PKP
PUBLIC KNOWLEDGE PROJECT

Journal of Biological Studies
ISSN 2209-2560

JBS
An Open Access Peer Reviewed Journal

The apoptotic effect of nandrolone on human gastric (AGS) and colon (HCT) cancer cells and evaluation of iNOS, MMP9 expression level, and caspase-3, -8 and -9 activity

Zahra Aalaei[1], Abdolhossein Shiravi[1*], Rahim Ahmadi[2], Vida Hojati[1]

[1] *Department of Biology, Damghan Branch, Islamic Azad University, Damghan, Iran*
[2] *Department of Biology, Avicenna International College, Budapest, Hungary*

Received: September 29, 2021; **Accepted:** November 15, 2021; **Published online:** December 22, 2021.

Abstract: Although androgens have been reported to induce apoptosis in cancer cells, there are few studies concerning with cytotoxic effects of nandrolone on human gastric (AGS) and colon (HCT) cancer cells. We evaluated the effects of nandrolone on apoptosis, iNOS, and MMP-9 expression level, and caspase-3, -8 and -9 activity in AGS and HCT cells. HCT and AGS cells were divided into control group and groups treated with 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml of nandrolone. Cytotoxicity was measured using MTT assay method. Flow cytometry was used to evaluate the apoptotic pathway occurred in cells. The expression levels of iNOS and MMP-9 genes were evaluated using real-time PCR, and caspases activity was measured by colorimetric method. Data were analyzed using ANOVA and independent t-test. Cell viability significantly decreased in AGS and HCT cells treated with 2.5 mg/ml, 5 mg/ml and 10 mg/ml of nandrolone. Treatment with the effective concentration of nandrolone (5.99 mg/ml) induced apoptosis in AGS and HCT cells and did not significantly alter the expression levels of iNOS and MMP-9 in AGS cells, however, led to significant decrease in iNOS and MMP-9 expression levels in HCT cells. Activity levels of caspase-3, -8 and -9 significantly increased in AGS and HCT cells treated with the effective concentration of nandrolone. Higher concentration of nandrolone had significant cytotoxic effect and apoptotic pathway in AGS and HCT cells mediated by intrinsic and extrinsic pathways. It migth also decrease the invasion and metastasis capability of colon cancer cells.

Keywords: Nandrolone; AGS; HCT; Apoptosis; MMP-9; iNOS.

*e-mail: shiravi738@yahoo.com

1 Introduction

Anabolic androgenic steroids are one of the largest synthetic derivatives of testosterone produced to increase anabolic effects and reduce androgenic effects. Nandrolone or 19-nortestosterone is in class II of the anabolic androgenic steroid groups (Patanè et al., 2020). Nandrolone has been used for the treatment of various diseases such as hypogonadism, anemia, kidney and hepatic failure, osteoporosis, burns, malnutrition, Cachexia, AIDS and metastatic breast cancer (Jurášek et al., 2015; Patanè et al., 2020). Recently, the use of sex steroids in the treatment of cancers, especially gastrointestinal cancers such as gastric and colon cancers, has been considered by many researchers. Gastric cancer ranks fifth among the most common cancers and the third leading cause of cancer death worldwide. Various factors such as old age, male gender, ethnicity, genetics, nutrition, smoking, alcohol consumption and *Helicobacter pylori* infection are involved in the development of gastric cancer (Poorolajal et al., 2020). The gastric mucosa is composed of cylindrical epithelial cells and glands. Inflammation causes gastritis, which leads to stomach ulcers and eventually stomach cancer (Rawla and Barsouk, 2019). Colon cancer is a serious type of gastrointestinal cancer that has a high prevalence and mortality in developed countries. It is the third leading cause of cancer in men and women in the United States. Colon cancer ranks third among the most common and fourth most deadly among cancers worldwide (Pacal et al., 2020).

Colon and stomach cancers have been reported to cause a very high mortality rate. Recent studies have shown that steroid hormones, especially estradiol, progesterone and testosterone, can stimulate or inhibit the proliferation of cancer cells. They can also play a role in preventing or stimulating metastasis in cancer cells (Simoes et al., 2015; Chen et al., 2015). The results of previous studies demonstrated that testosterone in cell culture medium reduces the viability of colon cancer (HT-29) cells (e.g., Farahmandlou et al., 2017). In a study that examined the effect of testosterone on the proliferation of AGS cancer cells, was shown that high concentrations of this hormone have an inhibitory effect on the proliferation of AGS cancer cells (Amani et al., 2019). Research has revealed that 19-nortestosterone analog 1 (nandrolone) has potent antiproliferative effects on cervical cancer cells (Kampa et al., 2008). In contrast, it has been reported that androgens can be a contributing factor in certain cancers including prostate cancer development by stimulating growth in the target organ (Dai et al., 1981). On the other hand, the results of some other studies indicated that androgenic steroids play a role in apoptosis of various cancer cells. Research has shown that testosterone has anti-proliferative and pro-apoptotic effects on endometrial cancer cells (Di Wu and Yang, 2020). It has been reported that testosterone induces apoptosis in lung cancer cells (Tishehyar et al., 2021). Roshan et. al., (2016), showed that testosterone-albumin compounds are selective for androgen membrane receptors (mARs) that cause apoptosis in colon cancer cells. In contrast, Kimura et al., (2001)reported that androgens protect cancer cells against apoptosis caused by various stimuli.

Inducible nitric oxide synthase (iNOS) and matrix metallopeptidase -9 (MMP-9) are expressed in many human tumors and are also involved in tumor growth, metastasis and malignancy in many cancer cells (Chen et al., 2004; Ala-aho et al., 2005). iNOS can induce angiogenesis in gastric tumors (Chen et al., 2004; Wang et al., 2005). It has also been shown that the addition of apocynin with testosterone is effective in inhibiting iNOS activity (Juliet et al., 2004). The findings showed that there was an association between increased expression level of MMP-9 and malignancy in colorectal cancer (Langenskiöld et al., 2005). Increased levels of MMP-9 in tumor tissue may be associated with the progression of colorectal tumors (De Clerck et al., 1994). The

results showed that testosterone significantly reduced the expression level of MMP-9 in HCT colon cancer cells, but did not change in AGS cells exposed to the cytotoxic dose of testosterone (Amani et al., 2021). In contrast, the use of synthetic MMPS (Matrix metalloproteinase) inhibitors in clinical studies has been shown to reduce the progression of cancer and metastasis (Sang, 1998).

