Ferdowsi University of Mashhad
ISSN 2008-9147
Numbers: 2

# JCMR

## Journal of Cell and Molecular Research

Volume 1, Number 2, Winter 2009

Issuance License No. 124/902-27.05.2008 from Ministry of Culture and Islamic Guidance

# Journal of Cell and Molecular Research (JCMR)

**Volume 1, Number 2, Winter 2009**





This Journal is indexed in the Islamic World Science Citation Center

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# Comparison of *pepck* gene expression in developing seeds and leaves of chickpea (*Cicer arietinum* L.) plant

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Received 28 August 2009 Accepted 19 September 2009

## Abstract

Phosphoenolpyruvate Carboxykinase, encoded by the *pepck* gene, plays an important role in gluconeogenesis. It also seems to be important in metabolism of nitrogenous compounds in developing seeds of legumes, including amides and ureides which are then transformed into amino acids, necessary for the synthesis of storage proteins. In this research, *pepck* gene expression in mRNA level, in different genotypes of chickpea (*Cicer arietinum* L.), was determined. Two low protein genotypes (MCC291 and MCC373) and two high protein genotypes (MCC458 and MCC053) out of 20 chickpea genotypes were selected. Total RNA were extracted through different stages of seed development, and the expression of the *pepck* gene was estimated by semi-quantitative RT-PCR. The results of the RT-PCR showed that two isoforms of this gene are expressed in high protein genotypes, whereas in the low protein genotypes, the expression of these isoforms was not obvious. Also this method showed a differential expression of *pepck* gene in different stages of flowering and seed development. *pepck* gene is expressed in higher levels during the sheet formation and developing seeds compared to the flowering and seed formation stages. Probably, the differential expression of *pepck* gene is related to its possible role in metabolism of seed components, particularly in determination of the protein content of chickpea seeds.

**Keywords**: chickpea, nitrogen metabolism, phosphoenolpyruvate Carboxykinase, seed development

## Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes in Western Asia, Northern Africa, Southern Europe, Central America and Southern Australia. This crop holds the third place among legumes as of the global cultivation acreage. Its grain is reach in carbohydrates (48.2-67.6%), protein (12.4-31.5%), starch (41-50%), fatty acids (6%), B-group vitamins, and nutritional elements (Icrisat, 2005); and is recommended to be consumed as an amino acids (esp. lysine) supplement to cereals.

Studies on the genes contributing to the seed filling in chickpea and its protein content might lead to engineer plants with seeds of a higher nutritional value. A gene of concern is phosphoenolpyruvate carboxykinase (*pepck*), encoding a protein with a substantial role in the gluconeogenesis pathway. It catalyses the conversion of oxaloacetate to phosphenolpyruvate (PEP) which will be further converted to sugar (Owusu-Apenten et al., 2002; Walker et al., 2003; Malone et al., 2007; McClements, 2007). An increase in PEPCK activity results in reduced organic acids and increased sugar content (Leegood et al., 1999). A probable role of PEPCK thus has also been recently reported in metabolism of nitrogen and nitrogenous compounds and their conversion into proteins in the grain of some legumes (Aivalakis et al., 2004). This gene is involved in growth, seed filling and amino acid content (esp. that of asparagine) of pea (*Pisum sativum*) seeds. The seed coat is recognized as the tissue reach in nitrogen transporter enzymes and invertases which contribute to amino acid and carbohydrate metabolism. The relationship between PEPCK and metabolism of amino acids and amides shows that it is a relatively sensitive enzyme to the presence of nitrogenous compounds in seed coat and cotyledons, with its content being affected by

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nitrate, ammonium and asparagine in seed coats, but only by asparagine in cotyledons of pea (ICRISAT, 2005).

Increased PEPCK content is combined with elevated levels of the enzymes involved in amino acid metabolism, such as mitochondrial asparagine amino-transferase, cytosolic alanine amino-transferase and acetyl-CoA carboxylase; thus contributing to metabolism, absorption and transmission of the nitrogenous compounds (Ruffner et al., 1975).

No studies on the expression and role of this gene and its possible relation to protein content has been reported in chickpea yet. In the present paper, the protein content (percentage) is measured in a number of cultivated chickpea genotypes, followed by comparison of the expression levels of *pepck* gene at different stages of seed filling in some of the genotypes. This study aims at revealing the relation between PEPCK transcript level with protein content of chickpea seeds which might, in the longer term, end at protein quality improvement of the crop through the gene manipulation procedures.

## Materials and Methods

### *Plant material and raw protein measurement*

The plant material included 20 genotypes of cultivated chickpea (table1), kindly provided by the seed bank of the plant science institute. The protein content was measured by Kjeldahl method (Roylet et al., 2003).

### *Cultivation of plant material and RNA extraction*

A number of intact seeds of four chickpea genotypes, having the farthest high/low protein content (MCC373 and MCC291 as lowest and MCC458 and MCC053 as highest) were selected and sterilized with 75% ethanol for 30 seconds. The seeds were then placed between two layers of the sterilized moist cloth to germinate in the laboratory. The germinated seeds were cultured in 12×12×12 cm pots containing equal amounts of clay, leaf compost and sand, at a depth of 3-4 cm, and maintained in the glasshouse for 3 months under photoperiod of 14 hrs light at 25±2 ºC and 10 hrs darkness at 15±2 ºC, until the genesis seed stage.

The *pepck* gene expression level was assessed using semiquantitative RT-PCR method. Total RNA was extracted from the mature seeds of the low and high-protein genotypes (referred to as L and H, respectively) by the guanidinum thiosianate protocol (Chomaczynski et al., 1987). The quality and quantity of the extracted RNA were assessed by agarose gel electrophoresis and spectrophotometry, and the loading volumes of the samples were adjusted accordingly.

### *Gene expression assessment at the mRNA level by RT-PCR*

The control primer pair, including a forward 19-mer oligonucleotide of 5'-TTTGTGAAAACTCTCACCG-3' and a reverse 18-mer of 5' GTCTGAGCACCAAATGGA-3', were designed based on the mRNA sequence available for ubiquitin protein as registered in the NCBI database. PEPCK primers were a 18-mer forward of 5'-GAAATCGGCACCTTCTAC-3' and a 19-mer reverse of 5'-CCTCATCCCTAACAACACG-3', designed based on the conserved regions identified in the cDNA sequences of PEPCK enzyme in alfalfa (*Medicago truncatula*)[1], tomato (*Solanum lycopersicum*)[2] and flaveria (*Flaveria trinervia*)[3].

The primers were intended to amplify a 208 bp fragment of the ubiquitin cDNA sequence, and fragments of 398 and 700 bp of the *pepck* cDNA and gDNA, respectively. All primers were designed using the Primer Premier (version 5) software.

cDNAs were constructed according to the standard recommendations (Fermentase) including; 0.4 µg of the template RNA together with 1 µl of the reverse primer (10 pmol/ml) and 8 µl of double distilled water incubating at 70ºC (in the thermocycler) for 5 minutes for denaturation; and immediate cooling on ice. The rest of the reaction was performed according to the standard protocols, 2µl of the first standard cDNA of each samples was subjected to ordinary PCR. The resulting cDNAs were stored at -20ºC.

The thermal scheme for PCR was an initial denaturation at 94ºC for 3 minutes, followed by 35 cycles of 94ºC, 52ºC and 72ºC for 1 minute each, and a final 6-minutes extension at 72ºC. The PCR products were loaded on 1.2% agarose gels. The sizes of the bands were estimated according to 100 bp DNA size marker. For equal loading of the first standard cDNA in RT-PCR reaction, the concentration must be adjusted according to an internal control gene, such as ubiquitin.continusely for equal loading of the samples, several PCR reactions were performed on serially diluted aliquots of the cDNA by using ubiquitin primers. In order to make a quantification of the results, gel photographs were analyzed by Lab Works software package which calculated a value for each amplified band (given in table 2) in form of a ratio

1 NCBI code AF212109.1
2 NCBI code AF327432.1
3 NCBI code AB050473.1

to the 400 bp band of the loaded commercial size marker (which was in turn given a value of 100 as the reference). The samples were then scaled by diluting the more concentrated cDNA.

## Results

### *Protein measurement*

The statistical results of protein measurements are presented in table 1 A remarkable variation in protein percentage is evident among the genotypes, with MCC458 and MCC053 having the highest (mean = 30.5) and MCC291 and MCC373 having the lowest (mean = 21.1) amount of protein. These were chosen as the extreme genotypes for further molecular analysis.

**Table 1.** The statistical results of protein content of different chickpea cultivars.

| Genotype | Seed Protein (percent)[1] | weight of hundred seeds (gr) | type |
|---|---|---|---|
| **MCC 067** | 23.50 | 33.2 | Kabuli |
| **MCC 099** | 25.85 | 9.8 | Desi |
| **MCC 165** | 25.24 | 16.8 | Kabuli |
| **MCC 291** | 21.13 | 19.0 | Dsei |
| **MCC327** | 25.66 | 21.4 | Kabuli |
| **MCC 333** | 24.72 | 30.8 | Kabuli |
| **MCC 476** | 23.12 | 28.2 | Kabuli |
| **MCC 495** | 26.12 | 27.2 | Kabuli |
| **MCC 510** | 24.68 | 32.0 | Kabuli |
| **MCC 053** | 30.41 | 33.2 | Kabuli |
| **MCC 202** | 23.60 | 15.8 | Kabuli |
| **MCC 258** | 25.90 | 31.4 | Kabuli |
| **MCC 332** | 28.46 | 15.0 | Desi |
| **MCC 426** | 28.31 | 33.2 | Kabuli |
| **MCC 458** | 30.57 | 25.4 | Kabuli |
| **MCC 477** | 25.42 | 27.4 | Kabuli |
| **MCC 496** | 24.80 | 25.6 | Kabuli |
| **MCC 498** | 26.30 | 23.6 | Kabuli |
| **MCC 207** | 27.40 | 17.6 | Desi |
| **MCC 373** | 20.92 | 17.4 | Desi |

### *RT-PCR results*

The total RNA extraction was successful, judged by two distinct bands of 26S and 18S rRNAs on agarose gel. The two selected high-protein genotypes showed two bands with sizes of 400 bp and 500 bp; whereas the two low-protein ones showed no detectable bands (figure 1). In order to exclude the possibility of DNA contamination of the extracted RNA samples, the total RNA was also incorporated as the negative control in each RT-PCR reaction (figure 1). PCR amplification of the extracted DNA resulted in a 700 bp band which is missing in the RT-PCR results. It could therefore be suggested that there must be intron/introns causing this deviation from the expected size of 400 bp. On the other hand, this is a confirmation of relatively gDNA free RNA extraction. RNA from alfalfa was used as the positive control to ensure the results throughout the experiments, for which a 400 bp band in the RT-PCR and a 2000 bp band in the gDNA amplification were detected (figure 2 and 3).


**Figure 1.** mRNA level of *pepck* gene in chickpea seeds in four different genotypes. Lanes 1-4 correspond to MCC 458, MCC053, MCC373, MCC291 RT-PCR results, respectively. Lanes 5 and 6 are negative controls of RNA and water, respectively. Lane 7 is size marker.

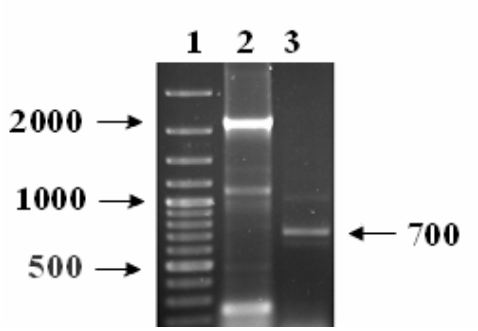


**Figure 2.** Optimization of polymerase chain reaction with pepck specific primers. Lanes 1, 2 and 3 are DNA 100bp Plus size marker, alfalfa gDNA amplified band, and chickpea gDNA amplified band, respectively.

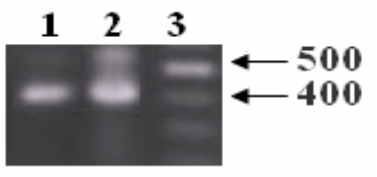


**Figure 3.** Optimization of polymerase chain reaction with pepck specific primers. Lanes 1, 2 and 3 are alfalfa cDNA amplified band (as positive control), chickpea cDNA amplified band and DNA 100bp Plus size marker respectively.

***Comparative analysis of pepck expression throughDifferent stages of chickpea growth and development***

The accumulation of *pepck* (as well as ubiquitin) encoding mRNAs were compared at 6 growth stages of 4 chickpea genotypes. As shown in fig 4, all samples were adjusted to reach equal ubiquitin bands upon PCR. This was also supported by comparison of the ribosomal RNA bands and equal volumes of each RNA were introduced into the RT-PCR reaction using the specific *pepck* primers. The RNA calibration based on the ubiquitin control is shown in figure 4.

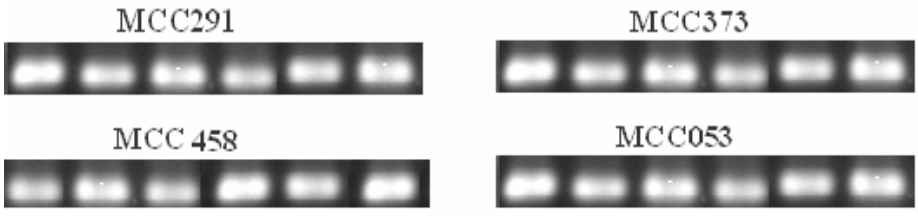


**Figure 4.** Scaling the total RNA concentration in four genotypes (MCC458,MCC053, MCC373, MCC291) of chickpea based on the ubiquitin amplified bands. Lanes 1-6 are loaded correspond to bearing few leaves, flower bud, flowering, sheet formation, seed formation, and full seed maturation, respectively.

The expected band was not amplified in any growth stage of MCC291 and MCC373 genotypes. For MCC458 and MCC053, however the differences in the intensity of the amplified bands are visually evident among different stages of growth and development, and especially in seed genesis stage (figure 5).

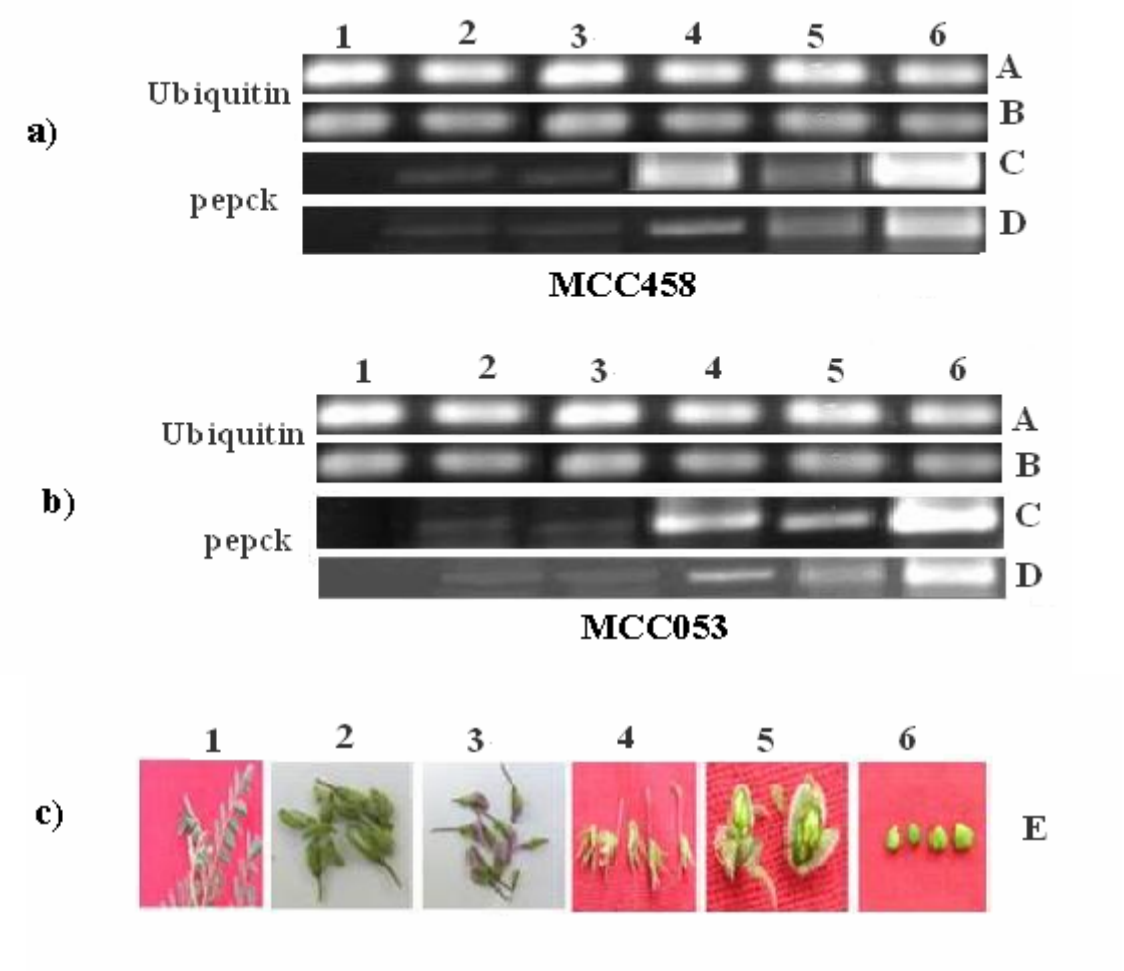


**Figure 5.** Comparative measurement of *pepck* mRNA levels at 6 growth stages of MCC458 (a) and MCC053 (b) chickpea genotypes, based on RT-PCR experiments: (A) calibration of ubiquitin amplified band after 25 cycles of PCR, (B) calibration of ubiquitin amplified band after 20 cycles of PCR, (C) amplified cDNA band related to *pepck* gene from each sample at the same conditions after 35 cycles of PCR, (D) amplified cDNA band related to *pepck* gene from each sample at the same conditions after 30 cycles of PCR, (E) developing stages of the examined samples including: 1) a few leaved 2) flower bud, 3) flowering, 4) sheet formation, 5) seed formation, 6) fully mature seeds (c).

**Table 2.** Quantification of the RT-PCR gel images for *pepck* (amplified at 35 PCR cycles) in MCC458 and MCC053 chickpea genotypes, using Lab Works software. Values are represented as percentage of the reference sample (size marker) which is given a value of 100.

| Genotype | Size marker | Flower bud | flower | Sheath formation | Seed formation | Seed development |
|---|---|---|---|---|---|---|
| **MCC 053** | 100 | 65.30 | 78.08 | 531.52 | 332.76 | 1288.40 |
| **MCC 458** | 100 | 59.07 | 65.13 | 498.57 | 284.36 | 985.50 |

## Discussion

Studies on mature seeds of pea and alfalfa has revealed involvement of the *pepck* gene in nitrogen storage, grain filling and amino acid enrichment, and thus metabolism of storage proteins during seed development (Aivalakis et al., 2004; Delgado et al., 2007). Similarly, our results suggest that in mature seeds of chickpea, the expression of this gene is related to the metabolism of nitrogenous compounds and increasement of seed protein content. It has also been shown that the Arabidopsis genome contains two related genes named *pck1* and *pck2* with the former being expressed at a higher level (Malone et al., 2007). It therefore appears that the two 400 and 500 bp bands, amplified in our studies might be counterparts of the *pck1* and *pck2*; and the higher amount of the 400 bp RT-PCR product could suggest that, in chickpea the same isoform could be of more involvement in chickpea development.

As shown here, *pepck* is not expressed at younger stage (a few – leaved), and experiences the lowest levels of expression at flowering and seed formation and the highest levels at bundle formation and full seed maturation stages. Table 2 also shows a higher expression level of *pepck* at seed filling stage. These results together with the fact that high protein plants are also high *pepck* ones, might add more weight on the notion that *pepck* is a key determinant of protein content in seeds.

