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Analysing the Radioprotective Effect of *Cotoneaster Nummularia* in Mouse Bone Marrow Cells Using Micronucleus Assay

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Abstract

Study of the different aspects of protection against the exposure of ionizing radiation has always been an active area of research. High cost and toxicity of radioprotective drugs have limited their use. So, search for new drugs with a high degree of protection and lower cost and side effects seem a necessity. In this study radioprotective effect of aqueous as well as alcoholic extracts of the Mann of *Cotoneaster nummularia*(Shirkhesht), regarding their high accessibility and possibly low side effects, against 2 Gy Gamma irradiation, was analyzed using micronucleus assay on bone marrow cells of male mice (Balb/c). Different doses of 250, 500, 1000 mg/kg/BW for aqueous and 3750, 7500, 15000 mg/kg/BW for alcoholic extract of Shirkhesht were administered IP for five constitutive days prior to 2 Gy gamma irradiation. The result compared with the known radioprotective effect of vitamin E after the same treatment schedule. High frequency of micronucleus was observed in non treated gamma-exposed mice, which represented the clastogenic effect of irradiation. Vitamin E, aqueous and alcoholic extracts of Shirkhesht treated mice represented a 5.56, 3.32 and 2.1 times decrease in the gamma-induced micronucleus frequency respectively. The data suggest a radioprotective effect of shirhkesht compared to vitamin E.

Keywords: Gamma irradiation, vitamin E, *Cotoneaster nummularia*, micronucleus, radioprotection

Introduction

Tensional or intentional irradiation in radiotherapy of cancers or from natural and industrial sources greatly damages the human tissues. The great efforts have been conducted to reduce the dangerous effects of ionizing irradiation to human body (Copeland, 1991; Durakovic, 1993; Narra and Harapanhalli, 1994).

The biological effects of ionizing radiation in living organisms are the result of long chain of reactions. The main part of the energy of radiation is absorbed by water, which comprises 70-80% of animal body. This produces significant amount of free radicals (Iyer and Lehnert, 2000).

Although one third of the damages caused by irradiation are the direct effects of ionizing radiation on DNA, many biological effects of ionizing radiation are believed to be the result of interaction of free radicals with DNA or other cellular macromolecules (Goodhead, 1994; Iyer and Lehnert 2000). Damages to DNA could be reduced by treatment of animals with sulphurous compounds, antioxidant vitamins, and plant natural products (Bonorden and Pariza, 1994; Hosseinimehr et al., 2003; Lee et al., 2005).

Antioxidant vitamins can protect the tissues from oxidative damages caused by irradiation. Although their detailed mechanism(s) yet to be explained, it is believed that they can significantly protect DNA and chromosomes of animal cells by their ability of scavenging the free radicals produced by ionizing irradiation (Narra and Harapanhalli, 1994; Konopacka and Wolny, 2001; Songthaveesin et al., 2004).

Seeking new radioprotective agents that conform to all criteria of an optimal radioprotectant is a very active line of research. An ideal radioprotective agent must provide different aspects for clinical applications, including effectiveness, low toxicity, availability, specificity and tolerance (Durakovic, 1993).

Recently, the herbal products which have been used in traditional medicine have become an attractive alternative as radioprotective agents. In several studies the radioprotective ability of these products have been investigated and many of those shown to be effective in protecting the cells from harmful effects of ionizing irradiation (Hosseinimehr et al., 2003; Lee et al., 2005;

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Rouhanizadeh and Khalkhali, 1971).

Other studies already have clarified the usefulness and reliability of *in vivo* micronucleuos assay, introduced by Schmid and Vol Ledebur (1973), in toxicological studies for assessing the structural and numerical chromosomal damages. Here chromosomal abnormalities, including structural damages and chromosomal loss are seen as tiny nucleus in cytoplasm, called Micronucleus (Mn) (Heddle and Salamone, 1981; Heddle and Hayashi, 1991). Analysing the radiation induced chromosomal aberrations has also been widely studied using this method (Gocke, 1996; Abramsson-Zetterberg et al., 1999).