Androgenic steroids can affect both intrinsic and extrinsic pathways of apoptosis in different cancer cells mediated by caspase cascade. It has been shown that testosterone increases the activity of caspase-3 in prostate cancer cells and can have an anti-tumor effect in prostate cancer (Papadopoulou et al., 2008). One study showed that testosterone increased caspase-9 and -3 activitiy in AGS cell line but had no effect on caspase-8 activity (Amani et al., 2019).The effect of 5 models of 17-19-nortestosterones (Chen et al., 2015; Poorolajal et al., 2020) on cancer cells including ovarian cancer, cervical cancer, multicellular breast cancer cell lines showed that caspase-3 and -9 activity significantly increased, however, there was not significant change in the activity level of caspase-8 (Gyovai et al., 2018).

Previous studies on the effects of anabolic androgenic steroids on various cancers are in many cases contradictory (Kampa et al., 2008; Dai et al., 1981; Gyovai et al., 2018). The studies have mainly focused on the effects of sex steroids on cancer cells and few research has been carried out to investigate the apoptotic effects of testosterone derivatives such as nandrolone on cancer cells. There is also insufficient information and reports concerning with the mechanism of apoptotic action of nandrolone on cancer cell lines. Therefore, the present study aimed to investigate the apoptotic effect of nandrolone on human gastric (AGS) and colon (HCT) cancer cells and evaluation of iNOS, MMP9 expression level, and caspase-3, -8 and- 9 activity.

2 Materials and Methods

2.1. Nandrolone:

Nandrolone was obtained from Abu Reyhan Pharmaceutical Company (Tehran-Iran) and dissolved in DMSO, toyeen and phosphate buffer saline (PBS). The preparation was carried out according to our previous studies (Amani et al., 2019) to produce different concentrations (0.625, 1.25, 2.5, 5 and 10) mg/ml of the hormone.

2.2. Cell culture:

Human gastric cancer (AGS) and colon cancer (HCT) cell lines were obtained from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were cultured in DMEM supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % antibiotics (Penicillin/Streptomycin). Cells were then cultured in incubator (37 °C, 5 % CO2 atmosphere).

2.3. MTT assay test:

MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide] assay was performed for assessing viability of AGS and HCT cells exposed to 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml of nandrolone for 24 h treatment. Cells were seeded in 96-well plates with 1×10^4 cells/well and placed at in a 5 % CO2 humidified incubator until 70-80 % confluency.

The complete growth medium was removed, and the cells were serum-starved for 24 h prior to treatment. Cells incubated in culture medium alone (untreated cells) served as control group. In experimental groups, the cells were treated with 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml of nandrolone for 24 h. The medium was then removed and 100 µL of MTT solution was added to each well, and the plates were incubated for 4 h. The MTT solution was removed and 100 µL aliquots of dimethyl sulfoxide (DMSO) were added to each well to dissolve the formazan crystals followed by incubation for 20 min. Treatments were performed in eight replicates, the absorbance of the resultant solution was calculated by a microplate reader (Bio-Rad, Hercules, CA) at wavelength of 570 nm. Cell viability was calculated as follows:

[Optical density (OD) of the sample/OD of the control] × 100 (Farahmandlou et al., 2017).

2.4. Apoptosis analysis by flow cytometry:

Apoptosis was assessed by flow cytometry using an annexin V-FITC apoptosis detection kit (Biolegend, USA), according to the manufacturer's instructions. Briefly, AGS and HCT cells (3 × 10^6 cells/well) were treated with effective concentration (5.99 mg/ml) of nandrolone. After 24 h cells were gently trypsinized, washed once with serum-containing the medium and re-suspended in 100 µL of binding buffer. Then, 5 µL of annexin V-FITC and 5 µL of propidium iodide were added. Following incubation at room temperature for 15 min in the dark, annexin V-FITC binding and propidium iodide staining were analyzed by flow cytometer (BD Falcon, USA) using the FITC signal detector (FL1) and phycoerythrin emission signal detector (FL3)(Jamalzadeh et al., 2017).

2.5. Real-time-PCR:

Cells were seeded into 6-well plates (5 × 10^5 cells/well) and incubated for 24 h. The cells were then exposed to effective concentration of nandrolone (5.99 mg/ml) and incubated for an additional 24 h. Total RNA was extracted from the cells by using RNA extraction kit (Transgen Biotech ER101-01, China), and reverse transcribed with the EasyScript® First-Strand cDNA Synthesis SuperMix Kit (Transgen Biotech AE301-02, China). The real-time experiments were conducted on the CFX96 real-time PCR system (Bio-Rad) by using the SYBR® Premix Ex Taq™ (TaKaRa). The primers for iNOS were 5'-GTGCCCTGCTTTGTGCG-3' (forward) and 5'-TCCTCCTGGTAGATGTGGTCCT-3' (reverse). The primers for MMP-9 were 5'-GGCGTCGTG GTTCCAACT-3' (forward) and 5'-CGGTCGTCGGTGTCGTAGT-3' (reverse). The primers for GAPDH, serving as the normalization controls, were 5'-CCCACTCCTCCACCTTTGAC-3' (forward) and 5'-CATACCAGGAAATGAGCTTGACAA-3' (reverse). The $2^{\Delta\Delta Ct}$ method was used to evaluate the relative gene expression. The effective concentration was calculated as follows:

Y = a*X + b, IC50 = (0.5-b)/ a (Zheng et al., 2017).

2.6. Caspase activity measurements:

In order to detect whether the nandrolone induce programmed cell death, the activity of caspase-3, -8 and -9 was determined by a colorimetric assay kit (Abnova, Taiwan) according to the manufacturer's instructions. AGS and HCT cells (3 × 10^6 cells/well) were treated with effective concentration of nandrolone (5.99 mg/ml) for 24 h. Following the treatment, the cells were harvested and the enzyme activities were determined by means of colorimetric assays mentioned

above (Gyovai et al., 2018). Concisely cells were washed with ice-cold PBS and lysed with 50 µL of chilled cell lysis buffer and incubated on ice for 10 minutes. Following centrifugation for 1 min at 10,000 g, then the supernatant (cytosolic extract) was transferred to a fresh tube and put on ice for immediate assay. By Bradford method, the protein concentration of the supernatant was evaluated and 200 µg of protein was then diluted to 50 µl of cell lysis buffer in each assay. In addition, 50 µL of 2X reaction buffer (containing 0.5 µl DTT) was added to each sample followed by adding 5 µl of the 4 mM DEVD-pNA ,4 mM IETD-pNA, and 4 mM LEHD-pNA substrate to assess the activity levels of caspase-3, -8 and -9, respectively. The samples were then incubated for 2 h at 37 °C and then read using the ELISA microplate reader (BioTek, USA) at 405 nm wavelength of each sample.The change in caspases-3,-8, and -9 activities involved in the apoptosis were determined by comparing these results with the level of the uninduced control Caspase Activity = (Absorbance $_{sample}$- Absorbance $_{blank}$)/(Absorbance $_{Control}$) × 100 (Jamalzadeh et al., 2017).