The *pepck* gene has been proven to be controlled spatially and temporally (in different tissues and at different growth stages) in other plants such as tomato, pea, alfalfa, cucumber, grape and arabidopsis (Ruffner et al.,1975; Ruffner,1982; Bahrami et al., 2001; Walker et al., 2001; Roylet et al., 2003; Aivalakis et al., 2004; Delgado et al., 2007; Malone et al., 2007). Western blots, mRNA-level analyses, and PEPCK-activity measurement have revealed the highest expression level of the gene to happen in the pericarp of the ripe fruits and the lowest in stems, roots, and germinating seeds (Ruffner et al.,1975; Bahrami et al., 2001). Several studies have also proven that elevated levels of the PEPCK enzyme through pea development coincides with higher absorption and transmition of the nitrogenous compounds, and consequently deposition of the storage proteins in the cotyledon (Delgado et al., 2007).

In addition, studies on alfalfa have revealed a differential expression pattern of this gene through seed development; with the highest level of expression at the torpedo embryo stage and the lowest level at bending cotyldons and mature embryo (Aivalakis et al., 2004). Here we similarly observed very obvious differences in the expression level among the stages of seed development in both genotypes (MCC458 and MCC053). This data, together with observation of the highest expression at the full seed maturation stage, empowers the conclusion that elevated expression of this gene contributes to increments in seed protein content.

As mentioned earlier, arabidopsis genome contains two genes namely *pck1* and *pck2* with different expression patterns. *pck1* expression is more abundant at pre-budding stage, roots, flowers and mature leaves; whereas *pck2* is expressed only in roots and flowers (Roylet et al., 2003; Malone et al., 2007). As results shown, the 500 bp PCR product seems more intense at the flowering and seed development stages, while at the same time the 400 bp product shows a much less intensity. It appears, in orchestra with the previously published reports, of existing two isoforms for PEPCK with different pattern of gene expression, as we also suggest for them to happen in chickpea. Meanwhile the results should be verified by immunohistochemical assays, Real Time-PCR and Western blot techniques in different tissues of the plant.

## Aknowledgement:

This study was supported by Institute of Biotechnology, Ferdowsi University of Mashhad.

## References

1- Aivalakis G., Dimou M., Flemetakis E., Plati F. and Katinakis P. (2004) Immunolocalization of carbonic anhydrase and phosphenolpyruvate carboxylase in developing seeds of *Medicago sativa*. Plant Physiology and Biochemistry 42.181-186.

2- Bahrami A. R., Chen Z., Walker R. P., Leegood R. C., and Gray J. E. (2001) Ripening-related occurrence of phosphoenolpyruvate carboxykinase in tomato fruit. Plant. Molecular Biology 47: 499-506.

3- Bajaj Y. P. S. (1990) Legumes and oilseed crops I. In: Biotechnology in Agriculture and Forestry 10: 3-37.

4- Carnel N. M., Agostino A. and Hatch, M. D. (1993) Photosynthesis in phosphoenolpyruvate carboxykinase – type C4 plants: mechanism and regulation of C4 acid decarboxylation in bundle sheath cells. Archives of Biochemistry and Biophysics 306: 360-367.

5- Chomaczynski P. and Sacchi N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanat-phenole chloroform extraction. Annual. Biochemistry 162:156-159.

6- Delgado A., Walker R. P., and Leegood R.C. (2007) Phosphoenolpyrovate carboxykinase in developing pea seeds is associated with tissues involved in solute transport and is nitrogen-responsive. Plant Cell and Environment 30: 225-235.

7- FAOSTAT (2007) Report. http: // faostat. fao. org/faostat/servelet.

8- Chickpea (2005) http://www.icrist.org/ chickpea/ htm.

9- Owusu-Apenten R. K., Marcel D., (2002) Quantitative effects on processing. In Food Protein Analysis, New York, 463 pp.

10- Leegood R. C. and Walker R. P. (1999) Phosphoenolpyruvate carboxykinase: its role and regulation. In Plant Carbohydrate Biochemistry (Eds. J. A. Bryant. M. M. Burrell and N. J. Kruger). 201-213. Bios Sientific Publishers, Oxford.

11- Malone S., Chen Z. H., Bahrami A. R., Walker R. P., Gray J. E. and Leegood R. C. (2007) Phosphoenolpyruvate carboxykinase in *Arabidopsis thaliana*: changes in gene expretion, protein and activity during vegetative and reproductive development. Plant Cell Physiology 48: 441-450.

12- McClements D. J. (2007) Kjeldahl method. In: Analysis of Proteins. University of Massachusetts, 442 pp.

13- Roylet E. L., Gilday, A. D. and Graham, I. A. (2003) The gluconeogenic enzyme phosphoenolpyruvate

carboxykinase in *Arabidopsis thaliana* is essensial for seedling establishment. Plant Physiology 131: 1834-1842.

14- Ruffner H. P. (1982) Metabolism of tartaric and malic acids in *Vitis*. a review - Part A. Vitis 21: 247-259.

15- Ruffner H. P. and kliewer W. M. (1975) Phosphoenolpyruvate carboxykinas activity in grap berries. Plant Physiology 56: 67-71.

16- Walker R. P. (2001) Using immunohistochemistry to study plant metabolism: the example of its use in localization of amino acids in plant tissues, and of phosphorylation of phosphoenolpyruvate carboxykinase and its possible role regulation. Jornal of Experimental Botany 52: 565-567.

17- Walker, R. P., Chen, Z. H., Acheson, R. M., Leegood, R. C. (2003) Effects of phosphorylation on phosphoenolpyruvate carboxykinase from the C4 plant, *Guinea grass*. Plant Physiology 128: 165–172.

*Journal of Cell and Molecular Research (2009) 1 (2), 68-71*

# Application of RAPD (Random Amplified Polymorphic DNA) marker for sex determination of *Pistacia vera* L.

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Received 22 August 2009 Accepted 19 September 2009

## Abstract

Sex determination in Pistacia as a dioecious plant is economically desirable. Identification of the male and female plants has a great value for Pistacia yield production. In the present study, leaf samples from four female plants from cultivars of Pistacia including Akbary, Akbar Aghaei, Fandoghi, and Kalleh Ghochi as well as male plants were used for RAPD PCR amplification. Among 32 primers with 10 mer two primers (FPKI106 and FPK105) were able to discriminate between female and male plants. Few bands were detected in the DNA pattern of male while they were absent in the female individuals. The PCR conditions were reproducible and can be recommended for sex determination of *Pistacia vera* cultivars

**Keywords:** *Pistacia vera*, RAPD-PCR, sex determination

## Introduction

Within the last decade, technological advancements have increasingly supported the use of genetics in determining population diversity of plants. Many molecular techniques are now available, which allow ecologists and other biologists to determine the genetic structure of a wide variety of closely related individuals. DNA markers that are known to be genetically linked to a trait of interest can be used for gene cloning, medical diagnostics, sex determination, and for trait introgression in plant and animal breeding programs (Lynch and Milligan, 1994).

Several different methods including isozyme analyses, restriction fragment length polymorphisms (RFLP), and random amplified polymorphic DNA (RAPD) are used for documenting genetic information. Although isozyme analysis and RFLP are a source of readily obtainable genetic information which is easily reproduced, they often do not show polymorphisms which are necessary to determine variation within a group of genetically similar individuals (Mulcahy, et al., 1993, Williams et al., 1990)

Because RAPD is a relatively straight-forward technique to apply, and the number of loci that can be examined is unlimited, RAPD analysis is viewed as having a number of advantages over RFLP's and other techniques (Lynch and Milligan, 1994), for example, no preliminary work such as probe isolation, filter preparation, or nucleotide sequencing is necessary (Williams et al.,1990). In many instances, only a small number of primers are necessary to identify polymorphism within species (Williams et al., 1990). Indeed, as Mulcahy et al., (1995) report, a single primer may often be sufficient to distinguish all of the sampled varieties. Williams et al. (1990).

The genus Pistacia, in the family Anacardiaceae, contains at least 12 tree and shrub species. P. vera L. (2n=32) the only edible nuts, is cultivated widely in the Mediterranean regions of Europe, Middle East and California. Pistacia breeding programs have been initiated to develop new cultivars (Irish and Nelson, 1989). Dioecy represents an inconvenience to pistachio breeding since the reproductive maturity of pistachio seedlings takes between 5 and 8 years. Currently there is no method for distinguishing between male and female pistachio seedlings prior to flowering. A method to determine the gender of plants before flowering would facilitate breeding and selection by enabling screening for gender at the seedling stage, thereby simplifying the breeding of male and female plants for different objectives, with a saving in time and economic advantages (Bawa, 1980). The presence of sex chromosomes has been claimed for other dioecious angiosperms, but in only a few cases has that claim been documented (Westergaard, 1958; Lewis and John, 1968; Parker and Clark, 1991). Examples include the genera Humulus, Rumex and, perhaps, Cannabis, although the presence of heterochromosomes in the is controversial

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(Westergaard, 1958; Durand and Durand, 1990).

Well documented examples include species of the genera Asparagus, Vitis, Spinacia and Mercurialis. In most of the species examined in details, the study of sex determination is complicated by the presence of additional alleles or factors that can modify the effects of the major sex determining genes (Durand and Durand, 1990). Pistacia vera shows perfect dioecy; mature female flowers have no trace of stamens and mature male flowers lack any evidence of female structures (Wannan and Quinn, 1991). This clear differentiation of sexual phenotype, combined with its perennial nature, an increasing economic importance of the crop and recent interest in breeding improved cultivars, makes the P. vera attractive for the study of different aspects of sex determination.

## Materials and Methods

### *Plant material and DNA extraction*

At least three fresh leaf segments from 7 years old individual female Pistacia plants cultivars Kalleh Ghochi (K), Akbari (A), Akbar Aghaei (AA), Fandoghii (F), and male plants were harvested from Pistacia field in Ardestan in Isfahan and kept in the freezer.

DNA was extracted according to the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987) with some modifications. Young leaf tissue (100 mg) was ground to fine powder in liquid nitrogen in 1.5 ml centrifuge tubes and mixed with 0.5 ml of CTAB extraction buffer (100 mM TRIS-HC1, 1.4 M NaC1, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% mercaptoethanol, 0.1% NaHSO3). The sample was incubated at 65°C for 1 h, mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 13000 rpm for 5 min in a desktop centrifuge. The aqueous phase was recovered and mixed with equal volume of isopropanol to precipitate the DNA. The nucleic acid pellet washed with 1 ml 10 mM ammonium acetate in 76% ethanol, dried overnight and re-suspended in 100 µl modified TE buffer (10 mM Tris-HCI, 0. 1 mM EDTA). DNA was extracted separately from each individual plant. In all cases extracted DNA (25 ng per 20 µ l reaction mix) was subjected to polymerase chain reaction (PCR) amplification. (All chemicals were supplied from Cinagene Company, Iran)

PCR Amplification: RAPD analysis was performed with 32 decamer primers and amplification reactions were carried out in an Eppendorf Master cycler gradient thermocycler (Eppendorf Netheler-Hinz, Hamburg, Germany). Amplification conditions used were 1 cycle at 94°C for 5 min followed by 39 cycles at 94°C for 30 sec, 30°C for 30 sec and 72°C for 1 min. RAPD reactions (Williams et al., 1990), were carried out in a volume of 20 µ l with 25 ng of genomic DNA, 2 µ M primer, 1 U of *Taq* polymerase, 0.2 mM dNTPs, and 2 mM $MgCl_2$ all from Cinagene Company, Iran. After electrophoresis of PCR products in 0.8% agarose (Cinagene) at 80 V, the gels were stained with 0.5 µ g/ ml ethidium bromide solution and visualized by illumination under UV light.

## Results

After screening 32 arbitrary 10-mer Operon primers, we identified FPKI106 and FPK105 primers as being able to differentiate sex type, when tested on four Pistacia male and female plants. These primers generated few bands in male samples which were absent in female plants (Figurs 1 and 2). These markers were reproducible under a broad range of amplification conditions (for example different $MgCl_2$ concentration and different annealing temperature), without any variation in the results. Additional experiments were applied for 4 weeks old seedlings of pistacia cultivars for sex determination. We found the same patterns of DNA bands after PCR amplification using FPKI016 and FPK105 primers for female and male seedlings similar to 7 years old plants.

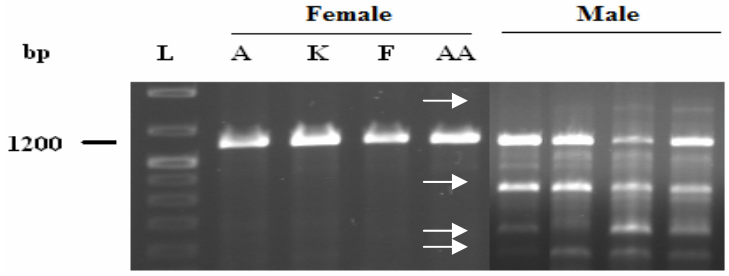


**Figure 1:** Identification of male and female individual plants of Pistacia cultivars by FPKI016 primer. L: DNA ladder, A: Akbary, K: Kalehghochi, F: Fandoghi, AA: Akbar Aghaii

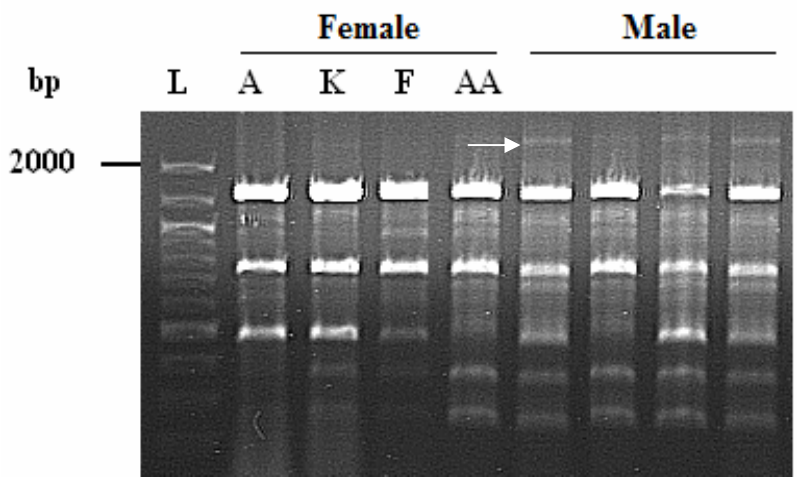


**Figure 2**: Identification of male and female individual plants of Pistacia cultivars by FPK105 primer. L: DNA ladder, A: Akbary, K: Kalehghochi, F: Fandoghi, AA: Akbar Aghaii

## Discussion

Efforts to identify dioecious plants sex type in an early stage of development is important for selecting female and male pistacia plants before transfer to the field in order to save time and reduce costs. To date, several molecular markers for sex type discrimination in dioecious plants, including Pistacia (Hormaza et al., 1994) and papaya (Urasaki et al., 2002) have been reported. In the present study, from the reported primer (OP008) by Hormaza et al., (1994), we tried to discriminate sex type in four Pistacia cultivars, but could not distinguish between male and female plants under several PCR amplification conditions (data not shown). Based on these unsatisfactory results we concluded that new markers are required to distinguish sex type of desirable Pistacia cultivars. In order to find molecular differences between male and female plants, different attempt such as immunochemistry, differences in proteins and enzymes, comparison of mRNA and tRNA populations or RNA hybridization kinetics, have been made in several dioecious species (Durand and Durand, 1990). These studies deal with gene expression and could just show a differential expression of common genes between males and females. A better approach to understand how sexual determination operates in dioecious species is to study the differences at the DNA level. The discovery of markers linked to sex determining genes could eventually allow us to clone the gene(s) involved in this process. Although promising results have been obtained with *Asparagus* (Jamsar et al., 2004) using RFLPs (Bracale et al., 1991), the RAPD technique appears to offer better prospects for rapid progress. Its advantages relative to RFLPs include technical simplicity, lower cost per data unit, small amount of DNA required and the higher level of polymorphism obtained with RAPDs (Waugh and Powell, 1992; Williams et al., 1993). Among 32 primers used in this study only 2 primers could reveal sex differentiation in pistacia cultivars. It has been proposed that the basic scheme of sex determination in animals involves a key gene that activates a cascade of regulatory genes (Truong et al., 1991). In plants, the system could be similar. If this is the case, of several to many genes could be involved in the differentiation of male and female flowers in dioecious plants, but sex determination could be controlled by a single locus acting as a trigger. In such a scenario, genes having the genetic information for carpels or stamens development would be present in both male and female plants with one major gene being the only difference between the two sexes (Irish and Nelson, 1989). Durand and Durand (1990) reported that a single major gene controls sex determination in some plant genera (e.g. *Asparagus* and *Vitis),* although there are exceptions like *Mercurialis* where the system is more complex with three genes involved in sex determination. Pistachio may have a similar system with a single major gene controlling sex determination Here we propose that, we may have identified a marker which possibly which is closely linked to a sex-determining chromosome segment in *P. vera* and this marker can be used to screen sex of pistacia seedlings well before they attain reproductive stage.

## Acknowledgment

Authors thanks the Research Council of the University of Isfahan for their support of research project No. 860411.

## References

1- Bawa K. S. (1980) Evolution of dioecy in flowering plants. Annual Review of Ecology and Systematic 11:15-39.

2- Bracale M., Caporali E., Galli M. G., Longo C., Marziani-Longo G., Rossi G., Spada A., Soave C., Falavigna A., Rafaldi F., Maestri E., Restivo F. M. and Tassi F. (1991) Sex determination and differentiation in *Asparagus officinalis* L. Plant Science 80:67-77.

3- Doyle J. J., and Doyle J. L. (1987) A rapid isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin 19:11-15.

4- Durand R. and Durand B. (1990) Sexual determination and sexual differentiation. Critical Review of Plant Science 9:295-316.

5- Hormaza J. I., Dollo L. and Polito V. S. (1994) Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked sergeant analysis. Theoretical and Applied Genetic 89: 9-13.

6- Irish E. E. and Nelson T. (1989) Sex determination in monoecious and dioecious plants. Plant Cell 1: 737-744.

7- Jamsari A., Nitz I., Reamon S. M. and Jung C. (2004) BAC-derived diagnostic markers for sex determination in asparagus. Theoretical and Applied. Genetic 108: 1140–1146.

8- Lewis K. R., and John B. (1968) The chromosomal basis of sex determination. International Review of Cytology 23: 277-379.

9- Lynch M. and Milligan B. G. (1994) Analysis of population genetic structure within RAPD markers. Mol. Ecol. 3: 91-99.

10- Lynch M. and Milligan B. G. (1994) Analysis of population genetic structure within RAPD markers. Molecular Ecology 3: 91-99.

11- Mulcahy D. L., Cresti M., Linskens H. F., Intrieri C., Silverstoni, Vignani O. R. and Pancaldi M. (1995) DNA fingerprinting of Italian grape varieties: a test of reliability in RAPDs. Advance in Horticulture Science 9: 185-187.

12- Mulcahy D. L., Cresti M., Sansavini S., Douglas G. C., Linskens H. F., Bergamini G., Vignani R. and Pancaldi M. (1993) The use of random amplified polymorphic DNAs to fingerprint apple genotypes. Science Horticulture 54: 89-96.

13- Parker J. S. and Clark M. S. (1991) Dosage chromosome systems in plants. Plant Science 80:79-92.

14- Truong A., Heinderyckx T., Install M. and Negrutiu P. (1991) Sex determination and differentiation in organisms other than higher plants. Plant Science 80:201-211.

15- Urasaki N., Tokumoto M. Tarora K., Ban Y., Kayano T., Tanaka H., Oku H., Chinen I. and Terauchi R. (2002) A male and hermaphrodite specific RAPD marker for papaya (Carica papaya L.) Theoretical. Applied Genetic 104: 281–285.

16- Wannan B. S. and Quinn C. J. (1991) Floral structure and evolution in the Anacardiaceae. Botany. Journal of Linneh Society 107:349-385.