The same method was followed in this study for assaying the radioprotective activity of alcoholic and aqueous extract of Shirkhesht.

Material and methods

Animals

The male Balb/c mice of 3-4 weeks old were kept for one week in animal house under a standard 12 h light: 12 h dark cycle and controlled temperature ($20 \pm 2\ ^{\circ}C$) conditions to adapt. They were divided in control and treated groups of 5 mice in each group. Throughout the experiment all the handling, keeping situation and working with animals were closely monitored by the local Society of Animal Rights of Iran, a member of the International Society of Animal Rights.

Treatment

Vitamin E was diluted with sesame oil to get the final treatment doses of 0.00, 100 and 200 mg/kg/BW (Konopacka et al., 1998).

The Mann of Cotoneaster nummularia (Shirkhesht) was purchased from local Herbal Medicine shop and its authenticity was approved by the experts and macerated. The alcoholic and aqueous components were extracted by 80% ethanol and deionised water respectively. After solvent evaporation, the remained powders were diluted with deionised water.

After determining the Lethal $Dose_{50}$ (LD50) of those extracts, the doses of 250, 500, 1000 mg/kg/BW and 3750, 7500 and 15000 mg/kg/BW of alcoholic and aqueous extracts were administered intraperitonealy for five constitutive days respectively. For vitamin E different doses of 50, 100, and 200 mg/kg/BW were also administered intraperitonealy for five constitutive days. The total volume of injection never exceeded 0.4 ml.

Irradiation

Whole body irradiation was performed one hour post-last treatment using Cobalt-60 in Mashhad Omid Hospital. Mice were irradiated in group of 5 with 2 Gy of gamma radiation (dose rate of 1 Gy/ Min).

Micronucleous assay

The assay was carried out according to Schmid and Vol Ledebur (1973). Briefly, the mice were sacrificed by cervical dislocation 24 hours post-irradiation. The bone marrow of both femurs was collected into a centrifuge tube using 5 ml of FCS. After centrifugation in 1000 rpm for 10 min, the supernatant was discarded and a drop of cell suspension was smeared on clean slides. The slides were stained by May Grundwald/Giemsa. The slides were coded to be scored blindly by the viewer. On each slide at least 1000 polychromatic erythrocytes (PCEs) were scored and the number of cells bearing micronucleus (MnPCE) was counted, the frequency of Normochromatic erythrocyte (NCE) also was calculated.

Statistical analysis

The data were analysed statistically and presented in tables and figures as Mean ± SEM. Interdifferences in each group were analysed by one-way variation analysis using Duncan software.

Results

To optimize the best time for obtaining the highest gamma-induced micronucleus frequency, a preliminary experiment was designed with un-treated mice. In this experiment the mice were exposed to 2 Gy gamma irradiation and harvesting took place after 24 and 48 hours. The Gamma irradiation statistically increased the frequency of MnPCEs ($P<0.01$) (Figure1). The induced-frequency of MnPCEs was decreased to half at the 48 hours post-irradiation. On the basis of this data the time course of 24 hours post-irradiation was selected as a time of harvesting the bone marrow cells throughout the experiment.

