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Testosterone Enanthate Induces Cell Death in Human Colon (HCT) and Gastric (AGS) Cancer Cells

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MS and RA suggested the idea of research and supervised all practical steps. NA carried out research plan including preparation of hormones, cell lines. MTT assay and performing the statistical analysis. MM and SK shared in the writing and revision processes.

Key words
Testosterone, AGS, HCT, Apoptosis, Caspase

ABSTRACT

Cytotoxic effects of testosterone on cancer cells have been reported in recent studies, however, the molecular pathway is unclear. This study aimed to investigate the effects of testosterone enanthate on expression level of iNOS, MMP9 and caspase-3, -8 and -9 in human colon (HCT) and gastric (AGS) cancer cells. Cell lines were divided into untreated (control) group and groups exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of testosterone enanthate. The cytotoxic effect of testosterone enanthate was measured using MTT assay method. iNOS, MMP9, caspase-3, -8 and -9 expression level were evaluated by real-time PCR and the caspases activity was measured by ELYSA method. Flow cytometry was used to detect and quantify the level of apoptosis in cell lines. The data were statistically analyzed between groups using ANOVA and student's t-test. Decreased viability was observed in AGS and HCT cells exposed to 0.1 and 1 mg/ml of testosterone. Exposure of AGS and HCT cells to cytotoxic dose of testosterone enanthate led to significant increase in gene expression and activity level of caspase-3 and -9, but no significant change in iNOS, MMP9 and caspase-8 expression level. MMP9 expression level significantly decreased in HCT cells, however, did not change in AGS cells exposed to cytotoxic dose of testosterone enanthate. Treatment of HCT and AGS cells with testosterone enanthate induces intrinsic - and not extrinsic- apoptosis pathway.

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and is the second leading cause of cancer-related death worldwide, and it is more common in developing countries (Ferlay *et al*., 2010, 2013). Epidemiological reports indicate that regardless of etiologies, there is a significant association between gender and GC incidence (Negi *et al*., 2006), which in part caused by sex steroid influence on GC development. Colon cancer is also one of the important causes of cancer-related morbidity and mortality (Marley and Nan, 2016) and is the second most commonly diagnosed cancer in women and the third in men worldwide. The rates vary more than 10 times across the world; high-income countries have approximately 2.5 times higher rates than do low-income countries (Lee *et al*., 2016). In view of remarkable gender disparity and the role played by male sex steroids in gastric and colon cancer development, some studies have explored the importance of the androgens in these cancers (Catalano *et al*., 2009; Tian *et al*., 2013; Zhang *et al*., 2014).

The findings have demonstrated the strong oncogenic properties of androgens in etiology of gastric and colon cancers (Marcinkiewicz *et al*., 2012). Wu *et al*., 1990 demonstrated the presence of androgen receptors in gastric cancer tissues. Androgen receptor not only mediates the effects of androgen but also functions as an oncoprotein by interacting with other molecules implicating the proliferation and metastasis of cancer cells (Miyamoto *et al*., 2007; Fang *et al*., 2011). Testosterone is the most important androgen in humans and has a significant impact on development of many different tissues including cancerous tissues. It has been revealed that testosterone is a transcription factor regulating various genes expression and protein synthesis, and hence, plays a significant role in tissue proliferation (Alkahtani, 2013). The pathophysiological effects have not been fully determined but it appears that testosterone has a key part in colon cancer cells proliferation *in vitro*, a mechanism that can be halted with anti-androgens (Ørsted *et al*., 2014). Most recent research findings indicated that androgens have a profound impact on gastric cancer development. It has been found that sex hormone-binding globulin is a blood plasma protein biomarker for gastric cancer (Cheng *et al*., 2018). Studies suggest that androgens influence gastric carcinogenesis (Gann *et al*., 1996). Despite attempts

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to clarify the effects of testosterone on proliferation and growth of cancer cells including digestive system cancer cells, the relationship between testosterone and development of cancer still remains highly controversial (Eisenberg *et al*., 2015).