17- Waugh R. and Powell W. (1992) Using RAPD markers for crop improvement. Biotechnology 10:186-191

18- Westergaard M. (1958) The mechanism of sex determination in dioecious flowering plants. Advance Genetic 9:217-281.

19- Williams J., Anne G., Kubelik K., Kenneth R., Livak J., Antoni R., Scott J. and Tingey V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18(22): 6531-6535.

20- Williams J. G. K., Hanafey M. K., Rafalski J. A. and Tingey S. V. (1993) Genetic analysis using random amplified polymorphic DNA markers. Method in Enzymology 218:704-740.

*Journal of Cell and Molecular Research (2009) 1 (2),72-76*

# Purification of Lipid Transfer Protein 2 ($LTP_2$) from Iranian rice paddy

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Received 26 August 2009 Accepted 19 September 2009

## Abstract

Plant nonspecific lipid transfer proteins (nsLTPs) are divided into nsLTP1 and nsLTP2. The existence of an internal hydrophobic cavity, is a typical characteristic of nsLTPs that serves as the binding site for lipid substrates. In this communication a simple, rapid and low-cost alternative method was developed for purification of nsLTP2 from rice paddy. After extracting, final supernatant was loaded on CM-Sepharose column, which had previously equilibrated with 0.05 M Tris-HCl buffer, pH 8. Bounded proteins were separated using a linear gradient of 0-0.5 M NaCl. Solution of separated proteins was dialyzed and applied on a Phenyl-Sepharose column which previously equilibrated with Tris-HCl 0.05 M, ammonium sulfate 1.5 M, EDTA 0.005 M and $NaHSO_3$ 0.3%, pH 8.4. Tris-Tricin SDS-PAGE of separated proteins, obtained from ion-exchange column, showed multiple bands in the range of 2-20 kDa. Further purification using hydrophobic column resulted in single band of nsLTP2 at about 7 kDa, reflecting a purified sample in the gel.

**Keywords**: purification, plant lipid transfer proteins, cation-exchange chromatography, hydrophobic chromatography

## Introduction

Cellular membrane biogenesis depends on the synthesis and transport of lipids. In eukaryotic cells, lipids are synthesized in the endoplasmic reticulum and golgi apparatus and transported to the cellular and organellar membranes. The synthesis of lipids in one organelle and subsequent movement through the cytosol to the cellular membrane of another organelle, suggest that water soluble transport proteins assisted in lipid transportation. These proteins are Lipid Transfer Proteins, LTPs (Cheng et al., 2004). The binding of lipids to LTPs can be both specific and nonspecific. These proteins have a great concern in pharmaceutical applications. They are good candidates for designing efficient drug delivery systems (Pato et al., 2001, Wang et al., 2005). The nonspecific lipid transfer proteins have affinity toward a variety of hydrophobic molecules (Charvolin et al., 1999). Nonspecific LTPs (nsLTPs) are ubiquitous proteins found in bacteria, yeast, plants and animals. They are the major lipid-binding proteins in plants have been isolated from rice, wheat, barley, maize, peaches and apricots (Cheng et al., 2004; Liu et al., 2002). Plant nsLTPs are basic (with pI 8–10) and disulfide-rich proteins divided into two subfamilies; nsLTP1 (molecular weight ~9 kDa) and nsLTP2 (molecular weight ~7 kDa) (Kader, 1996; Lin et al., 2005). All nsLTPs share a common structural fold stabilized by four disulfide bonds. Different nsLTPs have been purified from various plants. Douliez and colleagues, used cation-exchange, size-exclusion chromatography and RP-HPLC methods for purification of nsLTP2 from wheat seeds (Douliez et al., 2001). Other researchers has purified nsLTP2 from rice seeds using Sephadex (C25), 15S cation-exchange chromatography and HPLC methods (Liu et al., 2002). CM-Cellulose chromatography has been used in addition to gel filtration (Sephadex G50) and FPLC for gaining purified nsLTP2 from rice seeds (Dharmaraj et al., 2002). Gel filtration and HPLC as efficient methods for protein purification, require special and expensive equipments, usually are not available in any laboratories and time consuming as well. To this end, access to cost-effective and simple purification method will be a convenient issue. In this study an attempt was conducted to purify nsLTP2 from rice paddy kernels by using cation-exchange and hydrophobic chromatography, in order to develop a simple, low-cost and rapid procedure. Beside the simplicity of the used method, it provides a good opportunity for LTP purification elsewhere.

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## Materials and Methods

### *Materials*

Rice kernels were purchased from Lenjan local of Isfahan. CM-Sepharose and G50 Sephadex resins were obtained from Pharmacia. Chemical reagents were of analytical grade and purchased from Merck.

### *Purification procedure*

Rice LTPs were isolated and purified according to slightly modified previous procedures (Yu et al., 1988; Lin et al., 2005). Rice kernels were ground in a blender and its flour (250 g) was suspended in 400 ml of 50 mM sulfuric acid. After it stood at room temperature for 4 h, the particulate matter was removed by centrifugation at 5000 rpm for 30 min and supernatant was passed through a piece of cheesecloth. The pH of solution was adjusted to 8.00 with concentrated NaOH and after standing at 4°C for 12 h, the produced precipitate was removed by centrifugation at 5000 rpm for 30 min. 100 ml of final supernatant was loaded onto a CM-Sepharose column (1.5×20 cm) which had previously been equilibrated with 0.05 M Tris-HCl buffer pH 8.00 with the flow rate of 12 ml/h. Bounded proteins were separated by a linear gradient of 0 to 0.5 M NaCl in the same buffer (50 ml in each reservoir).Protein was monitored by on-line measurements of the absorbance at 280 nm. Solution of separated proteins was concentrated by freeze drying and dialyzed against 0.05 M Tris-HCl pH 8.4. Ammonium sulfate, EDTA and sodium bisulfite in final concentration of 1.5, 0.005 M and 0.3% was added to dialyzed solution, respectively. Final solution was loaded onto a Phenyl-Sepharose column (1.5×10 cm) which previously equilibrated with 0.05 M Tris-HCl, 1.5 M ammonium sulfate, 0.005 M EDTA and 0.3% NaHSO3 pH 8.4, with the flow rate of 40 ml/h. Elution performed by a decreasing non-linear gradient of buffer in five steps at 1.2, 0.9, 0.6, 0.3 and 0 M of ammonium sulfate. The eluted fractions at 1.2 M ammonium sulfate were pooled, desalted and freeze dried.

### *Tris-Tricin sodium dodecyl sulfate Polyacrylamide gel electrophoresis*

It was performed according to a slightly modified method of Schagger and Von Jagow (Schagger and Von Jagow, 1987). SDS-PAGE was carried out using gel with 3% C, 16.5% acrylamide for resolving gel, 10% acrylamide for spacer gel and 4% acrylamide for stacking gel and samples were then loaded onto the 1.0-mm slab gel followed by electrophoresis with 100 V. Gels were fixed in methanol 50%, acetic acid 10% and proteins were visualized by Coomassie Brilliant Blue G250 staining. Mioglobin (16.95 kDa), Mioglobin I+II (14.4 kDa), Mioglobin I (8.16 kDa), Mioglobin II (6.2 kDa) and Mioglobin III (2.5 kDa) were used as molecular weight markers.

### *ANS Fluorescence measurements*

To confirm the presence of hydrophobic surface on nsLTP2, emission spectral changes of ANS was studied during binding to the protein. ANS fluorescence intensity (400μM) was recorded between 400-600 nm after excitation at 380 nm. Then, ANS was added to solutions containing nsLTP2 (4 and 4.2 μM, respectively) in 0.05 M Tris-HCl buffer, pH 8.4 and fluorescence intensity recorded as described above.

## Results

***LTP2 purification*** NsLTP2 obtained from rice kernels was purified using a combination of ion exchange and hydrophobic interaction chromatography. A CM-Sepharose column was initially used for separation of basic peptides following extracting. The basic fraction (figure 1, P2 peak) represents the minor protein fraction. The electrophoretic analysis of P2 proteins obtained from cation exchange chromatography showed the presence of two major sets of bands in Tris-Tricin SDS-PAGE. The first set is comprised of low molecular weight proteins with apparent molecular masses between 6 and 8 kDa while the second one shows band with molecular weight of 2.5 kDa (figure 2, lanes 7-10).

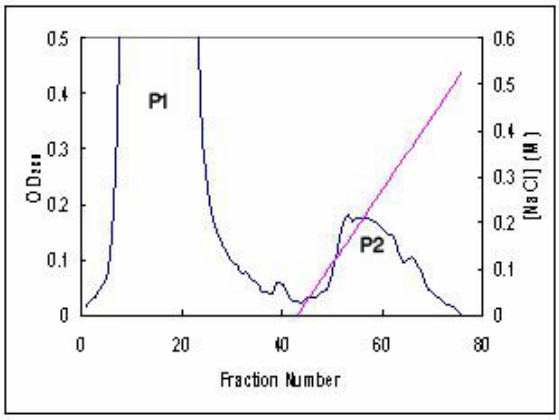


**Figure 1.** CM-Sepharose chromatography of extraction. The column was equilibrated with 0.05 M Tris-HCl pH 8.0 buffer and eluted with 0-0.5 M NaCl. The flow rate was 12 ml/h.

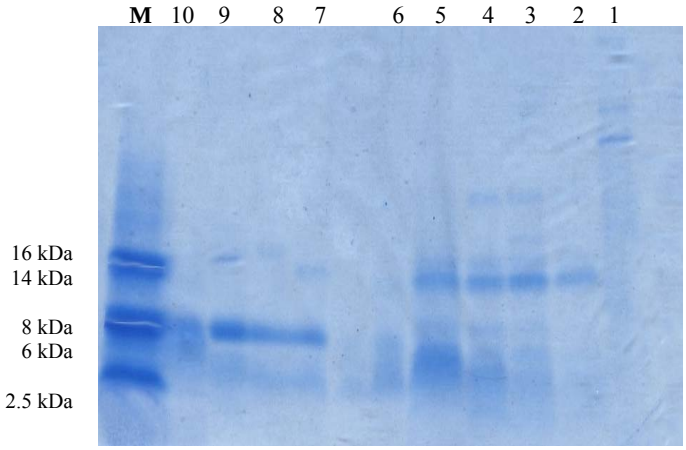


**Figure 2.** Tris-Tricin SDS-PAGE of P1 and P2 peaks. Lanes 1-6: P1 peak; lanes 7-10: P2 peak. Numbers in lane M refer to molecular mass markers.

For further purification, the P2 peak fractions were also subjected to hydrophobic interaction chromatography on Phenyl-Sepharose column.

The proteins were separated in several peaks (figure 3). Protein patterns were analyzed by Tris-Tricin SDS-PAGE and visualized by coomassie brilliant blue staining. The results showed the presence of a protein with a molecular weight of 7 kDa (figure 4, lanes 4-6).

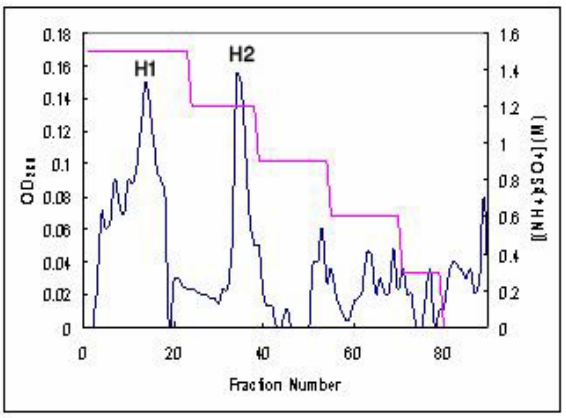


**Figure 3.** Phenyl-Sepharose chromatography of P2 fractions. The column was equilibrated with 0.05 M Tris-HCl, 1.5 M ammonium sulfate, 0.005 M EDTA and 0.3% NaHSO3 pH 8.4. Flow rate was 40 ml/h.

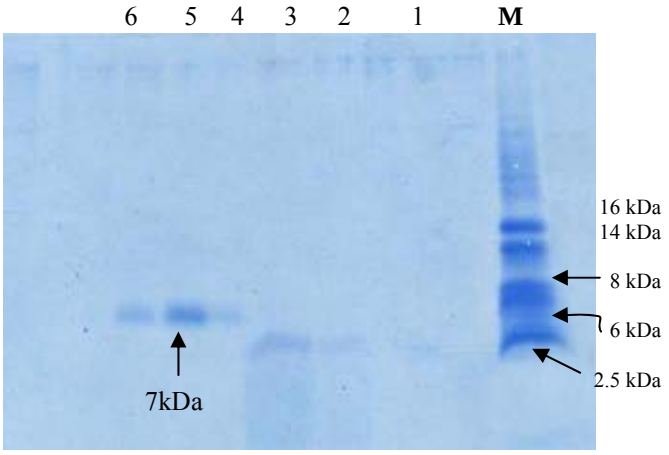


**Figure 4.** Tris-Tricin SDS-PAGE of protein fractions from Phenyl-Sepharose chromatography. Lanes 1-3: H1 peak; lanes 4-6: H2 peak. Numbers in lane M refer to molecular mass markers.

***ANS binding study***

ANS is a widely used hydrophobic probe for proteins and membranes characterization. The fluorescence intensity of ANS alone is low in water, while it places in hydrophobic medium its fluorescence intensity increases which is supplementary with a blue shift (Matulis et al., 1998). It has been shown that nsLTP's structure contains a hydrophobic cavity surrounded by four helices connected through disulfide bonds. The hydrophobic cavity is the binding pocket for lipid or fatty acid molecules (Liu et al., 2002). This naturally occurring feature of nsLTP should bring a useful opportunity for checking its presence in relevant solutions, via affinity toward hydrophobic molecules resembling ANS. Fig. 5 shows the ANS emission spectral changes in the absence and presence of nsLTP2 solution. It can be seen that ANS fluorescence intensity is low in the absence of nsLTP2 (curve 1) while its intensity is increased when nsLTP2 is present in the solution (curve 2 and 3), by way of a blue shift due to binding to the hydrophobic patches of the protein.

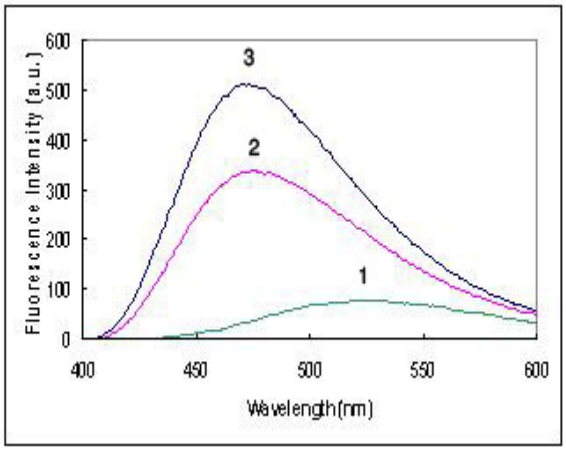


**Figure 5.** ANS fluorescence intensity (400 µM) in the absence of nsLTP2 (1) and in the presence of nsLTP2 (4µM) (2) and nsLTP2 (4.2 µM) (3).

## Discussion

Throughout the study of some articles had been purified LTP2 from various plants, we observed that some of those have loaded output of cation exchange chromatography onto that column in the same conditions and have repeated this stage one or two times over again (Ozaki et al., 1980; Yu et al., 1988). Since there were no sensible reasons for these repeats, we did not recreate this stage more times. Some other researchers have used gel filtration and/or RP-HPLC after ion exchange chromatography (Segura et al., 1993; Liu et al., 2002; Ooi et al., 2005). Using of these methods are

limited for the reason that of being costly and requirements of specific facilities. Consequently, in this study, relative to specific feature of LTP2 protein, presence of a hydrophobic cavity, we applied a hydrophobic chromatography method to separate the protein from rice paddy. The obtained results showed that this type of chromatography is useful and efficient tool for LTP2 purification.

In conclusion, given that other nsLTP2 purification methods have been used to date (Yu et al., 1988; Charvolin et al., 1999; Douliez et al., 2001; Liu et al., 2002) are intricate from the performance view point, due to high-cost, time overwhelming and instrumental dependency, the advantages of the developed procedure introduced in this article make it possible to access LTPs in a rapid and simple route. However, we proposed that ion-exchange chromatography along with hydrophobic interaction chromatography are convenient methods for feasible and rapid purification of nsLTP2.

## References

1- Charvolin D. (1999) The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 Å resolution. European Journal of Biochemistry 264: 562-568.

2- Cheng H. Ch. (2004) Cheng. Lipid binding in rice nonspecific lipid transfer protein-1 complexes from *Oryza sativa*. Protein Science 13: 2304–2315.

3- Dharmaraj S. (2002) Solution Structure of Plant Nonspecific Lipid Transfer Protein-2 from Rice *(Oryza sativa*). Journal of Biological Chemistry 277: 35267–35273.

4- Douliez J. P. (2001) Disulfide bond assignment, lipid transfer activity and secondary structure of a 7-kDa plant lipid transfer protein, LTP2. European Journal of Biochemistry 268: 1400-1403.

5- Kader J. C. (1996) Lipid transfer proteins in plants. Annul Review of Plant Physiology Plant Molecular Biology 47: 627–654.

6- Lin K. F. (2005) Characterization and structural analyses of nonspecific lipid transfer protein 1 from *Mung Bean*. Journal of Biochemistry 44: 5703-5712.

7- Liu W. J. (2002) Purification and characterization of a novel 7-kDa non-specific lipid transfer protein-2 from rice (*Oryza sativa*). Biochemistry & Biophysics Research Communications 294: 535–540.

8- Ooi L. (2005) Purification and characterization of non-specific lipid transfer proteins from the leaves of Pandanus amaryllifolius (Pandanaceae). Journal of Peptides 62:773-779.

9- Ozaki Y. (1980) Amino acid sequence of a purothionin homolog from barley flour. Journal of Biochemistry 87: 549-555.

10- Pato Ch. (2001) Potential application of plant lipid transfer proteins for drug delivery, Journal of Biochemistry & Pharmacology 62: 555-560.

11- Schagger H. (1987) Tricine-sodium dodecylsulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry 166: 368-379.

12- Segura A. (1993) Purification and antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach. Journal of FEBS 332: 243-24.

13- Wang Z. h. (2005) Identification of non-specific lipid transfer protein-1 as a calmodulin-binding protein in Arabidopsis, FEBS Letter 579: 1683–1687.

14- Yu Y. G. (1988) Amino acid sequence of a probable amylase/protease inhibitor from rice seeds. Archives of Biochemistry and Biophysics 265: 466-475.