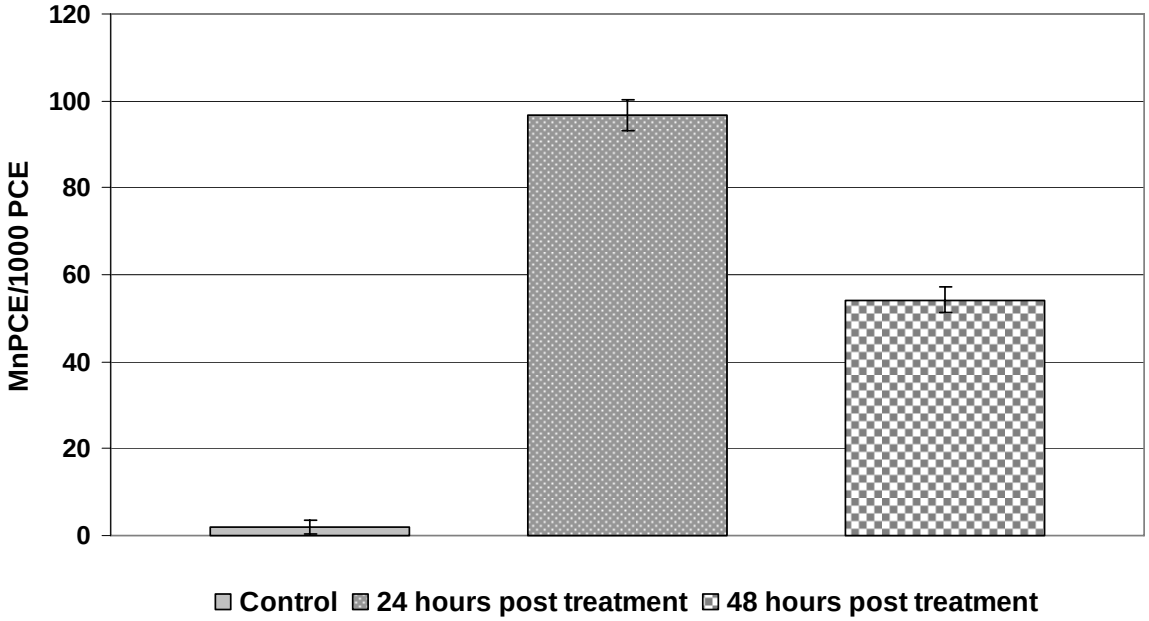

Figure 1. MnPCE induced by 2 Gy Gamma irradiation at different times post treatment

Effect of vitamin E on the frequency of Gamma-induced MnPCEs

The administration of different doses of vitamin E for five days before irradiation was resulted in dose-dependent decrease in the frequency of MnPCEs (P< 0.01) (Figure 2). Of all doses applied, 200 mg/kg/BW had the most effective result in reducing the MnPCEs frequency.

