Effect of Crocin on Cell Cycle Regulators in *N*-Nitroso-*N*-Methylurea-Induced Breast Cancer in Rats

Mahboobeh Ashrafi,^{1,2} S. Zahra Bathaie,¹ Saeid Abroun,³ and Mahshid Azizian¹

We previously showed the anticancer effect of crocin, a saffron carotenoid, in both breast and gastric cancers in animal models, but its mechanism of action is not clearly known, yet. In this study, the effect of crocin on cell cycle regulators is investigated. Female Wistar Albino rats were divided into two groups, with or without *N*-nitroso-*N*-methylurea (NMU) injection. After tumor formation, each group of rats was divided into two subgroups, receiving crocin or vehicle only. After 5 weeks, the rats were sacrificed and the tumors were retained for pathologic investigation and determination of the parameters. Before crocin treatment, the tumor volumes were 13.27 ± 3.77 and 12.37 ± 1.88 , but at the end of the experiment, they were 23.66 ± 8.82 and 11.91 ± 2.27 in the control and crocin-treated groups, respectively. Pathologic investigation indicated the adenocarcinoma induction by NMU. Reverse transcription–polymerase chain reaction and Western blot analysis showed overexpression of cyclin D1 and p21^{Cip1} in the NMU-induced breast tumors; however, the expression of both of them suppressed by crocin treatment. The previous studies indicated that crocin induces apoptosis in tumor tissue. In this study, we show that it also suppresses tumor growth and induces cell cycle arrest by down-regulation of cyclin D1. In addition, crocin suppressed p21^{Cip1} in a p53-dependent manner.

Introduction

RAT MODEL OF MAMMARY carcinoma using *N*-nitroso-*N*-methylurea (NMU) injection is a widely used animal model for breast cancer researches. Because of the similarities between these chemically induced mammary carcinomas and human breast cancers, including their histopathology, originating from mammary ductal epithelial cells and depending on ovarian hormones (estrogen and progesterone) for tumor development (Chan *et al.*, 2005, 2007), it is a good model to investigate the new strategies of breast cancer treatment.

Breast cancer is the most common malignancy in women and comprises 21% of all cancers in women in 23 areas (Parkin *et al.*, 1999). According to different epidemiologic studies in Iran, breast cancer is the first or second (after lung cancer) leading cause of death among cancerous women (Sadjadi *et al.*, 2009; Kolahdoozan *et al.*, 2010; Movahedi *et al.*, 2012; Taghavi *et al.*, 2012; Veisy *et al.*, 2015) and the rate of death was increased from 3.933 to 4.92 per 100,000 cases between 2006 and 2010 (Enayatrads *et al.*, 2015). Despite improvements in surgery and the use of adjuvant therapy, it continues to be fatal in many patients around the world. Furthermore, various side effects and chemoresistance are two important problems.

Therefore, there is a growing interest in using naturally occurring compounds in both chemopreventive and chemotherapeutic studies of breast cancer. There are evidences that medicinal herbs have anticancer properties, which are mediated through different mechanisms (Bathaie and Tamaioi, 2014), including alteration in the carcinogen metabolism, induction of DNA repair systems, immune activation, induction of apoptosis, and suppression of cell cycle progression.

Cyclins control the orderly progression of cells through the cell cycle by determining the timing of activation and the substrate specificity of a series of cyclin-dependent kinases (Cdks), which are sequentially activated during specific phases of the cell cycle. Three D-type cyclins (cyclin D1–3) act at mid-G1 by complexing with either Cdk4 or Cdk6 (Bui *et al.*, 1995; Peters *et al.*, 1995; Sherr, 1995). Among them, cyclin D1 overexpression was observed in many malignancies, including 40–90% of invasive breast cancers (Roy and Thompson, 2006).

The cyclin-dependent kinase inhibitors (CDKIs) identified in mammalian cells are classified into two major categories. The INK4 family includes p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}, which mainly inhibit Cdk4 and Cdk6 by binding to the Cdk subunit itself. The Cip/Kip family includes p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, which inhibit a broader

¹Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

²Department of Basic Sciences, Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran.

³Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

range of Cdks by binding to several cyclin/Cdk complexes. All of these CDKs cause G1 arrest when overexpressed in transfected cells. Because the CDKs are potent negative regulators of the cell cycle, it has been suggested that they may also function as tumor suppressor genes (Sgambato *et al.*, 1997; Nakayama and Nakayama, 1998; Roussel, 1999; Denicourt and Dowdy, 2004).

p53 as a tumor suppressor and cell regulator is also essential for preventing inappropriate cell proliferation and maintaining genome integrity that may occur following genotoxic stresses (Vousden and Lu, 2002). It can induce cell cycle arrest in the G1, G2, and S phases of the cell cycle. Thus, p53 provides additional time for the cell to repair genomic damage before entering the critical stages of DNA synthesis and mitosis. p21^{waf1/Cip1}, which is a CDKI, has been known as a downstream target of p53. Following a DNA damage, p21^{waf1/Cip1} (wild-type p53-activated fragment 1/Cdk-interacting protein 1) is the primary mediator of p53-dependent G1 cell cycle arrest (el-Deiry *et al.*, 1993; Xiong *et al.*, 1993). The expressions of p21^{waf1/Cip1} and p53 were evaluated in breast cancer patients. p21 was expressed in 49% and p53 in 32% of the patients. Inverse expression of these two proteins was seen in 73% cases. The percentage of Waf1-positive and p53-negative patients was more than Waf1-negative and p53-positive patients (Wakasugi *et al.*, 1997). However, overexpression of both p53 and p21 was reported in both NMU-induced (Ashrafi *et al.*, 2012) and 7,12-dimethylbenz[a]anthracene (DMBA)-induced (Crist *et al.*, 1996) breast cancer in rats.

Crocin as a water soluble carotenoid obtained from saffron showed various medicinal properties, including antioxidant (Papandreou *et al.*, 2006; Bathaie *et al.*, 2011), anti-inflammatory (Nam *et al.*, 2010), hypoglycemic, and antilipidemic activities (Shirali *et al.*, 2013), in addition to its anticancer effect (Escribano *et al.*, 1996; Hoshyar *et al.*, 2013). The biological properties of saffron and its components were recently reviewed by us (Bathaie and Mousavi, 2010; Bolhasani *et al.*, 2014; Bathaie *et al.*, 2014b). Previous studies have shown that crocin is safe and no toxic effect has been reported for its therapeutic usage (Bathaie and Mousavi, 2010). In addition, our recent study indicates its nontoxic effect, even in humans (Mousavi *et al.*, 2015).

Although the exact mechanism of action of saffron components is still not known, different studies indicated varieties in the molecular mechanism of the anticancer effect of saffron carotenoids. For example, induction of apoptosis in cancer cells (Hoshyar *et al.*, 2013; Bathaie *et al.*, 2013, 2014a; He *et al.*, 2014), telomerase inhibition (Noureini and Wink, 2012), interaction with DNA sequences and induction of conformational changes (Bathaie *et al.*, 2007; Hoshyar *et al.*, 2012), and decrease of histone-DNA complex formation (Ashrafi *et al.*, 2005) have been reported. Because of the importance of the cell cycle regulators in breast cancer and the observed effect of NMU on both p53 and p21, in the present study, the effect of crocin on cell cycle regulators such as cyclin D1, p53, p21^{Cip1}, and p27^{Kip1} was investigated.

Materials and Methods

Crocin separation and purification

Crocin was extracted and purified from the dried stigmas of *Crocus sativus* L. using the method previously described by us (Bolhasani *et al.*, 2005).

Animals and treatment

Forty 35-day-old female Wistar Albino rats were purchased from Pastor Institute, Tehran, Iran. All rats were housed under a controlled environment with a 12-h-light/12-h-dark cycle and temperature of 23°C ± 2°C, given a commercial diet with tap water *ad libitum*, and weighed weekly. The experimental protocol was approved by the Animal Ethics Committee in accordance with the Guidelines for the Care and Use of Laboratory Animals prepared by the Tarbiat Modares University. After a 2-week acclimatization period, rats were divided into two groups: A group of rats (#26) received three intraperitoneal injections of NMU (Sigma), dissolved freshly in 0.9% NaCl adjusted to pH 4.0 with acetic acid, at a dose of 50 mg/kg body weight, and at 50, 65, and 80 days of age (Ashrafi *et al.*, 2012). The remaining rats, representing the control groups, received the vehicle only.