Androgens can induce apoptosis in various cancer cells. The effect of androgens on apoptosis occurs predominantly by interference with caspase activation and the inhibition of caspase cleavage in both the extrinsic and intrinsic cell death pathways (Kimura *et al*., 2001). Some research findings indicate that androgens can inhibit activation of caspases-8, -7, and -9 and blocks apoptosis (Kimura *et al*., 2001), others show that androgens induce apoptotic responses via caspase-3 in cancer cells (Papadopoulou *et al*., 2008). It has also been reported that testosterone can induce apoptosis by PI3K/Rac1 signaling cascades regulations in colon cancer cells (Alkahtani, 2013).

The inducible enzyme nitric oxide synthase (iNOS) and matrix metallopeptidase 9 (MMP-9) expression, which have been implicated in cancer cells proliferation, metastasis or apoptosis (Kostourou *et al*., 2011; Shan *et al*., 2015), may be influenced by testosterone. Research have demonstrated that Finasteride, a type 2 5-alpha reductase inhibitor that blocks the conversion of testosterone into dihydrotestosterone (Cussenot *et al*., 2007) inhibits human prostate cancer cell invasion through MMP9 downregulation (Moroz *et al*., 2013). Association of MMP9 and iNOS expression with apoptosis in cancer cells has been reported in recent studies, however, there is not any report about direct role of testosterone on iNOS and MMP-9 expression in gastric and colon cancer cells. There are only very few studies examining the association of testosterone with cancer cells proliferation or apoptosis (Gann *et al*., 1996; Parsons *et al*., 2005; Hormones and Group, 2008; Daniels *et al*., 2010; Sawada *et al*., 2010; Hyde *et al*., 2012; Muller *et al*., 2012). The effects of testosterone on apoptosis in colon cancer cells have only been reported in few studies, and to our knowledge, never in gastric cancer cells. We, to clarify the apoptotic pathway involved, therefore, examined the effects of cytotoxic dose of testosterone enanthate on caspase-3, -8 and -9 expression and activity level, and also iNOS and MMP-9 expression in human colon (HCT) and gastric (AGS) cancer cells *in vitro*.

MATERIALS AND METHODS

Testosterone

Testosterone enanthate was obtained from the Abu Reyhan Pharmaceutical Company (Tehran-Iran). According to our previous studies (28) the hormone was solved in DMSO, Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) to produce different concentrations (0.001, 0.01, 0.1, 1 and 10 mg/ml).

Cell culture

AGS and HCT cells were purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (gentamicin) in an incubator (37 °C, 5% CO2 atmosphere). Cultured cells (passages 20 to 50) were detached and separated by 0.25% tryps in EDTA solution and washed by serum-free medium.

MTT proliferation assay

AGS and HCT cells were suspended in growth medium and sub-cultured at a density of 1×10^4 cells/well in a 96 well plate. Cytotoxicity of different doses of the testosterone was assayed using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide] method. Cells were left overnight at 37˚C to attach to the plate. Following day growth medium was replaced by growth medium containing hormone at different concentrations and incubated for 48 h. After 48 h, the medium was replaced with 100 µl of 1 in 10 diluted (in growth medium) MTT solution (DOBIO Biotech, Shanghai, China) and incubated for another 4 h at 37˚C. After 4 h, the MTT solution was removed. MTT was reduced by metabolically viable cells to a colored (purple) water-insoluble formazan salt. The purple color precipitate was solubilized by adding 100 µl of isopropanol and shaken for 20 - 30 mins at room temperature. The intensity of resultant purple color was measured at 570 nm in a DNM-9602G microplate reader.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from cells using a RNeasy RNA isolation kit (Roche, Germany) and was transcribed into first-strand cDNA using Easy Script First strand cDNA Synthesis Super Mix (Roche, Germany). Real-time quantitative RT-PCR was performed using ABI. Primer sets for PCR were as follows: *iNOS* forward: 5'-GTGCCCTGCTTTGTGCG-3', reverse: 5'-TCCTCCTGGTAGATGTGGTCCT-3', *MMP9* forward: 5'-GGCGTCGTGGTTCCAACT-3', reverse: 5'-CGGTCGTCGGTGTCGTAGT-3', *caspase-3* forward: 5'-GCCTGCCGTGGTACAGAACT-3', reverse: 5'-GCACAAAGCGACTGGATGAAC-3′, *caspase-8* forward: 5'-GACTGGATTTGCTGATTACCTACCTAA-3', reverse: 5'-CCTCAATTCTGATCTGCTCACTTCT-3', *caspase-9* forward: 5'-CCAGATGCCACCCCGTT-3',

reverse: 5'- CCCACTGCTCAAAGATGTCGT-3', *GAPDH* forward: 5'-CCCACTCCTCCACCTTTGAC-3', reverse: 5'-CATACCAGGAAATGAGCTTGACAA-3'. GAPDH was used as an internal control. We performed normalization and calculation steps as reported previously (Ueda *et al*., 2013). The relative expression level was determined by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Caspase colorimetric assay