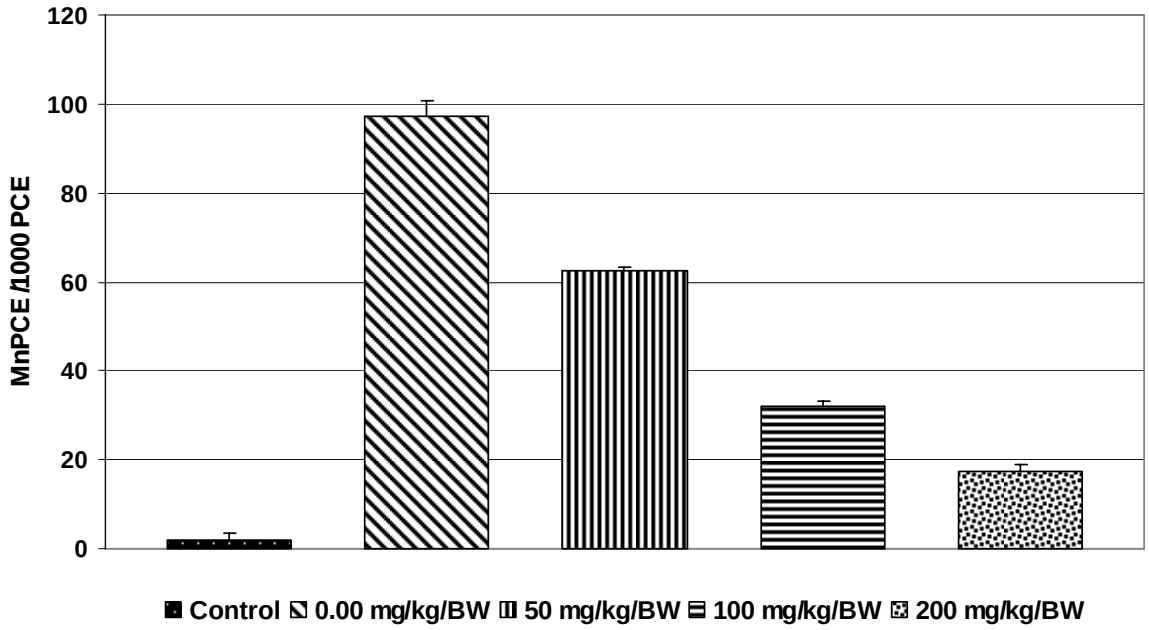

Figure 2. Effect of vitamin E on the frequency of Gamma-induced MnPCE

Effect of Aqueous extract of Shirkhesht on radiation induced-MnPCEs

The LD_{50} of aqueous extract of Shirkhesht was determined to be 2890 mg/kg/BW. Mice were injected with doses of 250, 500, and 1000 mg/kg/BW *ip* for five days. Aqueous extract of shirkhesht, in all doses, did statistically reduce the gamma-induced frequency of MnPCEs(P< 0.01) (Table 3). Despite statistical differences in the frequency of MnPCEs between 250 and 500 mg/kg/BW (P< 0.01), no statistical differences were observed between 500 and 1000 mg/kg/BW.

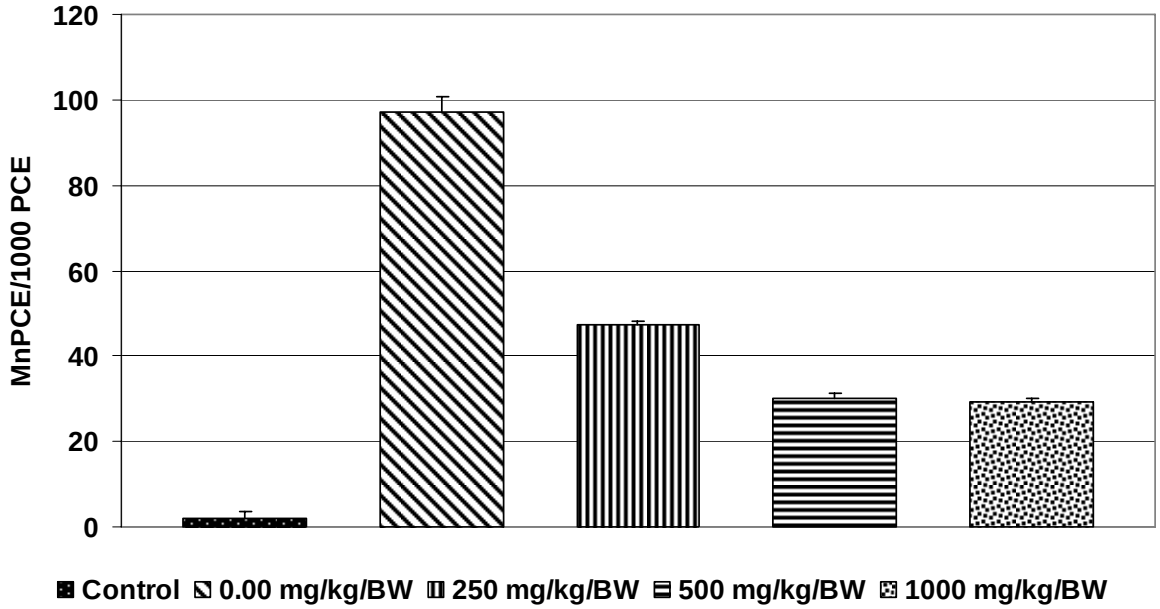

Figure 3. Effect of aqueous extract of Shirkhesht on the frequency of Gamma-induced MnPCE

Effect of Alcoholic extract of Shirkhesht on radiation induced-MnPCEs

The LD_{50} of alcoholic extract of shirkhesht was determined to be 17500 mg/kg/BW. On the basis of this, doses of 3750, 7500 and 15000 mg/kg/BW of alcoholic extract of Shirkhesht were selected and introduced *ip* to mice for five days. Alcoholic extract of shirkhesht caused a significant dose-dependent decrease in frequency of gamma-induced MnPCEs in all doses used($P< 0.01$)(Figure 4).