*Journal of Cell and Molecular Research (2009) 1 (2),77-83*

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

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Received 29 August 2009 Accepted 19 September 2009

**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.

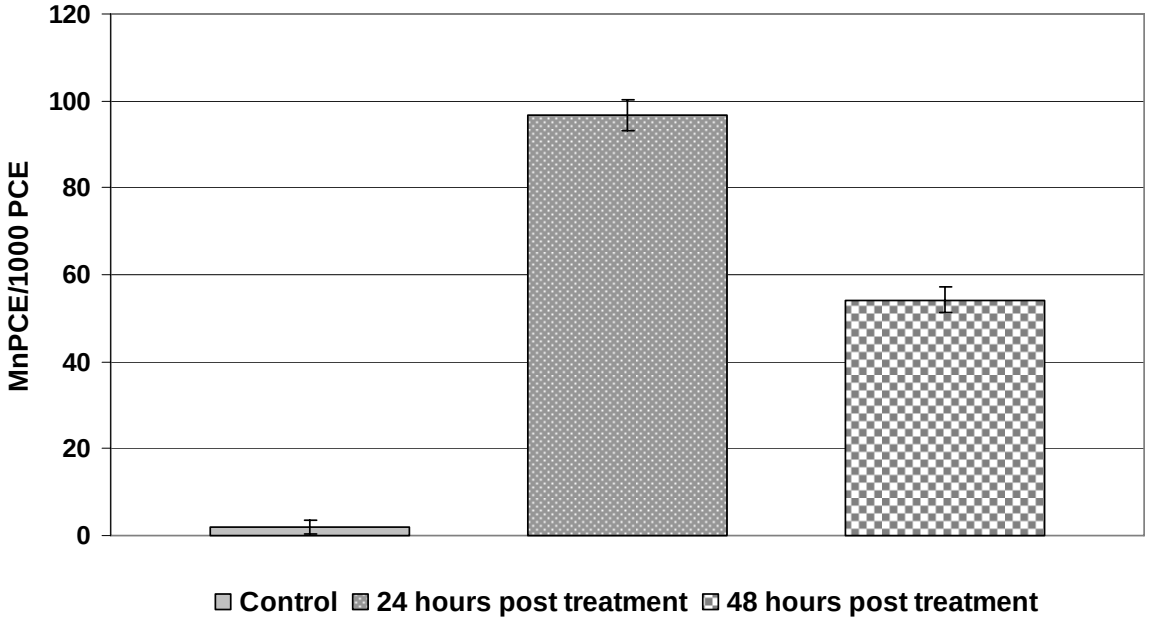


**Figure 1.** MnPCE induced by 2 Gy Gamma irradiation at different times post treatment

***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.

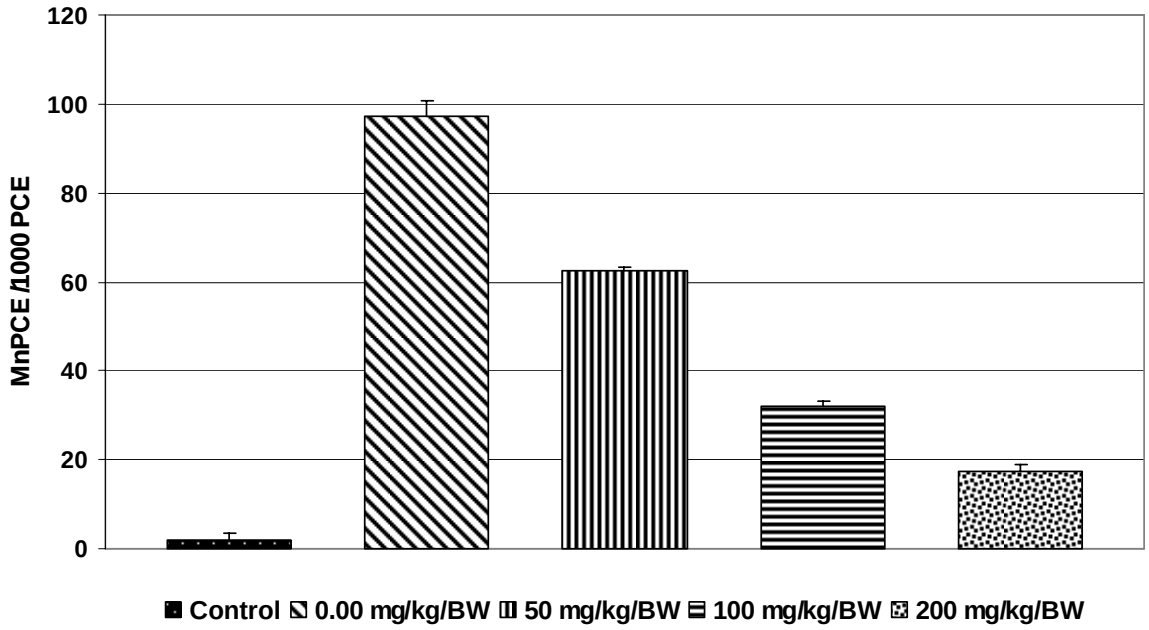


**Figure 2.** Effect of vitamin E on the frequency of Gamma-induced MnPCE

***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.

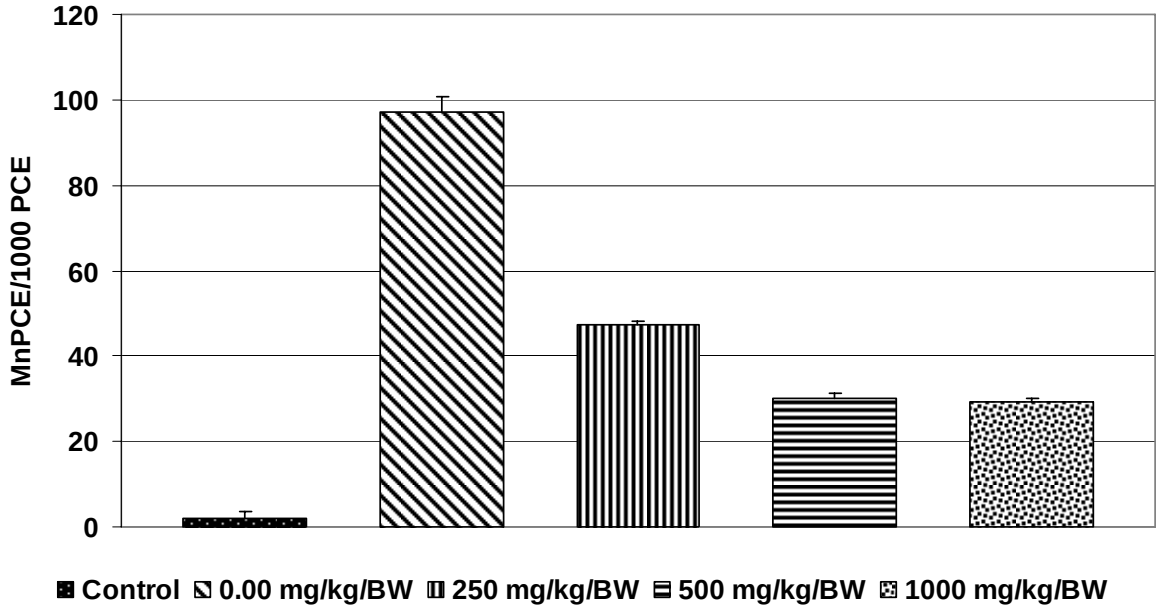


**Figure 3.** Effect of aqueous extract of Shirkhesht on the frequency of Gamma-induced MnPCE

***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).

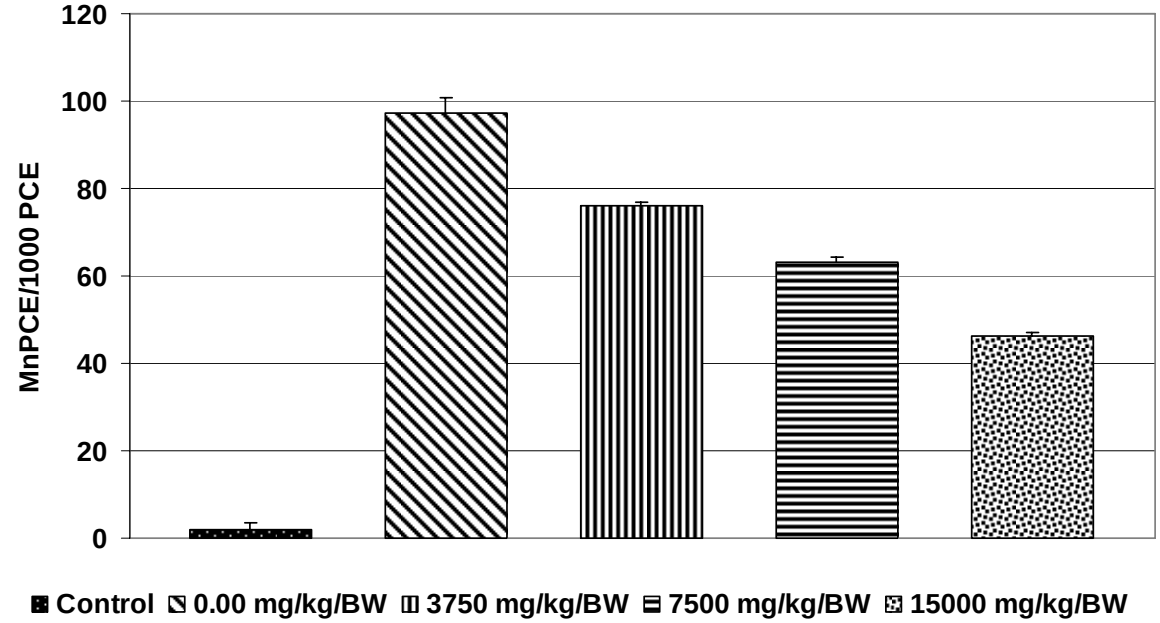


Figure 4: Effect of alcoholic extract of Shirkhesht on Gamma-induced MnPCE

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.

## References


1- Abramsson-Zetterberg L., Zetterberg G. and Grawe J. (1996) The time-course of micronucleated polychromatic erythrocytes in mouse bone marrow and peripheral blood. Mutation Research. 350: 349-358.

2- Abramsson-Zetterberg L., Grawe J. and Zetterberg G. (1999) The micronucleus test in rat erythrocytes from bone marrow, splleen and peripheral blood: the response to low doses of ionizing radiation, Cyclophosphamide and vincristine determined by flow cytometry. Mutation Research 423: 113-124.

3- Bektasoglu B., Esin Celik S., Ozyurek M., Guclu K. and Apak R. (2006) Novel hydroxyl radical scavenging antioxidant activity assay for water-soluble antioxidants using a modified CUPRAC method. Biochemist Biophysics Research Communication 345(3): 1194-200.

4- Bonorden W. R. and Pariza M. W. (1994) Antioxidant nutrients and protection from free radicals. Nutritional toxicology Reven press (Eds.), pp. 19-48.

5- Claycombe K. J. and Meydani S. N. (2001) Vitamin E and genome stability. Mutation Research 475: 37-44.

6- Copeland E. S. (1991) Mechanisms of radioprotection – A review. Photochemistry and Photobiology. 88: 839-844.

7- Durakovic C. A. (1993) Radioprotective agents in medicine. Arh Hig Rada Toksilokol. 44: 331-354.

8- Ghareman A. (1998) Flora of Iran Tehran: Research Inst. Of Forests and Rangelands. Code: 114/001/001/-114/013/001 (in Persian).

9- Gocke E. (1996) The micronucleus tests: its value as a predictor of rodent carcinogens versus its value in risk assessment. Mutation Research 352: 189-190.

10- Goodhead D. T. (1994) Initial events in the cellular effects of ionizing radiations: clustered damage in DNA. National Journal of Radiation Biology 65:7-17.

11- Hayashi M., Tice R. R., Mac Gregor J. T., Anderson D., Blakey D. H., Kirsch-Volders M., Oleson F. B., Pacchierotti F., Romagna F., Shimada H., Sutou S. and Vannier B. (1994) In vivo rodent erythrocyte micronucleus assay. Mutation Research 312: 293-304.

12- Heddle J. A. and Salamone M. F. (1981) Chromosomal aberrations and bone marrow toxicity. Environ Health Perspect 39: 23-27.

13- Heddle J. A. and Hayashi M. (1991) Micronuclei as index of cytogenetic damage. Environmental and Molecular Mutagenesis. 18: 277-291.

14- Hosseinimehr S. J., Tavakoli H., Pourheidari G., Sobhani A. and Shafiee A. (2003) Radioprotective effects of citrus extract against gamma-irradiation in mouse bone marrow cells. Journal of Radiation Research. 44(3): 237-241.

15- Iyer R., Lehnert B. E. (2000) Effects of ionizing radiation in targeted and nontargeted cells. Archives of Biochemistry and Biophysics. 376: 14-25.

16- Konopacka M., Widel M., Wolny J. R., (1998) Modifying effect of Vitamins C, E and beta – carotene against gamma–ray–induced DNA damage in mouse cells. Mutation Research 417: 85-94.

17- Konopacka M. and Wolny J. R. (2001) Antioxidant Vitamins C, E and beta–carotene reduce DNA damage before as well as after gamma-ray irradiation of human Lymphocytes in vitro. Mutation Research 491: 1-7.

18- Lee T. K., Johnke R. M., Allison R. R., O'Brien K. F. and Dobbs L. J. Jr. (2005) Radioprotective potential of ginseng. Mutagenesis 20(4): 237-243.

19- Mac Gregor J. T., Casciano D., Muller L. (2000) Strategies and testing methods for identifying mutagenic risks. Mutation Research 455: 3-20.

20- Matsuoka A., Yamazaki N., Suzuki T., Hayashi M. and Sofuni T. (1993) Evalution of the micronucleus test using a chinese hamster cell line as an alternative test. Mutation Research 272: 223-236.

21- Mutlu–Turkoglu U., Erbil Y., Oztezcan S., Olgac V., Toker G. and Uysal M. (2000) The effect of selenium and/or vitamin E treatments on radiation induced intestinal injury in rats. Life Sciences 66(20): 1905-1913.

22- Narra V. R. and Harapanhalli R. S. (1994) Vitamins as radioprotectors in vivo. Protection by Vitamin C against internal radionuclides in mouse testes:

implications to the mechanism of damage caused by the Auger effect. Radiation Research 137(3): 394-399.
23- Odin A. P. (1997) Vitamins as antimutagens: advantages and some possible mechanisms of antimutagenic action. Mutation Research 386: 39-67.
24- Peak M. J. and Peak J. G. (1990) Hydroxyl radical quenching agents protect against DNA breakage caused by both 365-nm UVA and by gamma radiation. Photochemistry and Photobiology. 51(6): 649-652.
25- Pouget J. P. and Mather S. J. (2001) General aspects of the cellular response to Low– and high–LET radiation. European Journal Of Nuclear Medicine. 28(4): 542-561.
26- Rouhanizadeh N. and Khalkhali Z. (1971) Evaluation biochimique de la propriete radioprotectrice du Shirkhesht. Ratioprotection 6(4): 279-283.
27- Rouhanizadeh N., Samii S. and Khalhkali Z. (1973) Degredation of desoxyribopolynucleotides in the lymphatic tissues of rats after irradiation: radioprotecting action of shirkhesht. Radioprotection 8(4): 277-281.
28- Schmid W. and Vol Ledebur M. (1973) The Micronucleus Test: methodological aspects. Mutation Research 19: 109-117.
29- Songthaveesin C., Saikhun J., Kitiyanant Y. and Pavasuthipaisit K. (2004) Radio-protective effect of vitamin E on spermatogenesis in mice exposed to gamma-irradiation: a flow cytometric study. Asian Journal of Andrology. 6(4): 331-336.
30- Tiku A. B., Abraham S. K. and Kale R. K. (2004) Eugenol as an in vivo radioprotective agent. Journal of Radiation Research 45(3): 435-440.
31- Tsou T. C., Chen C. L., Liu T. Y. And Yang J. L. (1996) Induction of 8-hydroxydeoxyguanosine in DNA by chromium(III) plus hydrogen peroxide and its prevention by scavengers. Carcinogenesis 17(1): 103-108.
32- Tsou T. C., Lai H. J. and Yang J. L. (1999) Effects of mannitol or catalase on the generation of reactive oxygen species leading to DNA damage by Chromium(VI) reduction with ascorbate. Chemical Research in Toxicology. 12(10): 1002-1009.
33- Umegaki K., Itoh T. and Ichikawa T. (1994) Effect of vitamin E on chromosomal damages in bone marrow cells of mice having received low dose of X-ray irradiation. International Journal of Vitamin and Nutrition Research. 64(2): 249-252.
34- Zargary A. (1999) Medicinal palnts (In Pharsi).
35- Zu Granetto C., Ottaggio L., Abbondanddo A. and Bonatti S. (1996) P53 accumulates in micronuclei after treatment with a DNA breaking chemical, methyl nitrosourea, and with the spindle poison, Vinblastine. Mutation Research. 352:61-64.

*Journal of Cell and Molecular Research (2009) 1 (2) 84-90*

# Some responses of dry farming wheat to osmotic stresses in hydroponics culture

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Received 14 May 2009 Accepted 19 September 2009

## Abstract

Osmotic stress is one of the major factors that significantly reduce yields in dry areas. Plants respond to this abiotic stress at physiological and molecular levels. Many genes are induced under stress conditions by transcription factors. Dehydration responsive element binding (DREB) protein is a subfamily of AP2/ERF transcription factors which control expression of many osmotic stress-inducible genes. In this study, 21 days old seedlings of Sardari cultivar, dry farming bread wheat transferred into hydroponics culture using Hoagland solution. Osmotic stress treatments performed with adding 100, 200 and 400 g/l poly-ethylene glycol 6000 to hydroponics culture to obtain –0.15, –0.49, and –1.76 MPa water potential, respectively. After the seedlings were withered and colorless, relative water content, dry weight, and photosynthesis measured. In addition, RT-PCR, and cDNA sequencing carried out. Molecular analysis of DREB translated protein sequence performed by DNAMAN, BLASTN, Pfam and PROSITE software. Results showed that osmotic stress decreased relative water content, root and shoot dry weight and net photosynthesis rate in comparison to control, significantly ($P < 0.05$). Sequence alignment indicated 98% homology with other *Triticum aestivum* DREB protein mRNA. There was an AP2 domain in the translated protein with three -sheets and one -helix and contains the $Val_{14}$ and $Glu_{19}$ amino acids. An EST Sequence deposited in NCBI GenBank database with the accession number of ES466900.

**Keywords:** AP2 domain, DREB, osmotic stress, physiological responses, wheat

## Introduction

Osmotic stress is one of the major factors that significantly reduce yields in dry areas (Trethowan et al., 2001). Wheat in some areas is usually grown on dry-agricultural fields and this often causes serious problems in wheat production (Tas and Tas, 2007). Acclimation to this stress, results from a series of integrated events occurring at physiological and molecular levels that helps in the retention and/or acquisition of water, protection of chloroplast functions and maintenance of ion homeostasis (Mohsenzadeh et al., 2006). Researchers have used various indices to categorize the symptoms of osmotic stress. These indices include changes in dry weight (Marcum et al., 1995; Huang et al., 1997), RWC (Schonfeld et al., 1988; Guan et al., 2004), net photosynthesis rate (Martin and Ruiz-Torres, 1992; Leidi et al., 1993; Flexas and Medrano, 2002) and gene expression (Shinozaki and Yamaguchi-Shinozaki, 1996; Bruce et al., 2002; Very and Sentenac, 2003).

AP2/ERF transcription factors are a large multigene family of plant-specific transcription factors with over 100 members, whose distinguishing characteristic is that they contain the so-called AP2 DNA-binding domain composed of 57-70 amino acid residues. They have been isolated from a variety of higher plants, such as *Arabidopsis thaliana*, tobacco, tomato, rice, maize, wheat and castor bean (Jofuku et al., 1994; Baker et al., 1994; Sakuma et al., 2002).

Many genes that function in stress tolerance are induced under stress conditions (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2003). Most of these stress-inducible genes are controlled by abscisic acid (ABA), but some are not, indicating the involvement of both ABA-dependent and ABA-independent regulatory systems in stress-responsive gene expression (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). Dehydration responsive element binding (DREB) protein is a subfamily of AP2/ERF transcription factors which contain one AP2 domain. It is known that the DREB genes are the key-genes conferring tolerance to water stress, in the ABA-independent pathway. The DREB transcription factor controls

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the expression of several target genes involved in the mechanism of osmotic stress tolerance (Liu et al., 2000; Latini et al., 2005; Badawi et al., 2007), by recognizing and binding to an essential cis-acting element, the dehydration responsive element (DRE) with a core motif of A/GCCGAC in the upstream of inducible genes (Yamaguchi-Shinozaki and Shinozaki, 1994; Cushman and Bohnert, 2000; Kizis et al., 2001; Shen et al., 2003; Liu et al., 2006; Wang et al., 2006).

Dry-farming is characterized by a somewhat short season and the nature of plant growth is modified by the arid conditions prevailing in dry-farming. So, the mechanisms involved in the response of plants to osmotic stress in dry-farming require further elucidation. We know that Sardari wheat is tolerant to drought because is a dry farming cultivar but the aim of this study was to get insight into some responses of dry farming wheat to osmotic stresses in hydroponics culture. It can help in understanding water stress tolerance mechanisms and defining conditions for identification of osmotic stress-inducible genes in the tolerant plants.

