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Reactive Oxygen Species Co-Operated with Sex Hormones Inhibit Proliferation of Hepa1-6 Cells

Ai-Guo Wang, Nam-Soon Kim and Dong-Seok Lee†

Laboratory of Human Genomics, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Korea

Reactive oxygen species (ROS) and sex hormones affect the proliferation of cells and are believed to play important roles in tumorigenesis. However, little is known regarding how these two factors interact to affect cell proliferation. In this study, hepa1-6 cells were treated with ROS and sex hormones (testosterone and steroidal) either separately or in combination. The sex hormones had no significant influence the cell proliferation up to a concentration of 1 μM. However, cell proliferation was inhibited when the cells were treated simultaneously with H_2O_2, which alone was found to promote cell proliferation at the concentrations of 15 μM. In conclusion, this study indicates that instead of promoting the cell proliferation, ROS interact with sex hormones to inhibit the Hepa1-6 cell proliferation.

Key Words: Cell proliferation, Hepa1-6, Reactive oxygen species (ROS), Sex hormones

INTRODUCTION

The liver is a hormone-sensitive organ. Both male and female livers contain androgen receptors (Nagasue et al., 1985; Roy et al., 1974; Sato et al., 1980) and high-affinity, low-capacity estrogen receptors (Porter et al., 1983; Rossini et al., 1989). Sex hormones are known to affect many functions of the mammalian liver (Eagon et al., 1985; Johnson, 1984). Hepatocellular carcinoma (HCC) is the most common malignancy found in humans. A general characteristic of HCC across different geographical areas is the striking male prevalence (Tanaka et al., 2000) Therefore, the gender factors (sex hormones) were suggested to play important roles in the regulation of hepatic cell proliferation.

It was reported that estradiol suppresses chemical carcinogens induced hepatic carcinogenesis and fibrogenesis (Shimizu et al., 1998; Yasuda et al., 1999). In addition, HCV-related cirrhotic women before menopause might have the ability to protect against developing HCC via hepatic ER (Shimizu et al., 2001). On the other hands, clinical studies have shown that men are prone to develop hepatocellular carcinoma compared with women and that androgen might be involved in the malignant transformation of the liver (Johnson et al., 1972). Some reports have showed that estrogen receptor decreased, however androgen receptor increased in HCC (Ohnishi et al., 1986). In addition, the HCC tissues could actively take up testosterones (Nagasue et al., 1986). Moreover, the elevated serum testosterone levels increase the risk of hepatocellular carcinoma (Yu et al., 1993) and the man have been identified as one of the most important risk factors for HCC (De Maria et al., 2002). So, the expression of androgen receptor may be augmented in association with malignant transformation of hepatocytes while the expression of estrogen receptor may be rather suppressed and that some of hepatocellular carcinoma may be androgen-dependent (Ohnishi et al., 1986).

Even, the oxidative stress induced by reactive oxygen species (ROS) can cause damage to proteins, lipids, carbohydrated, and nucleic acids (Cadenas, 1989; Davies, 1995; Fridovich, 1995), the proliferating mammalian cells exhibit a broad spectrum of responses to oxidative stress, depending on the stress levels encountered: mitogenic stimulation, temporary growth arrest, permanent growth arrest, and cell apoptosis or necrosis (Davies, 2001). The tumor cell showed characteristics of proliferation and elevated ROS

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†Corresponding author: Dong-Seok Lee, Laboratory of Human Genomics, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea.
Tel: 82-42-860-4349, Fax: 82-42-860-4608
e-mail: lee10@kribb.re.kr

levels. In addition, some tumors are sex hormone related or depended, such as liver tumor that have been detected for sex hormone receptors and male prevalence. However, there is no report to investigate if the ROS and sex hormone can function corporately to influence the proliferation of cells.

In this study, the regulated profiles of proliferation by ROS and sex hormones, separately and simultaneously, were investigated on the Murine Hepa1-6 liver tumor cells. The results showed that ROS and sex hormones co-operated to inhibit the proliferation of Hepa1-6 cells.

MATERIALS AND METHODS

1. Cell culture

Murine hepatoma Hepa1-6 cells were obtained from the American Type Cell Culture (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS).