The activity of caspases-3, -8 and -9 was determined using an Apo Target colorimetric protease assay kit (Abnova, Taiwan) according to the manufacturer's instructions. Briefly, apoptosis was induced in AGS and HCT cells by testosterone enanthate treatment while concurrently incubating a control culture without induction. Cells were counted as pellet 3×10^6 cells per sample. The cells were resuspended in 50λ of chilled Cell Lysis Buffer, incubated on ice for 10min, and then centrifuged for 1min in a micro centrifuge (10000×g). Supernatant was transferred to a fresh tube and put on ice. A 50λ of 2 × reaction buffer (containing 0.5λ DTT) was added to each sample followed by 5λ of the 4mM DEVD-pNA substrate, to sample 5λ of the 4mM IETD-pNA substrate. The samples were incubated in the dark at 37°C for 2 h. Samples were read in a microplate reader set at 405 nm. Fold increase in caspase-3, -8 and -9 activity was determined compared to that in untreated controls.

Apoptosis assay by flow cytometry analysis

Apoptotic cell death was assessed by flow cytometry using the Annexin V–FITC/PI Apoptosis Detection Kit according to the manufacturer's instructions (Biolegend, USA). After 72 h, cells were harvested and incubated with Annexin V-FITC and PI for 30 min in the dark at 4°C. Flow cytometric analysis was immediately performed. The data are presented as bi-parametric dot plots showing Annexin V-FITC green fluorescence versus PI red fluorescence.

Statistical analysis

Data were analyzed by SPSS and Excel software. The Kolmogorov-Smirnov test was used for normalized distribution of data. Then, the one-way ANOVA and student's t-test was used to analyze the data followed by Tukey's post hoc multiple comparison test. All data are expressed as the mean ± standard deviation (S.D.) and $P<0.05$ was considered significant.

RESULTS

Effect of testosterone on AGS and HCT cells viability

Viability of AGS and HCT cells did not significantly change when exposed to 0.001 and 0.01 mg/ml of testosterone enanthate compared with control group; however, exposure of AGS cells to 0.1 and 1 mg/ml of testosterone enanthate resulted in significant decrease in cell viability compared to control and other experimental groups (Fig. 1A). The HCT cells viability significantly decreased in groups exposed to 0.1 and 1 mg/ml of testosterone enanthate compared to control group (Fig. 1B). There was also significant difference in cell viability between group exposed to 10 mg/ml and groups exposed to 0.1 and 1 mg/ml of testosterone enanthate.