2.7. Statistical Analysis:

Data analysis was performed with SPSS software (version 21.0; SPSS, Chicago, IL, USA). Differences between cell viability in groups were tested using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. Independent samples t-test was used to detect differences in gene expression and caspase activity between experimental and control groups. All experiments were performed thrice. All data were expressed as the mean ± standard deviation (S.D.). Value differences were considered significant if the P value is < 0.05.

3 Results and Discussions

The cytotoxic effect of nandrolone on AGS and HCT cell lines was assessed using MTT assay with 3 replications to determine cell viability. Data on evaluation of iNOS and MMP-9 gene expressions in AGS and HCT cancer cells in the presence of effective concentration of nandrolone (5.99 mg/ml) were obtained using real time PCR. Data were obtained with 3 replications by measurement of caspase-3, -8 and -9 activity levels, and evaluating of internal, external and necrotic apoptosis using cytometry test in AGS and HCT cancer cells treated with effective concentration of nandrolone (5.99 mg/ml).

3.1. Effect of nandrolone on AGS and HCT cells viability:

The results of MTT test showed that treatment with 0.625 mg/ml of nandrolone resulted in significant increase and 2.5 mg/ml, 5 mg/ml and 10 mg/ml of nandrolone led to significant decrease in viability of AGS cells compared to control group (Figure 1).Viability of HCT cell line did not alter significantly when treated within 0.625 mg/ml and 1.25 mg/ml of nandrolone, however, significantly decreased when treated with 2.5,5 and 10 mg/ml of nandrolone compared the control group (Figure 2).

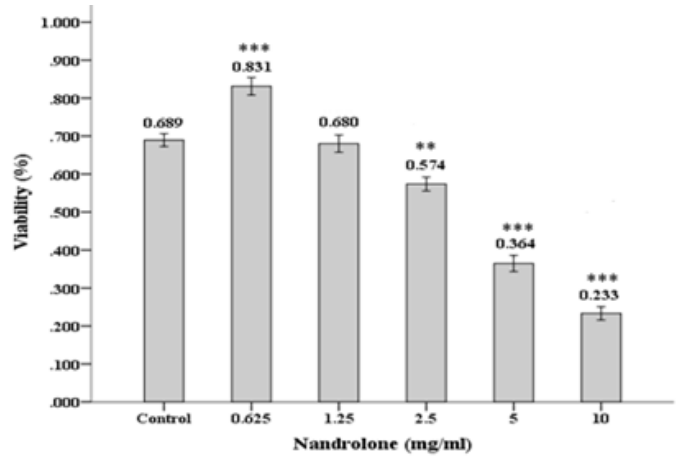

Figure 1: Viability of AGS cells treated with 0.625, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml of nandrolone. * indicates significant difference compared with control, and groups treated with 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 and 10 mg/ml of nandrolone (**: $P<0.01$, ***: $P<0.001$)

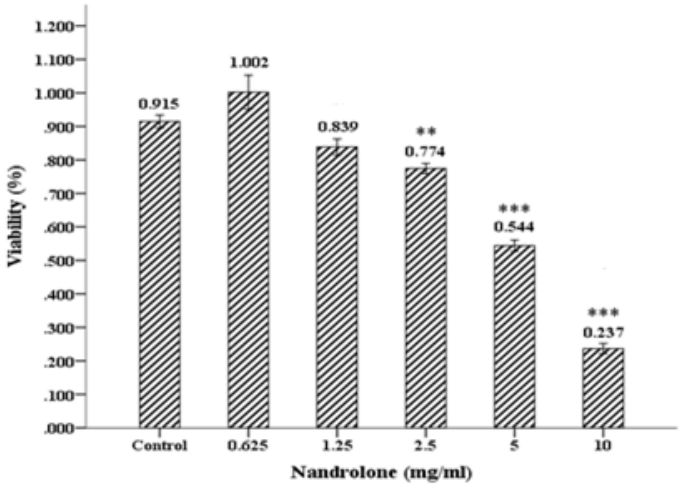

Figure 2: Viability of HCT cells treated with 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml of nandrolone.* indicates significant difference compared with control, and groups treated with 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml of nandrolone (**: $P<0.01$, ***: $P<0.001$).

3.2. To determine AGS and HCT cells apoptosis by flow ctometry:

The results of flow cytometry test showed that almost no apoptosis occurred in the control group of AGS and HCT cells (Figures: 3 and 4 (3a & 4a)), however, a significant increase in primary and secondary apoptosis and a significant decrease in viable cells were observed in AGS and HCT cells treated with effective dose of nandrolone (5.99 mg/ml). Cell population analysis had different population sets as shown in Figures 3 and 4 (3b & 4b). Annexin V + and propidium iodide-negative cells increased significantly by the treatment of AGS and HCT cells with effective dose (5.99 mg/ml) of nandrolone compared to control group, 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 (Figures: 3 and 4 (3c & 4c)).

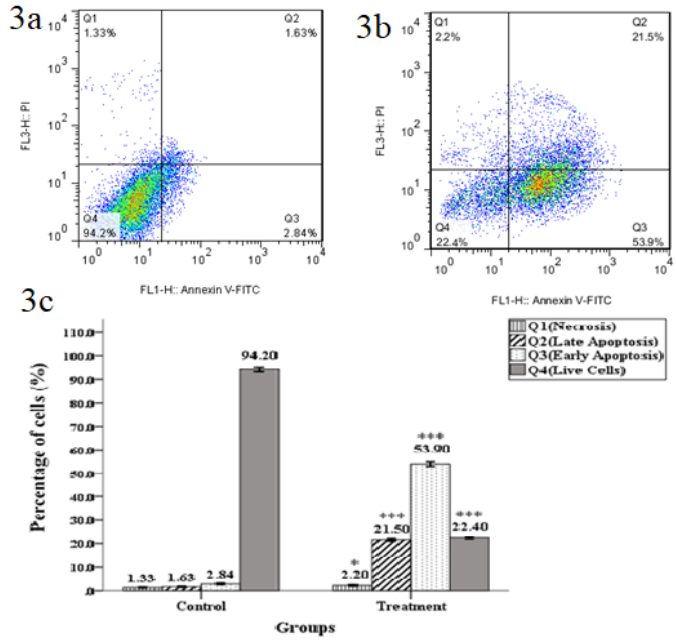

Figure 3: Necrosis, early and late apoptosis in AGS cell line induced by nandrolone (5.99 mg/ml): Q1: Necrosis; Q2: Late Apoptosis; Q3: Early Apoptosis; Q4: Viable cells. (3a) Control AGS cells; (3b) AGS cells treated with nandrolone. (3c) Q1, Q2, Q3 and Q4 phases in control and treated AGS cells. The analysis was done by FACSDiva Version 6.1.3. * and *** represent significant difference compared to control group (*: $P<0.05$, ***: $P<0.001$).