2. RT-PCR analysis

In order to detect if the ER and AR genes was expressed in Hepa1-6 cells, the RT-PCR analysis was performed. Total RNA was isolated from cultured Hepa1-6 cells using the TRIzol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA). RT-PCR was performed using a reverse transcription system (Promega Corp., Madison, WI) according to manufacturer's instructions. The primers used to detect the expression of the AR were the sense primer, 5'-AGTACCAGGGACCATGTTTTACC-3', and the anti-sense primer, 5'-AGAGATGATCTCTGCCATCATTT-3'; and the expression of the ER were the sense primer, 5'-ATGACATTCTACAGTCCTGC-3', and the anti-sense primer, 5'-TCACTGTGACTGGAGGTTCTGGG-3'. A separate RT-PCR using primer for the detection of Actin was used as a loading control. The production of a single band of the expected size on RT-PCR was recognized as amplification of the target genes.

3. Treatment

For treatment, the cells were re-plated at the concentration at 1×10^4 cells/well (96-well plate) and cultured for 24 hours. The medium was changed to fresh one with certain concentration of H_2O_2, estrogen and testosterone respectively or simultaneously. After treatment for 24 hours, the cell proliferation was analyzed by MTT assay. For controls, when the medium was changed to fresh one, the same volume of solvent that was used for solving H_2O_2, estrogen and testosterone was added and, after 24 hours culture, the controls was performed MTT assay with treated cells simultaneously.

4. MTT Conversion assay

After treatment, the medium was then replaced with 400 μl of fresh DMEM growth medium, and 40 μl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) was added. Following a 4-h incubation, the cells were lysed with 550 μl of lysis buffer (10% SDS, 0.1 M HCl). The relative level of MTT conversion in each sample was then measured by spectrophotometry ($\lambda = 595$ nm). The absorbance values were normalized to the mean absorbance of non-treated Hepa1-6 cells.

5. Statistical analysis

The differences between the experimental groups were tested for statistical significance using a Student's *t* test. *P*-values < 0.05 were considered to be significant.

RESULTS

1. The profile of ROS that influence the Hepa1-6 cell proliferation

It has been reported that the proliferating mammalian cells exhibit a broad spectrum of responses to oxidative stress, depending on the stress levels encountered: mitogenic stimulation, growth arrest, and cell apoptosis or necrosis (Davies, 2001). In order to confirm these results and find out the response spectrum of Hepa1-6 cells to ROS, the profile of responses of Hepa1-6 cells to H_2O_2 was determined. The results showed that when the concentration of H_2O_2 was lower than 15 μM, the growth of Hepa1-6 cells was significantly promoted; between 200 and 300 μM, the growth of cells was significantly inhibited; over 5 mM, the cells undergone necrosis or apoptosis (Fig. 1). These results showed that the Hepa1-6 cells also have the similar spectrum of responses to oxidative stress compared to the reported results.

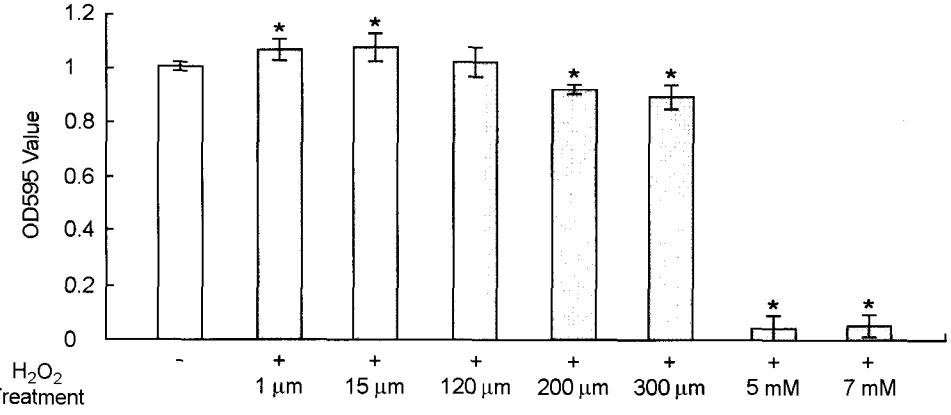

Fig. 1. The effect of H_2O_2 on the proliferation of Hepa1-6 cells. The Hepa1-6 cells were treated with H_2O_2 in a series concentrations and the proliferation of cells was determined by MTT analysis. * indicated significant difference compared with non-treated cells (P<0.05).