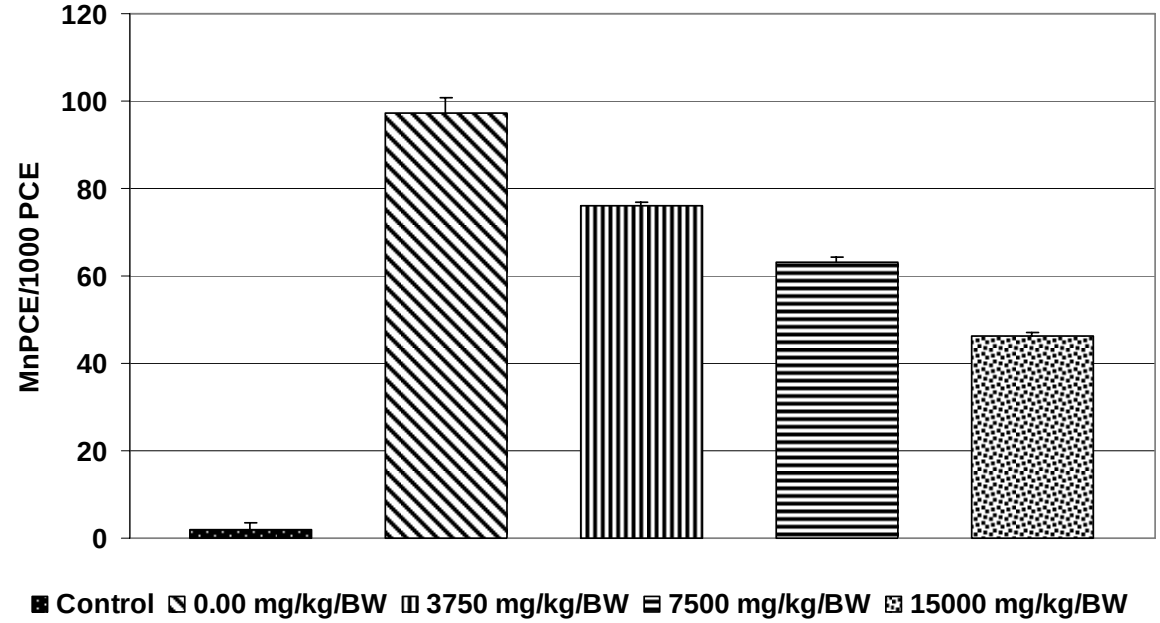

Figure 4: Effect of alcoholic extract of Shirkhesht on Gamma-induced MnPCE

In all experiments the frequency of PCE to PCE+NCE of gamma-irradiated mice was significantly lower than control (Table 1). Such reduction represented the toxicity of irradiation in all treated and untreated mice.

Table 1. Frequency of PCE/NCE[a]+PCE in all treatment which represents the toxicity of Gamma irradiation

	Treatment with Vitamin E	Treatment with aqueous extract of Shirkhesht	Treatment with alcoholic extract of Shirkhesht
Control	53.35 ± 0.95	53.35 ± 0.95	53.35 ± 0.95
0.00	48.57* ± 1.43	48.57* ± 1.43	48.57* ± 1.43
Treatment 1	50.86* ± 0.88	50.09* ± 0.88	48.45* ± 1.43
Treatment 2	48.12* ± 1.32	49.42* ± 1.32	49.86* ± 1.25
Treatment 3	49.13* ± 0.89	49.23* ± 0.89	50.52* ± 0.58

[a] Normochromatic eryhtrocyte
* Statistical differences(P<0.01)

Discussion

Ionizing radiation causes clastogenic chromosomal abnormalities in living cells (Goodhead, 1994; Iyer and Lehnert, 2000; Pouget and Mather, 2001). Gamma-irradiation of water molecules leads to production of different active elements such as Radical of hydroxyl (OH°), Hydrated electron [$e^{-}_{(aq)}$] and Radical of Hydrogen (H°). The main elements produced are the last two. It is believed that OH° is the most destructive to DNA (Goodhead, 1994; Konopacka et al., 1998). The hydroxyl radical scavengers are able to compete with DNA in interaction with free radicals (Copeland, 1991). Through their ability of scavenging the OH°, vitamins have the most important role in DNA protection against ionizing irradiation (Narra and Harapanhalli, 1994; Odin, 1997).