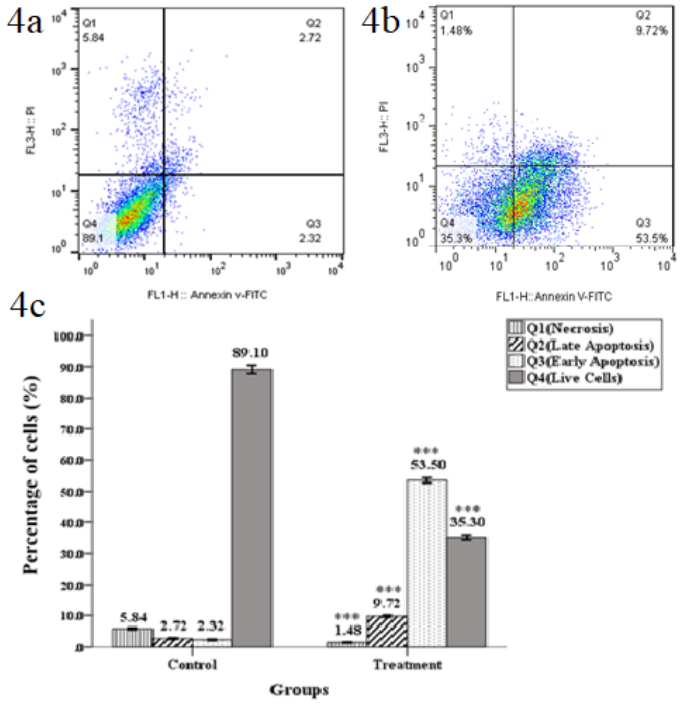

Figure 4: Necrosis, early and late apoptosis in HCT cell line induced by nandrolone (5.99 mg/ml): Q1: Necrosis; Q2: Late Apoptosis; Q3: Early Apoptosis; Q4: Viable cells. (4a) Control HCT cells; (4b) HCT cells treated with nandrolone. (4c) Q1, Q2, Q3 and Q4 phases in control and treated HCT cells. The analysis was done by FACSDiva Version 6.1.3. *** represent significant difference compared to control group (***: $P<0.001$).

3.3. Effect of nandrolone on expression of iNOS and MMP-9 genes:

Real-time PCR data showed that the relative expression levels of iNOS and MMP-9 were not significantly different from the control group found in AGS cells treated with effective dose of nandrolone (5.99 mg/ml), but significantly reduced in HCT cells treated with 5.99 mg/ml of nandrolone compared to control group (Figures: 5 and 6).

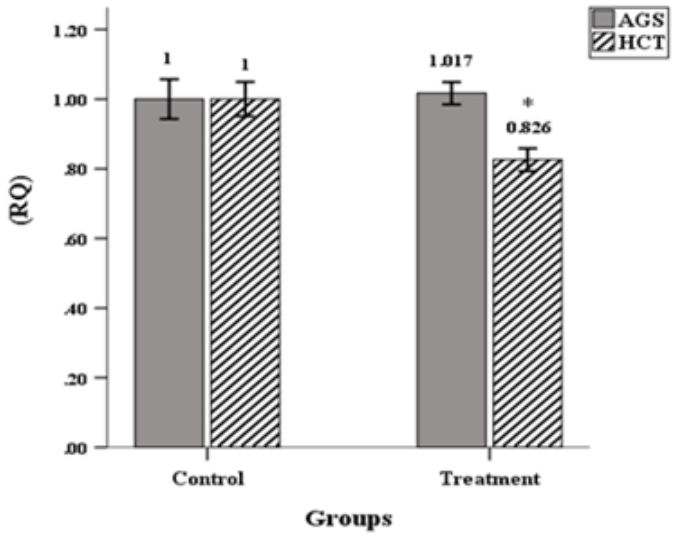

Figure 5: Relative quantitative expression (RQ) of iNOS in AGS and HCT cells treated with effective concentration of nandrolone compared to control group. * represents significant difference (*: $P<0.05$) compared to control group.

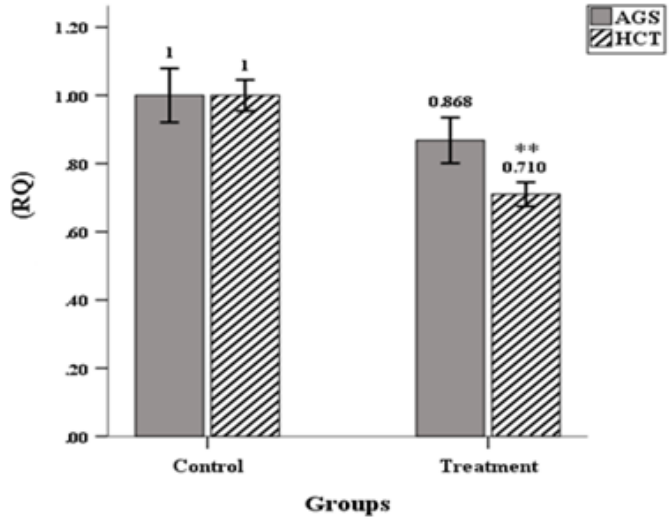

Figure 6: Relative quantitative expression (RQ) of MMP-9 in AGS and HCT cells treated with effective concentration of nandrolone (5.99 mg/ml) compared to control group. * represents significant difference (**: $P<0.01$) compared to control group.

3.4. Effect of nandrolone on caspase-3, -8 and -9 activity level:

The results of caspase test showed that the effective concentration at 5.99 mg / ml of nandrolone caused a significant increase in caspase-3, -8 and -9 activity in both AGS and HCT cell lines compared to the control groups ($P<0.001$, $P<0.01$, $P<0.001$, respectively) (Figures: 7 and 8).