## Materials and Methods

### *Plant material and growth conditions*

Nine days germinated seeds of Sardari cultivar, dry farming bread wheat (Triticum aestivum L.), were transferred into hydroponics culture using Hoagland solution and kept for 12 days in growth chamber conditions with 16 h light, 500 µ mol m− 2 s− 1 fluorescent light, 22/15 ◦ C day/night temperature and 60% humidity.

### *Osmotic stress treatments*

Osmotic stress treatments were performed with adding 100, 200 and 400 g/l poly-ethylene glycol 6000 (PEG 6000) to hydroponics culture to obtain –0.15, –0.49 and –0.76 MPa water potential (Michel and Kaufmann, 1973), respectively. The untreated culture was used as control. The seedlings were withered and colorless after 5 days. Seedlings from each treatment and control were harvested, quickly immersed in liquid nitrogen and stored at –20 °C for RNA extraction.

### *RWC and dry weight measurements*

The percentage of relative water content (RWC) was calculated as:

RWC = (FW DW) / (TW DW) × 100

Variables were the fresh weight of harvested leaves which were cut to 1cm segments (FW); the weight of leaf segments soaked in water at 4°C in the dark for 24 h (TW); and dry weight of the segments baked at 80 ◦ C for 24 h (DW).

### *Photosynthesis measurement*

Photosynthesis rate (AN) was determined by using Photosynthesis System HCM-1000 (Heinz Walz, Effeltrich, Germany). The middle part of the youngest, fully expanded leaf of all plants was placed in a gas-exchange cuvette (1010-M; Heinz Walz), while CO2 concentration and flow rate were kept constant at 360 ppm. Plants were illuminated in the cuvette with 1800 µ mol $m^{-2}$ $s^{-1}$ PAR at 20◦ C during measurements. Data were automatically collected every minute after photosynthesis rate was stabilized. The rate of photosynthesis was expressed as µ mol CO2 $m^{-2}$ $s^{-1}$.

### *DNA extraction and primer design*

Leaf tissue (200 mg) was ground to a fine powder in liquid nitrogen, and DNA was extracted using modified CTAB method. Primers were designed by Oligo5 software and using consensus of alignment of DREB genes for wheat from NCBI GenBank.

### *RNA extraction and RT-PCR analysis*

Seedling leaf samples were ground in liquid nitrogen and total RNA was extracted using Aurum™ Total RNA Mini Kit (Bio-RAD). First-strand cDNA was synthesized from extracted RNA and by cDNA Synthesis system Kit (Roche). Primer sequences for the DREB transcripts were forward primer:

5′-AAGAAGTGGAAGGAGGAAA-3′ and reverse primer: 5′-CTAAACCCATCATCACCA-3′.

Wheat actin was used as a positive control for the RT–PCR with 5′-GACCCAGACAACTCGCAACT-3′ as the forward primer and 5′-CTCGCATATGTGGCTCTTGA-3′ as the reverse primer. The PCRs were carried out in 20- L solution comprising 10 ng of samples cDNA, 1 x PCR buffer, 1.5 mM MgCl2, 0.25 mM each dNTP, 2 µM of each primer, and 1 U Taq DNA polymerase. The PCR profiles for both genes were an initial denaturation at 94 °C for 5 min followed by 35 amplification cycles (94 °C for 1 min, 50 °C for 1 min, and 72 °C for1 min) and final extension at 72 °C for 10 min. The PCR amplification products were separated in 1% (w/v) agarose gels.

### *Purification of PCR product and sequencing*

PCR products were purified using High Pure PCR Product Purification Kit (Roche), and then sequenced (MWG, Germany).

### *Cloning*

DREB sequence was cloned in pTZ57R/T plasmid and DH5α strain of E. coli using Ins TMPCR Product T/A Cloning Kit (Fermentas). Plasmid extraction was carried out by AccuPrep® Plasmid Extraction Kit (BIONEER) to confirm the cloning via PCR and gel electrophoresis.

### *Statistical analysis and computations*

All physiological experiment set-ups were randomized complete block designed with three replicates. Raw data were imported to Microsoft Excel program for calculation and graphical representation. SPSS version 11.5 program was used for analysis of variance and comparison of means by Duncan's method at $P < 0.05$.

Transeq software was used for translating the nucleotide sequence to amino acid sequence. The alignment of the deduced amino-acid sequence of AP2 domain with other AP2 domain-containing carried out by protein blast search databases. Sequence similarity and several structural features were predicted by online databases and related software including BLAST, Pfam and PROSITE.

## Results

### *Plant growth and osmotic stress treatments effects on RWC and dry weight*

Seeds of Sardari cultivar wheat were germinated at the rate of 88.5% in sufficient moisture and were grown under growth chamber conditions. RWC averages were 88.5% for control seedlings as the reference point for well watered plants and 84.9, 61.1, and 43.4 percent for –0.15, –0.49, and –1.76 MPa water potential, respectively. Results showed that osmotic stress decreased relative water content, significantly ($P< 0.05$) (Figure 1). In addition, osmotic stress decreased both root and shoot dry weight significantly ($P< 0.05$) from 0.015 and 0.033 g root and shoot dry weight, respectively in control to 0.009 and 0.019 g in plants which treated with 400 g/l polyethylene glycol 6000.

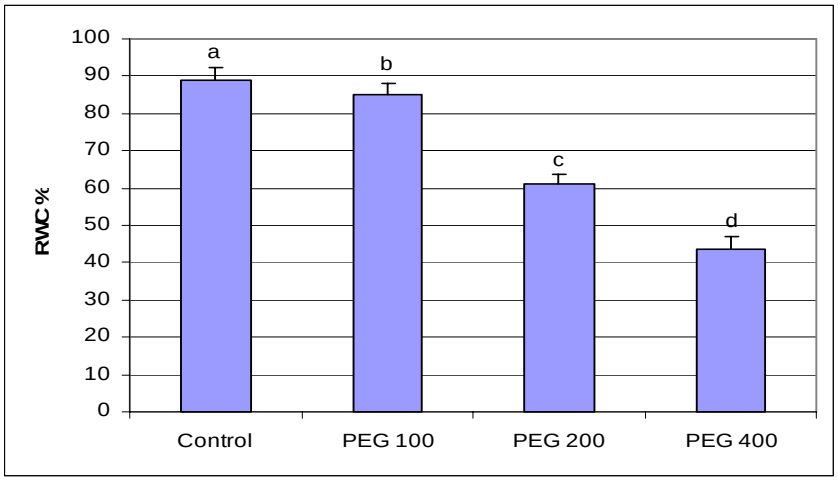


**Figure 1.** RWC in control and drought- treated plants. PEG 100, 200 and 400 g/l are equal to –0.15, –0.49, and –1.76 MPa water potential, respectively. Data are means ±S.E. of three replicates. Treatments with the same lowercase letters were not significantly different based on mean comparison by Duncan's method at $P < 0.05$.

### *Effects on photosynthesis rate*

Figure 2 shows a significant decrease in the net photosynthesis rate as drought became severe. The most declines were seen between 100 and 200 g/l PEG treatments. In 200 g/l PEG treatment with –0.49 MPa water potential the net photosynthesis rate was zero but in 400 g/l PEG with –1.76 MPa water potential, the net photosynthesis rate was negative because respiration is higher.

### *Gel electrophoresis display*

Agarose gel electrophoresis of amplified cDNA by specific primers showed 700-bp bands for DREB gene and a 500-bp band for positive control (wheat actin) (Figure 3).

### *Sequencing and bioinformatics analysis*

Sequencing reported a 645 nucleotide sequence which was deposited in NCBI GenBank database with the accession number ES466900. Alignment of TaDREB sequence using blast search, indicated that it has 98% homology with DREB1 genome A and B and DREB1 mRNA of wheat.

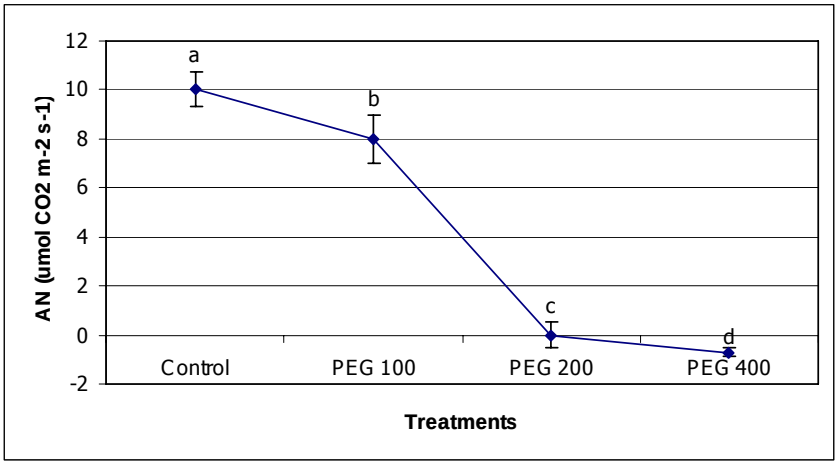


**Figure 2.** Rate of net $CO_2$ assimilation in control and drought- treated plants. PEG 100, 200 and 400 g/l are equal to –0.15, –0.49, and –1.76 MPa water potential, respectively. Data are means ±S.E. of three replicates. Treatments with the same lowercase letters were not significantly different based on mean comparison by Duncan's method at $P < 0.05$.

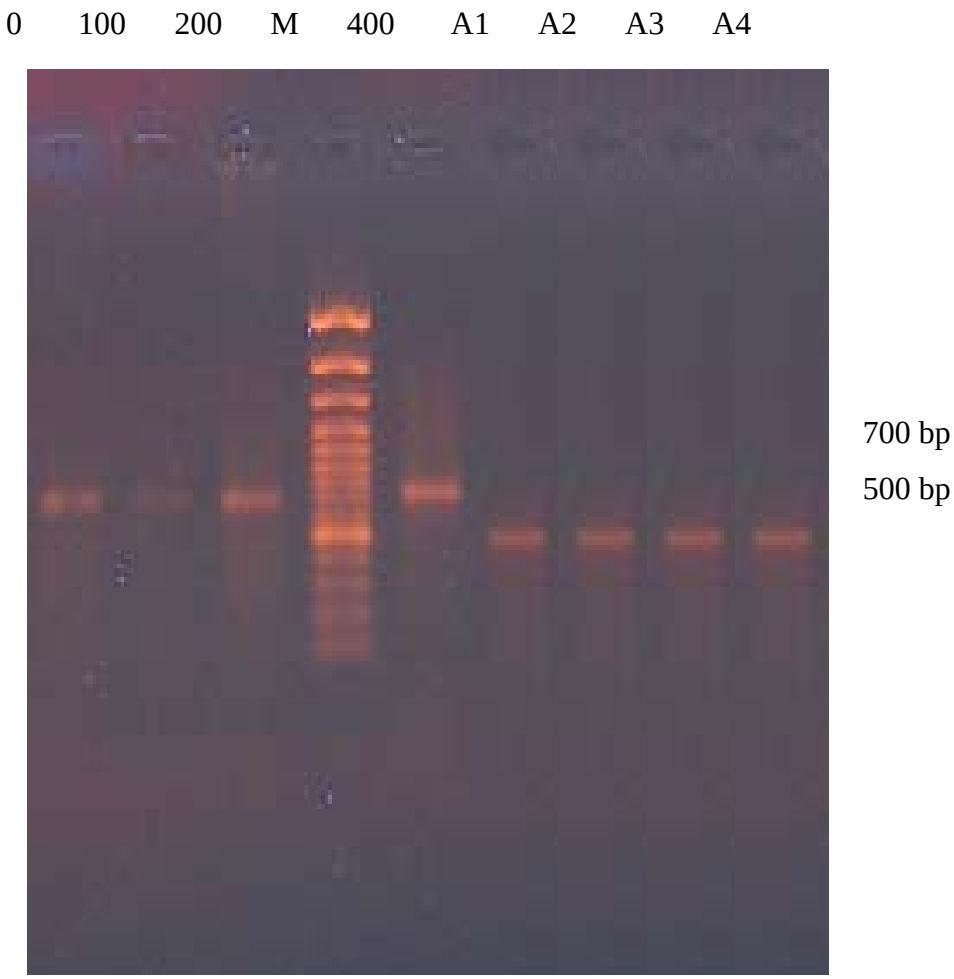


**Figure 3.** Agarose gel electrophoresis of amplified cDNA by specific primers. cDNA constructed via RT-PCR from seedling mRNA under conditions with adding 0, 100, 200, and 400 g/l poly-ethylene glycol 6000 to hydroponics culture. M column is 100-bp ladder and A1 to A4 are expression of the actin as housekeeping gene in the four treated tissue.

The Pfam program shows that the deduced amino-acid sequence of AP2 domain including three β-sheets and one α-helix with the valine14 and glutamic acid19 amino acids (Figure 4). According to protein blast search databases, our deduced amino-acid sequence of AP2 domain has 98 to 100 % identities with other wheat, 92 to 93 % identities with Avena sativa, Festuca arundinacea and Poa prantensis, 87 to 89 % identities with Sorghum bicolor, Cynodon dactylon and Zea mays and 80 to 83 % identities with Populus euphratica and Glycine max.

A Y R *G V R Q R T* W *G K W V A E I R E P* N R G N *R L W L G S F P T A V E A A R A Y D D A A R A M Y G* A K A R V N F S E Q S P D A N S

**Figure 4.** The deduced amino-acid sequence of AP2 domain analysed by Pfam program. AP2 domain has three β-sheets and one α-helix, respectively showed by italic. The valine$_{14}$ and glutamic acid$_{19}$ amino acids of AP2 domain are typed with bigger font.

## Discussion

Wheat crop responds to osmotic stress in the form of changes in various physiological and biochemical processes. As mentioned in results, osmotic stress decreased relative water content; both root and shoot dry weight and net photosynthesis rate, significantly. These results are confirmed by other researches (Mohsenzadeh et al., 2006; Gill et al., 2002; Flexas et al., 2002). Based on these data, it is possible to define three levels of drought stress: mild, moderate and severe. If RWC is reduced to below 5-7% of the control ones, plants looked healthy and decrease in growth rate was not significant. Moderate reduction of RWC for 10–25% affected biomass and the stress is severe when RWC decreased by more than 25%. Lawlor (2002) suggests that decreased ATP concentration at low RWC impairs protein synthesis, though it may increase the synthesis of certain types of proteins (Flexas, 2002). The moisture content of dry-farm wheat, the chief dry-farm crop, is more important. In this study, RWC as an indicator of the level of response to osmotic stress showed the mild, moderate and severe drought stress for 100, 200, 400 g/l PEG treatments conditions, respectively. Maintenance of high plant water status and the net photosynthesis rate in 100 g/l PEG treatment is an indication of osmotic stress resistance and it is necessary for Sardari cultivar as dry farming wheat and it may be due to gene expression induced by transcription factors like DREB protein. The recognition of the superior quality of wheat grown under osmotic stress stimulates faith in the great profitableness of dry-farming which is growth without irrigation under a limited rainfall.

As figure 3 shows, TaDREB gene in Sardari wheat is expressed both under osmotic stress and control conditions and it is suggested that this gene is also responsible for the non-stress physiology or growth and development of the plant. This observation is similar to that of Latini and co-workers (Latini et al., 2005). In addition, we know that the DREB genes are controlled by the ABA-independent pathway and the responsiveness of plants to osmotic stress is critically mediated by the increase in ABA levels.

The primary and secondary protein databases shows that our Sadari DREB deduced amino-acid sequence of AP2 domain is very similar to other wheat plant and slightly different with other plants. This similarity of the protein motif suggests similar functions. In particular, understanding regulatory gene networks in stress response cascades depends on successful functional analyses of cis-acting elements (Yamaguchi-Shinozaki and Shinozaki, 2005). According to Shen and co-workers research, in different wheat cultivars, the TaDREB1 gene is induced by low temperature, salinity and drought; and the expression of some genes that contains DRE motifs in its promoter is closely related to the expression of TaDREB1.The results suggest that TaDREB1 functions as a DRE-binding transcription factor in wheat (Shen et al., 2003). Four genes encoding putative ethylene-responsive element binding factor (ERF)/AP2 domains were cloned from *Brassica napus*, and these genes could be induced by low temperature, ethylene, drought, high salinity, abscisic acid and jasmonate treatments (Liu et al., 2006).

The obtained 645 nucleotide sequence from cDNA was also amplified from genomic DNA with the same primers and it means that this segment of sequence has no intron. This study is the first research of DREB gene on an Iranian plant.

## Acknowledgment

This study was supported by Shiraz University, Shiraz, Iran.

## References

1- Badawi M., Danyluk J., Boucho B., Houde M. and Sarhan F. (2007) The *CBF* gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal *CBF*s. Molecular Genetics and Genomics 277: 533-554.

2- Baker S. S., Wilhelm K. S. and Thomashow M. F. (1994) The 59-region of *Arabidopsis thaliana* cor15a has cis-acting elements that confer cold, drought and ABA-regulated gene expression. Plant Molecular Biology 24: 701-713.

3- Bruce W. B., Edmeades G. O. and Barker T. C. (2002) Molecular and physiological approaches to maize improvement for drought tolerance. Journal of Experimental Botany 53: 13-25.

4- Cushman J. C. and Bohnert H. J. (2000) Genomic approaches to plant stress tolerance. Current Opinion of Plant Biology 3: 117-124.

5- Flexas J. and Medrano H. (2002) Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. Annals of Botany 83: 183–189.

6- Gill P., Sharma A. D., Singh P. and Bhullar S. (2002) Osmotic stress-induced changes in germination growth and soluble sugar content of *Sorghum bicolar* (L.) moench seeds. Plant Physiology 28: 12-25.

7- Guan X. Q., Zhao S. J., Li D. Q. and Shu H. R. (2004) Photoprotective function of photorespiration in several grapevine cultivars under drought stress. Photosynthetica 42(1): 31-36.

8- Huang B., Duncan R. R. and Carrow R. N. (1997) Drought-resistance mechanisms of seven warm-season turfgrasses under surface soil drying. I. Shoot response. Crop Science 37: 1858-1863.

9- Jofuku K. D., Den Boer B. G.W., Van Montagu M. and Okamuro J. K. (1994) Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell 6: 1211-1225.

10- Kizis D., Lumbreras V. and Pages M. (2001) Role of $AP_2$/EREBP transcription factors in gene regulation during abiotic stress. FEBS Letters 498: 187-189.

11- Latini A., Rasi C., Chiavicchioni G., Chiaretti D., Sperandei M., Cantale C., Iannetta M., Dettori M., Ammar K. and Galeffi P. (2005) Expression study of a gene for a dehydration-responsive transcription factor in durum wheat. Proceedings of the XLIX Italian Society of Agricultural Genetics Annual Congress. Potenza, Italy.

12- Lawlor D. W. (2002) Limitation of photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP. Annals of Botany 89: 871–885.

13- Leidi E. O., Lopez J. M., Lopez M. and Gutierrez C. (1993) Searching for tolerance to water stress in cotton genotypes: photosynthesis, stomatal conductance and transpiration. Photosynthetica 28: 383-390.

14- Liu Q., Zhao N., Yamaguch-Shinozaki K. and Shinozaki K. (2000) Regulatory role of DREB transcription factors in plant drought, salt and cold tolerance**.** Chinese Science Bulletin 45(11): 970-976.

15- Liu Y., Zhao T. J., Liu J. M., Liu W. Q., Liu Q., Yan Y. B. and Zhou H. M. (2006) The conserved Ala37 in the ERF/AP2 domain is essential for binding with the DRE element and the GCC box. FEBS Letters 580: 1303-1308.

16- Marcum K. B., Engelke M. C., Morton S. J. and White R. H. (1995) Rooting characteristics and associated drought resistance of zoysiagrasses. Agronomy Journal 87: 534-538.