Tumor number, size, and volume were determined weekly by palpation, after 4 weeks of NMU administration up to the end of the experiment. Tumor volume was calculated according to the $V = (4/3)\pi R_1^2 R_2$ (radius $R_1 < R_2$) formula (Fendl and Zimmiski, 1992). When tumor size reached between 10 and 15 mm in the largest dimension, each of the NMU-treated group and control group of rats were divided into two groups; one group received crocin and the other received the vehicle only. The groups were named as follows: NMU-treated control group (T1, $n=7$), NMU-treated group receiving crocin (T2, $n=7$), control group with no treatment (C1, $n=7$), and a control group receiving crocin (C2, $n=7$). The rats without tumors were excluded from the study. The crocin-treated groups (T2 and C2) received four doses of 200 mg/kg body weight of crocin, dissolved in the serum physiologic, by intraperitoneal injection at 7-day intervals. Groups T1 and C1 were intraperitoneally injected with the serum physiologic.

Tissue sample preparation

The rats were sacrificed under anesthesia 7 days after the last injection of crocin. All mammary tumors and normal mammary glands of all the groups were dissected and fixed as follows: half of the tumors were fixed in 10% formalin, embedded in paraffin wax, and then stained with hematoxylin and eosin (H&E) for histological study, and the other half of the tumors were immediately frozen in liquid nitrogen and stored at -70°C for reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis.

Reverse transcription-polymerase chain reaction

Total RNA from excised rat mammary glands and tumors was isolated using the TRIZOL extraction reagent (Invitrogen) according to the manufacturer's recommendations. The integrity of mRNA was confirmed by electrophoresis in a 1% agarose gel and Thermo Scientific Nanodrop 2000C spectrophotometer. The cDNA was synthesized by using a Revert Aid™ H Minus first strand cDNA synthesis kit (Fermentas, Inc.) as described in the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out by amplification of genes together with the reference gene

TABLE 1. EXPERIMENTAL CONDITIONS FOR POLYMERASE CHAIN REACTION

<i>Gene</i>	<i>Primer sequence</i>	<i>Product size</i>	<i>Annealing temperature (°C)</i>
Cyclin D1	F: 5'-CAGACCAGCCTAACAGATTTC-3' R: 5'-TGACCCACAGCAGAAGAAG-3'	208	56
p21	F: 5'-CTGGATGCTAGAGGTCTGC-3' R: 5'-AGAGTTGTCAGTGTAGATGC-3'	105	58
GAPDH	F: 5'-CAAGGTCATCCATGACAACCTTTG-3' R: 5'-GTCCACCACCCTGTTGCTGTAG-3' F: 5'-AACGACCCCTTCATTGAC-3' R: 5'-TCCACGACATACTCAGCAC-3' F: 5'-AACGACCCCTTCATTGAC-3' R: 5'-TCCACGACATACTCAGCAC-3'	500, 190, 160	58, 60
P53	F: 5'-GTGGCCTCTGTCATCTTCCG-3' R: 5'-CCGTCACCATCAGAGCAACG-3'	290	60
P27	F: GAGGGCAGATACGAGTGGCAG R: CTGGACACTGCTCCGCTAACC	238	60

The primer sequence, annealing temperature, and expected size of PCR products.
GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.

(GADPH) using an equivalent cDNA template, PCR master mix (Fermentas, Inc.), and specific primers, in a MJ Mini™ Personal Thermal Cycler (Bio-Rad). PCR conditions for cyclin D1 amplification were as follows: 30 cycles of 95°C for 30 s, 56°C annealing for 45 s, and 72°C extension for 45 s; for p21, the conditions were as follows: 30 cycles of 95°C for 30 s, 58°C annealing for 45 s, and 72°C extension for 45 s; for p53 and p27, the conditions were as follows: 30 cycles of 95°C for 30 s, 60°C annealing for 45 s, and 72°C extension for 45 s. The primer sequences, product sizes, and annealing temperatures are given in Table 1. The primer sequences of p53, p27, and GAPDH were taken from the literature and the cDNA synthesis kit (Kohen *et al.*, 2003; Takamura *et al.*, 2003; Hsu *et al.*, 2006). Cyclin D1 and p21 primers were designed using Oligo 6, Genrunner, and AlleleID 07 software. Then, reaction products were separated on 2% agarose gel and visualized by ethidium bromide staining. The bands were quantified by densitometric analysis through image capturing system software. The relative target mRNA expression level was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample.