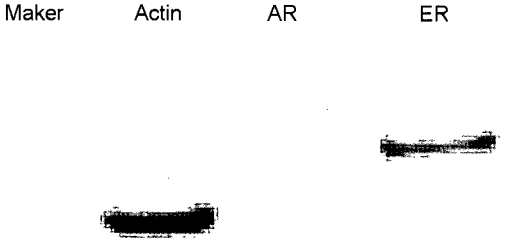

Fig. 2. The expression of androgen and estrogen receptors in Hepa1-6 cells. The total RNAs were extracted from Hepa1-6 cells and the expression of androgen and estrogen receptors was analyzed by RT-PCR. Actin was used as an internal control. AR: androgen receptor; ER: estrogen receptor.

2. The profile of sex hormones that influence the Hepa1-6 cell proliferation

The sex hormones have been reported having promoting functions to the proliferation of normal cells (De Maria et al., 2002). To certificate if it is also true for Hepa1-6 cells, firstly, we confirmed the expression of sex hormone receptors by RT-PCR. The results showed that the Hepa1-6 cells firmly expression ER and AR (Fig. 2). So, the treatment by sex hormones at 10 nM, 1 μM and 100 μM was performed. Data showed that no significant proliferation changes were detected up to 1 μM treatment for estrogen (Fig. 3A) and up to 100 μM for testosterone (Fig. 3B). But the proliferation was significantly inhibited when the treatment concentration was up to 100 μM for estrogen. These results indicated that, at proper concentrations, the sex hormones alone are not cytopathic to the Hepa1-6 cells, apart from the high concentration of estrogen that inhibit the Hepa1-6 cells proliferation at 100 μM.

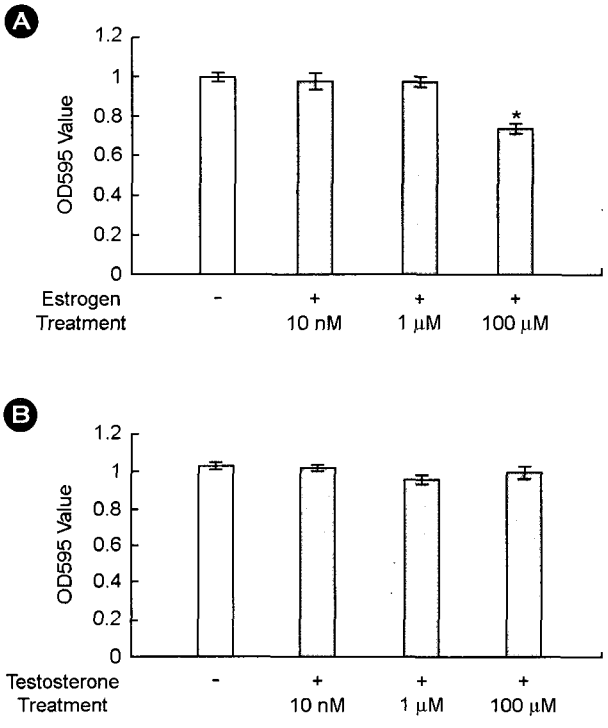

Fig. 3. The effect of estrogen and testosterone on the proliferation of Hepa1-6 cells. The Hepa1-6 cells were treated with estrogen **(A)** and testosterone **(B)** in a series concentrations and the proliferation of cells was determined by MTT analysis. * indicated significant difference compared with non-treated cells (P<0.05).