17- Martin B. and Ruiz-Torres N. A. (1992) Effects of water-deficit stress on photosynthesis, its components and component limitations, and on water use efficiency in wheat (*Triticum aestivum* L.). Plant Physiology 100: 733-739.

18- Michel B. E. and Kaufmann M. R. (1973) The osmotic potential of polyethylene glycol 6000. Plant Physiology 51: 914-916.

19- Mohsenzadeh S., Malboobi M., Razavi K. and Farrahi-aschtiani S. (2006) Physiological and molecular responses of *Aeluropus lagopoides* (poaceae) to water deficit. Environmental and Experimental Botany 56 (3): 314-322.

20- Sakuma Y., Liu Q., Dubouzet J. G., Abe H., Shinozaki K. and Yamaguchi-Shinozaki K. (2002) DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochemical and Biophysical Research Communications 290: 998-1009.

21- Schonfeld M. A., Johnson R. C., Carver B. F. and Mornhinweg D. W. (1988) Water relations in winter wheat as drought resistance indicators. Crop Science 28: 526-531.

22- Shen Y. G., Zhang W. K., He S. J., Zhang J. S., Liu Q. and Chen S. Y. (2003) An EREBP/AP2-type protein in Triticum aestivum was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. Theoretical and Applied Genetics 106: 923-930.

23- Shinozaki K. and Yamaguchi-Shinozaki K. (1996) Molecular responses to drought and cold stress. Current Opinion in Biotechnology 7: 161-167.

24- Shinozaki K. and Yamaguchi-Shinozaki K. (2003) Regulatory network of gene expression in the drought and cold stress responses. Current Opinion in Biotechnology 6: 410-417.

25- Tas S. and Tas B. (2007) Some physiological responses of drought stress in wheat genotypes with different ploidity in Turkiye. World Journal of Agricultural Science 3(2): 178-183.

26- Thomashow M. F. (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annual Review of Plant Physiology and Plant Molecular Biology 50: 571-599.

27- Trethowan R. M., Crossa J., Ginkel M., and Rajaram S. (2001) Relationships among bread wheat international yield testing locations in dry areas. Crop Science 41: 1461-1469.

28- Very A. A. and Sentenac H. (2003) Molecular mechanisms and regulation of $K^+$ transport in higher plants. Annual Review of Plant Biology 54: 575-603.

29- Wang J. W., Yang F. P., Chen X. Q., Liang R. Q., Zhang L. Q., Geng D. M., Zhang X. D., Song Y. Z. and Zhang G. S. (2006) Induced expression of *DREB* transcriptional factor and study on its physiological effects of drought tolerance in transgenic wheat. Acta Genetica Sinica 33: 468-476.

30- Yamaguchi-Shinozaki K. and Shinozaki K. (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6: 251-264.

31- Yamaguchi-Shinozaki K. and Shinozaki K. (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress responsive promoters. Trends in Plant Science 10: 88-94.

32- Zhu J. K. (2002) Salt and drought stress signal transduction in plants. Annual Review of Plant Biology 53: 247-273.

*Journal of Cell and Molecular Research (2009) 1 (2), 91-96*

# Pattern of collagen IV expression in glomerular and mesenchymal basement membrane during fetal and postnatal period of Balb/c Mice

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Received 5 Januaury 2009 Accepted 19 September 2009

## Abstract

Basement membrane of glomerular mesangium (BMG) is a thin membrane which helps to support the capillary loops in a renal glomerulus and type IV collagen is require for complete BM formation during glomerulogenesis. In this investigation specific antibody of type IV collagen has been used in light microscopy to study development of BMG of the embryonic and postnatal mouse glomerular mesangium. In this study, 20 female Balb/C mice were selected randomly and finding vaginal plug was assumed as day zero of pregnancy. 12 pregnant mice were sacrified by cervical dislocation in one of gestational days 13-18, their fetuses were fixed and serially sectioned. Immunohistochemical Study for tracing of collagen type IV in BMG was carried out. The same processes were carried out for kidneys preparation of pups on 5, 10, 15 and 20 days after birth (2 mothers for each day). The result of the present study revealed that collagen IV reaction was weak on day 15 of gestation. The amount of collagen increased continuously until next days of fetal life and primary of 10 days postnatal in BMG. After this period, collagen IV showed no significant change in newborns. These data indicate that collagen IV appears just during the glomerular vasculogenesis and because of continuity and glomerular endothelial cell differentiation, type IV collagen, is the major structural protein in BMG, have been implicated in these processes.

**Keywords:** collagen IV, glomerular basement membrane, kidney, mouse

## Introduction

Basement membrane is a specialized structure of extra cellular matrix and consists of different compositions such as proteins and sugars (Gulberg et al., 1995; Berkholtz et al., 2006). This structure contains several components such as types IV, V collagen, laminin, fibronectin, sulfated and nonsulfated glycosaminoglycan (Berkholtz et al. 2006; Mates et al., 2004). Among these compositions, collagen is the most abundant constituent and type IV collagen has been identified a main structure of it (Thesleff et al., 1979; Horacek et al., 2004). Basement membrane not only changes during embryonic period but also alters during later stages of life and its alterations considers as an index of tissue changes in pathologic studies (Poschl et al., 2004; Cosgrove et al., 2008). ECM components turn over continually in developmental organs (Carnegie, 1993; Chai et al., 2003). In other words, molecules and matrix components are required in cell differentiation. Among them, type IV collagen play complex roles in developmental phenomena such as migration, proliferation, morphogenesis and metabolism (Paralkar et al., 1991; Thesleff et al., 1979). The most prominent role of extra cellular matrix is migration and cell adhesion that type IV collagen serves them (12, 13, 14). Therefore, considering collagen plays role in vital organs changes, it necessary seems its investigation.

## Material and Methods

20 virgin female Balbc/c mice were selected randomly and finding vaginal plug were designated as day zero of pregnancy. 2 mices were anesthetized by chloroform and were sacrified by cervical dislocation in every gestational days 13-18. Their fetuses were collected and were processed for histological studies. The similar processes were used for newborns on 5, 10, 15 and 20 of postnatal days. Kidneys were isolated from newborns of 2 mothers for each day. Finally, all samples of fetuses and new borns were placed in paraffin blocks and sectioned serially at a thickness of 7 µm. After deparaffination and rehydration, sections of kidneys were washed twice for 5 min with Tris buffer

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(containing 1.5% sodium chloride at PH=7). None-specific antibodies were blocked with 3% Triton X-100 and goat serum for 3 hours. For blocking endogenous peroxides activity, sections were treated with 3% $H_2O_2$- methanol for 1 hour and were incubated with the monocolonal antibody against collagen IV (conjugated with Horse radish peroxidase) at a dilution 1:50 overnight. Then sections again were placed in Tris buffer solution containing 3%Triton and 2% goat serum and were washed three times for 10 min with Tris buffer. After this stage, sections were placed for 15 min in Di-aminobenzidine containing 0.03% $H_2O_2$ and after washing samples, were counterstained with hematoxylin. The sections were mounted with glycerol gel. In this method, collagen shows positive reaction according to amount of appearance and the coloring reaction is from light to dark brown. The coloring reaction of collagen is a proper index for determination of BMG. The images of glomerular regions of kidneys were obtained by a camera microscope and were saved as a file. The intensity of collagen IV reaction was graded by two separate individual according to *firth* method (Firth et al., 1996).

***Statistical analysis***

The data were analyzed by using SPSS software and Kruskal Wallis and Mann-Withney tests. *P*-values<0.05 was considered as significant.

## Results

Our finding revealed although mesenchymal cells are enclosed uretric bud and glomerular primordium and rudimentary tubules observed on day 13 of gestation (figure1), no collagen reaction was detected until this period of time. Glomerular development completed on embryonic day 14 and collagen just showed weak reaction in parenchym of vessels (PV) but not in glomerular basement membrane. Collagen IV showed first reaction on day 15 of gestation in cortical glomerulus (figure 2). The intensity of reaction increased continually during next days and detected on day 18 of gestation in basement membrane of cortical glomerulus (figure 2). The results of this stage showed that epithelial basement membrane not only appeared in cortical regions of gelumerulus but also observed in tufts of capillary. The observations on 5, 10 and 15 days of postnatal period indicated that collagen reaction was more intensive on day 5$^{th}$ of postnatal period in glomerular basement membrane (table 1) but this reaction was not increased showed significantly on day 10$^{th}$ and after that during postnatal days.

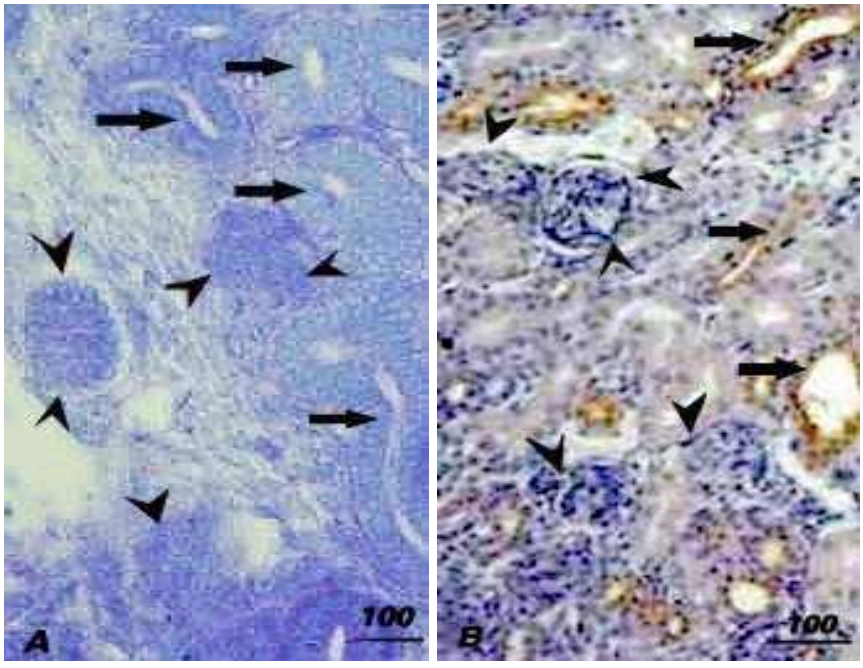


**Figure 1.** a. Transverse section through kidney paranchyme on day 13 of gestation that collagen indicated no reaction in glomerular primordium (arrow heads) as well as rudimentary tubules (arrows). b. Other transverse section during glomerular development on day 14 of gestation. Although glomerular structure have been completed, no reaction was observed in any area except for parenchyma vessles.

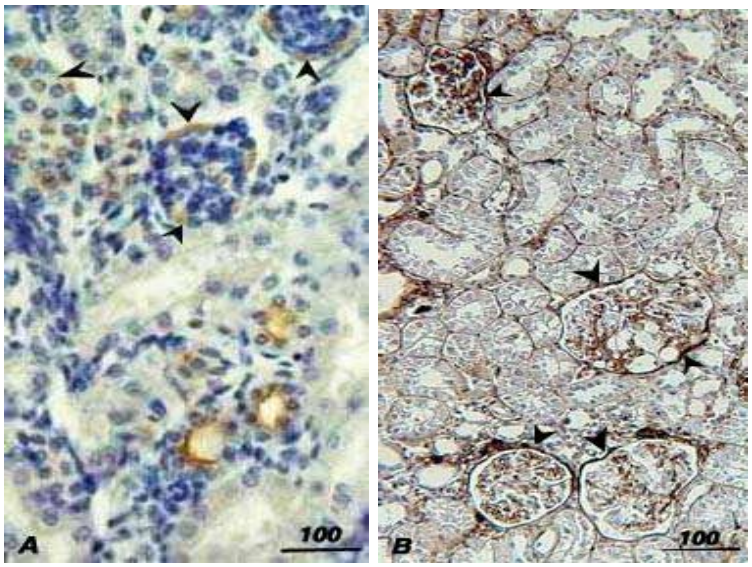


**Figure 2.** a. Transverse section of glomerulus on day 15 of gestation, The first reaction was observed in basement membrane of glomerulus of cortical regions (arrow heads). b. Sections of glomerulus on 18 day of gestation. In this image it have been showed labeling in both basement membrane of cortical glomerulus (arrow heads).

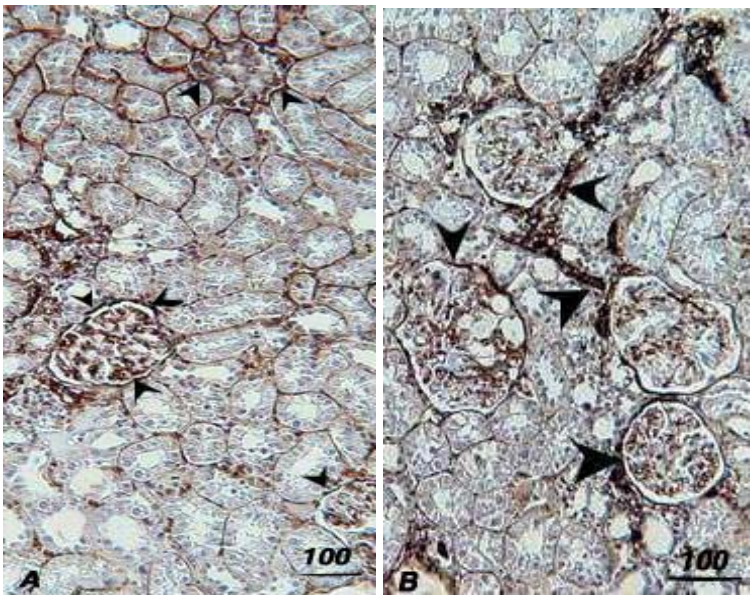


**Figure 3.** a. Transverse sections through glomerulus on 5$^{th}$ postnatal day, arrowheads refer to strong reaction in glomerular basement membrane. b. Glomerulus on 10$^{th}$ postnatal day, ECM and basement membrane labeling indicate more intensive reaction than previous image.

**Table 1.** Collagen type IV reaction during kidney glomerular morphogenesis

| Embryonic and postnatal days | BMG * | ECM ** | PV *** |
|---|---|---|---|
| 13$^{th}$ embryonic day | - | - | - |
| 14$^{th}$ = = = = | - | - | + |
| 15$^{th}$ = = = = | + | + | ++ |
| 18$^{th}$ = = = = | +++ | ++ | ++++ |
| 5$^{th}$ postnatal day | +++ | +++ | ++++ |
| 10$^{th}$ = = = = | ++++ | ++++ | ++++ |

Gradation of ranging from negative (zero) to 4 positive in conformity with the severe of reaction from negative, weak, moderate, strong and high strong.

Values represent means ± standard error of the mean (S.E.M), compared to embryonic with postnatal days: * ($P$ 0.002), ** ($P$ 0.005) and *** ($P$ 0.05).

## Discussion

Fibrillar proteins especially type IV collagen play different roles in limbs development and tendon junctions (Langmaier et al., 2001). These proteins not only act as conducting system for migration but also they are effective on wound repair, synthesis and turn over components of extra cellular matrix (such as fibronectin, tenasin, collagens, chondroitin sulfate and heparan sulfate), proliferation and cell differentiation (Holster et al., 2007). Interestingly, it has verified that fibrillar proteins such as type IV collagen play important role in repair embryonic wounds without remaining scar (Campos et al., 2008; Figus et al., 2007). Based on our data, it seems that proteins of basement membrane synthesis are required for glomerular epithelium formation. Among them, type IV collagen is the most remarkable component of glomerular basement membrane synthesizes under inductions mechanisms and it reserved in glomerular primordium.

Kidneys are the most important part of urinary tract that its mesenchyme forms tubular epithelium and renal glomerular during different stages of renal morphogenesis (Abrass et al., 2006; Barasch et al., 1999). The embryologic studies show that rudimentary glomerulus appears on 13.5 day of gestation in mouse (Sukhatme et al., 1993). The immunohistochemistry studies of glomerular endothelial formation showed that type IV collagen was first indicated weak reaction on 15 day of gestation. This represents that vessel endothelial formation in glomeruls is required macromolecules of basement membrane especially type IV collagen which plays a crucial rolein this process (Mejelle et al., 2007). Our data indicated that type IV collagen not only increased during final stage of embryonic but also followed on 1-10 of postnatal days.

Based on the previous studies it have been shown that basement membrane density may change by factors such as long-time activity of kidney and increasing age (Gulber et al., 2008). The studies also have revealed that in pathological condition, such as diabetes, renal failure influence on thickness of basement membrane and density of collagen (Funabiki et al., 1998). At this period of time, it have been proved that former protein, is effective on glomerular formation and renal filtration at early stages of kidney development and contributes in an immune response by signal transduction to adjacent tissues and extra cellular matrix that increase thickness of basement membrane and collagen density (Borza et al., 2007). Although this autoimmune response supports glomerular endothelium against chemical factors, it may results in decline of glomerular filtration and renal failure in acute cases. It have been believed that compositions of basement membrane play a structural role in epithelial cells, while have been distinguished basement membrane plays complexity roles in cell behavior such as development, proliferation, morphogenesis, metabolism and pathologic changes (Gullberg et al., 1995). Extra cellular matrix contributes in different cellular activities such as adhesion, migration and signal transduction (Hurle et al. 1989 and Chohen et al., 2007).

In extra cellular matrix, there are 2 groups of fibrillar proteins in extra cellular matrix, structural

proteins such as collagen and elastin. The second one are the proteins that play adhesion role such as laminin and fibronectin (Olson et al., 1991). Among these proteins, collagens are the most abundant component of extra cellular matrix. At least 19 types of collagen have been distinguished so far. Type IV collagen is the most important type of collagen in extra cellular matrix that its crucial role were discussed before. It has been identified that various types of collagen contributes in different morphologic process. The researches have shown that collagens induce endothelial cells to tubular ducts formation (Maeshima et al., 2006). Type II collagen may plays a role in epithelial-mesenchyme interactions that induce to form cytoskeleton (Ishibe et al., 1989). Type VI collagen probably causes cell-matrix interactions and cytoskeleton turn over in fibroblasts that increase cell surface during development (Doane et al., 1992). The studies indicate that extra cellular matrix causes specialized functions in different tissues. For example, the hepatocytes should contact to extra cellular matrix and basement membrane that synthesize specific proteins. Also, numerous rowth factors and hormones transduct signals via binding to extra cellular matrix (Zahang et al., 2007). On the other hand, it is important understanding relation between extra cellular matrix and functional mechanism of growth factors, pathogenesis of diseases and finding new therapeutics (Favor et al., 2007). The increasing type IV collagen represents that glomerular development is dependent to this type of collagen which appeared during mesangeal formation. Collagen detection on 15 days of gestation in glomerular basement membrane and its increase during next days, suggest that gelumerular development is dependent to basement membrane formation.

## Acknowledgement

This work was supported by research grant Number 87040 from office of Vice-Chancellor for Research Affairs of Zabol University of Medical Sciences and office of Vice-Chancellor for Research Affairs of Mashhad University of Medical Sciences and the authors wish to thank them for their financial support. We also wish to thank Mrs. Motejadded for her excellent technical assistance.

## References

1- Abrass C. K., Berfield A. K., Ryan M. C., Carter W. G. and Hansen K.M. (2006) Abnormal development of glomerular endothelial and mesangial cells in mice with targeted disruption of the lama3 gene. Kidney International 70(6): 1062-1071.

2- Barasch J., Yang J., Ware C. B., Taga T., Yoshida K., Erdjument-Bromage H., Tempst P., Parravicini E., Malach S., Aranoff T. and Oliver J. A. (1999) Mesenchymal to epithelial conversion in rat metanephros is induced by LIF. Cell 99: 377–386.

3- Berkholtz C. B., Lai B. E., Woodruff T. K. and Shea L. D. (2006) Distribution of extracellular matrix proteins type I collagen, type IV collagen, fibronectin, and laminin in mouse folliculogenesis. Histochemistry and Cell Biology 126(5): 583-592.