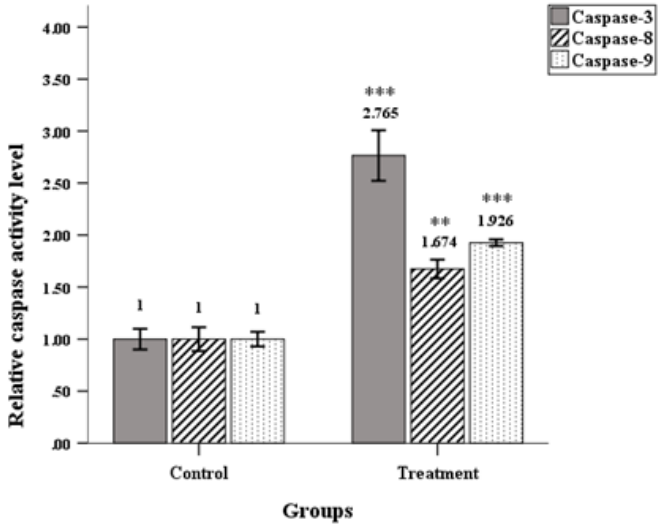

Figure 7: Activity level of caspase-3, -8 and -9 in AGS cells treated with effective concentration (5.99 mg/ml) of nandrolone compared to control group.* represents significant difference compared to control group (**: $P<0.01$, ***: $P<0.001$).

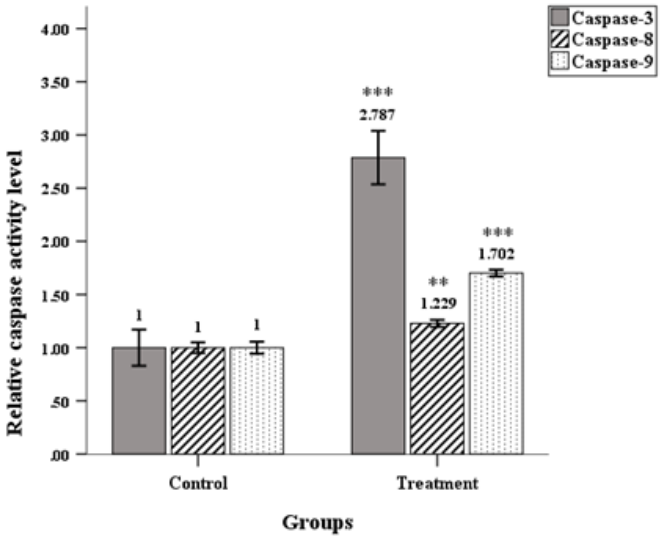

Figure 8: Activity levels of caspase-3, -8 and -9 in HCT cells treated with effective concentration (5.99 mg/ml) of nandrolone compared to control group.* represents significant difference compared to control group (**: $P<0.01$, ***: $P<0.001$).

Although the studies have shown that anabolic androgenic steroids can affect cell viability in various cancer cells (Dai et al., 1981; Kampa et al., 2008; Gyovai et al., 2018), a few studies have been performed to report the apoptotic effects of anabolic androgenic steroids, especially nandrolone, on cancer cells according to which the present study examined the cytotoxic and apoptotic effects of nandrolone on gastric and colon cancer cells to reveal whether nandrolone is involved in colon and gastric cancer cells proliferation and apoptosis in vitro.

3.5. The cytotoxic and apoptotic effects of nandrolone on AGS and HCT cancer cells:

The results of this study showed that 2.5 mg/ml, 5 mg/ml and 10 mg/ml of nandrolone have a significant cytotoxic effect on AGS and HCT cancer cells. In line with this finding, research has shown that anabolic androgenic steroids, which are synthetic derivatives of testosterone, can be effective in inhibiting the proliferation of various cancer cells (Ajduković et al., 2013; Di Wu and Yang, 2020). It has been reported that androstane analogues have significant anti-proliferative action against a wide range of cancer cell lines including prostate, breast, cervical, ovarian, leukemia, melanoma, colon and gastric cancers (Ajduković et al., 2015; Jakimov et al., 2015). Testosterone

has also inhibitory effects on the proliferation of various cancer cells, including breast cancer cells (Glaser and Dimitrakakis, 2015). The results of a study showed that high concentrations of testosterone had an inhibitory effect on the proliferation of AGS cancer cells (Amani et al., 2019). Research has shown that high concentrations of nandrolone agonists inhibit the proliferation of colon cancer cells (Verzola et al., 2004). Nandrolone has been also reported to have potent antiproliferative effects on cancer cell lines including cervical cancer cells (Kampa et al., 2008). However, by contrast, research has shown that elevated plasma androgen levels are associated with a higher risk of developing certain cancer cells in vivo (Ørsted et al., 2014). We have also shown that the effective concentration of nandrolone (5.99 mg/ml) induces primary and secondary apoptosis in both AGS and HCT cell lines. In line with the results of our study it has been reported that testosterone has pro-apoptotic effects on prostate and colon cancer cells (Anagnostopoulou et al., 2013). Testosterone also induces apoptosis in colon cancer cells by regulating PI3K/Rac1 signaling (Alkahtani, 2013). The results of a study showed that testosterone reduces the incidence of colon cancer by regulating the process of apoptosis (Gu et al., 2011). In contrast, a research suggests the protective role of androgens in cancer cells against apoptosis caused by various stimuli (Kimura et al., 2001).

3.6. The effects of nandrolone on iNOS expression level in AGS and HCT cells:

Our findings showed that the expression level of iNOS was not significantly alter in AGS cells, but significantly reduced in HCT cells treated with effective dose of nandrolone. Despite research carried out in vivo and in vitro to elucidate the effects of male sex steroid hormones on iNOS expression level in cancer cells, association of male sex steroid hormones with iNOS activity is still unclear. iNOS functions to produce nitric oxide (NO) (Alderton et al., 2001). Previous studies have shown that sex steroid hormones are involved in the expression of iNOS and NO production in breast cancer cells (Bentrari et al., 2005). Indeed, testosterone regulates the synthesis of nitric oxide synthase (NOS) and therefore is an important modulator in the production of nitric oxide (Zvara et al., 1995; Blute et al., 2009). By contrast, the results of a study showed that testosterone did not cause a significant change in iNOS gene expression level in gastric and colon cancer cells (Amani et al., 2021). However, testosterone progenitors have been reported to increase NOS expression and NO level in breast and ovarian cancer cells, respectively (Pance, 2006; Maleki et al., 2015). Intra-tumor iNOS activity promotes the growth of cancer cells including prostate cancer cells (Cronauer et al., 2007), however, high concentrations of NO suppress the protective effects of testosterone progenitors in human colon adenocarcinoma cells (Marino et al., 2006). iNOS also plays an important role in the expression and activity of VEGF (Vascular endothelial growth factor). VEGF and iNOS are associated with pathological features of gastric cancer such as invasion, lymphatic metastasis or hematogen, MVD (Microvessel density) increases iNOS expression level and VEGF expression. As a result, iNOS and VEGF can induce angiogenesis in gastric tumors (Chen et al., 2004; Wang et al., 2005) and nandrolone may stand against it by reducing iNOS expression level. Further research is needed to reveal the exact mechanism of action behind the nandrolone effect on colon cancer tumors in vivo.