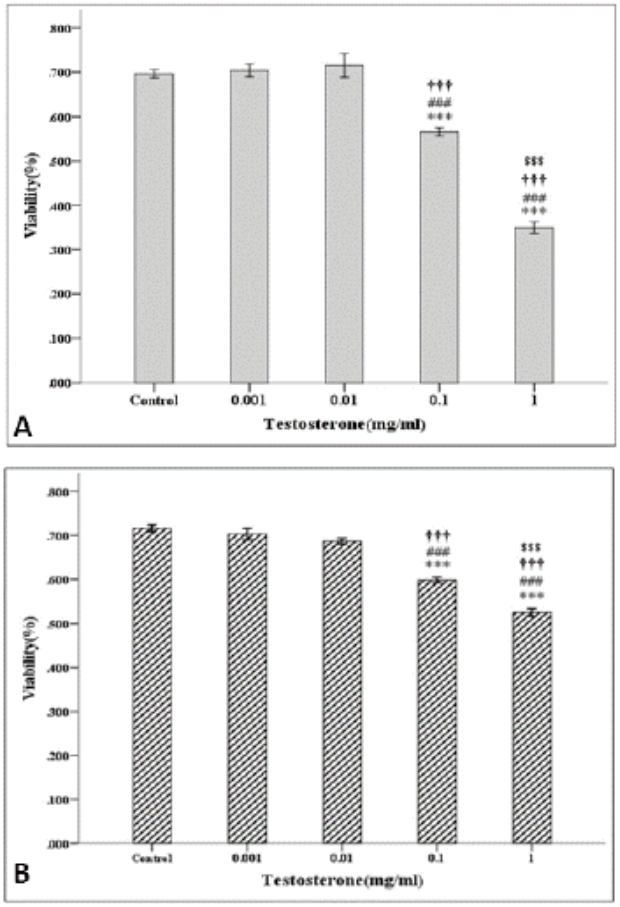

Fig. 1. Viability of AGS (A) and HCT (B) cells exposed to 0.001, 0.1, 1 and 10 mg/ml of testosterone enanthate. *, #, † and $ indicate significant ddifference compared with control, and groups exposed to 0.001, 0.01, 0.1 and 1 mg/ml of testosterone, respectively. (***, P<0.001; ###, P<0.001; †††, P<0.001 and $$$, P<0.001).

Effect of testosterone on expression of iNOS, MMP9, caspase-3, -8 and -9 in AGS and HCT cells

The cytotoxic concentration of testosterone enanthate (1mg/ml) was used to evaluate iNOS, MMP9, caspase-3, -8 and -9 expression in AGS and HCT cells. Exposure of

AGS and HCT cells to 1 mg/ml of testosterone enanthate led to non-significant change in iNOS and caspase-8 and significant increase in caspase-3 and caspase-9 expression level (P<0.01 and P<0.001, respectively). MMP9 expression level did not significantly change in AGS cells, but significantly decreased in HCT cells exposed to 1 mg/ml of testosterone enanthate compared with control group (P<0.001) (Figs. 2 and 3).

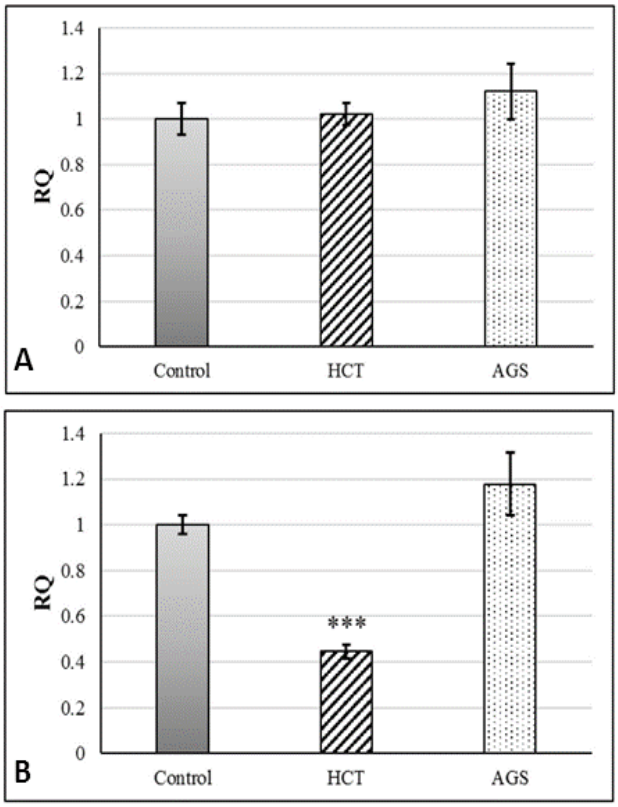

Fig. 2. Partial expression level (RQ) of iNOS (A) and MMP9 (B) in HCT and AGS cells exposed to cytotoxic concentration of testosterone enanthate compared with control group.

Effect of testosterone on expression of caspase-3, -8 and -9 activity level in AGS and HCT cells

Exposure of AGS and HCT cells to 1 mg/ml of testosterone enanthate showed no significant change in caspase-8 activity level, but significant increase in activity level of caspase-3 (P<0.001) and caspase-9 (P<0.05) compared to control group (Fig. 4).