4- Borza D. B. (2007) Autoepitopes and alloepitopes of type IV collagen: role in the molecular pathogenesis of anti-GBM antibody glomerulonephritis. Nephron. Experimental Nephrology 106 (2): 37-43.

5- Campos A. C., Groth A. K. and Branco A. B. (2008) Assessment and nutritional aspects of wound healing. Current Opinion in Clinical Nutrition and Metabolic Care 11(3):281-288.

6- Carnegie J. A. and Cabaca O. (1991) The influence of extracellular matrix components on the proliferation and migration of inner cell mass-derived parietal endodermal cells. Biology of Reproduction 45(4): 572-580.

7- Carnegie J. A. and Cabaca O. (1993) Extracellular matrix composition and resilience: two parameters that influence the in vitro migration and morphology of rat inner cell mass-derived cells. Biology of Reproduction 48(2): 287-299.

8- Chai Q., Krag S., Miner J. H., Nyengaard J. R., Chai S. and Wogensen L. (2003) TGF-beta1 induces aberrant laminin chain and collagen type IV isotype expression in the glomerular basement membrane. Nephron. Experimental Nephrology 94(4): 123-136.

9- Cohen M. P., Lautenslager G. T., Hud E., Shea E., Wang A., Chen S. and Shearman C. W. (2007) Inhibiting albumin glycation attenuates dysregulation of VEGFR-1 and collagen IV subchain production and the development of renal insufficiency. American Journal Physiology Renal Physiology 292(2): 789-795.

10- Cosgrove D., Meehan D. T., Delimont D., Pozzi A., Chen X., Rodgers K. D., Tempero R. M., Zallocchi M. and Rao V. H. (2008) Integrin alpha1beta1 regulates matrix metalloproteinases via P38 mitogen-activated protein kinase in mesangial cells: implications for Alport syndrome. American Journal Pathology 172(3): 761-773.

11- Doane K. J, Yang G. and Birk D. E. (1992) Corneal cell matrix interaction type VI collagen promotes adhesion and spreading of corneal fibroblast. Experimental Cell Research 200: 490-499.

12- Favor J., Gloeckner C. J., Janik D., Klempt M., Neuhäuser-Klaus A., Pretsch W., Schmahl W. and Quintanilla-Fend L. (2007) Type IV procollagen missense mutations associated with defects of the eye, vascular stability, the brain, kidney function and embryonic or postnatal viability in the mouse, Mus musculus: an extension of the Col4a1 allelic series and the identification of the first two Col4a2 mutant alleles. Genetics 175(2): 725-736.

13- Figus A., Leon V. J., Philip B. and Dziewulski P. (2007) Severe multiple extensive postburn contractures: a simultaneous approach with total scar tissue excision and resurfacing with dermal regeneration template. Journal of Burn Care and Research 28(6): 913-917.

14- Firth N. A. and Reade P. C. (1996) The prognosis of oral mucosal squamous cell carcinomas: a comparison of clinical and histopathological grading and of laminin and type IV collagen staining. Australian Dental Journal 41(2): 83-86.

15- Funabiki K., Makita Y., Yamamoto M., Shike T., Fukui M., Sumiyoshi Y. and Tomino Y. (1998) Dissociated expression of collagen type IV subchains in diabetic kidneys of KKAy mice. Nephron 80 (2): 208-213.

16- Gubler M. C. (2008) Inherited diseases of the glomerular basement embrane. Natural Clinical Practical Nephrology 4(1):24-37.

17- Gullberg D. and Ekblom P. (1995) Extracellular matrix and its receptors during development. The International Journal of Devevelopmental Biology 39: 845-854.

18- Holster T., Pakkanen O., Soininen R., Sormunen R., Nokelainen M., Kivirikko K. I. and Myllyharju J. (2007) Loss of assembly of the main basement membrane collagen, type IV, but not fibril-forming collagens and embryonic death in collagen prolyl 4-hydroxylase I null mice. The Journal of Biological Chemistry 282(4): 2512-2519.

19- Horacek M. J., Thompson J. C., Dada M. O. and Terracio L. The extracellular matrix components laminin, fibronectin, and collagen IV are present among the epithelial cells forming Rathke's pouch. Acta Anatomica 147(2): 69-74.

20- Ishibe T., Seongchae I. and Yoo J. (1989) Type II collagen distribution in the ear of the developing chick embryo. Laryngoscope 99: 547-553.

21- Khoshnoodi J., Pedchenko V. and Hudson B. G. (2008) Mammalian collagen IV. Microsc Reserch Technology 71(5): 357-370.

22- Langmaier F., Meladek M., Kolomaznik K. and Sukop S. (2001) Collagenous hydrolysates from

untraditional sources of proteins. Reaction condition and the yield of enzymatic hydrolysis of short cattle tendons. International Journal of Cosmetic Science 23(4): 201-206.

23- Maeshima A., Sakurai H. and Nigma S. K. (2006) Adult kidney tubular cell population showing phenotypic plasticity, tubulogenic capacity, and integration capability into developing kidney. Journal of American Society of Nephrology 17(1): 188-198.

24- Mates L., Nicolae C., Morgelin M., Deak F., Kiss I. and Aszodi A. (2004) Mice lacking the extracellular matrix adaptor protein matrilin-2 develop without obvious abnormalities. Matrix Biology 23(3): 195-204.

25- Ment L. R., Stewart W. B., Ardito T. A. and Madri J. A. (1991) Beagle pup germinal matrix maturation studies. Stroke 22(3): 390-395.

26- Mjelle J. E., Rekvig O. P. and Fenton K. A. (2007) Nucleosomes possess a high affinity for glomerular laminin and collagen IV and bind nephritogenic antibodies in murine lupus-like nephritis. Annal of the Rheumatic Disease 66(12): 1661-1668.

27- Olson A. D., Pysher T. and Bienkowski R. (1991) Organization of intestinal epithelial cells into multi cellular structure requires laminin and functional actin microfilament. Experimental Cell Research 192: 543-549.

28- Paralkar V. M., Vukicevic S. and Reddi A. H. (1991) Transforming growth factor beta type 1 binds to collagen IV of basement membrane matrix: implications for development. Developmental Biology 143(2): 303-8.

29- Poschl E., Schlotzer-Schrehardt U., Brachvogel B., Saito K., Ninomiya Y. and Mayer U. (2004) Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. Development 131(7): 1619-1628.

30- Thesleff I., Stenman S., Vaheri A. and Timple R. (1979) Changes in the Matrix Proteins, Fibronectin and Collagen, during Differentiation of Mouse Tooth Germ. Devmental Biology, 70: 116-126.

31- Sukhatme V. P. (1993). Renal development: challenge and opportunity. Seminars in Nephrology 13: 422–426.

32- Zhang H. J., Tai G. X., Zhou J., Ma D. and Liu Z. H. (2007) Regulation of activin receptor-interacting protein 2 expression in mouse hepatoma Hepa1-6 cells and its relationship with collagen type IV. World Journal of Gastroenterology 13(41): 5501-5505.

*Journal of Cell and Molecular Research (2009) 1 (2), 97-105*

# Relation between gonadal hormones and sexual maturity of female Kutum *Rutilus frisii kutum* during spawning season

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Received 3 June 2009 Accepted 19 September 2009

## Abstract

Relation between sexual maturity and levels of two main steroid hormones in gonads, 17-□ estradiol ($E_2$) and testosterone (T) were studied by using histological and radioimmunoassay in female kutum *Rutilus frisii kutum* during spawning season of the southern Caspian Sea. The study was carried out from February to May 2008 using 105 migrated fish specimens catched from the River sefid-rood by various tools of catching including (Gillnet, Cast net, Seine net and Sheyl or Kulham). The results revealed that changes in plasma levels of gonadal steroids, ($E_2$) and (T) were closely correlated with ovarian development and increased in Gonadosomatic index (GSI) ($P<0.05$). GSI was increased in March and reached the highest value (29.47±4.2) in April. It was then decreased sharply in early May. The highest peak of plasma level of (T) and ($E_2$) was during spawning season and it was associated with the GSI. The results showed that levels of ($E_2$) and (T) in female kutum at the stage IV of sexual maturity was significantly higher than immature gonads (ovary in stages II and III) ($P<0.01$). Plasma ($E_2$) and (T) levels increased in February, the highest levels were observed in March and the early of April (105.6±75.3 and 29.2±96.6 ng/ml), respectively. It was decreased in the late of April and in the early of May during the spawning season ($P<0.05$).

**Keywords:** kutum, *Rutilus frisii kutum* (Kamenskii, 1901), sex steroid hormones, spawning season, Caspian sea

## Introduction

Generally populations of Caspian Kutum, *Rutilus frisii kutum* (Kamenskii, 1901) recorded along near the coast, from the Trek River the north to the southern part of the Caspian Sea (Sharyati, 1993). This species is an endemic fish and more than 70% of fishermen catch it in Iranian coastal of the Caspian Sea. The catch ratio of catched Caspian Kutum of the southern coastal was about 17195 tons in 2007-08 (www.shilat.com). In natural environment, the fish spawning in groups, in slow moving rivers occur at the water temperature of 9-23$^{0C}$ (Sharyati, 1993). The *Rutilus frisii kutum* is an order of Cypriniformes representative of cyprinid and Spawning strategy in this fish has a group synchronous and single spawning behavior. The principal spawning age groups are 2-5 years for males ad 3-6 years for females.

Several studies have been made in female teleosts to correlate the processes of ovarian follicular development and gametogenesis with seasonal fluctuations in plasma steroid levels Fostier et al., (1983); Kobayashi et al. (1989); Pankhurst and Conroy (1988); Rinchard et al. (1993); Rosenblum et al. (1987); Ramesh et al. (2009). Maturation of the egg is a long process that involves complex physiological and biochemical changes. Vitellogenesis is a process in which yolk proteins are produced in the liver, transport to the ovary and stored in the egg; resulting in tremendous egg enlargement. When conditions are appropriate for final maturation, nuclear development resumes, and the germinal vesicle migrates to one side. Finally, the walls of the germinal vesicle break down and maturity development completes. The association of changes in gonadal development with plasma levels of gonadal steroids has proven to be a valuable tool for understanding the endocrine control of reproduction in teleosts. Moreover, in teleosts, vitellogenesis and final oocyte maturation are regulated by gonadotropins via steroids secreted by the granolosa and theca cells of developing and mature oocytes. The occurrence of steroid production in different cells of the ovary may be related to different phases of oocyte development. Estradiol, ($E_2$) stimulates in

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**Figure 1.** Kutum *Rutilus frisii kutum* migrated to river Sefid- Rood of the southern Caspian Sea during spawning season.

turn the hepatic synthesis and secretion of vitellogenin which is accumulated in the oocytes. Correlations between changes of plasma levels of gonadal steroids and oocyte development have been well documented in a number of freshwater species including salmon forms (Whitehead et al., 1983; Truscott et al., 1986), cyprinids (Kobayashi et al., 1987), catfish *Heteropneustes fossilis* (Lamba et al., 1983), goldeye *Hiodon alosoides* (Pankhurst et al., 1986), walleye *Stizostedion vitrum* (Malison et al., 1994) and marine species including orange roughly *Hoplostethus atlanticus* (Pankhurst and Conroy, 1988; Putheti et al., 2008), Japanese whiting *Sillago japonica* (Matsuyama et al., 1990), Japanese sardine *Sardinops melanostictus* (Matsuyama et al., 1991) and common snook *Centropomus undecimalis* (Roberts et al., 1999). Environmental conditions and seasonal cues begin the process of maturation in many fish, this can take up to several years. When the gametes have matured, an environmental stimulus may signal the arrival of optimal conditions for the fry, triggering ovulation and spawning. Some environmental stimuli are changes in photoperiod, temperature, rainfall, and food availability. A variety of sensory receptors detect these cues, including the eye, pineal gland (an organ in the dorsal part of the forebrain that is sensitive to light), olfactory organs, taste buds, and thermo receptors. The objectives of this study are to investigate the relation between gonadal steroid levels, the hormonal profiles of (T) and ($E_2$) with sexual maturity stages in kutum *R. frisii kutum* during spawning season.

## Material and methods

### *Experimental fish*

To investigate gonadal development during natural spawning season, each Thursday morning at 10:00, 105 female kutum *Rutilus frisii kutum*, were collected from February to May in 2008, fish specimens were catched by variious tools of catching (Gillnet, Cast net, Seine net and Sheyl or Kulham) with a mesh size length 22mm. The period of fish collection lasted for a full calendar year and water temperature was recorded whenever fish were collected. Scales were collected from the specimen in order to determine their age (Chungunova, 1959). Scales were measured to aging and total length and forke length measured the nearest 0.1cm and weighed (W) to the nearest 0.1 g. The ovaries were dissected out and weighed, the condition factor (CF) was determined using the following formula (Bagenal, 1978)

$$CF=W/L^b\times100$$

Where W=total fish weight (g); L=fish standard length (cm) and b=slop of length-weight relationship.

Gonadosomatic index (GSI) was determined using the following formula (Roff, 1983).

GSI = gonad weight_100/body weight) for each fish analyzed throughout the sampling period was calculated and recorded.

### *Steroid assay and histological analysis*

Fish were anaesthetized with clove oil (*Syzyglum aromaticum*) (75-115 ppm) and blood samples were taken from the caudal vessels by using heparinized disposable syringes. Sample was centrifuged for 10 min at 3000 rpm and the plasma was stored at -45 $^0$C until steroid analysis. Plasma levels of ($E_2$) and (T) were measured by radioimmunoassay using the procedure described by (Rinchard et al., 1993).

Ovaries were fixed in Bouin's solution, embedded in paraffin after dehydration-infiltration, sectioned at 5 µm and stained with Mayer's hematoxylin and eosin for histological examination under binocular microscope. The developmental stage and the diameter of the 20 largest oocytes were recorded. Each gonad was classified according to the most advanced type of oocyte present (Table 1).

**Table 1.** Maturity stages of the ovary of kutum.

| Ovarian stage | Oocyte stages present in the ovary | Description of the most advanced Oocytes |
|---|---|---|
| (I) Previtellogenic | Previtellogenic oocytes | Oocytes with vacuole-free cytoplasm |
| (II) Onset of endogenous vitellogenesis | Previtellogenic oocytes and oocytes in endo-genous vitellogenesis | Oocytes at primary yolk vesicle stage, glycoproteins appear and occupy 2 or 3 rings in the cytoplasm periphery (early endogenous vitellogenesis) |
| (III) Completion of endo-genous vitellogenesis | Previtellogenic oocytes and oocytes having complete endogenous vitellogenesis | Oocytes are full of glycoprotein inclusions. Follicular and cellular layers are differentiated (late endogenous vitellogenesis) |
| (IV) Exogenous vitellogenesis | Previtellogenic oocytes and oocytes at different stages of exogenous vitellogenesis | Oocytes accumulate yolk globules and yolk vesicles are in periphery of the cytoplasm |
| (V) Final maturation | Previtellogenic oocytes and oocytes in final maturation | Appearance of the micropyle and migration of the germinal vesicle to the micropyle |
| (VI) Post-spawning | Previtellogenic oocytes and pre- and post-avoulatory follicles | The follicle cells in the pre- and postovulatory follicles show hypertrophy, the yolk substance degenerates |

Since this was a field study in river condition and during catching process, which may not be controlled as in the laboratory, a degree of stress may have been encountered in fish individuals but the significance of the variation is not as great as in controlled laboratory conditions (Cornish et al., 1993). Therefore in this study fish were anaesthetized with clove oil (*Syzyglum aromaticum*) (75-115 ppm)

***Statistical analysis***

Data were statistically analyzed by analysis of variance (ANOVA) in SPSS software (Ver. 11.0.)

## Results

Results clearly show that, the following processes occurred in the ovaries of kutum females in the spawning seasonal migration from March to April 2008: in early February, the gonads of various individuals were at maturity stage IV. The cross section of different fish ovarian maturity stages are showed in figure 2-4 (H&E, X 40). The Table 1 Shows relationship between standard length and body weight for all individuals and shows positive allometric growth of kutum, *R. frisii kutum*. Figure 5 shows the relation between water temperature ($^{0C}$) with day length (hr) and gonadosomatic index (GSI). Relationship between values of monthly condition factor (CF) and gonadosomatic index (GSI) are presented in Figure 6. Monthly changes in the maturity stage (most advanced oocyte stage in the ovary) of kutum in Sefid- Rood river southern Caspian Sea was shown in Figure 7. In this study relation between Monthly condition factor (CF) and gonadosomatic index (GSI) with the maturity stage and monthly concentration levels of ($E_2$) with gonadosomatic index (GSI) of kutum in Sefid- Rood river southern Caspian Sea was shown in figure 8 and figure 9 respectively. Combination of monthly concentration of ($E_2$) and (T) related in (Figure 10). Circulatory plasma ($E_2$) and (T) concentration (ng/ml) values for the entire experimental period and female plasma estradiol levels were low from February but afterward they increased significantly to March coinciding with the preponderance of vitellogenic follicles in the ovary. In the case of female estradiol, the highest concentrations are seen during March and early April. Therefore in the female kutum, estradiol reaches a maximum in March (Figure 9, 10). Then plasma (E2) and (T) levels exhibit a sharp decline in early May when oocyte maturation takes place.

During this period, the females had an increased GSI and the GSI continued to increase further and reach high values in April (Figure 6, 8). The histological pictures show the clear synchronicity of oocyte maturation.

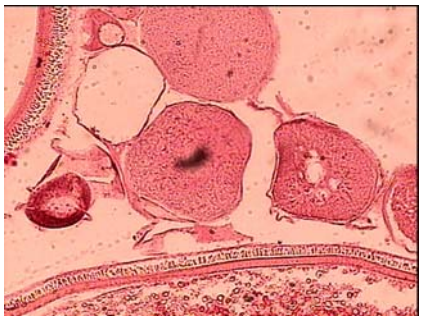
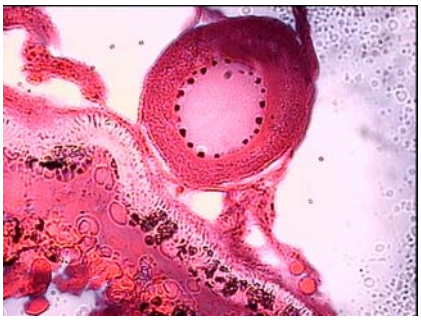
A   B

**Figure 2.** Histological picture, cross section of fish ovarian used in the study in 2008: A- maturity stage (Oogony) II (H&E, X 20). B- Maturity stage (Oogony) III in female kutum. (H&E, X 40).

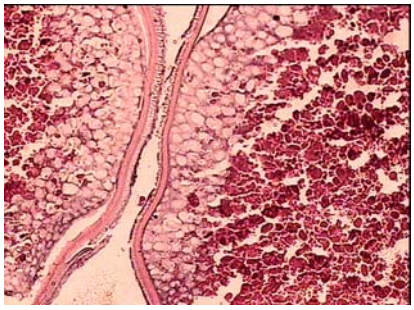
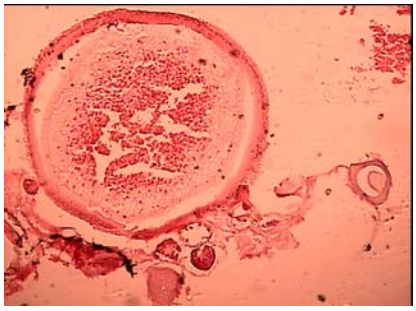
A   B

**Figure 3.** Histological picture, cross section of fish ovarian used in the study in 2008: A&B- maturity stage (Oogony) IV-V (H&E, X 40).