3.7. The effects of nandrolone on MMP-9 expression in AGS and HCT cells:

AGS cells showed no significant alteration in MMP9 expression, however, the expression level of MMP9 decreased in HCT cells treated with effective dose of nandrolone. Association between MMP9 and cancer development has been reported showing that MMPs have a significant role in

development and maintaining of tumors (Di Carlo et al., 2005). In line with our findings, it has been shown a link between androgens and expression of MMPs in certain cancer cells (Gonzalez et al., 2008; Morales-Vásquez et al., 2020). Androgen progenitors can modulate the activity of MMP-2/MMP-9 in vitro in breast cancer cells (Nilsson et al., 2007). Testosterone progenitors can also have anticancer properties by inhibiting the expression level of MMP-9 in colon cancer cells (Hsu et al., 2011; Grybos and Bar, 2014). In contrast to our findings, testosterone has been reported to increase the activity level of MMP enzymes in prostate hyperplasia (Muñoz et al., 2015). Increased MMP-9 activity is also one of the most important factors in liver metastasis due to colorectal cancer (Liabakk et al., 1996).

3.8. The effects of nandrolone on caspase-3, -8 and -9 activity in AGS and HCT cells:

The effective concentration (5.99 mg/ml) of nandrolone caused a significant increase in caspase-3, -8 and -9 activity in both AGS and HCT cells. Consistent with our findings, the results of a study showed that testosterone in high concentrations stimulates the apoptotic pathway through cytosolic receptors (Verzola et al., 2004) and increasing the activation of caspase-3 and caspase-8 in colorectal cancer cells (Sasso et al., 2019). Another study has also shown that testosterone progenitors can increase caspase-3 and -9 activity in HCT116 cell carcinoma cell line, leading to increased apoptosis (Jin et al., 2017). It has been also reported that testosterone and cortisol increase the activity of caspases -8 and -9 in colon cancer cells (Amani et al., 2019). In 2018, a study by Giovai and others which examined the effects of 5 models of 17-19-nortestosterones (Chen et al., 2015; Poorolajal et al., 2020) on several cancer cell lines, showed that caspase-3 and -9 activity increased in a concentration-dependent manner, while no change in caspase-8 activity was observed (Gyovai et al., 2018). In contrast, androgens have been reported to inhibit apoptosis and reduce caspase activity in human prostate adenocarcinoma LNCaP cells (Rokhlin et al., 2005).

The possible mechanism of action of nandrolone on apoptosis in AGS and HCT cancer cells appears to be that anabolic androgenic steroids (AAS) induce apoptosis in many different cancer cells and increase caspase activity in both intrinsic and extrinsic apoptotic pathways (Jin et al., 2017; Gyovai et al., 2018; Sasso et al., 2019). Therefore, in the present study, nandrolone probably increased the activity of caspases-3,-8 and -9 in both cell lines by affecting both external and internal apoptotic pathways in gastric and colon cancer cells. However, more research is needed to determine how nandrolone affects the expression of apoptotic genes, as well as factors affecting the expression of metastatic genes and enzymes involved in cancer progression.

4 Conclusion

Overall, the results of the present study show that nandrolone can have cytotoxic effects in high concentrations on AGS and HCT cell lines. The effective concentration of nandrolone causes primary and secondary apoptosis in both AGS and HCT cells and decreases growth and metastasis in HCT cells in vitro. Nandrolone has a capability to induce external and internal apoptosis pathways in AGS and HCT cells by increasing the activity levels of caspases-3, -8 and -9. More in vitro and in vivo experimental and clinical research are needed to elucidate the effects of nandrolone on colon and gastric tumors development.

5 Conflict of interests

The authors declare that there are no competing interests.

References

Ajduković, J.J., Djurendić, E.A., Petri, E.T., Klisurić, O.R., Ćelić, A.S., Sakač, M.N., Jakimov, D.S., & Gaši, K.M.P. (2013). 17 (E)-picolinylidene androstane derivatives as potential inhibitors of prostate cancer cell growth: antiproliferative activity and molecular docking studies. Bioorganic & medicinal chemistry, 21(23): 7257–7266.

Ajduković, J.J., Gaši, K.M.P., Jakimov, D.S., Klisurić, O.R., Jovanović-Šanta, S.S., Sakač, M.N., Aleksić, L.D., & Djurendić, E.A. (2015). Synthesis, structural analysis and antitumor activity of novel 17-picolyl and 17 (E)-picolinylidene A-modified androstane derivatives. Bioorganic & medicinal chemistry, 23(7): 1557–1568.

Ala-aho, R., & Kähäri, V-M. (2005). Collagenases in cancer. Biochimie, 87(3–4): 273-286.

Alderton, W.K., Cooper, C.E., & Knowles, R.G. (2001). Nitric oxide synthases: structure, function and inhibition. Biochemical journal, 357(3): 593–615.

Alkahtani, S. (2013). Testosterone induced apoptosis in colon cancer cells is regulated by PI3K/Rac1 signaling. Asian journal of andrology, 15(6):831–834.

Amani, N., Shariati, M., Ahmadi, R., Khatamsaz, S., & Mokhtari, M. (2019). The Cytotoxic Effects of Testosterone on Gastric Cancer Cell Line (AGS) and Evaluation of Caspase-3,-8, and-9 Activity. Qom University of Medical Sciences Journal,13(6): 8–17.

Amani, N., Shariati, M., Ahmadi, R., Khatamsaz, S., & Mokhtari, M. (2021). Testosterone Enanthate Induces Cell Death in Human Colon (HCT) and Gastric (AGS) Cancer Cells. Zoological Society of Pakistan, 1–9.

Anagnostopoulou, V., Pediaditakis, I., Alkahtani, S., Alarifi, S.A., Schmidt, E-M., Lang, F., Gravanis, A., Charalampopoulos, I., & Stournaras, Ch. (2013). Differential effects of dehydroepiandrosterone and testosterone in prostate and colon cancer cell apoptosis: the role of nerve growth factor (NGF) receptors. Endocrinology, 154(7): 2446–2456.

Bentrari, F., Arnould, L., Jackson, A.P., Jeannin, J-F., & Pance, A. (2005). Progesterone enhances cytokine-stimulated nitric oxide synthase II expression and cell death in human breast cancer cells. Laboratory investigation, 85(5): 624–632.