3. The co-operation of ROS and sex hormones to inhibit Hepa1-6 cell proliferation

To find out if the co-operation of H_2O_2 and sex hormones can influence the proliferation of Hepa1-6 cells, the cells were treated with H_2O_2 and sex hormones at different concentration series. For H_2O_2, the concentration of 15 μm

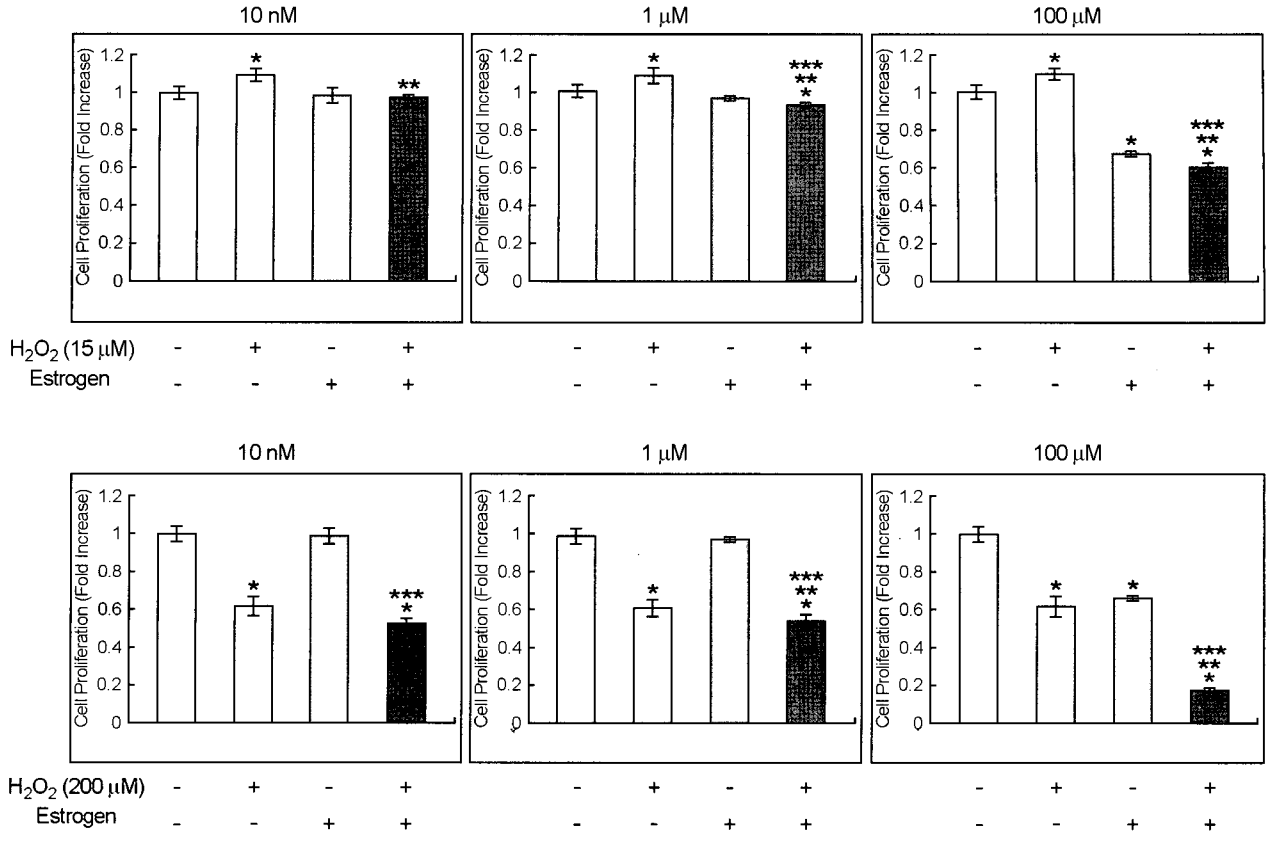

Fig. 4. The co-operative effect of estrogen and H_2O_2 on the proliferation of Hepa1-6 cells. The Hepa1-6 cells were treated with estrogen and H_2O_2 at the same times in a series concentrations and the proliferation of cells was determined by MTT analysis. * indicated significant difference compared with non-treated cells; ** indicated significant difference compared with H_2O_2 treated cells; *** indicated significant difference compared with estrogen treated cells ($P<0.05$).