In search for new source of radioprotective agent, the extracts of Shirkhesht were subjected to analysis. For this reason the *in vivo* Micronucleus assay was performed. Application of this method has also been widely reported in analysis of radioprotective ability of different agents (Hosseinimehr et al., 2003; Heddle and Hayashi, 1991).

Irradiation of mice in this study led to an extreme increase in the frequency of MnPCEs, compared to the control. The increase of the Micronucleus frequency could be explained by the clastogeneic ability of gamma-irradiation which in turn leads to structural chromosomal abnormality. The radiation-induced micronucleus frequency has been reported by others (Hayashi et al., 1994; Konopacka et al., 1998; Konopacka and Wolny, 2001; Hosseinimehr et al., 2003). The control frequency of micronucleus is at the same level of other studies, although the gamma irradiation-induced micronucleus frequency after 24 h is higher than the frequency report elsewhere (Hosseinimehr et al., 2003; Tiku et al., 2004). This difference could be the result of the higher dose of gamma-irradiation used in this study.

Time-dependent decrease in Gamma-induced micronucleus frequency was observed here. In similar studies also such decline in the Mn frequency has been reported (Matsuoka et al., 1993; Mac Gregor, 2000). The time-dependent decrease of Mn in anucleated polychromatic erythrocytes could be the end points of different pathways, degradation of micronucleus by cytoplasmic nucleases (Zu Granetto et al., 1996) or maturation and then releasing of the MnPCE into the blood stream.

The aim of the experiment was to study the effect of Shirkhesht extract on induced micronucleus frequency, so 24 hours post irradiation, which was exhibited the highest frequency of Mn, was selected for harvesting the bone marrow cells. Toxicological studies also suggest the same time course for harvesting the bone marrow in *in vivo* micronucleus assay (Hayashi et al., 1994; Abramsson-Zetterberg et al., 1996).

In this study, Five days tresatment with vitamin E decreases the gamma-induced MnPCE frequency. Administration of vitamin E by either oral or through intragastric gavage for 4 days could significantly elevate the level of vitamin E concentration in bone marrow of mice (Umegaki et al., 1994). Radioprotective effect of vitamin E in *in vivo* and *in vitro* studies has been documented in several studies. Due to its free radical scavenging ability and its capability in protecting DNA from direct effect of H2O2, the decrease in chromosomal damages induced by ionizing irradiation could be well explained (Konopacka *et al.* 1998, Mutlu–Turkoglu et al., 2000; Konopacka and Wolny, 2001; Claycombe and Meydani, 2001).

According to the results represented in Figure 3 and 4, administration of the aqueous as well as alcoholic extract of Shirkhesht has led to a significant decrease in the gamma-induced MnPCE frequency. The data suggest the radioprotective capability of extracts of Shirkhesht.

There is not enough information about the radioprotective ability of Shirkhesht. It is a natural

product that could be easily absorbed through mouth. It has been proposed that its radioprotective ability was because of its inhibiting effect on enzymes which were activated after irradiation and could break DNA (Rouhanizadeh and Khalkhali, 1971). Although so far there is not enough evidence to support this.

Extract of Shirkhesht contains 40-60% Manitol (Zargary, 1999). The free radical and activated oxygen scavenging ability of manitol has been documented in several studies (Peak and Peak, 1990; Tsou et al., 1996; Tsou et al., 1999; Bektasoglu et al., 2006). On the basis of those studies it is possible to explain the radioprotective capability of Shirkhesht and resulted reduction of MnPCE frequency through free radical scavenging of manitol present in its extracts. In mice exposed to gamma irradiation there was a competition between DNA and manitol to interact with free radicals which led to lower DNA damages and MnPCE frequency.

The medicinal properties of Shirkhesht as a natural product used in traditional medicine has been widely studied. Results of this experiment suggest that the Mann of Cotoneaster *nummularia* (Shirkhesht) could be an effective replacement for chemical radioprotective agents to reduce the damages to DNA of persons exposed to ionizing radiation because of their occupations or treatment protocols. Also once more this study reveals the usefulness of micronucleus assay in study of chromosomal damages induced by ionizing irradiation.

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