Blute, M., Hakimian, P., Kashanian, J., Shteynshluyger, A., Lee, M., & Shabsigh, R. (2009). Erectile dysfunction and testosterone deficiency. Advances in the management of testosterone deficiency, 37: 108–122.

Chen, R., Cui, J, Wang, Q., Li, P., Liu, X., Hu, H., & Wei, W. (2015). Antiproliferative effects of anastrozole on MCF-7 human breast cancer cells in vitro are significantly enhanced by combined treatment with testosterone undecanoate. Molecular medicine reports, 12(1): 769–775.

Chen, T., Nines, R.G., Peschke, S.M., Kresty, L.A., & Stoner, G.D. (2004). Chemopreventive effects of a selective nitric oxide synthase inhibitor on carcinogen-induced rat esophageal tumorigenesis. Cancer research, 64(10): 3714–3717.

Cronauer, M.V., Ince, Y., Engers, R., Rinnab, L., Weidemann, W., Suschek, C.V., Burchardt, M., Kleinert, H., Wiedenmann, J., Sies, H., Ackermann, R., & Kröncke, K-D. (2007). Nitric oxide-mediated inhibition of androgen receptor activity: possible implications for prostate cancer progression. Oncogene, 26(13): 1875–1884.

Dai, W.S., Kuller, L.H., LaPorte, R.E., Gutai, J.P., Falvo-Gerard, L., & Caggiula, A. (1981). The epidemiology of plasma testosterone levels in middle-aged men. American Journal of Epidemiology, 114(6): 804–816.

De Clerck, Y.A., Shimada, H., Taylor, S.M., & Langley, K.E. (1994). Matrix Metalloproteinases and Their Inhibitors in Tumor Progression a. Annals of the New York Academy of Sciences, 732(1): 222–232.

Di Carlo, A., Terracciano, D., Mariano, A., & Macchia, V. (2005). Matrix metalloproteinase-2 and matrix metalloproteinase-9 type IV collagenases in serum of patients with pleural effusions. International journal of oncology, 26(5): 1363–1368.

Di Wu, J.Z., & Yang, C. (2020). Effect of letrozole combined with testosterone on the proliferation and apoptosis of endometrial carcinoma cells. European Journal of Gynaecological Oncology, 41(4): 604–608.

Farahmandlou, N., Oryan, S., Ahmadi, R., & Eidi, A. (2017). Association of testosterone with colorectal cancer (ht29), human glioblastoma (a172) and human embryonic kidney (hek293) cells proliferation. Acta Endocrinologica (Bucharest), 13(2): 144–149.

Glaser, R., & Dimitrakakis, C. (2015). Testosterone and breast cancer prevention. Maturitas, 82(3): 291–295.

Gonzalez, L.O., Corte, M.D., Vazquez, J., Junquera, S., Sanchez, R., Alvarez, A.C., Rodriguez, J.C., Lamelas, M.L., & Vizoso, F.J. (2008). Androgen receptor expresion in breast cancer: relationship with clinicopathological characteristics of the tumors, prognosis, and expression of metalloproteases and their inhibitors. BMC cancer, 8(1): 1–10.

Grybos, A, & Bar, J. (2014). The relationships between the immunoexpression of KAI1, MMP-2, MMP-9 and steroid receptors expression in endometrial cancer. Folia histochemica et cytobiologica, 52(3): 187–194.

Gu, S., Papadopoulou, N., Nasir, O., Föller, M., Alevizopoulos, K., Lang, F., & Stournaras, Ch. (2011). Activation of membrane androgen receptors in colon cancer inhibits the prosurvival signals Akt/bad in vitro and in vivo and blocks migration via vinculin/actin signaling. Molecular Medicine, 17(1-2): 48–58.

Gyovai, A., Minorics, R., Kiss, A., Mernyák, E., Schneider, G., Szekeres, A., Kerekes, E., Ocsovszki, I., & Zupkó, I. (2018). Antiproliferative properties of newly synthesized 19-nortestosterone analogs without substantial androgenic activity. Frontiers in pharmacology, 9: 825.

Hsu, H-H., Hu, W-S., Lin, Y-M., Kuo, W-W., Chen, L-M., Chen, W-K., Hwang, J-M., Tsai, F-J., Liu, Ch-J., & Huang, Ch-Y. (2011). JNK suppression is essential for 17-Estradiol inhibits prostaglandin E2-Induced uPA and MMP-9 expressions and cell migration in human LoVo colon cancer cells. Journal of biomedical science, 18(1): 1–12.

Jakimov, D.S., Kojić, V.V., Aleksić, L.D., Bogdanović, G.M., Ajduković, J.J., Djurendić, E.A., Gaši, K.M.P., Sakač, M.N., & Jovanović-Šanta, S.S. (2015). Androstane derivatives induce apoptotic death in MDA-MB-231 breast cancer cells. Bioorganic medicinal chemistry, 23(22): 7189–7198.

Jamalzadeh, L., Ghafoori, H., Aghamaali, M., & Sariri, R. (2017). Induction of apoptosis in human breast cancer MCF-7 cells by a semi-synthetic derivative of artemisinin: a caspase-related mechanism. Iranian journal of biotechnology, 15(3):157–165.

Jin, P., Wang, D-z., Lyu, C-x., Wang, Y-t., He, Y-q, Sheng, J-q, & Li., X. (2017). Mismatch repair protein hMLH1, but not hMSH2, enhances estrogen-induced apoptosis of colon cancer cells. Journal of Cancer, 8(16): 3232–3241.

Juliet, P.A.R., Hayashi, T., Daigo, S., Matsui-Hirai, H., Miyazaki, A., Fukatsu, A., Funami, J., Iguchi, A., & Ignarro, L.J. (2004). Combined effect of testosterone and apocynin on nitric oxide and superoxide production in PMA-differentiated THP-1 cells. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1693(3):185–191.

Jurášek, M., Rimpelová, S., Pavlíčková, V., Ruml, T., Lapčík, O., & Drašar, P.B. (2015). Synthesis and biological evaluation of nandrolone–bodipy conjugates. Steroids, 97: 62–66.

Kampa, M., Pelekanou, V., & Castanas, E. (2008). Membrane-initiated steroid action in breast and prostate cancer. Steroids, 73(9–10): 953-960.

Kimura, K., Markowski, M., Bowen, C., & Gelmann, E.P. (2001). Androgen blocks apoptosis of hormone-dependent prostate cancer cells. Cancer research, 61(14): 5611–5618.