and 200 μm was selected, because 15 μm significantly promoted the cell proliferation and 200 μm inhibited it. For sex hormones, the concentration of 10 nM, 1 μM and 100 μM were selected to screen the possible influence on the cell proliferation dependent on the different concentration series. The results showed that the co-operation of the H_2O_2 and sex hormones significantly inhibit the cell proliferation. For estrogen, follow the concentration series, the higher concentration showed significantly higher inhibition functions to the cell proliferation at the background of either 15 or 200 μM H_2O_2 concentrations (Fig. 4). For testosterone, at the background of 15 μM H_2O_2 concentration, when testosterone was added, the stimulation function of H_2O_2 disappeared. In the case of 200 μM H_2O_2 concentration, adding testosterone promote the toxicity of H_2O_2 to the cells (Fig. 5). It indicated that, although the H_2O_2 alone at 15 μM concentration can stimulated the cell proliferation, the cell proliferation stimulating function of H_2O_2 disappeared or go to the opposition side, inhibition the cell proliferation when the sex hormones were added. For the 200 μM concentration, the toxicity of H_2O_2 was promoted by sex hormones.

DISCUSSION

Although the sex hormones have showed their proliferation stimulating functions in normal hepatocytes (De Maria et al., 2002), we can not detect this phenomenon in Murine Hepa1-6 liver tumor cells. It has been reported that neither anti-estrogen nor anti-androgen treatment is effective in unresectable HCC therapy (Grimaldi et al., 1998; Liu et al., 2000). These results may suggest that, once the tumor has developed, the anti-androgen and anti-estrogen treatment has probably no clinical relevance on the progression of the disease or that 'clinically' HCC is not a sex hormones responsive tumor. This may explain that why the sex hormones have no significant influence on the proliferation of Hepa1-6 tumor cells in proper concentration range. Even