To determine AGS and HCT cells apoptosis by flow cytometry

Flow cytometry was used in our study to discriminate early apoptotic cells from late apoptotic and necrotic ones. In the early stages of apoptosis, phosphatidyl serine (PS) is translocated from the inner side of the plasma membrane to the outer layer. Annexin V, a calcium dependent phospholipid-binding protein with a high affinity for PS, can therefore be used as a sensitive probe for the exposure of PS on the cell membrane and hence as a marker of apoptosis. Figures 5A, D are representative of control AGS and HCT cells, which almost no apoptotic cells were detected. However, in testosterone enanthate treated AGS and HCT cells (Figs. 5B, E, respectively), a significant increase in early and late apoptotic cells and significant decrease in live cells were shown. As shown in Fig. 5B, E, analysis of the cell population had distinct sets of population. Annexin V+ and propidium iodide-negative cells increased significantly by the treatment of AGS and HCT cells with cytotoxic dose of testosterone enanthate compared to control, indicating the translocation of phosphatidyl serine, an early event of the apoptotic process. The percentage of necrotic cell death was almost ignorable in control and treated groups (Figs. 5C, F).

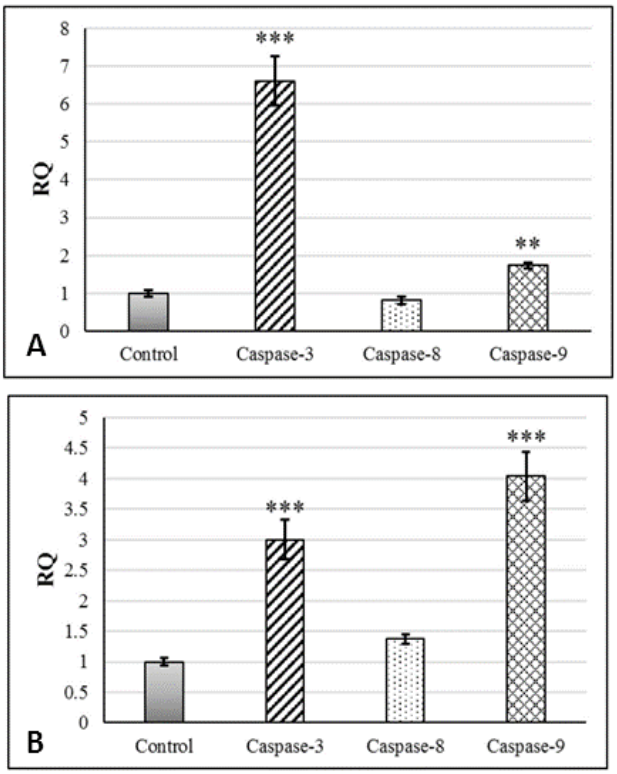

Fig. 3. Partial expression level (RQ) of caspase-3, -8 and -9 in AGS cells (A) and HCT cells (B) exposed to cytotoxic concentration of testosterone enanthate compared with control group. *** and ** represent significant difference compared to control group (***: P<0.001, **: P<0.01).

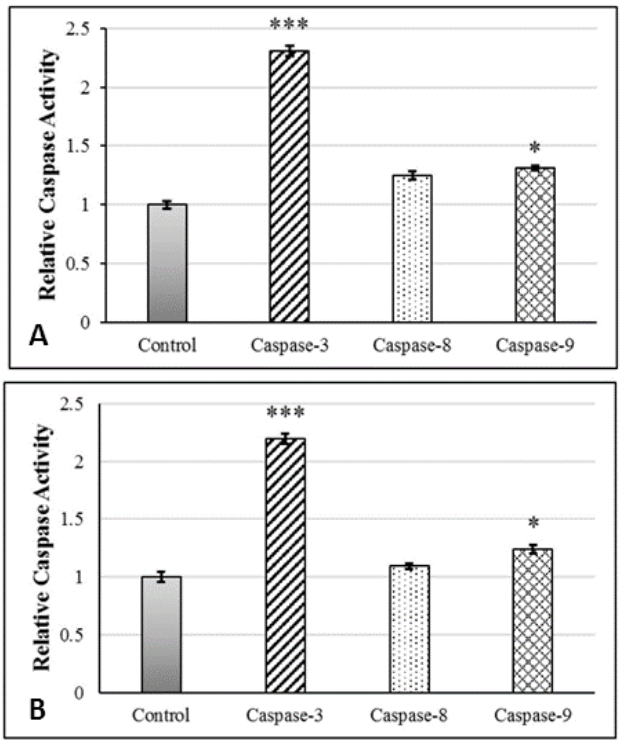

Fig. 4. Activity level of caspase-3, -8 and -9 in AGS cells (A) and HCT cells (B) exposed to cytotoxic concentration of testosterone enanthate compared with control group. *** and * represent significent difference compared to control group. (***: P<0.001 and *: P<0.05).