Western blot analysis

For Western blotting, frozen tumor and normal mammary gland tissues (about 100 mg) were homogenized in a lysis buffer (containing 150 mM NaCl, 50 mM EDTA, 1 mM NaF, 10 mM Na₂P₂O₇, 0.1% sodium dodecyl sulfate [SDS], 100 mM Tris-HCl, 1% glycerol, and 1% Triton X-100), containing a cocktail of protease and phosphatase inhibitors (Sigma). Equivalent amounts of protein were applied to 12% SDS-polyacrylamide gels, separated by electrophoresis, and electrotransferred to 0.45-μm-pore-size polyvinylidene difluoride membranes (Roche). Membranes were immersed in a blocking solution (5% nonfat dry milk, 0.05% Tween 20 in phosphate-buffered saline [PBS]) and were incubated overnight at 4°C. Primary incubation of the membranes was carried out, respectively, by using 1:100 and 1:200 dilution of mouse monoclonal anti-p21 and anticyclin D1 antibodies (#450 and #271610;

Santa Cruz Biotechnology, Inc.) for 2 h at room temperature in 3% nonfat dry milk. After washing with PBS-0.05% Tween 20, filters were incubated for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibody (1:7000 dilution, goat anti-mouse, #2005; Santa Cruz Biotechnology, Inc.). Protein bands were visualized using an ECL advance Western blotting detection kit (Amersham; GE Healthcare) after washing. An equal loading of proteins was assessed with a mouse monoclonal anti-β-actin antibody (#81178; Santa Cruz Biotechnology, Inc.).

Statistical analysis

Data are shown as mean ± standard deviation. For gene expression, differences between the data obtained in all groups, and tumor volume analysis, one-way ANOVA were used. All comparisons with *p*-values below 0.05 were considered significant.

Results

Treatment response and pathology of mammary tumors

Breast tumors were observed only in the NMU-treated rats (T1 and T2). Before crocin treatment, the mean volumes of tumors were about 13.27 ± 3.77 and 12.37 ± 1.88 in the T1 and T2 groups, respectively. At the end of the experiment, they were 23.66 ± 8.82 and 11.91 ± 2.27 in these groups (T1 and T2, respectively). As demonstrated in Figure 1, administration of crocin significantly suppressed tumor growth in the NMU-treated rats.

The H&E stained sections of the samples were analyzed by a specialist in the field of histopathology. As shown in Figure 2, the majority (about 91.9%) of tumors from the NMU-treated groups were malignant adenocarcinoma of different types, such as papillary and comedo carcinoma. About 8.1% were benign epithelial neoplasms such as lactating adenoma (adenomas with milk-like substance in the lumen) and papillary adenoma.

FIG. 1. Tumor volume in T1 and T2 groups before and after treatment. Analysis was performed by one-way ANOVA analysis. Values are presented as mean $\pm$ SD, and the same letters indicate the significance of the data that compares with another.

Expression of cyclin D1, p21^{Cip1}, p27^{Kip1}, and p53

The expression of cyclin D1, p21^{Cip1}, p27^{Kip1}, and p53 was examined in mammary tissues (normal and tumors) of all rats in both mRNA and protein levels. There was no alteration in the expression of these genes in the normal mammary glands, groups C1 and C2. (The Western blot data are not shown.) As shown in Figure 3A–C, a significant decrease in the mRNA and protein levels of cyclin D1 was observed in the tumors of group T2 in comparison with T1. A marked decrease in the mRNA and protein level of p21^{Cip1} and a nonsignificant decrease in the mRNA levels of p27^{Kip1} and p53 were observed in the tumors of group T2 in comparison with T1 (Figs. 4A–C, 5A, B, and 6A, B).