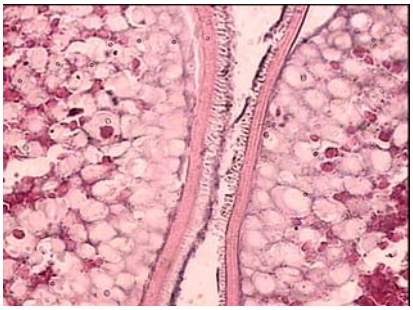
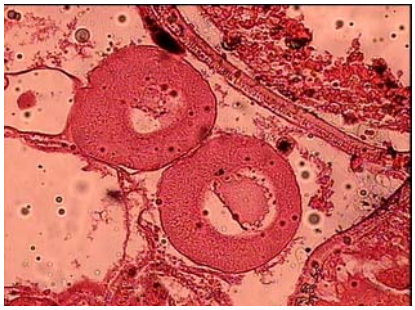
A   B

**Figure 4.** Histological picture, cross section of fish ovarian used in the study in 2008: A maturity stage (Oogony) IV; B-maturity stage(Oogony) III in female kutum with total length 423mm, 1784gr weight and aging 5.(H&E,X 40).

**Table 1.** Relationship between standard length and body weight for all individuals which is described by equation: $W= 0.0096 \times SL^{2.03735}$ ($r^2= 0.96$, n= 105), there is a positive allometric growth for the kutum specimen. MOSLS, mean observed standard length and standard deviation; MOWS, mean observed weight and standard deviation. A significantly different was MOSL and age (P= .027).

| Age | MOSLS (mm) | MOTWS (gr) | Number |
|---|---|---|---|
| 3 | 304 ± 6.12 | 549 ± 63 | 25 |
| 4 | 337 ± 10.24 | 896 ± 94 | 50 |
| 5 | 378 ± 13.58 | 1219 ± 85 | 18 |
| 6 | 441 ± 19.85 | 1593 ± 114 | 12 |

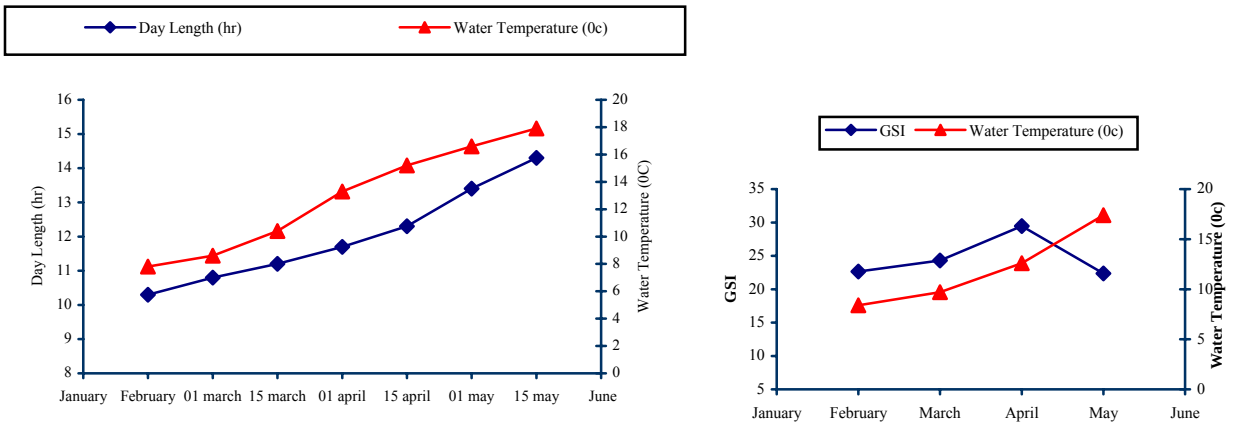



**Figure 5.** Relation between water temperature $^{(0C)}$ with gonadosomatic index (GSI) and day length $^{(hr)}$ in southern of Caspian Sea of River Sefid-Rood.

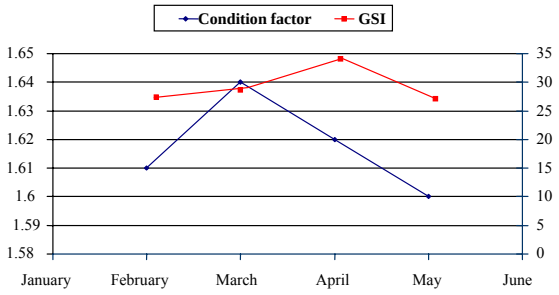



**Figure 6.** Monthly condition factor (CF) and gonadosomatic index (GSI) of kutum in River Sefid- Rood southern Caspian Sea.

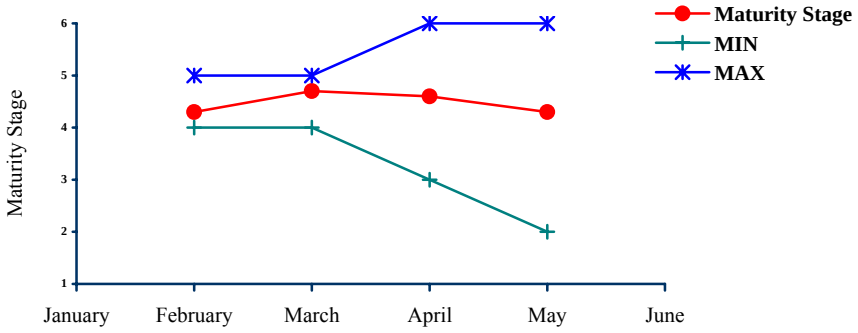



**Figure 7.** Monthly changes in the maturity stage (most advanced oocyte stage in the ovary) of 105 individuals kutum in River Sefid-Rood southern Caspian Sea.

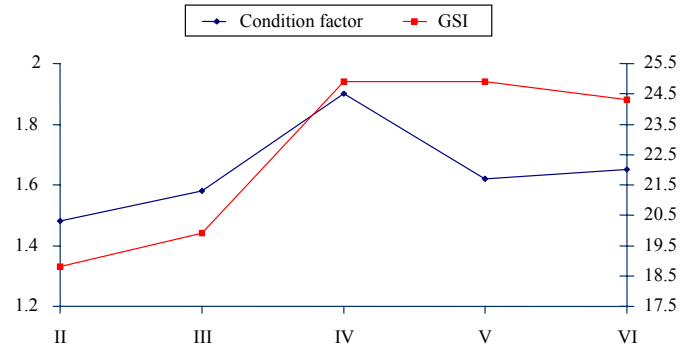



**Figure 8.** Relationship with Condition factor index (CF) and gonadosomatic index (GSI) with sexual maturity of 105 individuals kutum in River Sefid-Rood southern Caspian Sea. (n=105).

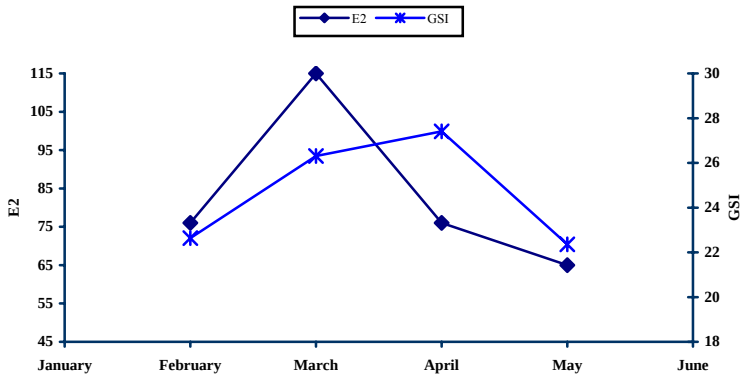



**Figure 9.** Monthly concentration of ($E_2$) and gonadosomatic index (GSI) of 105 individuals kutum in River Sefid-Rood southern Caspian Sea. (n=105).

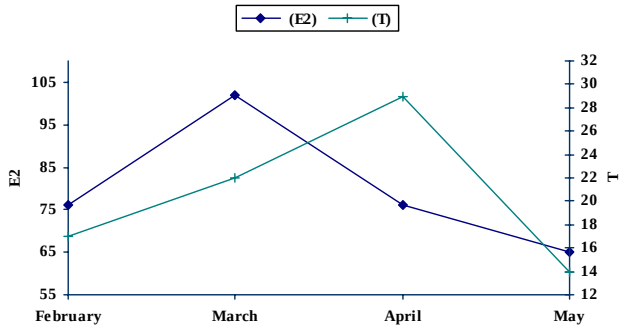



**Figure 10.** Monthly concentration of ($E_2$) and (T) of 105 individuals kutum in River Sefid-Rood southern Caspian Sea. (n=105).

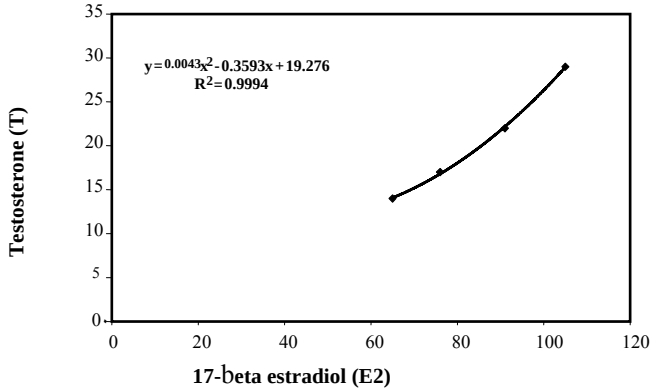



**Figure 11.** Relationship between levels of (T) and (E2) concentration in plasma which occurs at the same time in kutum of the River Sefid- Rood southern Caspian Sea. (n=105).

## Discussion

Khalko and Talikina 1993 described that in the ovaries of bream females *Abramis brama* in the Rybinsk reservoir from autumn to spring during winter months, trophoplasmic growth of eggs proceeds with a corresponding enlargement of yolk globules. Yolk deposition comes to an end, and oocytes become functionally mature in late March to early April. Generally, ($E_2$) is responsible for stimulating vitellogenesis and also released by female gonads during the pre-spawning period. According to the results Condition factor index (CF) and gonadosomatic index (GSI) related with sexual maturity stages and Monthly concentration of sexual steroids hormones demonstrated in (figure 8 and 10) reflects the importance of this hormone. Over the period from February to April a gradual increase in plasma levels was observed a bimodal increase from both the gonads and the inter-renal tissues. Estradiol is known to be secreted by the cells of the ovarian follicles that promote the development and maintenance of the female sexual characteristics. In humans this hormone (together with some other hormones) is responsible for controlling the female sexual cycle. Also ($E_2$) has been reported to stimulate vitellogenesis in teleosts changed the plasma levels of sex steroid hormones during gonadal maturation (Silversand et al., 1993); (Smith and Haley, 1988). These authors reported an increase in plasma (E2) levels once spawning commences and remains high throughout the period of oocyte growth. Sen et al. 2002 reported that concentration of plasma testosterone (T) in Indian major carp *Labeo labeo rohita* is expected to be high when it is no longer needed for aromatization, while (T) levels during postvitellogenic stage exhibited a quick decline in this fish, coinciding with the fall of plasma ($E_2$) concentration. A sudden drop in the plasma ($E_2$) level in *Labeo rohita* from vitellogenic to postvitellogenic stage may be explained in terms of switching off the aromatise (CYP19) activity as the oocytes progressed to maturation. Almost a similar profile of $E_2$ has been reported during the transition from vitellogenic to maturational stage in rainbow trout (Fostier et al., 1983). This drop in circulatory ($E_2$) levels probably reduces the intensity of sex steroid feedback, allowing the occurrence of hypothalamus-mediated gonadotropnes surge, which is required for the development of oocyte maturational competence. Rinchard et al. 1993 mentioned that in other teleosts such as gudgeon, *Gobio gobio*, there was no decrease of $E_2$ level during oocyte maturation; meanwhile this study has shown decreased $E_2$ in some specimens of kutum. (Rosenblum et al. 1987) observed a good correlation between circulating ($E_2$) and calcium levels in female teleosts. Increases in plasma ($E_2$) in female Tilapia *Oreochromis mossambicus* paralleled increases in both GSI and calcium levels (Cornish, 1993), thereby confirming a role for estradiol in vitelogenesis in present study for Kutum *R. frisii kutum*, is in agreement that correspond with those for most teleosts fish and vertebrates. The slight increase of testosterone (T) levels during oocyte development may be related to its role as precursor of ($E_2$) synthesis. At high concentration, (T) might also be involved in hepatic vitellogenin synthesis (Rinchard et al., 1993). The sudden peak was measured when most fish were in final maturation (stage V), an effect of the release of testosterone (T) into the plasma when this was no longer needed for aromatization. This acute rise in testosterone indicates that oocytes are fully mature and ready to ovulate (Kobayashi et al., 1989). Although the same relationship was

established between oocyte stages and testosterone levels in fish in river Sefid- Rood during spawning season. The present study shows that an increase in the level of (T) in the plasma could be associated with correlation between levels of (T) and (E2) concentration in Kutum of the River Sefid- Rood, southern Caspian Sea (Figure 1). There is also an increase in day length during this period, which has been shown to be an environmental cue to a preovulatory surge in hormonal secretion in cyprinids (Aida, 1988).

## Acknowledgment

The authors thank all colleagues Dr. Sarpanah, Khanipoor, Abdolmalaki, Shabai, Shaapoor, Sudagar, Hosseini, who helped us and for valuable comments during the preparing of the manuscript, and acknowledge M.Sc. Halajiyan, Kazemi for assistance in histological study and Mr Selseleh for assistance in fish sample collection. We thank also members of the Guilan, Fishing Technology Department, Mirza Kochak Vocation & Higher Education Center for Fisheries Sciences and Technology and Golestan, Gorgan University of Agricultural Sciences and Natural Resources. We appreciated Dr Sadeghi and Jalali in Sadeghi Clinical laboratory in Astaneh Ashrafiyeh and Mr. Maleki in Fadaei Pathology laboratory in Rasht.

## References

1- Aida K. (1988) A review of plasma hormone changes during ovulation in Cyprinid fishes. Animal physiology – Reproduction. Aquaculture (Netherlands) 74(1)11-21

2- Bagenal T. B., (1978) Aspects of fish fecundity. In ecology of freshwater fish production. Ed. S.D. Gerking. Blackwell Scientific, Oxford.75-101.

3- Chungunova N. I., (1959) Age and growth studies in fish. Translated by. D. Yassaki, 1963. Washington D. C. National Science Foundation. USA. 131P.

4- Cornish D. A., Smit G. L. and Campbell I. M. (1993) Seasonal fluctuation in gonadotropin levels in the plasma and gonads of male and female tilapia, *Oreochromis mossambicus*. Water SA 23 (3): 263-270.

5- Fostier A., Jalabert B., Billard R., Breton B. and Zohar Y. (1983) The gonadal steroids. In: WS Hoar, DJ, Randall and E.M Donaldson (Eds). Fish Physiology. Vol.9A. Academic Press, New York.

6- Khalko V.V. and Talikina M.G. (1993) Comparative description of prespawning condition of gonads in reproductive isolated of phytophilous fishes from the Rybinsk reservoir, Vopr. Ikhtiol. 33: 241-247.

7- Kobayashi M., Aida K. and Hanyu U. (1987) Hormone changes during ovulation and effects of steroid hormones on plasma gonadotropin levels and ovulation in goldfish. Gen. Comp. Endocrinol. Jul.67 (1):24-32.

8- Kobayashi M., Aida K. and Hanyu U. (1989) Involvement of steroid hormones in the preovulatory gonadotropin surge in female goldfish. Fish Physiology and Biochemistry (7): 1-6. 141-146.

9- Lamba V., Goswami S. V. and Sundararaj B. I. (1983) Circannual and circadian variations in plasma levels of steroids (Cortisol, estradiol-17β, estrone, and testosterone) correlated with the annual gonadal cycle in the catfish, *Heteropneustes fossilis* (Bloch). Gen. Comp. Endocrinology 50: 2. 205–225.

10- Malison J. A., Procarione L. S., Barry T. P., Kapuscinski A. R. and Kayes T. B. (1994) Endocrine and gonadal changes during the annual reproductive cycle of the freshwater teleost, *Stizostedion vitreum*. Fish Physiolology and Biochemistry 13 (6): 473–484.

11- Matsuyama M., Adachi S., Nagahama Y., Maruyama K. and Matsuura S. (1990) Diurnal rhythm of plasma steroid hormone levels in the Japanese whiting, *Sillago japonica*, and a daily-spawning teleost. Fish Physiolology and Biochemistry 8: 329–338.

12- Matsuyama M., Fukuda T., Ikeura S., Nagaham Y. and Matsuura M. (1991) Annual reproductive cycle of the captive female Japanese sardine *Sardinops melanostictus*: relationship to ovarian development and plasma levels of gonadal steroid hormones. Mar. Biol. 108: 21–29.

13- Pankhurst N. W. and Conroy A. M., (1988) Endocrine changes during gonadal maturation and spawning in the orange roughy (*Hoplostethus atlanticus* Colett), a teleost from the midslope waters off New Zealand. Gen. Comp.Endocrinology 70: 262–273.

14- Pankhurst N.W., Stacey N. E. and Van Der Kraak G. (1986) Reproductive development and plasma levels of reproductive hormones of goldeye, *Hiodon alosoides* (Rafinesque), taken from the North Saskatchewan River during the open-water season. Can. J. Zool. 64: 2843–2849.

15- Ramesh R. Putheti and Mahesh. C. Patel (2009) Pharmaceutical formulation and development of Floating and Swellable sustained drug delivery systems: a review". E-Journal of Science & Technology. (e-JST) 4: 2,1-12.

16- Ramesh Putheti R. N., Okigbo M. S., Advanapu R., Leburu. (2008) Ground water pollution due to aquaculture in east coast region of nelore district, andhrapraesh, india.AJEST.2 (3):p046-050.

17- Rinchard J., Kestemont P., Kuhn E. R. and Foster A. (1993) Seasonal changes in plasma levels of steroid hormones in an asynchronous fish the gudgeon *Gobio gobio* L; (Teleosti, Cyprinidae). Gen. Comp. Endocrinology 92: 168-178.

18- Roberts S. B., Jackson L. F., King W. V., Taylor R. G., Grier H. J., Sullivan C. V. (1999) Annual reproductive cycle of the common snook: endocrine correlates of maturation. Trans. Am. Fish. Soc. 128 (3): 436–445.

19- Roff D. A. (1983). An allocation method of growth and reproduction in fish. Can. J. aquat. Sci. (40): 9 1395-1404. Doi: 10.1139/f83-161

20- Rosenblum P. M., Pudney J. and Callard I. P. (1987). Gonadal morphology, enzyme histochemistry and plasma steroid levels during the annual reproductive cycle of male and female brown bullhead catfish, *Ictalurus nebulosus* Lesueur. J. Fish Biology 31 (3): 325-341.

21- Sen U., Mukherjee D., Bhattacharyya S. P. and Mukherjee D. (2002) Seasonal changes in plasma steroid levels in Indian major carp *Labeo labeo rohita*: influence of homologous pituitary extract on steroid production and development of oocyte maturational competence. Gen. Comp. Endocrinology 128: 123–134.

22- Sharyati A. (1993) Fishes of the Caspian Sea region. Iranian fisheries company, Iran, third edition. pp. 77-79 (in Persian).

23- Silversand C., Johan Hyllner S. and Haux C. (1993) Isolation, immunochemical detection, and observations of the instability of vitellogenin from four teleosts. Journal of Experimental Zoology (267): 6. 587-597.

24- Smith C. J. and Haley S. R. (1988) In vitro stimulation and inhibition of steroid hormone release from postovulatory follicles of the tilapia, *Oreochromis mossambicus*. Cell and Tissue Research 254: 439-447.

25- Truscott B., Idler D. R., So Y. P., Walsh J. M. (1986) Maturational steroids and gonadotropins in upstream migratory sockeye salmon. Gen. Comp. Endocrinology 62: 99–110. 26- Whitehead C., Bromage N. R., Breton B. (1983) Changes in plasma levels of gonadotropins, oestradiol 17b and vitellogenin during the first and subsequent reproductive cycles of female rainbow trout. Aquaculture 34: 317–326.

27- WWW.Shilat.Com (Shilat, Iranian Fisheries Organization)

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Journal of Cell and Molecular Research

Volume 1, Number 2, Winter 2009