DISCUSSION

The cytotoxic effects of testosterone on HCT and AGS cancer cells

Our findings indicated that lower testosterone enanthate concentrations) 0.001 and 0.01 mg/ml) did not have a significant impact on viability of AGS and HCT cancer cells in vitro, but higher testosterone enanthate concentrations (0.1 and 1 mg/ml) were shown to have anti-proliferative and apoptotic effects on AGS and HCT cancer cells. Our findings are in line with the studies showing that testosterone has inhibitory effects on the proliferation and development of cancer cells (Liu *et al*., 2017). Previous studies also have demonstrated that higher circulating level of androgen precursor has protective effects on digestive system and is associated with a lower risk for colon cancer (Alberg *et al*., 2000). By the contrast, it has been shown that high levels of androgens may increase the risk of cancer (Warburton *et al*., 2015). A 2014 study has suggested that increased plasma testosterone levels are associated with a high risk of cancer and premature death after cancer (Lin *et al*., 2015). Another study shows that increased levels of testosterone are probably related to the increased risk of lung cancer and the risk of prostate cancer, but this is not the case for colon cancer, and studies on men show that there is no relationship between testosterone levels and the risk of colorectal cancer (Yao *et al*., 2018).

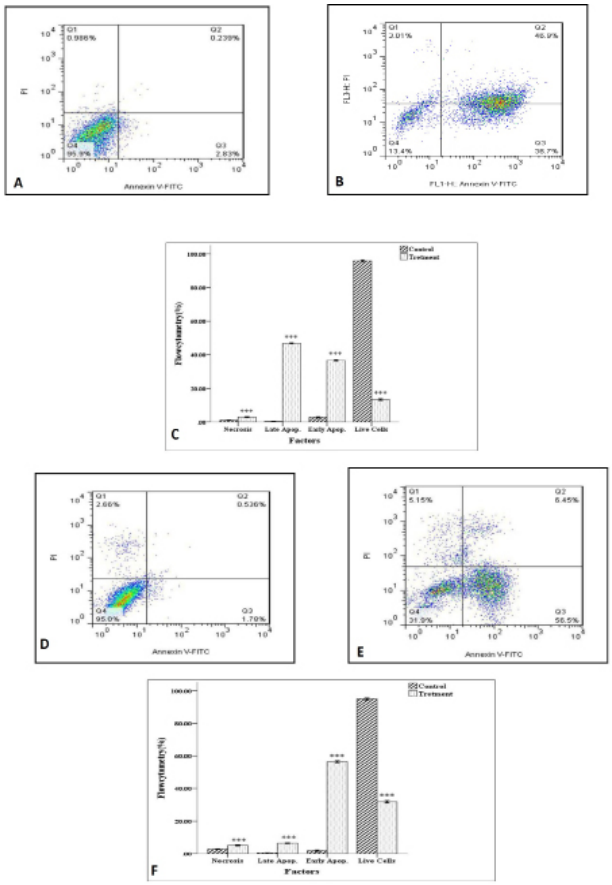

Fig. 5. Apoptosis in AGS and HCT cell lines induced by testosterone enanthate: Q1, Necrosis; Q2, Late apoptosis; Q3, Early apoptosis; Q4, Viable cells. (A) Control AGS cells; (B) AGS cells treated with testosterone; (C) Necrosis, early and late apoptosis and live cells in control and testosterone treated AGS cells; (D) Control HCT cells; (E) HCT cells treated with testosterone enanthate and (F) Necrosis, Early and late apoptosis and live cells in control and testosterone treated HCT cells. Percentage of apoptotsis increased in treated cells compared with control groups. The analysis was done by FACSDiva version 6.1.3. *** represents significant difference compared to control group (***P<0.001).