Langenskiöld, M., Holmdahl, L., Falk, P., & Ivarsson, M-L. (2005). Increased plasma MMP-2 protein expression in lymph node-positive patients with colorectal cancer. International journal of colorectal disease, 20(3): 245–252.

Liabakk, N-B., Talbot, I., Smith, R.A., Wilkinson, K., & Balkwill, F. (1996). Matrix metalloprotease 2 (MMP-2) and matrix metalloprotease 9 (MMP-9) type IV collagenases in colorectal cancer. Cancer research, 56(1): 190–196.

Maleki, J., Nourbakhsh, M., Shabani, M., Korani, M., Nourazarian, S.M., Dahaghi, M.R.O., & Moghadasi, M.H. (2015). 17-estradiol stimulates generation of reactive species oxygen and nitric oxide in ovarian adenocarcinoma cells (OVCAR 3). Iranian journal of cancer prevention, 8(3): e2332.

Marino, M., Galluzzo, P., Leone, S., Acconcia, F., & Ascenzi, P. (2006). Nitric oxide impairs the 17-estradiol-induced apoptosis in human colon adenocarcinoma cells. Endocrine-related cancer, 13(2): 559–569.

Morales-Vásquez, F., Castillo-Sánchez, R., Gómora, M.J., Almaraz, M.Á., Pedernera, E., Pérez-Montiel, D., Rendón, E., López-Basave, H.N., Román-Basaure, E., Cuevas-Covarrubias, S., Maldonado-Cubas, J., Villa, A., & Mendez, C. (2020). Expression of metalloproteinases MMP-2 and MMP-9 is associated to the presence of androgen receptor in epithelial ovarian tumors. Journal of ovarian research, 13(1): 1–10.

Muñoz, D., Serrano, M.K., Sampieri, C.L., Cocotle, L., Herrera, D., Rojas, F., Manzo, J., & Hernández, M.E. (2015). Effects of Prolactin and Testosterone on MT1-MMP mRNA Expression and Enzymatic Activity of MMP-2 and MMP-9 in the Rat Prostate. The FASEB Journal, 29: LB145.

Nilsson, U.W., Garvin, S., & Dabrosin, C. (2007). MMP-2 and MMP-9 activity is regulated by estradiol and tamoxifen in cultured human breast cancer cells. Breast cancer research and treatment, 102(3): 253–2561.

Ørsted, D., Nordestgaard, B., & Bojesen, S. (2014). Plasma testosterone in the general population, cancer prognosis and cancer risk: a prospective cohort study. Annals of oncology, 25(3):712–718.

Pacal, I., Karaboga, D., Basturk, A., Akay, B., & Nalbantoglu, U. (2020). A comprehensive review of deep learning in colon cancer. Computers in Biology and Medicine, 126: 104003.

Pance, A. (2006). Nitric oxide and hormones in breast cancer: allies or enemies? Future Oncol, 2(2): 275–88.

Papadopoulou, N., Charalampopoulos, I., Anagnostopoulou, V., Konstantinidis, G., Föller, M., Gravanis A, Alevizopoulos, K., Lang,F., & Stournaras, Ch. (2008). Membrane androgen receptor activation triggers down-regulation of PI-3K/Akt/NF-kappaB activity and induces apoptotic responses via Bad, FasL and caspase-3 in DU145 prostate cancer cells. Molecular cancer, 7(1):1–13.

Patanè, F.G., Liberto, A., Maria Maglitto, A.N., Malandrino, P., Esposito, M., Amico, F., Cocimano, G., Li Rosi, G., Condorelli, D., Di Nunno, N., & Montana, A. (2020). Nandrolone decanoate: use, abuse and side effects. Medicina, 56(11): 606.

isk Poorolajal, J., Moradi, L., Mohammadi, Y., Cheraghi, Z., & Gohari-Ensaf, F. (2020). Risk factors for stomach cancer: a systematic review and meta-analysis. Epidemiology and health, 42: e2020004.

Rawla, P., & Barsouk, A. (2019). Epidemiology of gastric cancer: global trends, risk factors and prevention. Przeglad gastroenterologiczny, 14(1): 26–38.

Rokhlin, O.W., Taghiyev, A.F., Guseva, N.V., Glover, R.A., Chumakov, P.M., Kravchenko, J.E., & Cohen, M.B. (2005). Androgen regulates apoptosis induced by TNFR family ligands via multiple signaling pathways in LNCaP. Oncogene, 24(45): 6773–6784.

Roshan, M.H., Tambo, A.,& Pace, N.P. (2016). The role of testosterone in colorectal carcinoma: pathomechanisms and open questions. Epma Journal, 7(1): 1–10.

Sang, Q.X.A. (1998). Complex role of matrix metalloproteinases in angiogenesis. Cell research, 8(3): 171–177.

Sasso, C.V., Santiano, F.E., Arboccó, F.C.V., Zyla, L.E., Semino, S.N., Guerrero-Gimenez, M.E., Creydt, V.P., Fontana, C.M.L., Carón, R.W. (2019). Estradiol and progesterone regulate proliferation and apoptosis in colon cancer. Endocrine connections, 8(3): 217–229.

Simoes, B.M., Alferez, D.G., Howell, S.J., & Clarke, R.B. (2015). The role of steroid hormones in breast cancer stem cells. Endocrine-related cancer, 22(6): T177–T86.

Tishehyar, A., Ahmadi, R., Mahmoudi, M., & Mohamadnia, A. (2021). The Apoptotic Effects of Testosterone on Lung Cancer (A549) Cells. Journal of Animal Biology, 13(4): 37–45.

Verzola, D., Gandolfo, M.T., Salvatore, F., Villaggio, B., Gianiorio, F., Traverso, P., Deferrari, G., & Garibotto, G. (2004). Testosterone promotes apoptotic damage in human renal tubular cells. Kidney international, 65(4): 1252–1261.

Wang, G-Y., Ji, B., Wang, X., & Gu, J-H. (2005). Anti-cancer effect of iNOS inhibitor and its correlation with angiogenesis in gastric cancer. World journal of gastroenterology: WJG, 11(25): 3830–3833.

Zheng, N., Chen, J., Liu, W., Liu, J., Li, T., Chen, H., Wang, J., & Jia, L. (2017). Mifepristone inhibits ovarian cancer metastasis by intervening in SDF-1/CXCR4 chemokine axis. Oncotarget, 8(35): 59123–59135.

Zvara, P., Sioufi, R., Schipper, H.M., Begin, L., & Brock, G. (1995). Nitric oxide mediated erectile activity is a testosterone dependent event: a rat erection model. International journal of impotence research., 7(4): 209–219.