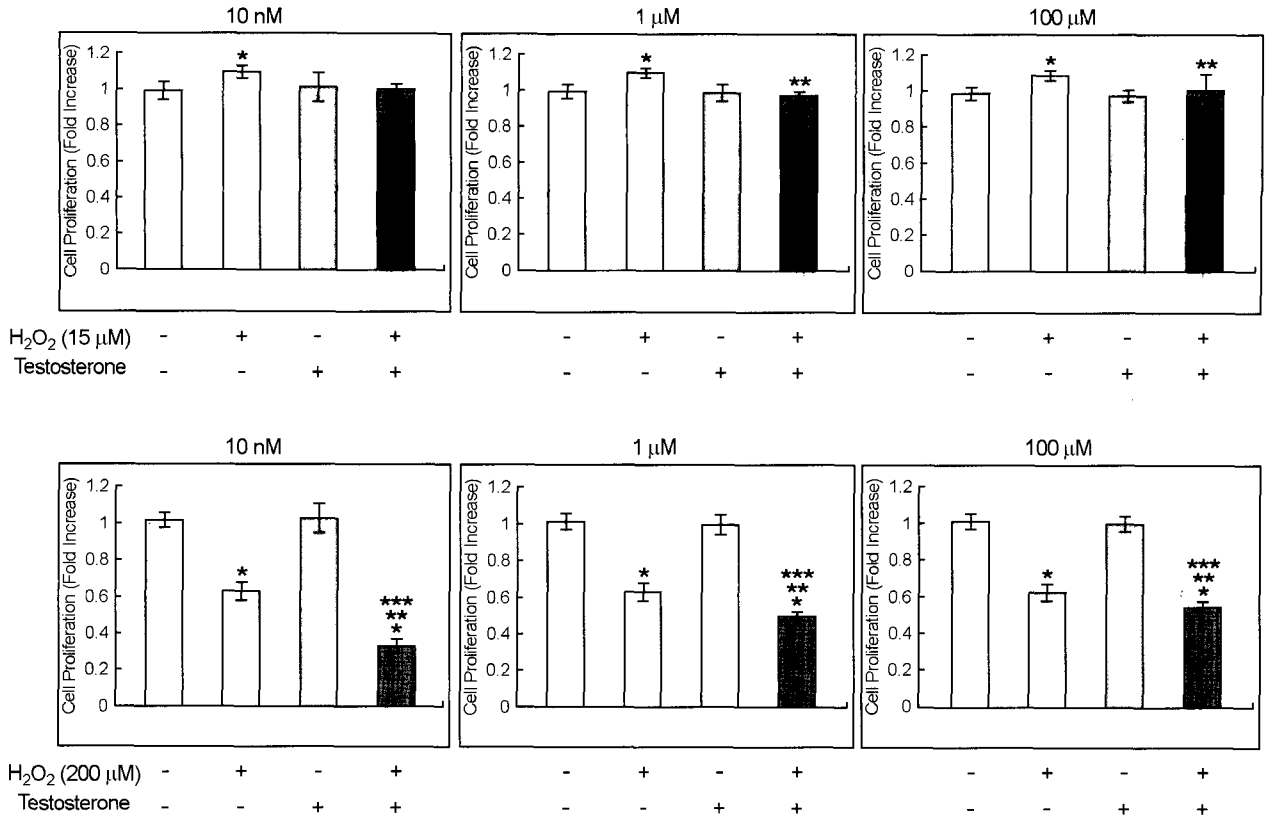

Fig. 5. The cooperative effect of Testosterone and H_2O_2 on the proliferation of Hepa1-6 cells. The Hepa1-6 cells were treated with testosterone and H_2O_2 at the same times in a series concentrations and the proliferation of cells was determined by MTT analysis. * indicated significant difference compared with non-treated cells; ** indicated significant difference compared with H_2O_2 treated cells; *** indicated significant difference compared with testosterone treated cells ($P<0.05$).

the sex hormone receptors were all expressed in this cell line. Another possible is that the functions of sex hormones on liver cancer development in human beings are different from the Murine.

The variant type of ER have been reported in human liver cancer and thought to play an important role in liver cancer development (Johnson, 1984; De Maria et al., 2002). However, in the Murine Hepa1-6 liver tumor cells, we can not detect variant ER. In addition, we also can not find variant type ER in the liver tumor tissues of our H-ras12V transgenic mice (wang, et al., 2005) (data not shown). This indicated that, in the case of Murine liver tumors, the relationship of estrogen and ER on liver tumor development may different from human being.

The influence of ROS on the cell proliferation in our study is consistent to the previous reports (Davies, 2001). The lower ROS level can stimulate the cell proliferation. It has been reported that in cancer cells, the ROS are higher compared to normal cells (Pelicano et al., 2004). So, we proposed that, when cancer cells overcome the ROS stress, the higher ROS level may stimulate the cancer cell proliferation. In this study, we showed that the co-operation of sex hormones and ROS firmly inhibit the cell proliferation even in concentration of 15 μM H_2O_2 that showed stimulating function to the cell proliferation alone. It was reported that estradiol suppresses chemical carcinogens induced hepatic carcinogenesis and fibrogenesis (Shimizu et al., 1998; Yasuda et al., 1999). In addition, HCV-related cirrhotic women before menopause might have the ability to protect against developing HCC via hepatic ER (Shimizu et al., 2001). Our results may offer one explain that the inhibition function of estradiol to the liver cancer are partly by the co-operation with the ROS. However, for the androgen, the more detailed mechanisms remained to be elucidated.

In conclusion, in this study, the co-operation of sex hormones and H_2O_2 to inhibit the hepa1-6 cell proliferation was determined. In our view, this is the first time to show that the sex hormones can co-operated with ROS to inhibit

cell proliferation. It showed a new view in the relationship of sex hormones and ROS related signaling pathways that co-function to the cell proliferation, even the mechanisms remained to be elucidated.

Acknowledgements

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REFERENCES

Cadenas E. Biochemistry of oxygen toxicity. Annu Rev Biochem. 1989. 58: 79-110.

Davies KJ. Oxidative stress: the paradox of aerobic life. Biochem Soc Symp. 1995. 61: 1-31.

Davies KJA. The spectrum of responses of oxidants in proliferating cells: concentration. Oxygen Society Annual Meeting. 2001.

De Maria N, Manno M, Villa E. Sex hormones and liver cancer. Mol Cell Endocrinol. 2002. 193: 59-63.