Discussion

The results of the present study indicate the anticancer effect of crocin in NMU-induced breast cancer in female rat. Before and after crocin treatment, the volumes of tumors were determined in the T1 and T2 groups. The results showed that the rate of tumor growth was twice greater in the NMU-injected group with no other treatment than the similar group receiving crocin; it means that crocin significantly suppressed the growth of tumors. Similar results were reported by us in our previous study (Chitsazan *et al.*, 2006). In addition, pathologic analysis showed both comedo and papillary types of adenocarcinoma with areas of necrosis in NMU-induced breast tumors (T1), however in the crocin-treated group only papillary-type tumors have been observed. Since the growth of comedo-type tumor is faster than the papillary type, this result is completely consistent with the observed changes in the tumor volume. These results are in accordance with the reported data on the therapeutic effect of crocin on colon adenocarcinoma in rats (Garcia-Olmo *et al.*, 1999), bladder cancer in mice (Zhao *et al.*, 2008), in leukemia HL-60 cells both *in vitro* and xenografted in mice (Sun *et al.*, 2013), and in both rat model of gastric cancer and human AGS cells (Hoshyar *et al.*, 2013).

FIG. 2. Histopathology images of tumors. (A) Comedo-type tumor in T1 group with areas of necrosis (shown by the arrow); this area is indicating the presence of debris from dead (necrotic) cancer cells. (B) Papillary-type tumor in T2 group, without necrotic area. H&E staining of mammary tumors shows invasive intraductal carcinoma in NMU-induced tumors. H&E, hematoxylin and eosin; NMU, N-nitroso-N-methylurea.

We also showed that crocin induced apoptosis in AGS cells and MNNG-induced gastric cancer in rats. Crocin increased the Bax/Bcl-2 ratio and the activity of caspases in AGS cells, in addition to the increased sub-G1 population of cells (Hoshyar *et al.*, 2013). In addition, the induction of apoptosis and activation of caspases have been reported in MCF-7 cells after treatment of cells with saffron extract (Mousavi *et al.*, 2009). The present study was also investigating the effect of crocin on cell cycle regulators in NMU-induced breast cancer.

As shown in Figure 3, the results of both RT-PCR and Western blot analysis indicate that cyclin D1 expression was increased in the NMU-treated group. The cyclin D family comprises key proteins involved in facilitating the entry of cells into the cell cycle and progression through S phase. The overexpression of cyclin D1 and activation of Cdks in G1 phase may be the key factors for shortening the G1 phase, increasing the cell proliferation rate and oncogenesis

FIG. 3. The results of cyclin D1 expression. (A) RT-PCR and (B) Western blot results of rat tumors with or without treatment by crocin in comparison with the normal mammary tissue. (C) Densitometric analysis of cyclin D1 expression. Values are presented as mean $\pm$ SD, and the same letters indicate the significance of the data that compares with another. M, DNA marker; C1 and C2, control groups with or without crocin treatment, respectively; T1 and T2, NMU-injected groups with or without crocin treatment, respectively; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation.

(Hinz *et al.*, 1999). An immunohistochemical study in 64 breast cancer patients demonstrated that, the cyclin D1 gene is amplified in $\sim 24\%$ of mammary carcinomas and its protein is overexpressed in more than 50% of cases (Barbareschi *et al.*, 1997).

As indicated in the results, crocin treatment significantly suppressed cyclin D1 overexpression in both mRNA and protein levels. Thus, in addition to the induction of apoptosis and activation of caspases that were shown in previous studies, it is the third mechanism of crocin anticancer effect. These results are in accordance with the reported data on the effect of crocin on downregulation of cyclin D1 expression

in bladder cancer (TCCB) T24 cell line and *in vivo*, in BALB/c xenograft tumor (Zhao *et al.*, 2008).