The effects of testosterone on caspase-3, -8 and -9 expression and activity in HCT and AGS cells

The results of the present study revealed that the testosterone enanthate-induced apoptosis in gastric and

colon cancer cells is mediated by caspase-9 and -3, but not caspase-8. Caspase-8 acts as initiator caspase of the extrinsic apoptosis pathway and caspase-9 acts as an initiator caspase of the intrinsic apoptosis pathway (Yoo *et al*., 2002), according to which, treatment of HCT and AGS cells with testosterone enanthate induces intrinsic - and not extrinsic-apoptosis pathway. Association of caspase-3 and -9 activation with apoptosis in cancer cells of digestive system has been reported in previous studies (Chen *et al*., 2015). It has been shown that the effect of androgen on cell death occurs predominantly by interference with caspase activation in both extrinsic and intrinsic cell death pathways (Kimura *et al*., 2001). However, in a study, treatment of LNCaP cells with testosterone resulted in reduced caspase-3 activation (Rokhlin *et al*., 2005). Androgen treatment also inhibited activation of caspases-8 and -9 by TNF-alpha +/- irradiation (Kimura *et al*., 2001). A relationship between caspase-3 expression and apoptosis in gastrointestinal neoplasmic cells was demonstrated so that that neoplasmic cells were able to inhibit apoptosis through inhibition of caspase activity (Isobe *et al*., 2004). In a study it was observed that apoptosis is induced via caspase-3 and caspase-8, but not caspase-9 activation (Gong *et al*., 2009). However, another study showed that in certain cells, the onset of apoptosis was mediated by caspase-9 and not by caspase-8 activation (Mueller *et al*., 2003). Apoptosis in AGS cells has been reported to be mediated by caspase-dependent apoptotic pathway (Saralamma *et al*., 2015). Apoptosis in AGS and HCT cancer cells may be mediated by caspase-dependent or caspase-independent pathway, however, our experiment has shown that treatment of various types of cancer cells with testosterone induces caspase-dependent pathway.

The effects of testosterone on iNOS expression in HCT and AGS cancer cells

In mammals, NO is synthesized by the enzyme nitric oxide synthase (NOS). The inducible NOS (iNOS) epession is high in many tumors, however, the role of iNOS during tumor development is very complex and quite perplexing, with both promoting and inhibiting actions being described (Vannini *et al*., 2015). NO directly inhibits the activity of caspases, providing an effective means to block apoptosis. There is an enhanced expression of iNOS in human colorectal cancers (Choudhari *et al*., 2013); however, in our study we have shown that iNOS expression level did not significantly change in colon (and gastric) cancer cells treated with testosterone enanthate, indicating that testosterone may inhibit iNOS expression in HCT and AGS cancer cells, which in turn, caspase-3 and -8 are activated to induce apoptosis pathway. Recently, it was also reported that the iNOS expression is not regulated by the androgen receptors (Moledina *et al*., 2001), therefore, there is another possibility that testosterone does not involve in inducing of iNOS expression in HCT and AGS cells. Further research is required to clarify the association of testosterone with iNOS expression in colon and gastric cancer cells.

The effects of testosterone on MMP9 expression in HCT and AGS cancer cells

In the present study, MMP9 expression level significantly decreased in HCT cells, however, did not change significantly in AGS cells treated with testosterone enanthate. The MMPs substantially contribute to angiogenesis, differentiation, proliferation and apoptosis in cancer cells. The implications of MMPs in cancers are no longer mysterious; however, the mechanism of action is yet to be explained (Verma *et al*., 2014). The expression of MMP9 seems to be negatively regulated by the androgen pathway. In a study androgen supplementation significantly reduced the secretion and activity of MMP9 in cancer cells (Illemann *et al*., 2006). However, in another study it has been shown that MMP9 expression is not stimulated by androgens (Liao *et al*., 2003). While MMP9 expression has important prognostic implications in certain cancer cells, the significance of the more significant expression of MMP9 expression by tumor cells has not been clarified (Mehner *et al*., 2014). Although previous evidence has shown that apoptosis in cancer cells is associated with significant increase in MMP9 expression level, in our study, interestingly, the results showed that treatment of HCT and AGS cancer cells with testosterone enanthate induces apoptosis without significant change in MMP9 expression.

CONCLUSION

High doses of testosterone has significant anti-proliferative as well as apoptotic effects on AGS and HCT cancer cells in vitro. Exposure of HCT and AGS cells with testosterone induces intrinsic and not extrinsic- apoptosis pathway.

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Statement of conflict of interest

The authors have declared conflict.

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