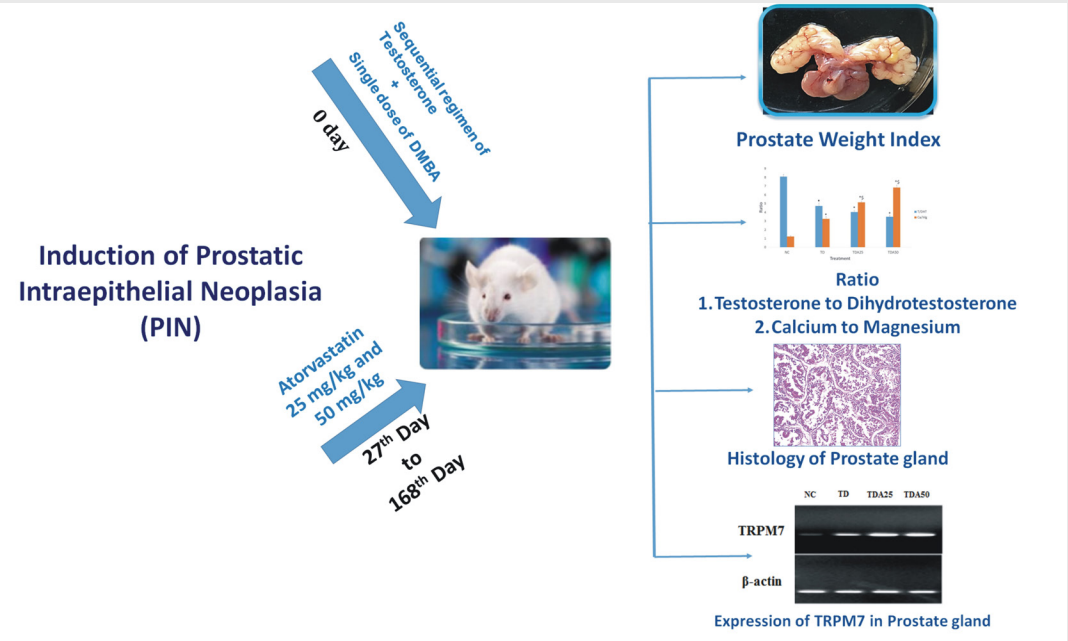
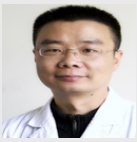
PICTORIAL ABSTRACT



SUMMARY

- Present study investigated the effects of atorvastatin (25 and 50 mg/kg/BW p.o.) on cell proliferation of prostate gland induced by a sequential regimen of testosterone plus single administration of 7,12-Dimethylbenz(α) anthracene.
- Histopathology, prostate weight index, serum testosterone to dihydrotestosterone (DHT) ratio, $Ca2+/Mg2+$ ratio and expression of TRPM7 channel in prostate tissue homogenate were done to estimate effect of atorvastatin on 7,12-Dimethylbenz (α) anthracene and testosterone-induced prostatic intraepithelial neoplasia in the prostate of Wistar rats.
- Results of present study revealed that the different pattern of prostatic intraepithelial neoplasia with associated hyperplasia in model control group while concurrent treatment with atorvastatin increases this effect by developing a pattern of high grade prostatic intraepithelial neoplasia (PIN) with dysplasia in prostatic histoarchitecture.
- Furthermore, atorvastatin treatment produced elevated levels of TRPM7 protein expression with higher calcium to magnesium ratio, low ratio of serum testosterone to dihydrotestosterone and enlargement in prostatic weight index.
- The current research revealed that atorvastatin has a promoting role in intraepithelial neoplasia induced by DMBA-testosterone in rats through increased expression of the TRPM7 channel.

About Authors



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