Eagon PK, Porter LE, Francavilla A, DiLeo A, Van Thiel DH. Estrogen and androgen receptors in liver: their role in liver disease and regeneration. Semin Liver Dis. 1985. 5: 59-69.

Fridovich I. Superoxide radical and superoxide dismutases. Annu Rev Biochem. 1995. 64: 97-112.

Grimaldi C, Bleiberg H, Gay F, Messner M, Rougier P, Kok TC, Cirera L, Cervantes A, De Greve J, Paillot B, Buset M, Nitti D, Sahmoud T, Duez N, Wils J. Evaluation of antiandrogen therapy in unrespectable hepatocellular carcinoma: results of a European Organization for Research and Treatment of Cancer multicentric double-blind trial. J Clin Oncol. 1998. 16: 411-417.

Johnson FL, Lerner KG, Siegel M, Feagler JR, Majerus PW, Hartmann, JR, Thomas ED. Association of androgenic-anabolic steroid therapy with development of hepatocellular carcinoma. Lancet 1972. 2: 1273-1276.

Johnson PJ. Sex hormones and the liver. Clin Sci (Lond). 1984. 66: 369-376.

Liu CL, Fan ST, Ng IO, Lo CM, Poon RT, Wong J. Treatment of advanced hepatocellular carcinoma with tamoxifen and the correlation with expression of hormone receptors: a prospective randomized study. Am J Gastroenterol. 2000. 95: 218-222.

Nagasue N, Ito A, Yukaya H, Ogawa Y. Androgen receptors in hepatocellular carcinoma and surrounding parenchyma. Gastroenterology 1985. 89: 643-647.

Nagasue N, Yukaya H, Chang YC, Ogawa Y, Kohno H, Ito A. Active uptake of testosterone by androgen receptors of hepatocellular carcinoma in humans. Cancer 1986. 57: 2162-2167.

Ohnishi S, Murakami T, Moriyama T, Mitamura K, Imawari M. Androgen and estrogen receptors in hepatocellular carcinoma and in the surrounding noncancerous liver tissue. Hepatology 1986. 6: 440-443.

Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resist Update 2004. 7: 97-110.

Porter LE, Elm MS, Van Thiel DH, Dugas MC, Eagon PK. Characterization and quantitation of human hepatic estrogen receptor. Gastroenterology 1983. 84: 704-712.

Rossini GP, Baldini GM, Villa E, Manenti F. Characterization of estrogen receptor from human liver. Gastroenterology 1989. 96: 1102-1109.

Roy AK, Milin BS, McMinn DM. Androgen receptor in rat liver: hormonal and developmental regulation of the cytoplasmic receptor and its correlation with the androgen-dependent synthesis of alpha2u-globulin. Biochim Biophys Acta. 1974. 354: 213-232.

Sato N, Ota M, Obara K. Presence of binding component(s) for testosterone in rat liver cytosol. Endocrinol Jpn. 1980. 27: 315-319.

Shimizu I, Inoue H, Yano M, Shinomiya H, Wada S, Tsuji Y, Tsutsui A, Okamura S, Shibata H, Ito S. Estrogen receptor levels and lipid peroxidation in hepatocellular carcinoma with hepatitis C virus infection. Liver 2001. 21: 342-349.

Shimizu I, Yasuda M, Mizobuchi Y, Ma YR, Liu F, Shiba M, Horie T, Ito S. Suppressive effect of oestradiol on chemical hepatocarcinogenesis in rats. Gut 1998. 42: 112-119.

Tanaka K, Sakai H, Hashizume M, Hirohata T. Serum testosterone: estradiol ratio and the development of hepatocellular carcinoma among male cirrhotic patients. Cancer Res. 2000. 60: 5106-5110.

Yasuda M, Shimizu I, Shiba M, Ito S. Suppressive effects of estradiol on dimethylnitrosamine-induced fibrosis of the liver in rats. Hepatology 1999. 29: 719-727.

Yu MW, Chen CJ. Elevated serum testosterone levels and risk of hepatocellular carcinoma. Cancer Res. 1993. 53: 790-794.