Based on the RT-PCR and Western blot results presented here, crocin significantly suppressed the p21^{Cip1} overexpression due to the NMU injection and cancer induction in breast tissue. As mentioned above, p21 regulates several cell cycle proteins, including cyclin D1, inhibits Cdk, and induces cell cycle arrest (Xiong *et al.*, 1993; Archer *et al.*, 1995; Wakasugi *et al.*, 1997). Although p21 has been known as the inhibitor of CDKs, the coexpression of cyclin D1 and p21 protein is required for the initial steps of tumor development. The overexpression of cyclin D1 and p21 are often

FIG. 4. The results of p21^{Cip1} expression. (A) RT-PCR and (B) Western blot results of the rat tumors with or without crocin treatment in comparison with the normal mammary gland. (C) Densitometric analysis of p21^{Cip1} expression. Values are expressed as mean $\pm$ SD, and the same letters indicate the significance of the data that compares with another.

FIG. 5. The results of p27 expression. (A) RT-PCR and (B) densitometric analysis of P27 expression. Values are expressed as mean $\pm$ SD.

reported in human cancers and is correlated with a high tumor grade and poor prognosis (Dai *et al.*, 2013).

p53 as a tumor suppressor is known to play a key role in practically all types of human cancers, especially breast cancer (Oren and Rotter, 1999). Our results also indicated its overexpression in NMU-induced breast cancer in Wistar Albino rat that is similar to that reported in Sprague Dawley rat (Crist *et al.*, 1996). It has also shown that in this type of cancer induction, there is no mutation in the p53 gene (Kito *et al.*, 1996). On the other hand, p21 has been known as a critical downstream mediator of wild-type p53 (Dotto, 2000); thus, overexpression of p21^{Cip1} after NMU administration and its downregulation due to crocin treatment may occur through the pathways dependent on p53.

The anticancer effect of crocin on three human colorectal cancer cells (HCT-116, SW-480, and HT-29) has been reported. The represented data indicated a significantly higher sensitivity of HCT-116 to crocin than the other two cell lines. HCT-116 cells contain wild-type p53, while the p53 tumor suppressor gene is mutated in SW-480 and HT-29 cells. Therefore, it was concluded that the stronger anticancer activity of crocin on HCT-116 cells is related to the wild-type p53 (Aung *et al.*, 2007). These results are affirmative reasons for the above-mentioned explanation of the relation between wild-type p53 and p21 and the effect of crocin.

Increased expression of cyclin D1 and p21^{Cip1} in association with decreased expression of p27^{Kip1} has been re-

ported in DMBA-induced mammary tumors in rats (Jang *et al.*, 2000). p27^{Kip1} has been known as another cell cycle regulator that inhibits G1 progression through Cdk2 inhibition. In most cancers, mitogen stimulates the elimination of p27^{Kip1} by decreased translation and increased ubiquitin-directed degradation (Alessandrini *et al.*, 1997; Vlach *et al.*, 1997), a cause for lower concentration of this cell cycle inhibitor in tumor tissues. Our results also indicated a significant decrease in p27 mRNA expression in some tumors; however, there were no changes in some others. Thus, the average mRNA expression of p27 in T1 was not significant in comparison with the average in C1. In addition, a non-significant reduction in p27 mRNA expression in T2, in comparison with T1, was also observed (Fig. 5). These nonsignificant changes are possible due to the limitations in our experiment that we could not investigate by Western blot. As mentioned above, the expression of p27^{Kip1} is mainly controlled by the rate of translation and degradation rather than by changes in the transcriptional activities (Slingerland and Pagano, 2000). The subject should be investigated in the future.

In conclusion, crocin, a carotenoid and a main metabolite of saffron, induces growth inhibition (decreased TV) in the NMU-induced breast cancer in rats through at least two different mechanisms; not only by inducing apoptosis but also by inhibiting cell cycle progression through down-regulation of cyclin D1 and p21^{Cip1} expression in a p53-dependent manner.

FIG. 6. The results of p53 expression. (A) RT-PCR and (B) densitometric analysis of P53 expression. Values are expressed as mean $\pm$ SD.

Acknowledgments

The authors are thankful to the Research Council of Tarbiat Modares University for supporting this project. We especially thank the Avicenna Research Institute for their assistance to do some experiments.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
S. Zahra Bathaie, MSc, PhD
Department of Clinical Biochemistry
Faculty of Medical Sciences
Tarbiat Modares University
Tehran 14115-111
Iran

E-mail: bathai_z@modares.ac.ir

Received for publication May 26, 2015; received in revised form June 28, 2015; accepted July 14, 2015.